Paul L. H. McSweeney
James A. O'Mahony
Alan L. Kelly *Editors*

Advanced Dairy Chemistry

Volume 3: Lactose, Water, Salts and Minor Constituents

Fourth Edition

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Editors
Paul L. H. McSweeney
School of Food and Nutritional Sciences
University College Cork
Cork, Ireland

James A. O'Mahony
School of Food and Nutritional Sciences
University College Cork
Cork, Ireland

Alan L. Kelly
School of Food and Nutritional Sciences
University College Cork
Cork, Ireland

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Preface to the Fourth Edition

This volume completes the fourth edition of *Advanced Dairy Chemistry* and follows publication of Volume 1A (*Proteins: Basic Aspects*) in 2013, Volume 1B (*Proteins: Applied Aspects*) in 2016 and Volume 3 (*Lipids*) in 2020, all by Springer, New York. Originating in the 1980s as *Developments in Dairy Chemistry* and then edited by Prof. P. F. Fox, the *Advanced Dairy Chemistry* series is the largest source of authoritative information on the chemistry of milk and has become an important conduit for reviews in emerging topics. Like its predecessors, this edition of the series is intended for academics, researchers and senior students, and each chapter is written by an acknowledged expert in the topic and is extensively referenced to facilitate further investigation of details.

The structure of this volume of the fourth edition differs somewhat from those of earlier editions. While retaining in-depth coverage of the major constituents of milk covered by this volume, more emphasis than in earlier editions has been placed on aspects unique to milk and more general topics have been omitted (e.g. the chemistry of Maillard browning) or rationalized (e.g. nutritional aspects of salts and vitamins). While certain authors updated their chapters from the third edition, a number of new authors were invited to contribute to this volume bringing their fresh perspectives. A new chapter is included also on partitioning of milk constituents.

We express our sincere thanks to our 26 contributors for sharing so willingly their expertise and knowledge of dairy chemistry and which made our task as editors a pleasure. We also thank colleagues at Springer Nature for their assistance in the production of this volume.

Cork, Ireland — Paul L. H. McSweeney
Cork, Ireland — James A. O'Mahony
Cork, Ireland — Alan L. Kelly

Preface to the Third Edition

This volume completes the third edition of *Advanced Dairy Chemistry*, a series which commenced as *Developments in Dairy Chemistry* in 1982. This book provides an update of many of the topics covered in the second edition of *Advanced Dairy Chemistry* Volume 3, published in 1997, and complements Volumes 1 and 2 of the third edition (*Proteins*, 2003, and *Lipids*, 2006), making the *Advanced Dairy Chemistry* series the most comprehensive treatise on the topic.

Six (Chaps. 1–4, 6, 7) of the 15 chapters in this volume are devoted to various aspects of lactose, including its chemical properties, solid and solution states, its significance in various dairy products, production and utilization, syndromes associated with lactose malabsorption and its reaction chemistry. In recent years, galactooligosaccharides produced from lactose by the transferase activity of β-galactosidase have become important due to their prebiotic activity, and Chap. 5 is devoted to this topic. The indigenous oligosaccharides in the milk of various species are discussed in Chap. 8.

The chemistry and technological aspects of milk salts and water are discussed in Chaps. 9 and 11, respectively. The nutritional and health aspects of lactose, minerals and vitamins are assessed in Chaps. 6, 8, 10, 12 and 13. Flavours and off-flavours in dairy products and the physico-chemical properties of milk are reviewed in Chaps. 14 and 15, respectively.

Like its predecessors, this volume is intended for lecturers, senior students and research personnel working in the field of dairy chemistry and technology. Each chapter is written by an expert and is thoroughly referenced to facilitate further study of specific points.

We would like to express our sincere appreciation to the 35 authors from nine countries who contributed to this volume for sharing so willingly their knowledge of dairy chemistry, which made our task as editors a pleasure.

Cork, Ireland — P. F. Fox
Cork, Ireland — P. L. H. McSweeney

Preface to the Second Edition

This book is the third volume of *Advanced Dairy Chemistry*, which should be regarded as the second edition of *Developments in Dairy Chemistry.* Volume 1 of the series, *Milk Proteins*, was published in 1992 and Volume 2, *Milk Lipids*, in 1994. Volume 3, on lactose, water, salts and vitamins, essentially updates Volume 3 of *Developments in Dairy Chemistry* but with some important changes.

Five of the eleven chapters are devoted to lactose (its physico-chemical properties, chemical modification, enzymatic modification and nutritional aspects), two chapters are devoted to milk salts (physico-chemical and nutritional aspects), one to vitamins and one to overview the flavour of dairy products. Two topics covered in the first editions (enzymes and other biologically active proteins) were transferred to Volume 1 of *Advanced Dairy Chemistry* and two new topics (water and physico-chemical properties of milk) have been introduced.

Although the constituents covered in this volume are commercially less important than proteins and lipids covered in Volumes 1 and 2, they are critically important from a nutritional viewpoint, especially vitamins and minerals, and to the quality and stability of milk and dairy products, especially flavour, milk salts and water. Lactose, the principal constituent of the solids of bovine milk, has long been regarded as essentially worthless and in many cases problematic from the nutritional and technological viewpoints; however, recent research has created several new possibilities for the utilization of lactose.

Like its predecessor, this book is intended for lecturers, senior students and research personnel; each chapter is written by an expert on the particular subject and is extensively referenced.

I wish to express my sincere thanks and appreciation to all the authors who contributed to this book and whose cooperation made my task as editor a pleasure.

Cork, Ireland P. F. Fox

Preface to the First Edition

This volume is the third in the series on the chemistry and physical chemistry of milk constituents. Volumes 1 and 2 dealt with the commercially more important constituents, proteins and lipids, respectively. Although the constituents covered in this volume are of less direct commercial importance than the former two, they are nevertheless of major significance in the chemical, physical, technological, nutritional and physiological properties of milk.

Lactose, the principal component of the milks of most species, is a rather unique sugar in many respects—it has been referred to as one of Nature's paradoxes. It is also the principal component in concentrated and dehydrated dairy products, many of the properties of which reflect those of lactose. The chemistry and principal properties of lactose have been thoroughly researched over the years and relatively little new information is available on these aspects; this new knowledge, as well as some of the older literature, is reviewed in Chap. 1.

Although lactose has many applications in the food, pharmaceutical and chemical industries, not more than 10% of the potentially available lactose is actually recovered as such. Like other sugars, lactose may be modified by a multitude of chemical reagents; some of these are reviewed in Chap. 2 and some applications of the derivatives discussed. The enzymatic hydrolysis of lactose to glucose and galactose has considerable technological as well as nutritional significance, and the recent literature on this subject is reviewed in Chap. 3. Lactose is not digestible by the majority of the world's population, and the current views on this nutritionally important problem are discussed in Chap. 4. A deficiency of either of two enzymes involved in the Leloir pathway for galactose metabolism leads to the inability to metabolize galactose produced from lactose (or other galactose-containing sugars) and causes two relatively rare congenital diseases referred to as galactosaemia, the literature on which is reviewed in Chap. 5.

Quantitatively, the salts of milk are minor constituents but they play a disproportionately important role in many of the technologically important properties of milk, some of which have been discussed in Volume 1 of this series. Recent literature on the rather complex chemistry of the milk salts per se is reviewed in Chap. 6. Many

of the inorganic constituents of milk, some of which are present only at trace levels, are also of very considerable nutritional significance. Since a variety of minerals are required for proper growth and development, and milk is the sole source of these requirements at a critical stage of infant growth, the significance of milk as a source of dietary minerals is discussed in Chap. 7.

The flavour/off-flavour of milk and dairy products is undoubtedly technologically important and extremely complex. This topic could easily occupy a full volume in this series but a comprehensive summary is presented in Chap. 8.

Many people may regard milk simply as a source of lipids, proteins, carbohydrates and minerals, with very little biological activity as such. This, in fact, is not the case; milk contains a great variety of biologically active species, some of which, e.g. enzymes, may cause undesirable changes in milk and dairy products during storage, while others, e.g. vitamins, immunoglobulins, are of very considerable nutritional and biological significance. Chapters 9–11 review the recent literature on the indigenous enzymes in milk, indigenous antibacterial systems and vitamins, respectively. The importance of at least some of the indigenous enzymes and vitamins is well established but the indigenous antibacterial systems may be of much greater significance than considered heretofore, and it is hoped that Chap. 10 will stimulate further research in this area.

I wish to thank sincerely the 13 authors who have contributed to this volume; their cooperation and effort made my task as editor rather simple.

Cork, Ireland P.F. Fox

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Contributors

H. Douglas Goff Department of Food Science, University of Guelph, Guelph, ON, Canada

Rita M. Hickey Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

T. R. Hill Population Health Sciences Institute, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, UK

S. A. Hogan Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

David S. Horne 2 Boghall Farm Steadings, West Lothian, Scotland, UK

E. H. Hynes Instituto de Lactología Industrial, Facultad de Ingeniería Química, Universidad Nacional del Litoral (UNL), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina

Catherine J. E. Ingram Research Department of Genetics, Evolution and Environment, University College London, London, UK

D. N. De. S. Jayasinghe Produce Delivered Ltd., Auckland, New Zealand

P. Jelen Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, AB, Canada

P. M. Kelly Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

M. J. Lewis Department of Food and Nutritional Sciences, University of Reading, Reading, UK

John A. Lucey Department of Food Science, University of Wisconsin-Madison, Madison, WI, USA

Hannah K. Masterson Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

Nicolás Montalva Society and Health Research Center, Faculty of Humanities, Universidad Mayor, Santiago, Chile

School of Public Health, Universidad Mayor, Santiago, Chile

Eoin Murphy Teagasc Food Research Centre, Co. Cork, Ireland

D. E. Otter Auckland University of Technology, Auckland, New Zealand

Rebecca A. Owens Department of Biology, Maynooth University, Maynooth, County Kildare, Ireland

Anthony H. J. Paterson School of Food and Advanced Technology, Massey University, Palmerston North, New Zealand

M. C. Perotti Instituto de Lactología Industrial, Facultad de Ingeniería Química, Universidad Nacional del Litoral (UNL), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina

Naritchaya Potes School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Yrjö H. Roos School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Dallas M. Swallow Research Department of Genetics, Evolution and Environment, University College London, London, UK

Tadasu Urashima Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

S. Wu Massey University, Auckland, New Zealand

Chapter 1
Lactose: Occurrence, Properties, Reactions, and Significance

P. Jelen

1.1 General Introduction and History

Lactose, commonly called milk sugar, is a carbohydrate uniquely associated with milk of almost all mammals, including humans. It is a reducing disaccharide, composed of two monosaccharides glucose and galactose, linked by a β1 → 4 glycosidic bond. Its molecular formula is $C_{12}H_{22}O_{11}$, its systematic name being β-D-galactopyranosyl-(1 → 4)-D-glucose. As the glucose can exist in two forms (the α-pyranose form or the β-pyranose form), in contrast to galactose (which only has the β-pyranose form), lactose in aqueous solutions can be present in two anomeric forms, α-lactose and β-lactose. The lactose content in human milk is higher than that of all industrially relevant farm animals the milk of which is used in human nutrition, as shown in Table 1.1.

The first documented historical record mentioning lactose as "salt of milk whey" was published in 1633 by Italian physician Fabrizio Bartoletti (often referred to as Bartolettus, 1576–1630). He isolated a "curious whitish salt" by evaporation of water from milk whey, followed by repeated dissolution and coagulation. His method was reprinted in 1688 by the German physician Michael Ettmüller (1644–1683) and mentioned in 1700 by the Venetian pharmacist Lodovico Testi (1640–1707) in his booklet on milk sugar (saccharum lactis). The Swedish German chemist Carl Wilhelm Scheele (1742–1786), remembered mainly for his discovery of oxygen, had wide research interests leading to isolating and characterizing, for the first time, many organic compounds including lactose in 1780. Other notable chemists of the past also contributed to the early knowledge of lactose; thus, Heinrich Vogel (1778–1867) proved in 1812 that glucose will be produced by

P. Jelen (✉)
Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, AB, Canada
e-mail: psjelen@interbaun.com

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Table 1.1 Lactose and water content in milk of mammals used in human nutrition, in comparison to human milk (as is, % w/w, data averaged from various sources)

Source	Lactose	Water
Cow	4.6	87.3
Water buffalo	4.8	82.8
Goat	4.3	86.7
Sheep	4.8	82.0
Camel	4.5	87.0
Donkey	6.3	90.5
(Human)	(7.5)	(87.1)

hydrolyzing lactose, while Louis Pasteur in 1856 isolated galactose as the other lactose component. The exact configuration of the two component sugars was proposed in 1894 by another German chemist, the 1902 Nobel Prize recipient Emil Fischer (1852–1919). The name lactose was coined by the French chemist Jean Baptiste André Dumas (1800–1884).

The interest in lactose as a commercial commodity, as well as a subject of scientific research, increased considerably during the nineteenth century. The foundations of the present knowledge of lactose, especially regarding its chemistry, molecular structure, physical properties, and crystallization behavior were laid during the early twentieth century, including the systematic work of Hudson (1904). This rapidly expanding knowledge has been reviewed numerous times (Whittier 1925; Whitter 1944; Weisberg 1954; Zadow 1984, 1992; Schaafsma 2008; Ganzle et al. 2008; Wong and Hartel 2014). In 2012, the International Dairy Journal published a special issue containing 9 reviews focusing on various aspects of technology, nutrition, and health of lactose and its derivatives (Jelen and Smithers 2012). Most of the major textbooks on Dairy Chemistry, Technology, or Nutrition include a separate chapter on lactose, its properties and/or its nutritive value (e.g., Jenness and Patton 1959; Webb and Johnson 1965; Webb et al. 1974; Renner 1983; Walstra and Jenness 1984; Fox 1985, 1997; Wong et al. 1988; Fox and McSweeney 1998; Walstra 2002; Walstra et al. 1999, 2006; Miller et al. 2007). In the four volumes of the Encyclopedia of Dairy Sciences (Fuquay et al. 2011), there are almost 200 entries related to lactose. The book Lactose and Its Derivatives (Sinelnikov et al. 2007) appeared to be until recently the most comprehensive source of information on the subject, unfortunately available only in Russian. The most recent book, Lactose (Paques and Lindner 2019) presents reviews of the evolutionary role, health effects, and the most current industrial topics related to lactose and some of its commercially interesting derivatives.

The objective of this introductory chapter is to provide a brief summary of the main traditional building blocks of knowledge concerning lactose, leading to better understanding of the next few chapters reviewing selected topics where active lactose-related research is generating new results. Although crystallization has been a main topic of lactose research studied for a long time, the new glass transition data provide additional research angles in this regard. This subject is covered in Chapter 2, while in Chapters 3 and 4 the production and significance of lactose in various dairy

products are reviewed in detail. Current research concerning lactose is especially active in areas related to nutrition, including lactose ingestion and malabsorption (Chapter 6), while the subject of oligosaccharides (Chapters 5 and 7) is of great industrial significance presently.

1.2 Lactose Biosynthesis and Functions in Milk

It may be of interest to note that, in contrast to most other sugars being of plant origin, the only significant source of lactose in nature is the mammary gland of lactating mammals. Several million tons of isolated lactose are produced annually as a valuable industrial product just from milk of the few species of mammals used as farm animals for human nutrition purposes; the overall production of lactose by all lactating mammals is obviously many times higher.

The biosynthesis of lactose in the epithelial mammary gland cells has been studied extensively and several exhaustive reviews of the information have been published, mainly towards the end of the last century (Brew and Hill 1975; Jones 1978; Larson 1985, others). These seem to serve as the basis of information found in later texts on lactose, including the previous editions of this series. The latest, rather extensive, review of this topic can be found in the book Lactose (Paques and Lindner 2019) mentioned above. In the following, only the most salient aspects of this somewhat unusual biosynthetic pathway are summarized. It involves two molecules of glucose being absorbed from the blood, one being converted (epimerized) to galactose via the Leloir pathway which is widespread in animal tissues and bacterial cells. This metabolic process also eliminates the galactose toxicity which could exist in its free form. The galactose is phosphorylated and coupled to the second molecule of glucose by a β-1,4-glycosidic linkage, through the action of a unique two-component enzyme, lactose synthase.

Lactose serves two important functions in milk. It is a ready source of energy for the neonate, providing about 30% of the caloric value of bovine milk, and it influences osmotic pressure of milk, which is isotonic with blood and hence is essentially constant. The lactose contributes about half of the total milk osmotic pressure, with the diffusable ions (especially K^+, Na^+, and Cl^-) being the other contributors. There is a strong inverse relationship between the concentration (mM) of lactose and the osmolality (mM) of milk (Holt and Jenness 1984; Holt 1985).

An interesting question has been posed frequently as to whether there is a readily explainable evolutionary reason for lactose to be the carbohydrate of milk, considering the complex pathway of formation. It was proposed that, since a given weight of lactose exerts only half the osmotic pressure that the same weight of a monosaccharide would, a given osmotic effect provides twice as much energy. Since the osmotic pressure is fixed in milk due to the osmotic pressure of blood being constant, the inclusion of a disaccharide seems advantageous on this account, but this does not explain a reason why specifically galactose is formed by the rather convoluted pathway and included in the disaccharide molecule. A recent review on galactose metabolism (Coelho et al. 2015) offers a detailed explanation, as it indicates the

biological importance of galactose, not only for the neonatal development but generally as a crucial structural element in macromolecules. A recent factsheet issued by the International Dairy Federation (IDF 2017) has brought further attention to the positive role of galactose in various aspects of human health and nutrition.

The principles of lactose synthesis in the mammary gland are the same for all mammals. However, the concentration of lactose in milk of various species of mammals fluctuates significantly, even though in average values, the lactose content in milk of most mammals used as milking animals worldwide is similar, 4.3–5.0%, w/w (Table 1.1). The average lactose content in milk of bovine breeds most often used for industrial milk production varies only slightly, while the variations among individual animals of a given breed can be much larger. The synthesis of lactose draws water osmotically into the Golgi vesicles from the blood and hence affects the total milk yield; milk production would increase with increased lactose synthesis. At the same time, this also explains the relatively constant lactose content (% w/w) of milk, and the variations in lactose levels between cows, which is much smaller than the variation in the other two macronutrients, fat and protein. It is also important to realize that lactose synthesis is only one of the numerous processes of the total milk synthesis, all occurring in the mammary gland. The synthesis of other milk components, particularly the triglycerides and milk proteins, also proceeds at the same time, based on the availability of glucose from blood. One of the milk proteins being secreted by the mammary epithelial cells is α-lactalbumin, one of the main whey proteins, which is a part of the lactose synthase system. The other component of this complex is a galactosyltransferase enzyme; the lactose synthase complex catalyzes the final formation of lactose from glucose and galactose. All these reactions proceed simultaneously and depend on blood glucose availability and thus on the overall metabolic status of the lactating female, with the lactose synthesis playing several key roles in the overall milk synthesis in the mammary gland.

Thus, the key roles of lactose in the overall milk production can be summarized as follows. Lactose determines the volume of milk produced; its significant contribution to the milk osmotic pressure is a crucial factor in milk yield; and the production of oligosaccharides is inextricably linked to lactose synthesis. In addition, the lactose synthesis process, drawing on the available glucose, exerts significant effects on the production of other major milk components, the synthesis of which is also based on the supply of glucose; this includes the secretion of α-lactalbumin, indispensable for lactose production, illustrating the interrelationships of all these reactions, with lactose being the center-piece element.

1.3 Properties and Reactions

The properties of lactose are generally similar to those of other sugars but differ in some technologically important respects. Lactose is a reducing sugar, i.e., it has a free, or potentially free, carbonyl group (an aldehyde group in the case of lactose). Like other reducing sugars, lactose exists partially as an open-chain form with an

aldehyde group which can form a hemi-acetal and, thus, a ring structure. The formation of a hemi-acetal creates a new chiral center (asymmetric carbon) which may exist as two isomers (enantiomorphs), α or β. In an aqueous solution, the equilibrium between the α and β forms will be established by the mutarotation process, i.e., by alternatively opening and forming the ring structure. The α and β anomers of lactose have very different properties, the most important of which are specific rotation, $[a]^{20}_{D}$ (+91.1° and + 33.2° for α- and β-lactose, respectively; Walstra et al. 2006) and solubility (74 and 480 g/1000 g H_2O, for α- and β-lactose, respectively, at 20 °C; Sienkiewicz and Kirst 2006). These significantly differing properties will lead to establishment of the equilibrium ratio of β- to α-lactose in aqueous solutions, which at 20 °C is 1.68 (62.7/37.3), the corresponding equilibrium $[a]^{20}_{D}$ being about +55.3° and the final lactose solubility at this equilibrium about 192 g/1000 g H_2O. The α-lactose solubility (and thus also the final solubility) increase steadily with temperature, being 96.5 and 248 g/1000 g H_2O at 30 °C and 233.5 and 584 g/1000 g H_2O at 60 °C (Schuck 2011). Mutarotation is a first-order reaction, the rate of which increases sharply with increasing temperature. The mutarotatory equilibrium is established almost instantaneously at 75 °C, while it takes several hours at ambient temperatures, as first estimated by Bell (1930) from the seminal data of Hudson (1904).

The aqueous solubility of lactose, especially of the α-enantiomorph, is low at ambient temperature compared to other sugars, but increases rapidly with increasing temperatures.

The solubility of α-lactose is significantly more temperature-dependent than that of the β anomer. At temperatures >93.5 °C, α-lactose becomes more soluble that β-lactose. Hence, it is the β form of lactose which crystallizes above this temperature, while the usual commercial form of lactose (the α-lactose) is being crystallized at <93.5 °C, such conditions being much more easily attainable industrially. For special purposes, the β form of lactose may be manufactured by crystallization well above the cross-over temperature. The α-lactose crystallizes as a monohydrate ($C_{12}H_{22}O_{11}\cdot H_2O$), while β-lactose forms anhydrous crystals; thus, the yield of α-lactose is ~5% higher than that of β-lactose. Some details concerning solubility, crystallization, mutarotation, and hygroscopicity of lactose are found in Chapters 2, 3, and 4, or in specialized texts, e.g., that of Schuck (2011). These interrelationships need to be well understood as they play a significant role in the production of isolated lactose, or in various industrial problems, especially concerning concentration and drying of products containing substantial amounts of lactose, as discussed later.

The phenomena related to growth kinetics and morphology of especially α-lactose monohydrate crystals have been studied extensively in the past, and various crystal forms have been published. The tomahawk appearance of the fully developed crystal has been accepted since its first appearance in the literature (Hunziker and Nissen 1927) and reprinted innumerable times, especially as redrawn in the crystallographic canon by Kreveld and Michaels (1965) even though such a complex form is rarely if ever encountered in real situations. Rather, the crystals can assume many other shapes, prisms, needles, pyramids, flat triangles, “half-moons,” etc.; pictures of all these and similar forms can be found in the literature. In the constant supersaturation conditions attainable especially in laboratory studies using

Fig. 1.1 Single crystals of α-lactose monohydrate produced by growing them individually in aqueous lactose solutions of constant supersaturation and used in single crystal experiments (Jelen 1972; weight of the crystals approx. 0.25 g, length about 1.5 cm)

the single crystal methodology (Bhargava and Jelen 1996; Jelen, 1972), the α-lactose monohydrate crystal grows in a quasi-pyramid-like form, with the only active face of the pyramid being the bottom (Fig. 1.1). However, the constant supersaturation conditions are rarely encountered in industrial conditions, where the crystal shape will be affected by the varying conditions during the crystallization process, including the supersaturation, temperature, cooling rate, and especially the presence of impurities in the lactose solution (Jelen and Coulter 1973; Bhargava and Jelen 1996, others). Understanding the mechanism of crystalline growth and its kinetics is of particular importance for production of isolated lactose by the usual industrial crystallization process, as well as in various situations where crystallization constitutes one of the technological steps for ensuring optimal product quality, particularly concerning dried lactose-rich powders or concentrated products with high lactose content.

Several other physical properties of lactose can be found in specialized treatises; these include density, melting point, heat of combustion, specific heat, etc. and are of lesser importance in understanding the subjects covered in subsequent chapters.

Compared to other sugars, lactose has a low level of sweetness; at least 3 times more lactose is needed to achieve equal sweetness in comparisons to sucrose standards of 1% or 5% aqueous solutions (Wong et al., 1998). However, even with the limited sweetness, adding small amounts of isolated lactose to compensate for the lower total solids when ultrafiltration permeates were used for protein down-standardization seemed to have a noticeable sweetening effect with as little as 0.75% added (Jelen and Michel 1999). β-lactose has been shown to be sweeter than α-lactose, but after reaching the mutarotatory equilibrium the sweetness difference became insignificant. The difference in sweetness of the two anomers is too small to be of practical significance in food applications like coating sugar in baking (Wong et al., 1998). In general, lactose has limited value as a sweetening agent in foods but

isolated lactose, UF whey permeate (with the lactose content over 80% dry matter), or even dried whey (about 73% lactose) are useful in applications where excessive sweetness is undesirable, e.g., bulking agent, protein standardization of milk, etc.

Like all reducing sugars, lactose can participate in the non-enzymatic browning reaction referred to as the Maillard reaction (MR), resulting in the production of (off-) flavor compounds and brown polymers and causing the color change, sedimentation, and other potential problems. Milk, containing both a reducing sugar and proteins (the two compound species participating in one of the several forms of the MR), offers suitable conditions for the reaction to proceed, most noticeably during the application of heat in production of concentrated milk products such as evaporated or sweetened condensed milk. The rate of the reaction is dependent on the process temperature and the concentration of the principal reactants. However, at the very high temperatures, such as used in UHT processing of milk, the reaction is initiated even though the reactant concentrations are much lower than in the milk concentrates. The reaction between the lactose and the available milk protein α- and ε- amino groups proceeds not only during the heating process but, once initiated, continues also during the storage, even at or below ambient temperatures. This could lead to formation of melanoidins, known to be formed at the late stages of the very complex series of intermediate MR steps. Formation of melanoidins leads to increase in molecular size and loss of solubility in storage of, e.g., spray-dried whey. The same general MR-type pathways, involving polymerization of casein and whey proteins, were used in the past to explain the age gelation phenomenon, but later studies suggested that the interaction between lactose and the ε-NH_2 lysine residues (the reaction sometimes referred to as lactosylation), occurring at the initial stages of the MR, may be of importance for sedimentation in highly heated liquid milk. This was recently confirmed by Malmgren et al. (2017) in storage experiments with directly heated UHT milk at accelerated storage temperature of 40 °C where no age gelation (but heavy sedimentation) occurred.

Several other reactions involving lactose take place during heat treatment of milk or other lactose-containing dairy products. One such reaction is the isomerization of lactose into lactulose, caused by conversion of the glucose moiety into fructose. The reaction of lactulose with the free ε- amino group of lysine produces an Amadori compound called lactulosyl-lysine (galactosefructoselysine), found in milk during the early stages of the MR. Quantification of lactulose has been proposed in the past to estimate the intensity of the heat treatment used in dairy processing. A more recent development in this regard involves an indirect determination of lactulosyl-lysine after its acid hydrolysis into pyridosine and furosine and measuring the furosine value by high performance liquid chromatography (HPLC). A study by Rattray, Gallmann, and Jelen Rattray et al. (1997) showed that the lactulosyl-lysine production upon heating of milk is affected by the lactose and protein concentrations, as well as the heating process and the temperatures used for storage of the final product.

Direct determination of lactose in various materials including foods may be accomplished by several chemical reactions including the redox titration using alkaline $CuSO_4$ (Fehling's solution) or chloramine-T, as the principal standard method for the quantitative determination of lactose. The phenol-sulfuric acid method of

Dubois et al. (1956) is simple but can only be used for determination of lactose in systems where no other reducing sugars are present, e.g., in cheese, milk, whey, or experimental pure lactose solutions. Nowadays, in large laboratories, lactose is usually determined by HPLC or by infra-red spectrophotometry. It may also be determined by polarimetry, enzymatically (using an enzyme assay kit), or by cryoscopy. The enzymatic methods are based on the hydrolysis reaction, which converts lactose to its monosaccharide constituents, glucose and galactose.

The enzymatic hydrolysis reaction is catalyzed by the enzyme β-galactosidase (EC3.2.1.23), obtained from a multitude of microbial sources. The exact chemical structures of these enzymes can differ widely, as can the optimum reaction conditions (especially pH); the unifying property for the whole β-galactosidase family is the catalytic effectiveness in the lactose hydrolysis reaction. The reaction can be accomplished by adding the free soluble enzyme preparation to the milk or other solution containing lactose, by using the immobilized enzyme column, or in an enzyme reactor. Bacteria fermenting lactose have the capability of producing lactase, and they can be utilized as a crude source of the enzyme as well (Vasiljevic and Jelen 2003). Enzymatic lactose hydrolysis can be used in production of lactose-free dairy foods, and as one method to increase the sweetness of certain products (e.g., yogurts), as the glucose–galactose mixture resulting from the hydrolysis reaction is at least 3× sweeter than the original lactose solution. Significant research and marketing emphasis has been expended in Australia and elsewhere on developments of sweetening hydrolyzed lactose syrups in 1980s, and numerous reviews on the lactose hydrolysis technologies appeared at that time. However, the present economic relationships make this method of using lactose for sweetening purposes doubtful.

Application of severe heat (about 120 °C or more) and very low pH (about 1.5) will also hydrolyze lactose. However, at these drastic conditions, other reactions will also take place and thus the acid hydrolysis of lactose is of academic interest only. Interestingly, lactose is much more resistant to acid hydrolysis in comparison to other disaccharides, e.g., sucrose.

1.4 Production and Uses of Lactose and Lactose Derivatives

In comparison to other macronutrients of milk (proteins, fat), lactose has been often called the least valuable milk component. It is also contained in milk in amounts significantly greater than any of the other components except water, and, as it is discarded in whey in the manufacture of cheese and industrial casein, it presents a major waste problem for the dairy industry. The search for industrially and economically viable uses of lactose has been an ongoing subject of scientific and industrial research for a very long time.

Much basic research elucidating the lactose properties and reactions described above has been aimed at defining conditions suitable for production of isolated lactose and various lactose-rich or lactose-based products. Earlier, these were made almost invariably from whey but nowadays the source of choice is often the

protein-free permeate resulting from whey or milk processing by membrane technology. The newest contributions to the scientific underpinning of some of these processes are explored in Chapters 2, 3, and 4.

About 95% of the world's whey stream originates from cheese production (Brewster, 2020). This is the single most important source of isolated lactose and other lactose-rich ingredients. The principal products from unmodified whey are various dry whey powders containing about 71-73 % lactose. The UF permeates resulting from fractionation of whey into whey protein concentrates or isolates – or by concentration and fractionation of milk – contain over 80% lactose in dry matter and are being used as the source material from which lactose is being produced by crystallization, as well as for other applications in liquid, concentrated or dried form. The various permeates have become an important ingredient and its use by various sectors of the food industry is rapidly increasing, at an average compound annual growth rate of 17% for the bakery industry, by far the most important user of the permeates presently (Brewster, 2020). Other segments of the food industry where permeates are making significant inroads are confectionery, dairy, and producers of hot drinks and snacks. The main types of dried products originating from whey are listed in Table 1.2, and most of these contain lactose as their main component.

The traditional crystallization technology and the adjunct downstream processing steps are essentially similar to those used for sucrose or other sugars, but the principal crystallization step is usually accomplished in batch crystallizers upon controlled cooling. The use of evaporative continuous crystallizers used routinely in sucrose crystallization is much less common, due to the mineral impurities found in whey (or milk) permeates or whey itself, necessitating additional steps such as demineralization (Wong and Hartel, 2014). Also, as most of the growth occurs on only one (the bottom) face of the lactose crystal pyramid (Dincer et al., 2009; Bhargava and Jelen, 1996), the rate of lactose crystal growth is much slower than that of a sucrose crystal, which grows on all faces of its hexagonal prism structure.

Table 1.2 Composition of the main types of whey-based dried products (% w/w, orientation values, averaged from various sources)

Product	Lactose	Protein	Ash
Dried whey	71–73	12.5	8.5
Demineralized dried whey	83	15.0	1.0
Deproteinated whey[a]	75–83	2.0–6.8	8.4–11.0
WPC "34"[b]	50	34.0–35.0	7.0
WPC	4–21	65.0–80.0	3.0–5.0
WPI	<1	88.0–92.0	2.0–3.0
Edible ("crude") lactose	99.0	0.1	0.2
Refined (USP) lactose	99.85	0.01	0.03

WPC Whey protein concentrate
WPI Whey protein isolate
USP United States Pharmacopeia
[a]UF permeate or ion exchange treatment
[b] Skim milk substitute

Thus, with higher levels of supersaturation, often reached during evaporative crystallization, spontaneous secondary nucleation ("false grain") would predominate, rather than the growth of the crystals needed for ease of the downstream operations, especially centrifugal separation and washing.

Owing to its relatively low sweetness and low solubility, the applications of lactose are different from those of sucrose or glucose. One of the principal applications of isolated lactose is as ingredient in the production of "humanized" infant formulae based on bovine milk, which has a significantly lower lactose content than human milk. The lactose used may be a crystalline product or demineralized whey (for physiological reasons, it is necessary to reduce the concentration of inorganic salts in bovine whey).

Lactose, either in its isolated form or as the main component of dried whey or UF permeate, is used in a number of special applications in the food industry, e.g., as a free-flowing or agglomerating agent, to accentuate/enhance the flavor of some foods, to improve some desired functionality, and as a bulking agent in many processed foods including products of the dairy industry such as ice cream. As a reducing sugar with limited sweetness, it is widely used in the bakery and confectionery industries for production of the golden crust of many baked goods, a desirable effect of the otherwise detrimental Maillard reaction.

One of the important traditional non-food uses of the crystalline lactose is by the pharmaceutical industry for pill formation, due to its ease of molding, tablet compression, and low hygroscopicity. Several companies produce isolated lactose especially formulated for tableting efficiency. This special subject is reviewed in detail in one of the chapters of the Paques and Lindner's (2019) book Lactose. However, in general, the global market for both isolated lactose and the dried whey is rather static and new approaches to utilization of the ever-increasing supply of lactose worldwide continue to be actively sought. Some other novel uses of lactose will be reviewed in later chapters.

Similar to other sugars, the lactose molecule has a number of functional groups with reactivities that can be used to convert lactose to several food-grade derivatives using either chemical or enzymatic pathways. The following groups are the primary targets in the derivatization processes: (a) the glycosidic linkage between glucose and galactose; (b) the free hydroxyl groups; (c) the reducing group of glucose; and (d) the carbon–carbon bonds. There are several commercially viable lactose derivatives being produced industrially. Reviews of the main lactose derivatives of interest have been published in the recent past, and these can be consulted for more specific information. In addition to the glucose-galactose syrups mentioned above, the following is a summary of the main derivatives of interest.

- Lactulose. Probably the most commercially successful derivative of lactose, produced by the epimerization of the glucose moiety of lactose to fructose under mildly alkaline conditions. Lactulose has many applications including use as a bifidogenic factor in infant formulas and health foods, and as a mild laxative. It is listed in the US Pharmacopoeia, European Pharmacopoeia, and Japanese Pharmacopoeia. A major portion of the Seki and Saito's (2012) review of the main lactose derivatives is devoted to details of the production, properties, and applications of this probiotic.

- Lactosucrose, a trisaccharide comprising galactose, glucose, and fructose, is a potential prebiotic oligosaccharide produced by enzymatic polymerization with sucrose. Its importance in maintaining human gastrointestinal homeostasis has been reported.
- Lactobionic acid is a saccharic acid comprising galactose and gluconic acid; it is a sweet-tasting acid, which is a rare property that can be exploited in processed foods. Lactobionic acid has application as a bifidus factor and as calcium chelator in dietary supplements. The most interesting non-food use is for preservation of transplant organs and as a humectant in skincare products.
- Lactitol. The carbonyl group of lactose can be reduced to lactitol (the alcohol of lactose). Since the aldehyde group of the Glu moiety is reduced to the OH group, it does not participate in a Maillard reaction and its heat stability is high. Its application can be as a sweetener as its taste is similar to that of sucrose. As a special use of lactitol, its effectiveness in protection of water logged archeological relics has been mentioned.
- Tagatose, the keto analogue of galactose, is usually included in the group of lactose derivatives, even though it is not produced from lactose directly but from the galactose obtained from lactose. Tagatose is nearly as sweet as sucrose, has a good quality sweet taste, and enhances flavor of other sweeteners. It is absorbed poorly from the small intestine and thus is considered as a low calorie sweetener.
- Galactooligosaccharides (GOS) are a special group of lactose derivatives, produced as a result of transferase activity of the β-galactosidase (lactase) enzyme, used normally for its hydrolytic activity in splitting lactose to its monosaccharide constituents as discussed above. Under certain conditions (mainly at high lactose concentrations) the transgalactosylation activity will predominate and oligosaccharides, usually containing 2 to 9 monosaccharides, will be produced. The GOS are composed of galactose, with glucose or galactose at the reducing end. In its transgalactosylation function, the lactase enzyme is catalyzing the addition of galactose units to the lactose molecule. The relative rates of the hydrolysis vs. transgalactosylation reactions depend on the enzyme source and other variables. Detailed GOS reaction pathways have been described in a number of publications, e.g., Chan and Ganzle, Chen and Gänzle 2017. Over 30 different di-, tri-, and tetrasaccharides with defined structures were identified as products of enzymatic transgalactosylation. The main reason for the currently keen interest in the GOS is their similarity with the native human milk oligosaccharides (HMO) present in relatively large quantities in human milk. The GOS used in infant formula to mimic the functions of HMO oligosaccharides, have other interesting physico-chemical and probiotic properties and may be useful also as food ingredients. Chapter 5 provides an up-to-date review of these two related but substantially different subjects.

In addition to the above described lactose derivatives obtained by chemical or biochemical reactions, lactose can serve as a substrate fermentable by some bacteria or yeasts. Using the classical microbial fermentation technology, various products can be obtained if the economies of the applicable processes are favorable. The value of lactose and some byproducts of the modern whey processing (especially the protein-free UF permeates containing relatively large amounts of what can be

termed "crude lactose") has been fluctuating rather wildly in the recent times and may signal revitalization of some of the fermentation processes that are presently economically non-competitive.

Lactic acid bacteria, capable of using lactose as the main fermentation substrate, must possess the ability of producing intracellular β-galactosidase to hydrolyze the lactose first, before turning it into the various metabolic byproducts. Some lactose fermenting bacteria are capable of combining the two monosaccharide molecules produced by the hydrolysis into long carbohydrate chains referred to as exopolysaccharides. This is being exploited by the contemporary dairy industry for improving texture of some products such as yogurt in using these types of bacteria as starter cultures. What has been in the past considered as one of the major defects of yogurts, the so-called ropiness, has become a desirable trait. Similarly, other lactic acid bacteria are capable of producing specific flavor compounds. As an example, diacetyl, a desirable flavor compound in buttermilk, sour cream, or cultured butter, is produced by *Leuconostoc* spp. co-starter bacteria for these products. This flavor compound can also be produced by fermenting lactose separately, isolating the diacetyl, and adding to the sweet butter to improve its sensory impact without the need to ferment the cream first.

The production of ethanol from lactose by fermentation using *Kluyveromyces lactis* or *K. fragilis* has been at a commercial level for at least 40 years. If the ethanol is used in potable products, this process is economically viable but whey-derived ethanol may not be classified as potable in some countries. The continued interest in new bioenergy sources could open new opportunities for lactose-derived industrial ethanol but such applications may not be cost-competitive and will depend strongly on local taxation policy. The oxidation of ethanol by *Acetobacter aceti* to acetic acid for vinegar or other applications is technically feasible but in most cases presently not cost-effective.

The in situ fermentation of lactose by lactic acid bacteria to lactic acid is widespread in the production of fermented dairy products. The same pathway can be used in large scale lactose fermentation to lactic acid for food or industrial applications (including the biodegradable plastic, polylactic acid), but once again, its cost-competitiveness with other fermentation substrates or with the chemical synthesis is problematic.

1.5 Biological, Technological, and Nutritional Significance of Lactose

Contrary to being considered the least important of the main milk components, the lactose plays several very significant roles in the whole agri-food chain. In the primary milk production, it determines the milk yield and influences other reactions in the mammary gland. The significance of lactose for the newborn is both as a source of energy and a source of the galactose important for the cerebral and

neurological development of the infant, as well as a principal building block for the equally nutritionally important galactooligosaccharides. The relative ease of hydrolysis in the digestive system of all neonates is an added benefit of lactose in the early life nutrition.

The ability of the digestive system of young mammals to hydrolyze lactose is an evolutionary trait necessitated by the natural selection of the lactose to be the principal carbohydrate for the neonate. Lactose provides about 40% of the energy needs of the young, but for its digestion it must be first converted to the two monosaccharides. Thus, the secretion of intestinal lactase by cells in the brush border of the small intestine is essential for neonatal development. The intestinal lactase secretion decreases with progressive weaning until it stops entirely, as commonly observed with almost all adult mammals which do not use milk as food after weaning. In the case of humans, when milk became a component of regular daily diet (about 9–10,000 years ago, in the Neolithic period) this had an evolutionary impact, whereby the β-galactosidase secretion sometimes does not cease but continues into the adulthood. As a result, two phenotypes of adults emerged; those that can digest lactose due to the continued ability to produce the intestinal β-galactosidase, and those that cannot. It has been estimated that about 35% of total world population are of the former type; however, the geographical distribution of the lactase persistence (LP) condition varies widely, with over 90% of northern Europeans being considered LP, in contrast to as little as 11% LP in Southern Europe and about 1% among native Americans. The lactase non-persistence (LNP) condition leads to one of the several complications that lactose causes for the dairy and food industry, referred to as lactose malabsorption or, in lay language, lactose intolerance. This subject is explored fully in Chap. 6.

The dairy industry has at its disposal several technological avenues to offer consumers, experiencing the LNP condition, dairy products that avoid the problem. These were listed and discussed by Harju, Kallioinen, and Tossavainen Harju et al. (2012) and include, apart from the practically lactose-free cheese, many other regular dairy products in which the lactose is either hydrolyzed or has been removed. The hydrolysis route, using exogenous, industrially produced β-galactosidase preparations, has the disadvantage of leading to products with increased sweetness. As mentioned above, the glucose–galactose mixture thus obtained is several times sweeter than the original lactose solution. Especially in the case of liquid milk products (pasteurized or UHT milk), the sweetness is not readily acceptable to regular consumers. However, the process is rather simple and does not require special equipment. A whole family of hydrolyzed lactose products in which the sweetness is not a problem (or may even be advantageous) has been developed (Jelen and Tossavainen 2003). The alternative route, removing lactose from the liquid milk altogether, using chromatographic columns or by membrane filtration, may lead to products indistinguishable from the regular liquid milk, if enough residual lactose is left in the milk and hydrolyzed to produce the same sweetness as lactose does in the original milk. The disadvantage is the higher cost and the need for additional specialized equipment; the added benefit, with the contemporary concern regarding obesity, is a substantially lower caloric content than in the regular milk.

The amounts of lactose in the fermented dairy foods like yogurt, sour cream, or cultured buttermilk are reduced by the bacterial fermentation by about 30–40%, but not entirely eliminated. Still, there is some evidence that the residual lactose in these products may be tolerated better by the LNP consumers than in the liquid milk where the pH is much higher. Some of the possible explanations of this still controversial subject are related to the presence of the live lactic bacteria, possibly combined with the higher viscosity of these products, resulting in longer oro-cecal transit time during which the hydrolysis can be sufficiently advanced in the upper gastrointestinal tract. When the unhydrolyzed lactose enters the lower intestine, it results in extra water being drawn into the large intestine, causing diarrhea; the lactose is then metabolized by intestinal bacteria with the production of gas (carbon dioxide, methane, and hydrogen) causing cramps and flatulence.

A different category of complications that lactose may cause in some industrially processed dairy foods and ingredients is related to its physical properties, in particular low solubility, crystallization behavior, and hygroscopicity. In products such as sweetened condensed milk, Dulce de Leche, or the Norwegian brown cheese Mysost, the concentration of the dry matter containing lactose has been significantly increased by partial removal of water. As a result, the lactose-in-water concentration greatly exceeds the maximum solubility limit, causing crystallization. If not counteracted by the appropriate processing techniques developed over many years, the appearance of the α-lactose monohydrate crystals could be detected and, if these are large enough, would make the product unacceptable. This is also true for ice cream, where much of the water, converted to pure ice crystals, is no longer available as a solvent and the lactose concentration in the unfrozen portion of the ice cream mix again exceeds the lactose solubility limit. If not properly managed by the mix formulation, the appearance of lactose crystals large enough to be detected in the mouth may cause a serious sensory defect termed sandiness. This is much more serious than appearance of large ice crystals caused sometimes by temperature fluctuation in the ice cream storage. Large ice crystals, even though also detectable in the mouth, will melt quickly upon the consumption of the ice cream, while the poorly soluble lactose crystals will persist and will cause a very unpleasant sensation in the throat while swallowing the ice cream.

In production of dry products such as skim milk, whey, or permeate powders, the fast drying process does not allow the slow growing crystals to develop. Any lactose which has not been pre-crystallized before the drying process will be present in the powder in the amorphous “glass” form, which is very hygroscopic. Powders made without the pre-crystallization step need to be packaged using water-vapor-impermeable packaging; otherwise, α-lactose crystals will form gradually by interacting with water vapor from the surrounding air. Firstly, liquid bridges between the powder particles will be formed. This will reduce the glass transition temperature, leading in turn to sticky surfaces of the particles, and finally formation of α-lactose crystals whereby the water binding the particles together enters the monohydrate crystal structure. The resulting interlocking mass of clumps causes the package to cake irreversibly.

To produce a non-caking, non-hygroscopic powder, the lactose must be pre-crystallized before the drying step. This is accomplished by holding the concentrate for several hours under the conditions suitable for production of small crystals, not to interfere with the spray-drying technology (see Chap. 3).

Crystalline lactose in the α-hydrate form has very low hygroscopicity and can be used, e.g., in icing sugar blends. The free moisture in isolated crystalline α-lactose or dried permeate powders, needs to be rigorously controlled below 3 g/kg powder. The free moisture is not bound by the crystalline material and may cause excessive mold development. The problem is less acute in dried whey powders with pre-crystallised lactose where the presence of whey protein with its strong water binding properties will counteract the free water effect. The free moisture naturally does not include the water of crystallization contained in the α-lactose crystals (approx. 45 g/kg lactose); this has no effect on the a_w in the package.

Yet another possible difficulty that lactose poses for processed dairy powders is its propensity for Maillard reaction. The MR proceeds not only during elevated heat processing but also, at a much slower rate, in milk and whey powders, especially during storage under adverse conditions of temperature and high in-package humidity (see Chap. 3). The rate of MR is the fastest at approx. $a_w = 0.6$, so the proper packaging and/or proper lactose pre-crystallization before the drying step is doubly important. The MR in these powders especially if stored for extended periods of time can lead to significant discoloration and loss of nutritive value.

Similar effects of the MR in terms of development of browning are noticeable in the concentrated fluid or semi-solid dairy products mentioned above – sweetened condensed milk, the South American specialty Dulce-de-Leche ("milk honey"), and the Norwegian specialty whey cheeses known as Gjetost, Gudbrandsdalsost or simply mysost (whey cheese) or brunost (brown cheese). The brown colour development is inevitable, especially in the latter product, as the final water removal step to reach the approx. 85 % dry matter typical of the sliceable product, or about 70% in the case of mysost spread, is carried in an atmospheric kettle. This high temperature evaporation is necessary to keep the highly viscous mass flowable during the important rapid cooling step, resulting in forced nucleation producing lactose crystals small enough to avoid the problem of sandiness. In some products being offered as speciality ingredients, such as caramelized sweetened condensed milk, the MR effects are advantageous.

The monosaccharides, glucose and galactose, are much more reactive than lactose; thus, powders containing hydrolyzed lactose are even more susceptible to MR. The hydrolysis of lactose by β-galactosidase markedly increases the heat stability of milk and concentrated milk, especially around the pH of minimum solubility (Tan and Fox 1996). The mechanism of stabilization has not been elucidated fully but is probably due to the carbonyls formed in the Maillard reaction; unfortunately, such lactose-hydrolyzed milk products are very susceptible to intense browning.

The nutritional significance of lactose and its most important derivatives, the oligosaccharides, has been long recognized for the human infant. The acceptance of its positive role in human nutrition in general is emerging much more slowly, mostly

as a pushback against its negative image due primarily to the lactose intolerance. Some of the positive nutritional claims made previously, especially as enhancer of calcium absorption, are still unsettled. The available data, although showing a positive tendency, have not been accepted by the European Food Safety Authority as sufficient to support the health claim that lactose improves calcium absorption (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2011).

There are other possible positive effects of lactose that are only now being slowly recognized, including its immunomodulatory functions and the various important effects of its monosaccharide component galactose. The IDF has been stepping up its advocacy of lactose as an important nutrient by the publication of a "position paper" on lactose (IDF 2020).

1.6 Conclusion

Systematic knowledge about lactose has been developing for more than 200 years, but is still incomplete. While the earlier research concentrated mainly on the physical properties and classical lactose chemistry, present investigations are focused predominantly on the biochemical reactions and the role that lactose plays in the nutrition of the young and old. As one of the unique, naturally occurring disaccharides, lactose has become a valuable commodity for both the food and non-food uses. However, due to the ever-increasing production of cheese worldwide, the availability of lactose keeps growing faster than the opportunities of its use, both as a pure carbohydrate and as a principal component of whey, the by-product of cheese production. The industrial processes used in the lactose production and utilization contribute to the generation of new knowledge through synergy between basic and applied research. Some of the new discoveries coming from both the basic and applied research streams are described in the following chapters of this volume.

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Chapter 2
Solid and Liquid States of Lactose

Naritchaya Potes and Yrjö H. Roos

2.1 Introduction

Lactose in dairy materials can exist in various crystalline and non-crystalline forms. These forms of lactose affect its behaviour, particularly, in the processing and storage of low-water dairy foods. Crystalline α-lactose monohydrate and anhydrous β-lactose are well-known solid forms of lactose. Lactose crystals have relatively poor solubilities in water. Lactose occurs in two anomeric forms, α- and β-lactose, which makes its solubility a complex function of temperature. α-Lactose has low solubility in water at room temperature, but mutarotation to temperature-dependent equilibrium quantities of the α and β forms influence the overall solubility of lactose. Lactose solubility increases rapidly with increasing temperature, with a greater increase in the solubility of α-lactose. Liquid dairy foods contain dissolved lactose in a complex chemical environment and lactose is likely to exist in a composition-, temperature- and process-dependent α:β ratio. On rapid removal of solvent water from dairy liquids on dehydration or freezing, lactose molecules retain their solution structure and, therefore, amorphous, non-crystalline solid forms of lactose are typical of dairy powders and frozen dairy desserts (Hartel 2001; Roos and Drusch 2016).

Amorphous lactose in dairy solids may exist in a glassy, solid state or in a syrup-like, supercooled liquid state. The apparent glass-like solid state results from a very high viscosity, exceeding 10^{12} Pa s and is typical of lactose in dairy powders and ice cream (White and Cakebread 1966). The state transition of amorphous solid- and liquid-like states occurs over a second order-type state transition known as the glass transition (White and Cakebread 1966), as described in Fig. 2.1. The glass transition involves no latent heat, but it can be observed from changes in heat capacity, thermal expansion coefficient, dielectric properties, various mechanical and flow

N. Potes · Y.H. Roos (✉)
School of Food and Nutritional Sciences, University College Cork, Cork, Ireland
e-mail: yrjo.roos@ucc.ie

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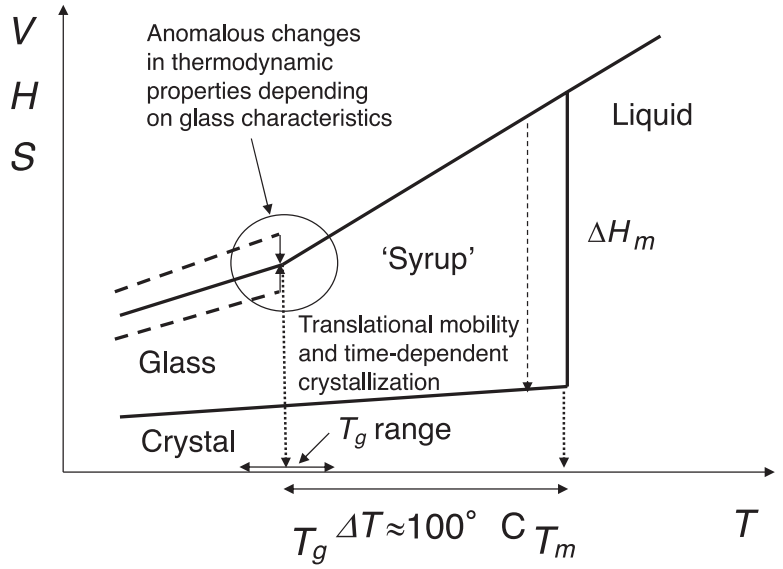

Fig. 2.1 A schematic presentation of changes in enthalpy, *H*, entropy, *S*, and volume, *V*, around the glass transition temperature, T_g, and melting temperature, T_m. The glassy state is a non-equilibrium state and the glass transition occurs over a temperature range and results in a change of a solid-like material to a syrup-like liquid of sugars

properties, and molecular mobility (White and Cakebread 1966; Lai and Schmidt 1990; Slade and Levine 1991; Kalichevsky et al. 1993a; Roos and Drusch 2016). The glass transition of hydrophilic dairy solids is dominated by that of lactose, in which water acts as a softener or "plasticizer" (Jouppila and Roos 1994a, 1994b; Jouppila et al. 1997). Plasticization by water can be observed as a decrease in the glass transition temperature with increasing water content.

Water plasticization is an important factor contributing to the dehydration characteristics and storage stability of dairy foods. A dramatic and well-documented decrease in the stability of dairy powders occurs above a critical water content and corresponding critical water activity (Supplee 1926; Troy and Sharp 1930; Herrington 1934; Lea and White 1948; King 1965; Labuza and Saltmarch 1981; Jouppila et al. 1997; Haque and Roos 2006). These values of critical water content and water activity correspond to those at which the glass transition of lactose occurs at the storage temperature (Fig. 2.2). Exceeding the glass transition conditions of lactose results in dramatic changes in the flow properties of dairy powders and the time-dependent crystallization of lactose (Roos and Karel 1991c; 1992; Jouppila et al. 1997; Paterson et al. 2005; Haque and Roos 2006). Many other physical and chemical changes observed in dehydrated and frozen dairy foods have been shown to result from water plasticization and the glass transition of lactose (Roos and Karel 1991a; Slade and Levine 1991; Jouppila et al. 1997; Hartel 2001).

The objective here is to highlight properties of non-crystalline lactose and its impact on characteristics of dairy food materials at low water contents and in the

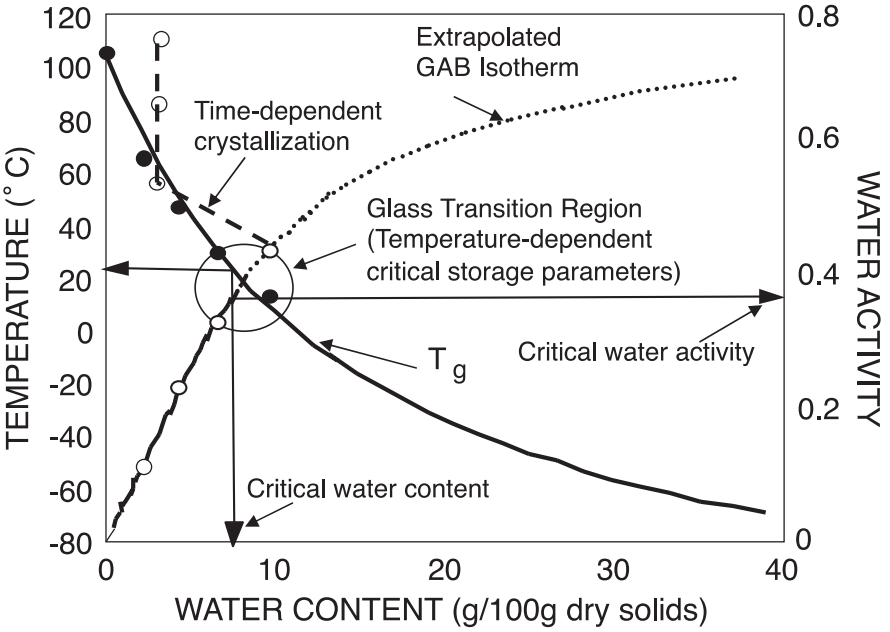

Fig. 2.2 Water plasticization and glass transition temperatures of lactose at various water contents. Depression of the glass transition temperature, T_g, with water content was predicted with the Gordon–Taylor equation. The critical water content and water activity correspond to plasticization depressing T_g to room temperature. Higher water levels result in stickiness, caking, increased browning rates and time-dependent lactose crystallization. The Guggenheim–Anderson–de Boer (GAB) water sorption isotherm was used to derive critical water activity. Data from Haque and Roos (2004)

frozen state. The presence of the crystalline form of lactose in dairy and other foods at low water contents, and its impact on properties and stability of dehydrated materials are also discussed. The non-crystalline state of lactose is often a non-equilibrium state, showing time-dependent characteristics which may be observed, for example, from changes in flow properties and time-dependent lactose crystallization. On the contrary, the crystalline state of lactose is an equilibrium state that often contributes to solids characteristics, textural properties, and functionality of food and pharmaceutical materials.

2.2 State Diagram of Lactose

A state diagram may be considered as a "map" which describes conditions at which non-crystalline materials appear as solid glasses or as supercooled liquids at various water contents and temperatures. State diagrams describe water plasticization behaviour of hydrophilic amorphous solids and the concentration dependence of the

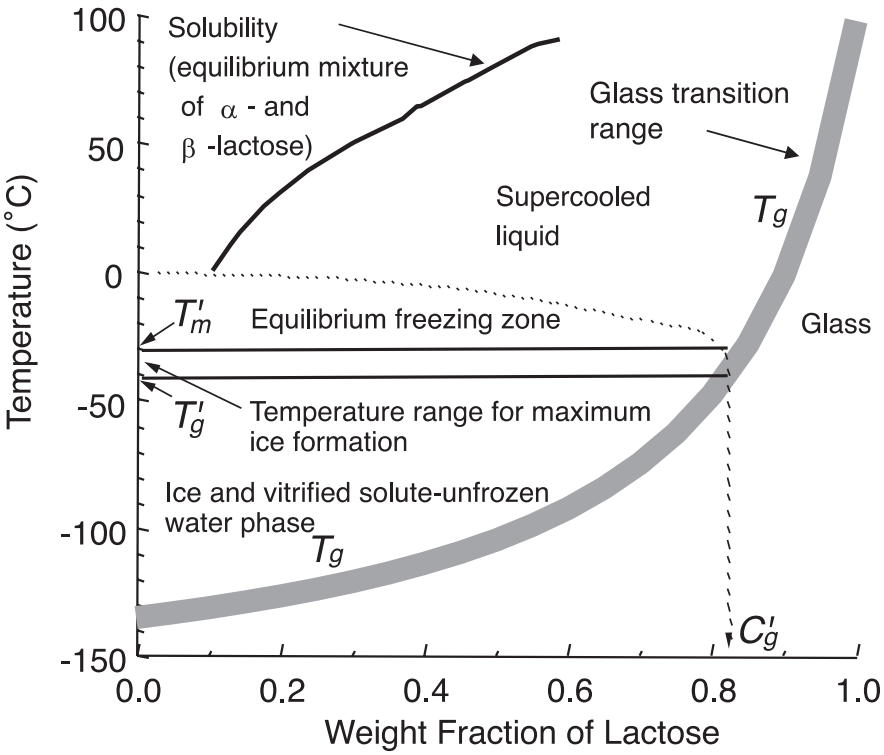

Fig. 2.3 State diagram of lactose. The glass transition temperature (T_g) curve (glass transition temperature range) at high solids content explains the physical state dependence on temperature and water plasticization. Solvent water crystallization is controlled by equilibrium freezing as defined by solute concentration and kinetically by vitrification at a solute concentration of C'_g with a glass transition of a maximally freeze-concentrated lactose at T'_g and onset temperature of ice melting at T'_m

glass transition of solutes, taking into account ice formation (solvent crystallization) and its effect on solute concentration at low temperatures. The state diagram of lactose (Fig. 2.3) is useful for characterizing the physical state and physical properties of common dehydrated and frozen dairy foods (Vuataz 2002).

State diagrams have been used by Levine and Slade (1988a, 1989) to characterize the effects of frozen storage temperature on food quality, which is particularly important to understand the frozen state properties of ice cream and other dairy desserts. State diagrams are available for lactose, milk powders with various fat contents and with hydrolysed lactose (Jouppila and Roos 1994b; Roos 2002), lactose-protein mixtures (Haque and Roos 2006) and lactose-salt blends (Omar and Roos, 2006a, 2006b). It appears that lactose governs the solid state of lactose-containing powders but the hydrolysis of lactose results in a significant change to solid properties, with this change being a result of the hydrolysis of lactose to glucose and galactose, which differ greatly in their sensitivity to water from that of lactose

(Jouppila and Roos 1994a, 1994b). It is also important to note that the glass transition of dairy solids is a property of the hydrophilic, miscible components, often dominated by lactose or its mixtures with added sugar components and products of lactose hydrolysis. Water plasticization occurs only in the solids-non-fat fraction and state diagrams describe the solids-non-fat properties of dairy solids.

The lactose-water blend is a binary solute-solvent mixture. Water, as a small molecular solvent, acts as a strong plasticizer and a significant depression of the glass transition temperature, T_g, occurs at low water contents (Slade and Levine 1991). The plasticization behaviour of amorphous polymer-solvent blends is often modelled using the Gordon–Taylor relationship (Gordon and Taylor 1952), which allows modelling of the glass transition temperature depression with increasing water content. Water plasticization of lactose has been shown to follow this equation which allows its use for establishing the glass transition curve in the state diagram of lactose (Roos and Karel 1991a; Potes et al. 2012). The Gordon–Taylor equation has also been applied to predict water plasticization of dairy powders (Jouppila and Roos 1994b; Haque and Roos 2006), casein (Kalichevsky et al. 1993a, 1993b) and a number of other foods (Roos and Drusch 2016). The Gordon–Taylor relationship is shown in Eq. (2.1), where w_1 and w_2 are weight fractions of solids and water, respectively, T_{g1} and T_{g2} are the glass transition temperatures of respective components and k is a constant.

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \tag{2.1}$$

The constant, k, in Eq. (2.1) can be derived from experimental data for T_g at various water contents (Roos 1995). Although numerous values have been reported for the glass transition temperature of non-crystalline water, the glass transition temperature for amorphous water is often taken as −135 °C (Sugisaki et al. 1968). Several equations other than the Gordon–Taylor relationship are available for predicting the effects of water plasticization and composition on the T_g of dairy solids (Roos and Drusch 2016).

Most state diagrams show equilibrium melting temperatures of ice at various water contents and kinetic limitations for ice formation. Ice formation ceases at temperatures where the equilibrium ice melting temperature approaches the glass transition of the freeze-concentrated solutes in an unfrozen solute matrix. Kinetically limited ice formation may be described as non-equilibrium ice formation. Non-equilibrium ice formation is a typical phenomenon in rapidly cooled carbohydrate solutions and is probably the most common form of ice formation in frozen dairy foods, including ice cream and frozen yoghurt. One of the first studies reporting non-equilibrium freezing was that of Troy and Sharp (1930), who found that rapid freezing of ice cream resulted in freeze-concentration and supersaturation of lactose which, at a sufficiently low temperature, would not crystallize. Several sugars, including lactose, and sugar-protein mixtures form such supersaturated amorphous

matrices in frozen foods (Bellows and King 1973; Roos and Karel 1991a; Slade and Levine 1991; Goff et al. 1993; Roos 1993; Goff 2002; Singh and Roos 2005).

State diagrams show the T_g at various water contents. Freezing of water results in the separation of ice and the concentration of solutes in unfrozen water. Freezing of water ceases as the glassy state of the unfrozen water-solute phase is approached (Roos 2021). State diagrams often include data for the glass transition temperature of the maximally freeze-concentrated solute (temperature at which ice formation ceases) with corresponding solute concentration, C'_g, onset temperature for ice melting in the maximally freeze-concentrated solution, T'_m, equilibrium ice melting temperature, T_m curve, and solubility. The state diagram of lactose, with transition temperatures and corresponding lactose concentrations, is shown in Fig. 2.3. The most precise C'_g values and corresponding unfrozen water contents, W'_g, can be derived from state diagrams established with experimental T_g values (Roos and Karel 1991b). The solute concentration of maximally freeze-concentrated solute matrices, including that of non-fat milk solids, has been found to be about 80% (w/w), i.e. the unfrozen water content (W'_g) is 20% (w/w). These values correspond to solute and water concentrations, respectively, at which ice formation may not occur in freezing, i.e. ice formation is not possible in a solution composed of 20% (w/w) water and 80% (w/w) solutes (Roos and Karel 1991a, 1991b; Roos 1993; Jouppila and Roos 1994b). Higher unfrozen water levels may exist in maximally freeze-concentrated matrices of food polymers such as starch and proteins, due to their much higher T'_g values (Roos and Karel 1991d; Roos, 1995; Singh and Roos 2005).

2.3 Stickiness and Caking

Stickiness and caking are phenomena which may occur when amorphous powder components are plasticized thermally as a result of heating or by exposure to a high humidity, resulting in water sorption and plasticization (Peleg 1977, 1983; Lloyd et al. 1996; Paterson et al. 2005; Fitzpatrick et al. 2007; Roos and Drusch 2016). Stickiness and caking of dairy powders are often related to water plasticization of amorphous lactose. Water plasticization may result in glass transition and viscous flow of the non-crystalline lactose at particle surfaces, which is observed as stickiness and caking. The surface viscosity of particles is an important property of amorphous powders. Downton et al. (1982) showed that surface viscosity governs the flow properties, stickiness and caking of amorphous powder particles. Levine and Slade (1988b) suggested that as the viscosity decreases rapidly above the glass transition, amorphous solids may undergo numerous time-dependent structural transformations. These changes in food materials include stickiness and caking of powders, plating of particles on amorphous granules and structural collapse of dehydrated structures.

Williams et al. (1955) found that the viscosity of amorphous glucose above its glass transition was similar to the viscosity of other inorganic and organic

glass-forming compounds. Viscosity was related to relaxation times above T_g and followed an empirical relationship known as the William–Landel–Ferry (WLF) Eq. (2.2), which was derived from the viscosity data for a number of compounds.

$$\log \frac{\eta}{\eta_s} = \frac{-C_1\left(T - T_s\right)}{C_2 + \left(T - T_s\right)} \tag{2.2}$$

where η is viscosity at temperature, T, η_s is viscosity at a reference temperature, T_s, and C_1 and C_2 are constants.

The main cause of stickiness is water or thermal plasticization of particle surfaces, which allows a sufficient decrease in surface viscosity and enhances liquid-like behaviour and the development of surface tension for adhesion. Downton et al. (1982) suggested that an increase of temperature or water content caused the formation of an incipient liquid state of a lower viscosity at the particle surface, which resulted in stickiness. Downton et al. (1982) proposed that particles may stick together if sufficient liquid can flow to build strong enough bridges between the particles and that the driving force for the flow is surface tension, which was confirmed for dairy foods by Adhikari et al. (2007).

Stickiness is a time-dependent property and since viscosity in the glassy state is extremely high, the contact time must be very long to allow adhesion. A dramatic decrease in viscosity above T_g reduces the contact time and causes stickiness which can be related to the time scale of observation. Downton et al. (1982) estimated that a surface viscosity lower than 10^6 to 10^8 Pa s at a contact time of 1–10 s was sufficient for stickiness. The sticky point was found to decrease with increasing water content. The critical viscosity for stickiness was almost independent of water content, ranging from 0.3×10^7 to 4.0×10^7 Pa s, which agrees well with the predicted viscosity range. Wallack and King (1988) reported that the critical viscosity range also applied to other amorphous powders.

Stickiness and caking may also be related to the hygroscopicity of non-crystalline sugars. Brennan et al. (1971), who studied the stickiness properties of powders during spray drying, pointed out that two approaches may be used to reduce the thermoplasticity and hygroscopicity, and therefore to solve problems caused by wall deposition in spray drying. These methods were the use of additives as drying aids and the use of specially designed equipment. The sticking point, which describes particle adhesion and stickiness temperature, of amorphous food solids against water content follows an isoviscosity curve with essentially constant temperature difference to T_g (Downton et al. 1982; Roos and Karel 1991a) and the measurement of the sticky point by the method of Lazar et al. (1956) can be considered as a method which, in fact, locates the glass transition within the food solids (Chuy and Labuza 1994).

Dairy solids-non-fat are plasticized by both temperature and water. Water at a constant temperature may affect physical properties, similar to temperature at a constant water content. Assuming that the WLF-type temperature dependence applies, the viscosity at a constant water content decreases with increasing temperature. The

WLF equation with the "universal" constants $C_1 = 17.44$ and $C_2 = 51.6$ when T_g is the reference temperature (Williams et al. 1955), predicts that an isoviscosity state of 10^7 Pa s exists at about 20 °C above T_g, which agrees with the experimental and predicted critical viscosity values for stickiness reported by Downton et al. (1982). The particular importance of the relationship between the sticky point and T_g is that the T_g of amorphous dairy powders can be used as a stability indicator. Thus, knowledge of the T_g and its dependence of water content can be used to evaluate causes of stickiness problems, especially in the production and storage of dairy and other amorphous powders, as described in Fig. 2.4.

Caking of sticky powders occurs when sufficient time is allowed for surface contact. According to Peleg (1977), liquid bridging is one of the main inter-particle phenomena which results in the caking of food powders. Factors that may cause liquid bridging include water sorption, melting of component compounds (e.g. lipids), chemical reactions that produce liquids (e.g. non-enzymatic browning), excessive liquid ingredients, water released due to crystallization of amorphous sugars, and wetting of the powder or equipment. The most common caking mechanism in food powders is plasticization due to water sorption and subsequent inter-particle fusion (Peleg and Mannheim 1977; Peleg 1983). Caking of amorphous powders often results from the change of the material from the glassy to the less viscous liquid-like state, which allows liquid flow and the formation of inter-particle liquid bridges. Peleg (1983) pointed out that "humidity caking" is the most common

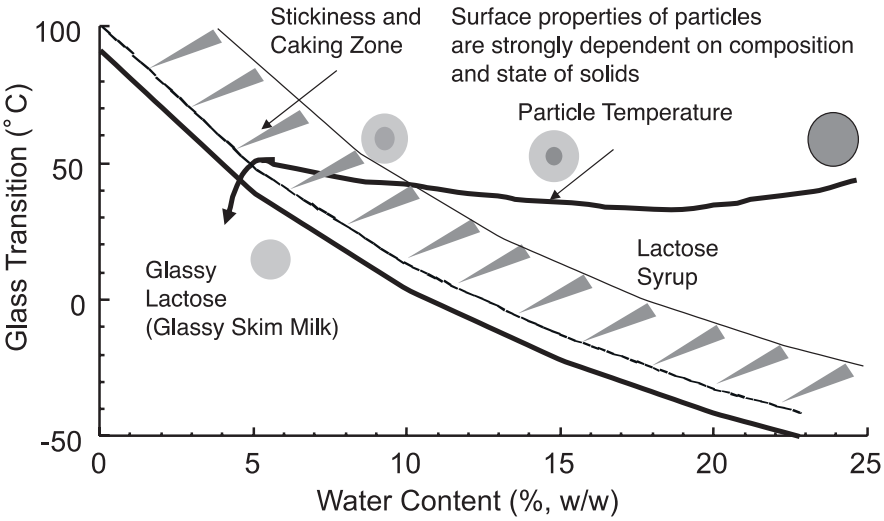

Fig. 2.4 Glass transition temperature, T_g, of skim milk with a schematic representation of liquid droplets during dehydration. Dehydration to a glassy state is required for free-flowing amorphous lactose component. The presence of lower molecular weight sugars would reduce the stickiness and caking zone, shown for lactose as a zone at 20 °C above T_g, to lower temperatures and water contents while a shift to higher temperatures can be achieved by mixing lactose with higher molecular weight components

mechanism of caking, with humidity caking being a consequence of an increasing water content, plasticization and depression of T_g to below ambient temperature (e.g. Slade and Levine 1991). The close relationships between stickiness and glass transition suggest that caking also occurs above the T_g, at rates which are defined by the temperature difference, $T - T_g$, which for dairy powders are highly dependent on solids composition, which is of particular importance in materials with hydrolysed lactose or modified sugar composition (Jouppila and Roos 1994a, 1994b; Vega et al. 2005).

2.4 Crystallization and Recrystallization

The non-crystalline state of lactose is a non-equilibrium state with a high level of supercooling and a large driving force towards the crystalline, equilibrium state. Lactose crystallization and recrystallization in dairy powders and frozen desserts are glass transition-related, time-dependent phenomena which are governed by the mobility of lactose molecules. Crystallization in the solid, glassy state may not occur as translational mobility of lactose is not possible and crystallization is kinetically limited. Molecules in the glassy state are not able to change their spatial arrangement to the highly ordered, crystalline equilibrium state. At temperatures and water contents exceeding the critical values for the glass transition, molecular mobility increases rapidly and results in lactose crystallization into various forms depending on temperature and water content (Haque and Roos 2005; Fan and Roos 2015).

Crystallization of amorphous lactose in dairy powders and in ice cream during storage is one of the principal causes of loss of product quality. Crystallization of amorphous lactose in these materials was related to quality defects by Supplee (1926) and Troy and Sharp (1930). These studies, in agreement with numerous other studies, such as that of Jouppila et al. (1997), have reported that dairy powders with amorphous lactose sorb large quantities of water at low relative humidities. Storage of dairy powders above a critical relative humidity results in substantial water plasticization and crystallization of the amorphous lactose. Dehydration of milk and whey by spray drying and roller drying produces a lactose glass which is often composed of a non-crystalline mixture of α- and β-lactose (Troy and Sharp 1930; Jouppila et al. 1997).

Herrington (1934) found that lactose glasses were stable at room temperature if they were protected from water. The existence of lactose in the glassy state in dairy foods and lactose crystallization at high storage humidities have been confirmed in numerous studies. These studies have used polarized light microscopy, electron microscopy, differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR) and X-ray techniques to analyse the physical state of lactose in dairy powders (King 1965; Lai and Schmidt 1990; Roos and Karel 1990; Jouppila et al. 1997; Haque and Roos 2005). As shown in Fig. 2.5, water sorption by most dehydrated dairy products, which contain lactose, show a characteristic break in the sorption

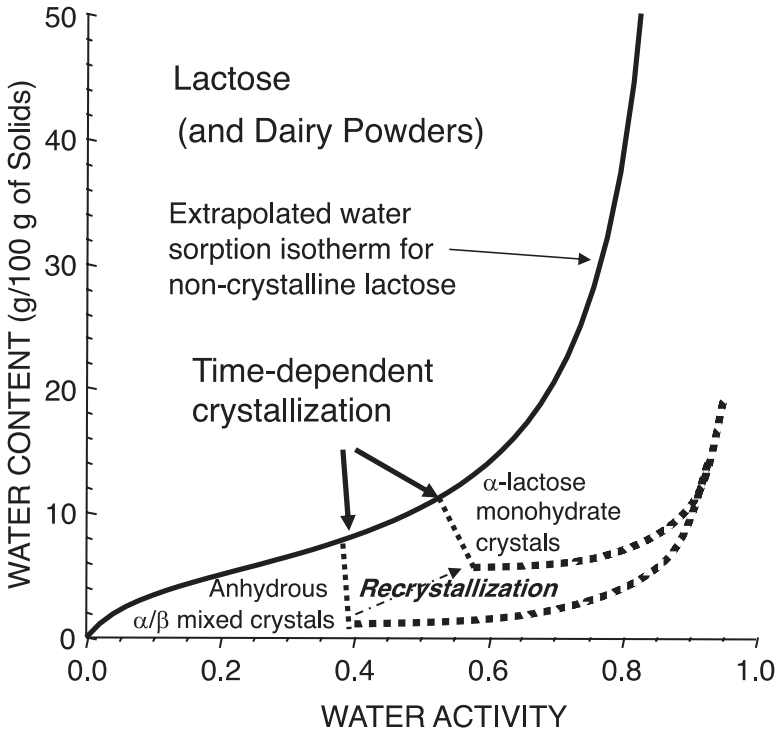

Fig. 2.5 Sorption isotherm of amorphous lactose. A break in water sorption occurs as a result of lactose crystallization above the critical water content. Crystallization can be observed at varying rates at different storage relative humidities. Recrystallization of anhydrous crystals to α-lactose monohydrate crystals may be observed at higher water activities (Haque and Roos 2005)

isotherm, indicating lactose crystallization (Berlin et al. 1968a, 1968b; Jouppila and Roos 1994a, 1994b; Haque and Roos 2006).

The crystallization behaviour of amorphous lactose in milk products is also temperature dependent. Berlin et al. (1970) observed that the relative humidity at which the break in sorption isotherms appeared was dependent on temperature, which was confirmed by Warburton and Pixton (1978); an increase in storage temperature shifted the break to a lower relative humidity. The temperature dependence of the water sorption properties of crystallizing amorphous sugars can be explained by changes in their physical state. DSC thermograms of milk powders show a glass transition and a crystallization exotherm for the amorphous lactose fraction (Jouppila and Roos 1994b). Water plasticization decreases the T_g of lactose and a higher water content causes lactose crystallization at a lower temperature. Water plasticization of non-crystalline lactose and the associated depression of the T_g to a lower temperature indicates that the break in the lactose sorption isotherm is both temperature and time dependent.

Amorphous lactose may crystallize in a complex manner into a number of crystalline forms. The crystalline form produced depends on the relative humidity and temperature. According to Vuataz (1988), lactose crystallizes as the anhydrous β-form at relatively low water activities, or as α-lactose monohydrate, above a_w of 0.57 at room temperature. As shown in Fig. 2.5, at intermediate water contents, recrystallization of β and α/β mixed forms appears to occur and produces higher amounts of α-lactose monohydrate during storage (Haque and Roos 2005). Structure of materials or food matrices and various other components in milk, particularly proteins and salts, also affect the crystallization properties and the crystalline form produced at different temperature and water conditions (Darcy and Buckton 1997; Haque and Roos 2006; Omar and Roos 2006a, 2006b).

The kinetics of crystallization at a constant temperature above T_g can be related to water content and water activity, which define the temperature difference, $T - T_g$. Therefore, lactose crystallization may occur above a critical water content or water activity at a constant temperature at a rate defined by the corresponding $T - T_g$ (Roos and Karel 1992). The rate of lactose crystallization in dairy powders increases also with increasing relative humidity of the storage environment (Saltmarch and Labuza 1980; Vuataz 1988, 2002; Jouppila et al. 1997). Increasing relative humidity increases water sorption and water activity, which causes water plasticization and increases the temperature difference, $T - T_g$. The $T - T_g$ of lactose defines the rate of crystallization, as shown in Fig. 2.6.

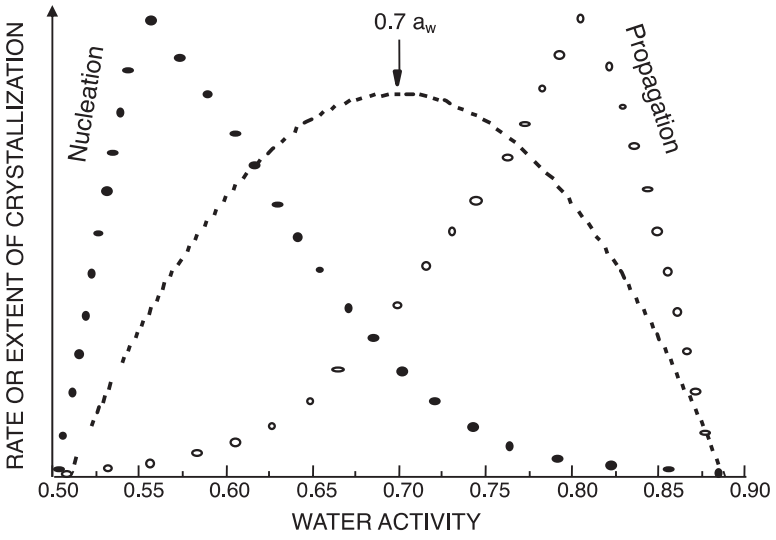

Fig. 2.6 Relative nucleation and crystallization rates for lactose at various water activities at room temperature. The glass transition of lactose is defined by water activity and crystallization occurs above the critical water activity. The rate of nucleation at a low water activity is high but crystal growth occurs slowly which results in a low overall rate of crystallization. The maximum rate and extent of crystallinity is achieved around 0.7 a_w (Jouppila et al. 1997)

Jouppila and Roos (1994b) determined glass transition temperatures for freeze-dried milk powders, which contained various amounts of fat. The T_g of non-fat solids at various water contents was almost the same as that of lactose (Fig. 2.2). The water sorption properties of the non-fat solids were not affected by the fat component. Jouppila and Roos (1994b) developed state diagrams for milk powders, which defined critical values for water content and water activity for stability. Combined T_g and water sorption data suggested that a water content of 7.6 g/100 g of non-fat solids depressed T_g to 24 °C. The corresponding water content for pure lactose was 6.8 g/100 g of solids. The critical a_w was 0.37. These values, being similar to those shown in Fig. 2.2, were in good agreement with several studies which have found critical water contents and storage relative humidities for milk powders based on water sorption properties (Warburton and Pixton 1978).

Milk powders with lactose hydrolysed to galactose and glucose showed no break in their sorption isotherms (San Jose et al. 1977; Jouppila and Roos 1994a). It was suggested that crystallization of individual sugars in the protein-glucose-galactose mixture was delayed in comparison to lactose crystallization in skim milk and whey powders. Skim milk powders containing hydrolysed lactose show a T_g well below that of amorphous lactose. Powder produced from skim milk containing galactose and glucose, as a result of enzymatic hydrolysis of lactose, had anhydrous T_g at 49 °C and a water content of 2.0 g/100 g of solids reduced the T_g to 24 °C (Jouppila and Roos 1994b). Our studies have shown that the T_g of lactose-containing anhydrous skim milk powders is close to that of lactose at 105 °C (Haque and Roos 2006). However, a number of T_g values for amorphous lactose have been reported, which reflects the sensitivity of the transition to composition and water. Various criteria are also used to locate the transition temperature in DSC thermograms and it may be taken from the onset or midpoint of the transition.

Galactose and glucose show glass transitions at 30 and 31 °C (Roos 1993), respectively. Although Kalichevsky et al. (1993a, 1993b) found that sugars had only a small effect on the T_g of casein, the T_g of milk powder containing hydrolysed lactose seems to be higher than is suggested by the T_g values of the component sugars. The T_g of milk powder is significantly reduced by lactose hydrolysis, which presumably is the main cause of stickiness during processing and storage, as well as of hygroscopic characteristics and higher susceptibility of the powder to non-enzymatic browning reactions. It should also be noted that although lactose is a reducing sugar, the hydrolysis of one mole of lactose produces two moles of more reactive reducing sugars (monosaccharides).

Lactose crystallization in dairy powders, including baby foods, results in higher rates of non-enzymatic browning and other deteriorative changes (Labuza and Saltmarch 1981; Saltmarch et al. 1981; Miao and Roos 2004). Saltmarch et al. (1981) found that the rate of browning at 45 °C increased rapidly above a_w 0.33 and showed a maximum between a_w 0.44 and 0.53. The maximum rate of browning occurred at a lower a_w than was found for other foods. The maximum rate was coincident with extensive lactose crystallization which was observed from scanning electron micrographs. The rate of browning was significantly lower in a whey powder which contained pre-crystallized lactose. The loss of lysine was also found

to be most rapid at water activities which allowed lactose crystallization (Saltmarch et al. 1981). Crystallization of amorphous lactose in closed containers increases water activity very rapidly and accelerates the browning reaction in comparison with the rate of the reaction at the same temperature but at a constant water activity (Kim et al. 1981). Compositional factors and crystallization behaviour of different sugars may also enhance oxidation (Shimada et al. 1991) and browning reactions (Miao and Roos 2004; Nasirpour et al. 2006). It is interesting to note that water sorption in dried dairy foods appears as an additive property of solids components (Potes et al. 2012; Fan and Roos 2015). On the other hand, the amount of water sorbed by amorphous lactose at various storage conditions of materials containing non-crystalline lactose may be established from a full sorption isotherm (Potes et al. 2012).

2.5 Crystallization and Recrystallization in Frozen Materials

The viscosity of a freeze-concentrated solute phase affects time-dependent crystallization phenomena, ice formation and material properties. Levine and Slade (1988a) pointed out that the retarding effect of added maltodextrins on ice recrystallization in ice cream was based on the elevation of the glass transition of a maximally freeze-concentrated solute phase, T'_g. At a sufficiently low temperature, the viscosity of a freeze-concentrated solute matrix becomes high enough to retard diffusion and delay ice formation (Roos and Karel 1991b). Maximum freeze--concentration may occur at temperatures slightly below the onset temperature of ice melting, T'_m, in the maximally freeze-concentrated material (Fig. 2.3). Generally, the T'_g' and T'_m increase with increasing molecular weight of the solute fraction (Slade and Levine 1991; Roos and Karel 1991d; Roos 2021).

Lactose crystallization in frozen dairy foods may occur above the glass transition temperature of the maximally freeze-concentrated solute matrix, T'_g. Lactose is one of the least soluble sugars and the loss of quality, including a sandy mouthfeel, resulting from lactose crystallization is well known (Troy and Sharp 1930; White and Cakebread 1966). The solubility of lactose at 0 °C is only about 12 g/100 g of water and it decreases substantially below the freezing temperature of water as a result of freeze-concentration (Nickerson 1974). The solubility of lactose decreases also in the presence of other sugars, e.g. sucrose (Nickerson and Moore 1972), which may facilitate lactose crystallization in frozen dairy desserts and ice cream. However, crystallization of freeze-concentrated solutes can be retarded and greatly reduced by the use of sugar blends and syrups and by the addition of polysaccharides (Hartel 2001).

Both lactose crystallization and recrystallization of ice in frozen desserts can be reduced by the addition of stabilizers which increase the viscosity of the unfrozen, freeze-concentrated solute phase. Singh and Roos (2005) also showed that in blends of polysaccharides, proteins and sugars, the T'_g was decreased but the T'_m increased as a result of retarded ice formation. The polysaccharide, protein (including

polysaccharide and protein stabilizers) and sugar composition seem to be the most important factors in formulation of frozen dairy foods with improved stability against solute crystallization and ice recrystallization.

2.6 Deliquescence of Lactose

Lactose crystals can be found in various forms: α-lactose monohydrate (stable hydrated crystal form), anhydrous β-lactose, anhydrous α-lactose and as mixed crystals, but the major crystalline forms of lactose are α-lactose monohydrate and anhydrous β-lactose. Formation of various crystalline forms or isomers depend on the temperature, concentration, pH, presence of foreign substances including impurities in lactose solution during the crystallization process, and food structure (porosity, density and compactness) (Hartel and Shastry 1991; Haque and Roos 2006b; Wong and Hartel 2014). Each crystal form has different properties. The α-form has characteristic tomahawk- and prism-like shapes with hard and brittle crystals. That is the most stable crystalline form of lactose, and it can be obtained by evaporation of a highly concentrated lactose solution at a low temperature, slow cooling during the precipitation step (crystallization), and high temperature drying (>160 °C) or dehydrating in dry hygroscopic solvents (for example methanol). The β-form has an appearance of kite-like or uneven-sided diamond shape and it crystallizes at temperatures above 93.5 °C or in the presence of methanolic potassium methoxide. It is more brittle and higher in solubility (500 g/L at 20 °C) than the α-form (70 g/L at 20 °C). The anhydrous crystal form is obtained after evaporation of a high concentration of lactose solution at 100 °C.

Both crystalline and non-crystalline forms of lactose are widely used in food, dairy, confectionary, and pharmaceutical industries as a carrier, excipient (water-sensitive solids using anhydrous lactose), filling, tableting (using anhydrous β-lactose or partial β-lactose anhydrate for a better compactibility) and binding agent. In recent years, dairy and beverage powders, and infant formula industries have used crystalline lactose compounds as a dry component in powders. In this way, the final product can be achieved without reformulation of the liquid dispersion, optimization of the wet mixing and dehydration process. The crystalline state of lactose is considered as the equilibrium state and it can provide certain characteristic textural properties, and functionality to food and pharmaceutical materials. But the state transition of lactose crystals or dissolving of crystalline lactose to the saturated aqueous solution (liquid state) may become a critical factor and affect flow behaviour (stickiness, agglomeration and caking), stability, and other physical and chemical properties of powder materials. A phenomenon known as “deliquescence transition” results in appearance of a liquid surface and occurs during increasing the relative humidity (RH) or above critical RH (deliquescence point) of lactose crystals as well as other crystalline solid materials (including sugars, organic acids, inorganic salts and vitamins). During increasing RH to above deliquescence point of a crystalline solid, the first mechanism of this phenomenon is the crystalline solid

starting to sorb water (surface interaction between water and solid), then it dissolves in the condensate film, and solid continues to dissolve and saturate the film leading to further absorption of water until the vapour pressure of saturated solution reaches equilibrium or the vapour pressure of the surrounding atmosphere (Salameh et al. 2006; Mauer 2020). The crystalline solid will dissolve until the saturated liquid is achieved or until completion of dissolution of solid, and further increasing of vapour pressure will result in dilution of solution (Zografi and Hancock 1994; Mauer and Taylor 2010).

Crystalline substances typically sorb only small amounts of water at the surface by virtue of hydrogen bonding during hydration, which may lead to microstructural rearrangements and compaction of the particle (including change of shape or porosity and decrease of particle mobility diameter depending on crystalline materials) due to the capillary condensation effects or partial dissolution and recrystallization (Mikhailov et al. 2009). But a high amount of absorbed water into the crystalline material may occur in hydrate crystallization (water in crystal structure). It is important to note that multi-component of crystalline mixtures can exhibit a stepwise of deliquescence transitions and each step follows by gradual water uptake until completion of dissolution (Mikhailov et al. 2009).

Different forms of crystalline lactose have different non-hygroscopicity levels. Salameh et al. (2006) found that crystalline solids of α-lactose monohydrate and anhydrous β-lactose were stable below 0.99 and 0.97 a_w at 25 °C. More recently, Allan et al. (2020) found that the deliquescence points of α-lactose monohydrate, anhydrous β-lactose and anhydrous α-lactose were 99, 88 and 87% RH, respectively. Allan et al. (2020) also reported that α-lactose monohydrate had the highest deliquescence point, while the deliquescence point of anhydrous β-lactose and anhydrous α-lactose were similar. Their results showed the deliquescence point of α-lactose monohydrate (99% to 98% RH), anhydrous β-lactose (89% to 82% RH) and anhydrous α-lactose (87% to 82% RH) decreased with increasing temperature from 20 °C to 50 °C (Salameh et al. 2006; Allan et al. 2020). Their findings indicated increasing solubility of lactose polymorphs at higher temperature. Furthermore, the deliquescence point of crystalline solid mixtures was found at lower values than the critical RH of the respective pure individual ingredients. This finding has been confirmed in various crystalline solid mixtures: for example, in binary mixtures such as sugar-sugar (Salameh et al. 2006), sugar-acid (Salameh et al. 2006; Salameh and Taylor 2006a; Salameh and Taylor 2006b), sugar-salt (Allan and Mauer 2016); in tertiary and quaternary mixtures such as sugars-acid (Salameh et al. 2006; Salameh and Taylor 2006b), sugar-salts (Allan and Mauer 2016). The binary mixture of crystalline materials showed greater impact on lowering critical RH than other multiple compound mixtures. Nevertheless, all the studies showed similar trends, with the addition or mixing of a greater number of crystalline compounds decreasing the deliquescent point of the overall blend. The material containing blending or mixing of different crystalline ingredients (multiple deliquescent compounds) could lead to more hygroscopic behaviour at a high RH or lowering value of the critical RH, resulting in increased caking, stickiness, agglomeration and chemical reactivity (including oxidation, hydrolysis, Maillard reaction) of

deliquescent powdered foods and ingredients (Salameh et al. 2006; Salameh and Taylor 2006a; Salameh and Taylor 2006b; Allan and Mauer 2016).

We can conclude that the deliquescence or critical RH of crystalline lactose solids is based upon polymorphs of lactose crystals. Deliquescence often occurs at high RH, and it has potential impact on the physical (flowability, caking and agglomeration, compressibility, dissolution) and chemical stability and quality of the food powders and the ingredients. It can be controlled by applying appropriate storage conditions (including RH, temperature and consolidation time) or maintaining atmospheric or environmental conditions to below the deliquescence point aiming to retain lactose crystal polymorphs in the material. But it needs to be emphasized that deliquescence depends on the composition or presence of other deliquescent compounds in the material or in contact with the crystal.

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Chapter 3
Significance of Lactose in Dairy Products

H. Douglas Goff, E. H. Hynes, M. C. Perotti, P. M. Kelly, and S. A. Hogan

3.1 Significance of Lactose in Dairy Products: Ice Cream

H. D. Goff
Department of Food Science, University of Guelph, Guelph, ON, Canada

3.1.1 Overview of Ice Cream Ingredients and Manufacture

This section will present a brief review of the sources and functionality of lactose in ice cream. The major issues surrounding lactose in ice cream include freezing point depression, lactose crystallisation and lactose digestibility. Readers are referred to previous chapters on ice cream in the *Advanced Dairy Chemistry* series for specific information related to proteins (Goff 2016) and lipids (Goff 2020) or to more general references on ice cream technology (Clarke 2012; Goff and Hartel 2013; Tharp and Young 2013) for further information.

The term 'ice cream' in its generic sense is used here to include all whipped dairy products that are manufactured by freezing and are consumed in the frozen state, including ice cream that contains either dairy or non-dairy fats, premium, higher-fat versions, 'light', lower-fat versions, ice milk, sherbet and frozen yogurt. Ice cream

H. D. Goff (✉)
Department of Food Science, University of Guelph, Guelph, ON, Canada
e-mail: dgoff@uoguelph.ca

E. H. Hynes · M. C. Perotti
Instituto de Lactología Industrial, Facultad de Ingeniería Química, Universidad Nacional del Litoral (UNL), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina

P. M. Kelly · S. A. Hogan
Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

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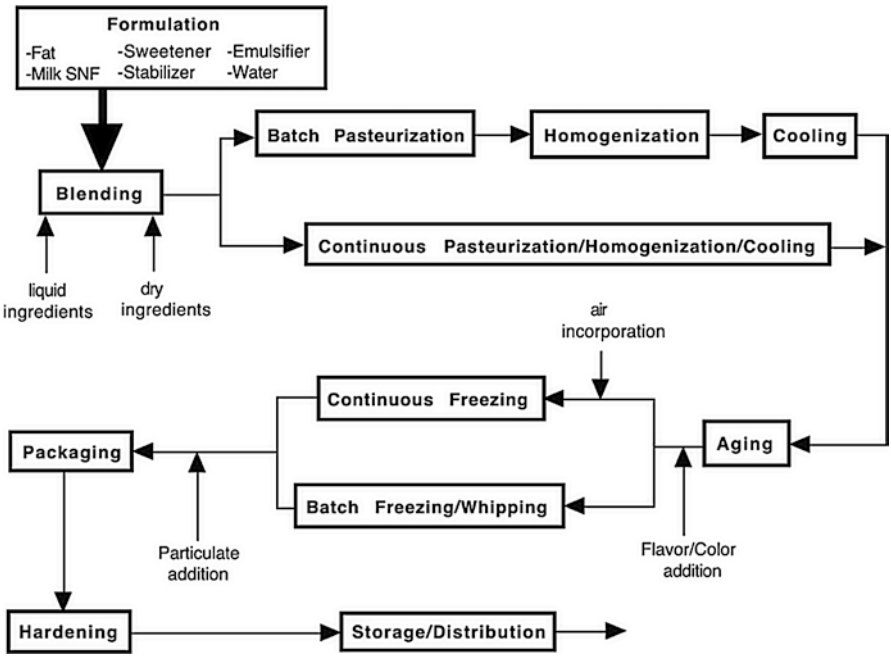

Fig. 3.1 Flow diagram for the production of ice cream

mix formulations specify the content of fat, milk solids-not-fat (MSNF), sweeteners, stabilisers, emulsifiers and water that are desired (Fig. 3.1). Dairy and other ingredients used to supply these components are chosen on the basis of availability, cost, legislation and desired quality. Common ingredients include: cream, butter or vegetable fats, as the main sources of fat; condensed skim or whole milk, skim milk powder, milk or whey protein concentrates and/or whey powder, as the sources of concentrated MSNF; sucrose and corn starch hydrolysates, as the sweeteners; polysaccharides, such as locust bean gum, guar gum, carboxymethyl cellulose, and/or carrageenan, as stabilisers; egg yolk or mono- and di-glycerides with or without polysorbate 80, as emulsifiers; and skim or whole milk or water, as the main sources of water in the formulation, to balance the total solids of the components (Goff and Hartel 2013). Usually, the same formulation can be used for the production of a variety of flavours.

The manufacturing process for most of these products is similar and involves the following steps (Fig. 3.1): preparation of a liquid mix by blending of ingredients, pasteurisation (>65 °C for 30 min or >80 °C for 25 s), homogenisation, cooling to 4 °C and ageing of the cold, liquid mix for 4–24 h; simultaneously whipping and freezing this mix dynamically under high shear to a soft, semi-frozen slurry with an air phase volume of 45–52% (overrun of 80–110%) at a temperature of about −5 °C; incorporation of flavouring ingredients to this partially frozen mix; packaging the product; and further quiescent freezing (hardening) of the product in high-velocity air to −30 °C (Goff and Hartel 2013). Homogenisation is responsible for the formation of the fat emulsion by forcing the hot mix through a small orifice under a

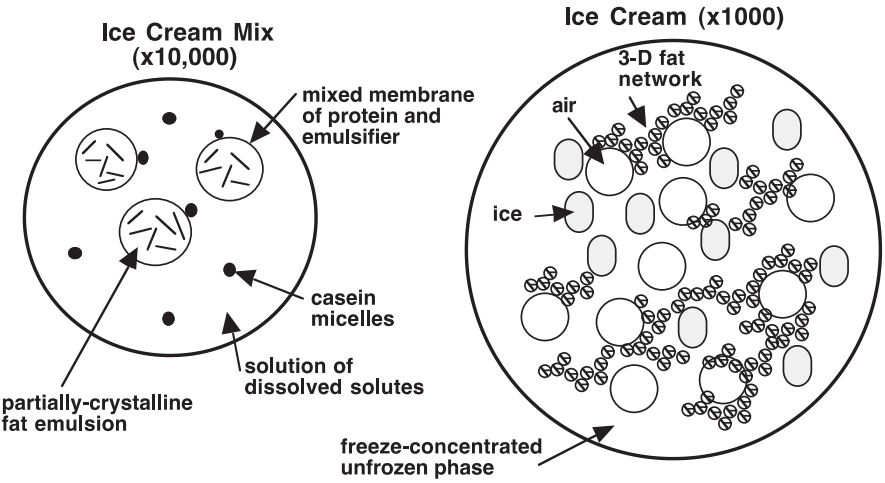

Fig. 3.2 Highly schematic illustration of the structure of ice cream mix and ice cream. Ice cream mix contains partially crystalline fat globules and casein micelles as discrete particles in a solution of sugars (including lactose), salts, dispersed whey protein and stabilisers, etc. The surface of the fat globule demonstrates the competitive adsorption of casein micelles, globular fat, partially denatured whey proteins, β-casein and added emulsifiers. Ice cream contains the ice crystals, air bubbles and partially coalesced fat globules as discrete phases within an unfrozen serum containing the dissolved material (including lactose). The partially coalesced fat agglomerates adsorb to the surface of the air bubbles, which are also surrounded by protein and emulsifier, and link the bubbles through the lamellae between them

pressure of 14–18 MPa, perhaps with a second stage of 3–4 MPa. Ageing allows for hydration of milk proteins and stabilisers (some increase in viscosity occurs during the ageing period), crystallisation of the fat globules, and a membrane rearrangement due to competitive displacement of adsorbed proteins by small-molecule surfactants. The concomitant aeration and freezing processes involve numerous physical changes, including the action of proteins and surfactants in forming and stabilising the foam phase, partial coalescence of the fat emulsion, causing both adsorption of fat at the air interface and the formation of fat globule clusters that stabilise the lamellae between air bubbles, and freeze-concentration of the premix by the removal of water from solution in the form of ice. The structure of ice cream is illustrated in the diagram in Fig. 3.2 (Goff and Hartel 2013). Lactose is dissolved in the unfrozen phase (see further discussion regarding freezing point depression and freeze-concentration below).

3.1.2 Sources of Lactose in Ice Cream

Lactose enters into mix formulations with the MSNF ingredients. Traditionally, the best sources of MSNF for high quality products have been fresh concentrated skimmed milk or spray dried low-heat skim milk powder; a typical formulation

might have 10–12% MSNF and consequently 6% or more lactose. Other sources include those containing whole milk protein (e.g. condensed or sweetened condensed whole milk, dry or condensed buttermilk, milk protein concentrates), those containing casein (e.g. phosphocasein or sodium caseinate) or those containing whey proteins (e.g. dried or condensed whey, whey or serum protein concentrate, whey or serum protein isolate) (Goff and Hartel 2013; Goff 2016). Dried whey has been investigated as an ingredient for ice cream for many years (for example Leighton 1944), principally due to reduced cost. However, it is high in lactose (~75–80%), which is a major limitation. It has now become quite common to supplement the traditional sources of MSNF (condensed skim milk or skim milk powder) with blended MSNF sources that contribute excellent functionality from the contribution of the protein (emulsification, foaming and water holding) while at the same time reducing total protein content in the dry blended ingredient from 36%, as found in skim milk powder, to 20–25%, to maintain mix costs at a level lower than they would be if skim milk powder was used.

Ingredients in the blends include milk or whey protein concentrates or isolates, perhaps also some caseinates, and whey powder or lactose, for standardisation. Much experience has been gained at blending these ingredients (Goff and Hartel 2013; Goff 2016) and the quality of ice cream resulting from their use can be very good. However, since most legal jurisdictions require a minimum total solids level in ice cream mix formulations, care must be taken to ensure that the lactose content in the formulation is not too high when formulating with these high-lactose ingredients, due to issues of freezing point depression and lactose crystallisation.

3.1.3 Contribution of Lactose to Freezing Point Depression

Freezing point depression is a colligative property, governed by Raoult's law and influenced by the collective number of moles of solute in solution. Thus, freezing point depression is a function of both the concentration of all the solutes and their molecular weight. Consequently, in an ice cream mix, the major contributors to freezing point depression are the sugars and milk salts (Leighton 1927; Smith and Bradley 1983; Jaskulka et al. 1993, 1995). Lactose is a disaccharide with a molecular weight of 342 Da and is usually present at a concentration of 6% or greater, and thus it contributes approximately 30% (although this varies with formulation) of the total freezing point depression of a mix (Goff and Hartel 2013). As the mix is frozen, solvent is removed in the conversion of water to ice, so that the effective concentration of solutes in the unfrozen phase continues to rise with decreasing subzero temperature, leading to the process of freeze-concentration and establishing the equilibrium ratio of ice to water as a function of temperature (see Fig. 3.2). This can be plotted on the freezing curve (Fig. 3.3), which is unique to each formulation (Bradley and Smith 1983; Bradley 1984; De Cindio et al. 1995; Livney et al. 2003; Whelan et al. 2008). The significance of freezing point depression and freeze-concentration is that they dictate the hardness of the ice cream as a function of temperature. In scooping or retailing operations, it is extremely important to have

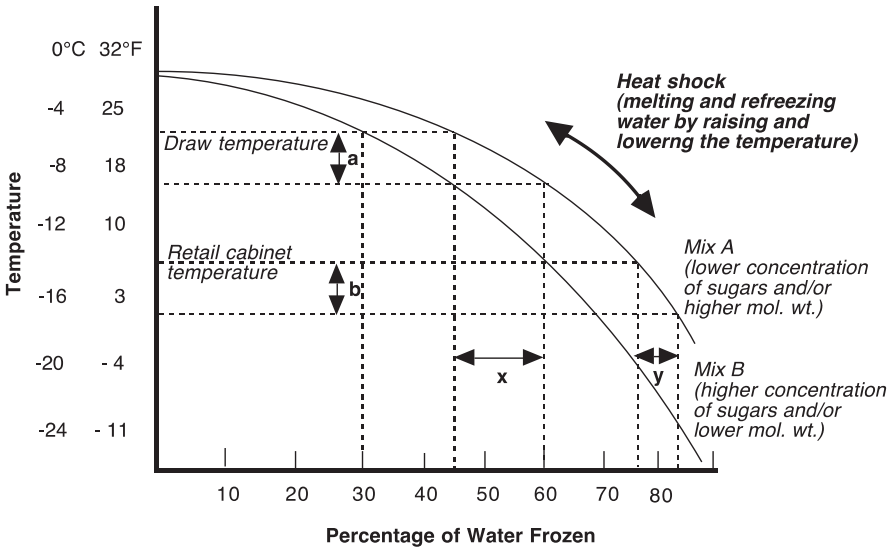

Fig. 3.3 Typical freezing curve for ice cream mixes of varying composition, showing the percentage of water frozen at various temperatures

all ice creams close to the same level of hardness. Hence, formulations must be adjusted to account for variable levels of sugars and/or types of sugars to ensure constancy in hardness.

Lactose is not very sweet and is contributed by the MSNF ingredients, as discussed above, rather than being considered as a sweetener. Nevertheless, excess levels of lactose, for example with high concentration of whey powder, can lead to ice cream that is too soft for typical storage/distribution temperatures and retail operations. As the amount of unfrozen water increases, the ice cream becomes more prone to problems like ice recrystallisation and the development of coarse or icy textures, and product that is more prone to lactose crystallisation (see below) and shrinkage (loss of air), all which limit its shelf life (Goff and Hartel 2013). These are all a result of enhanced mobility of constituents within the ice cream structure. This is one of the major limitations of the use of high levels of lactose in ice cream/frozen dairy dessert formulations.

3.1.4 Potential for Lactose Crystallisation

The crystallisation of lactose in general has been well studied and is reviewed elsewhere in this volume. The crystallisation of lactose in ice cream has also been well studied over many decades because, in this specific application, crystallisation leads to the serious texture defect known as sandiness (Zoller and Williams 1921; Nickerson 1954, 1956, 1962; Livney et al. 1995). The solubility of α-lactose is

7 g/100 g water at 20 °C. The solubility of β-lactose is 50 g/100 g water. The mutarotation equilibrium is 1.6 β: 1 α, and so the final total solubility of lactose is 18.2 g/100 g water at 20 °C at this ratio (Fox et al. 2015). Solubility and mutarotation are both a function of temperature so at 0 °C, the solubility of α-lactose is also limiting at approximately 2–3 g/100 g water to provide a total lactose solubility closer to 11 g/100 g water (Nickerson 1956) and this continues to decline into the subzero region. The initial concentration of lactose in ice cream may be expected to be approximately 9–10 g/100 g water, depending on formulation (Goff and Hartel 2013). However, the process of freeze-concentration due to the formation of ice is critical to an understanding of lactose crystallisation in ice cream, as it contributes much more strongly to supersaturation than does decreasing temperature in the absence of freezing. Solutes become freeze-concentrated in an ever-decreasing volume of solvent as temperature is lowered and more ice is formed. The water in this unfrozen phase forms an equilibrium ratio with ice at any given temperature. The removal of solvent (water) by freezing results in a doubling of the lactose concentration at the temperature of extrusion from the ice cream freezer (−5 °C) and concentrations of 3–5× at −10 °C to −20 °C as freeze-concentration continues.

It should be obvious from the above discussion that lactose has greatly exceeded its solubility (saturation) level in frozen ice cream and, from a thermodynamic point of view, could easily crystallise. Increasing supersaturation favours crystallisation (Hartel 2001). However, the first step of crystallisation is nucleation of the lactose and this process is constrained kinetically by both high viscosity and low temperature in the unfrozen phase, thus maintaining lactose in the supersaturated, non-crystalline state. This increased viscosity and decreased temperature, which decreases the driving force for crystallisation, and overwhelms the effect of increased supersaturation, which would increase the driving force (Hartel 2001). If α-lactose does nucleate, then there exists a threshold size of detection (crystals of 16–30 μm, Nickerson 1954, Hartel 2001, Goff and Hartel 2013) beyond which the textural defect of sandiness becomes increasingly evident. The typical trapezoidal wedge (tomahawk) shape of the α-lactose crystals is readily detected as very sharp, rough particles (Fig. 3.4), which are easily differentiated from ice crystals as the lactose crystals do not readily melt in the mouth or between fingers. Once nucleation has occurred, crystallisation can proceed quite quickly and once this level of lactose has been exceeded in packaged and flavoured ice cream, then disposal is the only recourse. Therefore, it is imperative that formulation, processing and storage conditions are all optimised to completely inhibit the nucleation of lactose.

The mix formulation is the first consideration for the minimisation of lactose crystallisation. Higher lactose concentration leads to greater supersaturation and hence the ice cream would be more prone to crystallisation. Recommendations regarding maximum levels of total MSNF to inhibit lactose crystallisation have been suggested to ice cream manufacturers for many years (Sommer 1944; Nickerson 1962); however, the modern use of polysaccharide stabilisers, as discussed below, offsets some of the older MSNF formulation recommendations. Lactose content is certainly a consideration in the use of MSNF ingredients such as whey powder, permeate or other MSNF sources that contain elevated levels of

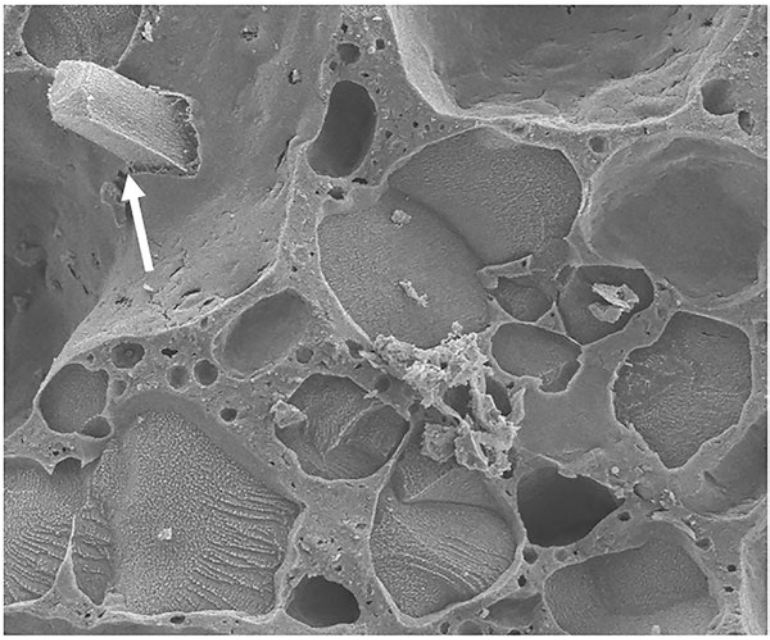

Fig. 3.4 Lactose crystal (arrow) protruding through an air bubble, as seen in sandy ice cream by cryo-scanning electron microscopy (width of photograph = 150 μm)

lactose. Nickerson and Moore (1972) showed that increasing sucrose concentration decreases lactose saturation, exacerbating the propensity for crystallisation. On the other hand, sucrose may inhibit the lactose nucleation process, thereby promoting supersaturation (Livney et al. 1995). Ingredients like dextrins from low DE corn syrup solids, soluble milk proteins and polysaccharide stabilisers, all of which promote solution viscosity, would be expected to have an inhibitory effect on lactose crystallisation. This is one of the important contributions of the polysaccharide stabilisers to ice cream quality (Goff and Hartel 2013). Some flavours, especially those with nuts, seem to be associated with a higher incidence of lactose crystallisation (Nickerson 1954, 1962). Explanations may be the inclusion of fine particles that act as nucleation sites for lactose or localised differences in water concentration as nuts absorb water during storage.

The most important storage parameter is temperature. Livney et al. (1995) showed that the time required to induce lactose crystallisation was reduced to a minimum (highest propensity for sandiness) as temperature was lowered from −5 to −12 °C, but induction times increased again at temperatures lower than −12 °C. Increasing supersaturation increased the driving force as temperature was reduced but at −12 °C this was offset by increasing viscosity. Crystal growth rates also followed a similar trend. Nickerson (1962) and Livney et al. (1995) also showed the increasing effect of temperature fluctuation on lactose crystallisation. Lactose

crystallisation is completely inhibited as the unfrozen phase approaches or enters into the amorphous solid (glassy) state, at approximately −25 to −30 °C.

3.1.5 Development of Lactose-Reduced Products

The consumption of lactose can be problematic for many people, due to lactose malabsorption or intolerance (see details elsewhere in this volume). Consequently, there is a significant market for lactose-reduced ice creams and frozen dairy desserts (Dekker et al. 2019a). In addition, lactose reduction alleviates the concerns of crystallisation. The two approaches are enzymatic lactose hydrolysis, either in the mix itself or from the use of lactose-hydrolysed ingredients, and the selection and blending of milk fat and MSNF ingredients to reduce lactose level.

Hydrolysis of lactose either in mix or in MSNF ingredients for use in ice cream has been studied by several researchers (Guy 1980; Huse et al. 1984; El-Neshawy et al. 1988; Lindamood et al. 1989; Matak et al. 2003; Abbasi and Saeedabadian 2015; Mahmood and Mahmood 2017; Tsuchiya et al. 2017). This makes the products digestible for those who are lactose-intolerant and also is a strategy to reduce the potential for lactose crystallisation (sandiness) development; however, hydrolysed lactose contributes twice the freezing point depression of an equal concentration of lactose, so that softness at storage or retailing temperatures and greater rates of ice recrystallisation become issues to overcome if hydrolysis is to be considered. Lactose hydrolysis levels can easily be controlled from 25% to 100% based on source and activity of the enzyme preparation, concentration, time and temperature of treatment (Lindamood et al. 1989; Matak et al. 2003). Successful hydrolysis can be obtained either at elevated temperatures for short time (Mahmood and Mahmood 2017; Tsuchiya et al. 2017) or during refrigerated temperatures over 24 h (Matak et al. 2003; Horner et al. 2011; Dekker et al. 2019a), depending on the enzyme used. Sweetness of lactose-hydrolysed ice cream is increased when compared to its unhydrolysed control (El-Neshawy et al. 1988; Lindamood et al. 1989), and this fact allows for a 15–25% reduction in sucrose or blending of alternative sweeteners, such as sugar alcohols, to obtain the optimal formulation for sweetness and freezing point depression (Abbasi and Saeedabadian 2015; Mahmood and Mahmood 2017; Dekker et al. 2019a).

It is also possible to formulate a lactose-free (or -reduced) or a sugar-free product by selection of anhydrous milkfat or butter as the dairy fat source, or vegetable fats as the non-dairy fat source, together with high-concentration milk protein concentrates, either whole milk proteins or caseinates, as the MSNF source (Alvarez et al. 2005; Whelan et al. 2008). With such a formulation, the presence of lactose in the product can be avoided. Cream could not be used to supply any fat and milk could not be used to supply the water, for formulation balancing, as either of these would also contain lactose. The milk protein concentrate or caseinate blend must supply all of the desired functionalities of the proteins, including emulsification of the fat, aeration of the foam and water holding capacity in the unfrozen phase.

3.2 Lactose in Dulce de Leche

E. H. Hynes and M. C. Perotti
Instituto de Lactología Industrial, Facultad de Ingeniería Química, Universidad Nacional del Litoral (UNL), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Santa Fe, Argentina

Summary Dulce de leche (DL) is part of the gastronomic and cultural heritage of the Río de la Plata region of South America. DL is a sweet, creamy and viscous dairy product consumed as a dessert, as a spread or as an ingredient for confectionery or ice cream. It is prepared by concentrating a mixture of milk with sucrose, and with the addition of neutraliser, among other ingredients, under variable conditions of temperature and pressure. The characteristics of different types of DL, technologies of manufacture, the significance of lactose on texture, colour, flavour and acceptability related to non-enzymatic browning reactions, and nutritional value are explored in this chapter.

3.2.1 Introduction

Dulce de leche (DL) is a traditional product originally hailing from the Río de la Plata region of South America. It was initially a homemade food, but its rapid industrial development made it a very popular and widely accepted commercial product. The main producer and consumer countries are Argentina and Uruguay, where DL is consumed alone as a dessert, spread like jam on bread or toast, or as an ingredient, filler or topping in confectionery (cookies, crepes, cakes, ice creams, fruits, sweet snack such as alfajores) and in the preparation of food products (ice creams, desserts). Annual per capita consumption in Argentina is about 3 kg, with a fairly stable production of around 120,000 tonnes/year (www.magyp.gob.ar/sitio/areas/ss_lecheria/estadisticas/_02_industrial/index.php). Of the total Argentinean milk production in 2019, 2.2% was used to make DL (www.ocla.org.ar/contents/newschart/portfolio/?categoryid=46). DL is mainly destined for the domestic market in Argentina and Uruguay but exports are growing slowly from year to year. Uruguay has a minor share of the market, with a production of 16,000 tonnes/year (www.gub.uy/ministerio-ganaderia-agriculturapesca/DIEA/Anuario2019). DL is also produced and consumed in other Latin American countries (Chile, Paraguay, Brazil) but to a lesser extent.

Dulce de leche is a creamy and viscous dairy product prepared by concentrating a mixture of milk and sucrose (hence the name), and with the addition of other ingredients, including sodium bicarbonate, under variable conditions of temperature/time and pressure according to the manufacturer. This confers differential sensory, physicochemical and nutritional characteristics affecting the consumer perception (Giménez et al. 2008; Gaze et al. 2015; Penci and Marín 2016).

Its high content of sugars and low moisture (minimum solids content of 70% w/w) leads to water activity below 0.85 (Ferramondo et al. 1984; Ranalli et al. 2012); therefore, microbial growth is reduced and shelf life is increased. The rheological behaviour of DL is intermediate between a concentrated solution and a gel, depending on the type and the solids content (Pauletti et al. 1990; Navarro et al. 1999). As a consequence of the composition of the mixture and the conditions of manufacture, extensive non-enzymatic browning occurs in the form of the Maillard reaction and caramelisation of sugars. Both reactions are responsible for the typical characteristics of the product: bright surface sheen, reddish-brown colour, creamy texture and pleasant caramel-like aroma. Several types of DL are available, the most popular called 'Familiar' is consumed mainly at home. Other types are intended for confectionery, ice cream and other industrial uses; the production of DL for ice cream has grown due to the increasing demand for frozen desserts. Variants with additional ingredients (cream, chocolate, dried fruits, cereals, almonds, coconuts, etc.) are allowed; these products are very common in the Brazilian market (Stephani et al. 2019). The main physicochemical and microbiological characteristics of DL are set in a regulation harmonised with the other countries belonging to Grupo Mercado del Sur (MERCOSUR), in an attempt to standardise identity requirements and quality parameters. DL is defined as 'the product obtained by the heat concentration, at normal or reduced pressure, of milk or reconstituted milk apt for human consumption, with the addition of sugar' (Res. GMC No. 137/96, Código Alimentario Argentino 2020). In addition, to obtain the quality certification 'Argentine Food a natural choice' (Res. SAV No. 12-E, 2018, Ministerio de Agroindustria de la Nación), DL must meet certain attributes related to product and process. For example, milk has to be obtained from cows fed mainly pasture and the time between milking and processing should be <1 h.

3.2.2 Technology

In the manufacture of DL, the initial mixture is composed of milk and sucrose; cream or dairy solids like whole or skimmed milk powder are legally allowed as optional ingredients according the type of DL; for example, for the DL with cream variety, the fat content in the final product is higher than 9%. Neutralisers are also used to maintain the pH of milk during concentration, glucose in 'Familiar' type DL for partial substitution of sucrose (up to 40%), vanillin or ethylvanillin as flavouring, potassium sorbate (maximum 600 mg kg^{-1}) and natamycin (maximum 1 mg $(dm^2)^{-1}$, surface sprinkled before the packaging is closed) as preservatives (Código Alimentario Argentino 2020). Contrary, the use of preservatives is not allowed in DL with quality certification 'Argentine Food a natural choice'.

The quantity of nutritive sweeteners added to the milk is calculated to give 68–70% total solids in the final product, of which milk solids account for at least 24%. Sucrose is most commonly used in Argentina, but glucose may also be used, in particular for 'Familiar' type DL, giving brightness to the product. The

technological advantages and drawbacks of glucose addition will be discussed later. To avoid a pH decrease during evaporation, the mixture of milk and sugars is first neutralised with sodium bicarbonate or calcium hydroxide to achieve a titratable acidity between 4 and 12 mg lactic acid 100 mL^{-1} according to the type of DL; the lower values of acidity are employed in the manufacture of familiar DL and the higher values for the confectionary-type DL.

Neutralisation avoids the destabilisation of casein micelles as a consequence of the decrease in pH during evaporation, which in turn is due to the concentration of calcium phosphate and its precipitation into the casein micelle forming H^+ (Zalazar 2003). Failures in manufacture with the separation of phases (solid proteins and liquid 'whey') are due mainly to inadequate neutralisation. Excess acidity may be detrimental to the Maillard reaction. On the contrary, an excess of neutraliser would cause strong colouration and a fluid texture (Penci and Marín 2016). The amount of alkali is calculated taking into account the acidity of the milk, and is added as solid sodium bicarbonate powder or, preferably, as an aqueous solution to improve mixing.

DL can be produced by the traditional process in open kettles (batch process, Fig. 3.5), or by semi-continuous or continuous processes. The first and the last methods are used most frequently for familiar type DL, and batch and semi-continuous methods for confectionary-type DL. The neutralised mixture prepared in a mixing tank is then gradually transferred to the evaporating kettle (in general, 1000 L capacity). The volume in the kettle is low initially (20% of its capacity); when the volume has been partially reduced, the rest of the mixture is fed slowly while boiling continues. A stirrer with scrapers is used to prevent the product sticking to the wall of the kettle and to improve the release of steam from the hot mass.

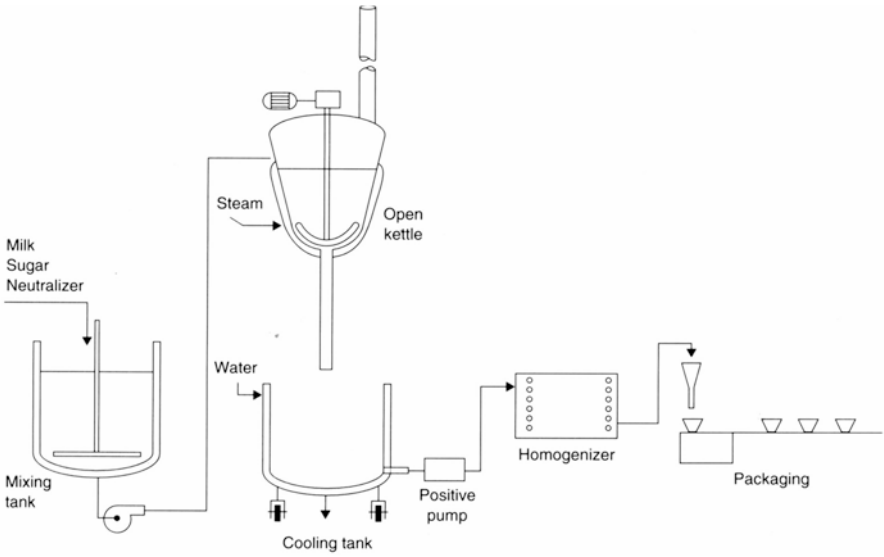

Fig. 3.5 Batch production of dulce de leche in open kettles

As the solids content increases, Maillard and other non-enzymatic browning reactions occur and the product attains its typical colour, flavour and viscosity. Heating is stopped when the solids content is 68%, which is measured by refractometry. Legislation requires 70% total solids but this target value is normally attained during discharge of hot DL and cooling. After that, familiar DL is homogenised to avoid clumps and improve texture and surface sheen. Finally, the product is packaged while still hot (65 °C), to stop the browning process and avoid microbiological contamination, in plastic (0.25, 0.5 and 1 kg), glass or tin containers. These last types of containers are preferred for premium DL. Paste-board packages (10 or 20 kg) are employed for confectionery purposes. DL is stably unopened at room temperature during its shelf life, but it must be refrigerated when it is opened.

In the semi-continuous process, the neutralised mixture is concentrated in a multiple-effect evaporator to improve the efficiency of the evaporation step; afterwards, the desired final solids content is obtained by boiling in an open kettle, where the colour and flavour develop. In the continuous process there is an inversion of the steps, as colour development is first attempted in a heat exchanger by heating and rapid cooling while the product is still liquid; the control of several variables such as temperature and pH is critical in order to obtain the desired colour. The coloured blend is then fed into a multiple-effect evaporator and finished in a scraped-surface evaporator before cooling to 60 °C in a tubular heat exchanger (Stephani et al. 2019; Penci and Marín 2016; Zalazar 2003). The production of DL by the continuous process has increased in recent years. Quality of DL from continuous process was not optimum in previous decades but now is similar to that of the traditional product.

3.2.3 Significance of Lactose in Dulce de Leche

3.2.3.1 Lactose Mutarotation and Crystallisation

Lactose crystallisation can occur in DL as the moisture is supersaturated with lactose. If the crystals of α-lactose monohydrate grow to exceed 10 μm, they will be perceived in the mouth; as they do not dissolve readily, they produce a rough or gritty sensation. Taking into consideration the average composition of the raw milk (fat: 3.6%, total proteins: 3.2%, lactose: 4.7%), and the usual concentrations of the added sugars (max. 30 kg sucrose per 100 L of milk, of which a maximum 40% w/w glucose may be used in replacement of sucrose), DL will have an average composition shown in Table 3.1. Among all sugars present, lactose has the lowest solubility in water (18% w/w, at 20 °C). For a mean concentration of lactose of 10%, lactose in the moisture (30%) of DL is around 33% w/w. Therefore, the aqueous phase of DL would be supersaturated with lactose (ca. 1.84 times higher than saturation solubility), even without considering the presence of glucose and sucrose in the system. It places in the intermediate equilibrium zone, defined by the solubility curves of lactose (Fig. 3.6).

Table 3.1 Typical composition of commercial Dulce de leche (familiar type)

Component	Percentage (w/w)
Sucrose	32.0–47.0
Lactose	8.5–11.8
Fat	6.0–7.9
Protein	5.0–7.1
Ash	1.8–2.0
Moisture	24.7–30.0
pH	5.70–6.37

Adapted from Hynes and Zalazar (2009), Gaze et al. (2015), Ranalli et al. (2012), Zarpelon et al. (2016), Oliveira et al. (2009) and Da Silva et al. (2020)

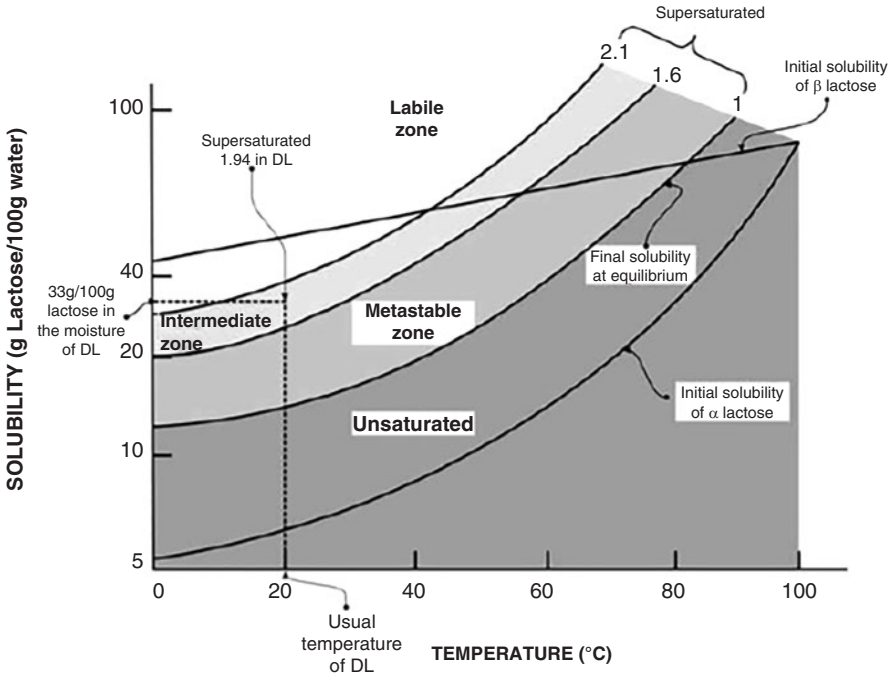

Fig. 3.6 Lactose solubility curves. Adapted from Fox and McSweeney (1998)

In DL, the anomeric forms of lactose mutarotate to an equilibrium: $\alpha \leftrightarrow \beta$, with an equilibrium ratio of β/α lactose of 1.68:1.00. If the mean concentration of lactose ($\alpha + \beta$) in DL is 10% w/w, the concentrations of α- and β-lactose would be 3.7% and 6.3% w/w, respectively. On the other hand, taking into account the final solubility of lactose (18% w/w at 20 °C), 15 g of lactose per 100 g of the aqueous phase of DL (i.e. 33–18 g) would be available for crystallisation; this is equivalent to 4.5 g of lactose per

100 g DL. With these considerations, 5.5 g of lactose (i.e. 10–4.5 g) would eventually remain soluble in 100 g DL after the crystallisation of the excess lactose.

From the value of the equilibrium ratio, it can be calculated that of 5.5 g of lactose in solution ($\alpha + \beta$), 2.0 g and 3.4 g would be α- and β-lactose, respectively. Summarising: 3.7 − 2.0 = 1.7 g of α-lactose would crystallise and 6.3 − 3.4 = 2.9 g of β-lactose would first mutarotate to α-lactose and then crystallise. In this context, the rate of crystallisation of lactose in DL depends not only on the rate of crystal formation itself but also on the mutarotation rate, and the slowest reaction will limit the crystallisation kinetics.

Depending on the characteristics of the system containing lactose, the slowest reaction may be mutarotation or crystallisation. Haase and Nickerson (1966a, b) found that mutarotation is very fast and consequently crystal formation is the rate-limiting step under the environmental conditions that exist in most dairy products. Under other conditions, for example when an extensive nucleation area is available for lactose crystallisation, it has been observed that neither of the two steps can be clearly identified as the rate-controlling step (Tweig and Nickerson 1968).

Kinetics of mutarotation is influenced by different factors. The presence of salts and sugars in the system impacts in opposite directions. Citrate and phosphates, at the concentrations found in milk, accelerate reaction to a rate twice as high as in pure water, whereas in contrast, high level of sucrose slows it down (Holsinger 1988). This latter effect is only slight up to 40% (w/v) of sucrose, but above this level, the rate decreases and the catalytic impact of milk salts is counteracted. Temperature and pH also influence the reaction rate. At 75 °C, the mutarotation reaction is completed in 1 h, while as the temperature decreases the reaction is slowed down. The effect of pH is small in the range of 2.5–7.5 but increases dramatically outside this range (Holsinger 1988). Therefore, taking into account the environmental conditions in DL and the data above mentioned, rate of lactose mutarotation should be low, as sucrose is in high concentration, the pH is between 6.20 and 7.00 and the storage temperature is always below 20 °C (Gaze et al. 2015; Francisquini et al. 2019). On the other hand, the formation of lactose crystals in the intermediate zone (Fig. 3.6) may be very slow. In this zone, seeding with lactose crystals can induce crystallisation but otherwise supersaturated solutions will be stable (Holsinger 1997; Ganzle et al. 2008).

The discussion above is an approach to the problem of lactose crystallisation in DL; in the real food matrix, the situation is even more complex than described. Mutarotation as well as crystal formation may be impaired in DL because of the high viscosity of the medium. In contrast, the high concentration of sucrose and glucose may reduce the solubility of lactose significantly, increasing its rate of crystallisation. In fact, a sucrose concentration of 70% has been reported to reduce lactose solubility to 42% of its solubility in water (Nickerson and Moore 1974), but the combined impact of sucrose and glucose on lactose solubility has not been studied. In addition, the influence of the Maillard products on the rates of lactose mutarotation and crystallisation has not been reported to date.

The available knowledge on lactose crystallisation in DL is mainly empirical, based on decades of observation and experience, and there are few systematic studies

on the subject. In good quality DL, the formation of lactose crystals larger than 10 μm and detectable in the mouth does not occur before 120 days of storage at room temperature (always below 25 °C). Consequently, industry has adopted this period as the usual shelf life for the product. However, due to a high demand, the food is generally consumed earlier. According to microscopy analysis performed by Oliveira et al. (2009), DL samples stored at 4 °C did not show crystals initially, but visible crystals of 60 μm started appearing after 10 days, the number of crystals increased on 35 days after which numbers remained constant until 125 days. Crystal growth was observed until 50 days and the size remained at 230 μm until the end of storage. In DL stored at 30 °C, a similar pattern was observed; although fewer crystals were formed, they were larger. The fact that crystallisation occurred more rapidly at 4 °C suggests that solubility of lactose is the rate determining process, while the fact that large crystals were formed at 30 °C is consistent with a lower driving force for crystallisation. In addition, at both storage temperatures, the majority of crystals had the characteristic truncated tomahawk shape associated with α-lactose monohydrate.

On the local market, DL is most often retailed in plastic packages. It has been observed that the occurrence of plastic flavour in the DL is the first sensory defect detected determining the shelf life of the product, before the appearance of gritty texture or 'sandiness'. Garitta et al. (2004) reported that the sensory shelf life was limited to 146 days after storage at 25 °C because a plastic flavour note was detected; sandiness was not reported in the product during this period. Premium DL, on the contrary, is often packaged in glass containers and the shelf life is generally required to be longer (180 days). In this type of product, sandiness probably determines its sensory shelf life, and its development should be delayed as much as possible. One of the most commonly used approaches to avoid lactose crystallisation and the occurrence of sandiness is the enzymatic hydrolysis of lactose to glucose and galactose, which is performed before manufacture by adding β-galactosidases to the milk and incubating for several hours. The hydrolysis of 30% of the lactose is enough to avoid crystallisation during a 180-day period. Glucose and galactose are sweeter than lactose and contribute as nutritive sweeteners to DL; also, they can participate in the Maillard reaction increasing the tendency to browning. Besides, it has been reported that lactose crystallisation can be retarded by increasing the proportion of glucose in DL (Ferramondo et al. 1984; Navarro et al. 1999; Kurlat 2010). Glucose forms a strongly hydrated complex with protein, increasing the viscosity which interferes with the formation of lactose crystals perceptible in the mouth (Oliveira et al. 2009). But in order to prevent drastic changes in DL characteristics, this addition should be done in the last stage of the manufacturing process when the solid content reaches 55–60% (Zalazar 2003), or when the temperature tends to decrease (Oliveira et al. 2009).

3.2.3.2 Non-enzymatic Browning Reactions

Unlike most food products, milk contains both proteinaceous material and a reducing sugar, which leads to non-enzymatic browning and changes in the nutritional value of the food when it is heated even at mild levels (Holsinger 1997). In DL, in addition, sucrose and glucose are usually present, while sucrose is a non-reducing sugar, glucose readily reacts with proteins according to the Maillard reaction, which is a complex cascade of reactions. It has been suggested that a small proportion of sucrose is hydrolysed to its reducing monomers, glucose and fructose, during the cooking of DL in the kettle (Rozycki et al. 2010); although such transformation is not significant during manufacture as environmental conditions are not favourable (Malec et al. 2005), small amount of fructose has been detected in DL (Ferramondo et al. 1984). Lactose is usually the main reducing sugar in DL, depending on the proportion of sucrose that is replaced by glucose syrup; lactose and milk proteins are the main reactants for Maillard browning. Non-enzymatic browning in DL also includes caramelisation reactions; in this case, sucrose has been reported to be the main sugar involved, although lactose may contribute also (Rozicky 2003). Caramelisation and Maillard reactions continue during the storage of the product after manufacture (Garitta et al. 2004).

Colour, appearance, flavour and texture are essential characteristics and critical quality parameters that define the acceptability of consumers. A reddish-brown colour, a caramel-like flavour and a smooth texture without grittiness are major sensory attributes of DL. Typical colour and texture correlate positively with the overall acceptance of DL (Garitta et al. 2004; Ares et al. 2006). Although, an excessively dark brown colour can lead to product rejection (Garitta et al. 2004; Giménez et al. 2008). Gaze et al. (2015) indicated that the DL colour is a combination of red and yellow, and suggested that instrumental colour measurement is an efficient and fast analytical tool to control products at industrial level, without additional sample preparation steps.The negative changes in the desirable attributes may be due to an inappropriate mixture formulation, too high a pH during manufacture, storage conditions, amongst other factors. The techniques most frequently adopted by the industry to avoid or delay the defects of gritty texture involve the increase of amounts of reducing sugars in the system. The strategy consisting of hydrolysing lactose with β-galactosidases leads to the formation of two molecules of reducing sugars (glucose and galactose) from each molecule of lactose. Another option is to replace part of the sucrose by glucose. Glucose or glucose syrup added to the formulation also brings bright, soft and smooth texture. However, the product may become very dark since the Maillard reaction is favoured (Morales and van Boekel 1998). Variable results have been reported using different indices to evaluate non-enzymatic browning. The accumulation of fluorescent uncoloured intermediates allows detection of the progress of reaction before any visual change occurred (Morales and van Boekel 1997; Rozycki et al. 2007). In addition, hydroxymethylfurfural (HMF) is an indicator compound of Maillard reaction and can be employed to measure the colour formation during production and storage of DL (Francisquini et al. 2018).

If alkaline conditions prevail in the mixture after neutralisation (pH > 7.50), there is an active participation of monosaccharides in the Maillard reaction, increasing the tendency to browning of product (O'Brien 1997; Rozycki et al. 2010). Temperature and protein concentration were the variables that more affected the colour and fluorescence development (Rozycki et al. 2010).

DL can darken during storage at room temperature due to continuing non-enzymatic browning. However, acceptability tests have shown that the colour changes during storage for the usual period (up to 180 days) are not determinants for product rejection by consumers (Garitta et al. 2004). Changes in sensory profiles were detected in DL containing different levels of reducing sugars due to lactose hydrolysis: increase in brown colour intensity and gloss, decrease in firmness, adhesiveness, peaks (length of time that peaks hold their shape after introducing the spoon vertically into the sample and raising it vertically from the sample once) and mouth coating, increase in ropiness and creaminess, increase in caramel flavour and in acid, pungent and aftertaste duration defects. Consumer overall acceptability decreased for products manufactured with 40% and 50% lactose hydrolysis (Giménez et al. 2008). Gaze et al. (2015) observed higher brightness for DL containing higher contents of glucose and they also revealed differences in the colour parameters attributed to variations in composition and technologies applied (time, temperature and pressure).

HMF index tends to increase with the sucrose substitution by glucose, with higher pH value in the mixture (Francisquini et al. 2018), and also with the level of lactose hydrolysis (Francisquini et al. 2019). Melanoidins, polymeric nitrogenous and brown-coloured macromolecules, are heterogeneous compounds mainly generated in the last stages of the Maillard reaction, contributing to colour of DL (Newton et al. 2012; Rodríguez et al. 2019). In addition, their beneficial (chelating properties, antimicrobial and antioxidant activities) and detrimental (mutagenic, carcinogenic) biological effects and the implications on human health are of great interest. But because the large number of different compounds formed and the difficulties in their purification and identification, these aspects are not fully understood (Wang et al. 2011; Echavarría et al. 2012). Melanoproteins—water-insoluble fraction of high molecular weight composed of melanoidins linked with protein—are the form of melanoidins in the DL matrix (Alves et al. 2020). They are polydisperse with nominal molecular weight ranging from 400 to 1800 Da. According to the UV-VIS and NMR spectra, the main components are aromatics. However, further studies are necessary for the complete elucidation of the structures of melanoidins in DL (Rodríguez et al. 2019). On the other hand, antioxidant activity was detected in DL samples (Cortés Yáñez et al. 2018; Alves et al. 2020) and was correlated with the formation of compounds in the intermediate and the final stages of the Maillard reaction (Cortés Yáñez et al. 2018).

Several volatile compounds belonging to different chemical families (acids, ketones, aldehydes, alcohols, hydrocarbons, furans, lactones and sulfurs) have been identified in DL (Gaze et al. 2015). Sulphur-containing compounds (such as dimethyldisulphide) are powerful odorants that are related to the flavour of cooked milk. Furans, lactones and furfural are associated with the Maillard reaction

(Zabbia et al. 2012), and the latter is also derived from the caramelisation reactions (Paravisini et al. 2012).

The impact of lactose on the nutritional properties of DL may be evaluated from two different points of view, the main one is the loss in nutritive value by damaging essential amino acids by Maillard reaction, lysine being the most affected amino acid. However, the rate of loss of available lysine depends on the DL formulation and the temperature-time of the process. Remaining available lysine was about 70% at the end of the process (102–103 °C for 120 min) for a formulation containing only sucrose. The replacement of 10% of sucrose by glucose increased by 90% the rate of lysine glycation and consequently the loss of lysine was higher (Malec et al. 2005). The second aspect is the nutritional concern for lactose-intolerant people, as DL is rich in lactose.

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3.3 Sweetened Condensed Milk

P. M. Kelly and S. A. Hogan
Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

Sweetened condensed milk (SCM) is a traditional long-life dairy product that relies on the addition of a critical amount of sugar (sucrose) to reduce the risk of bacterial spoilage. Thus, the preservation effect is accomplished when the so-called sugar ratio is exceeded. The reliance on sugar addition as a form of preservation has diminished over time as new processing treatments such as ultra-high temperature (UHT) heating and spray drying have become available.

3.3.1 Markets

Today, while SCM may not be classed as a mainstream consumer dairy product compared to fresh and fermented milks that dominate throughout chilled cabinets, it nevertheless occupies niche markets as a functional dairy product/ingredient for use in both domestic cooking and industrial confectionery production, e.g. the formulation and processing of toffees and fudges. Asian and South American markets have a particular affinity for SCM. Local manufacture in Asia relies on the importation of functionally suitable skim milk powder to facilitate SCM production by recombination technology (abbreviated RSCM) due to a shortage of local fresh milk supplies. A related SCM product ‘Dulce de Leche’ is widely consumed in South America (Sect. 3.2). Consumer preference in emerging dairy markets for the use of sweetened condensed milk in beverages such as coffee and tea is contributing

to market growth, an observation highlighted by Clarke (1999), who recognised that recombined sweetened condensed milk (RSCM) was one of the major product outputs by the recombination industry. These products are popular with processors who, in the absence of local fresh milk suppliers, depend on imported dairy commodities to formulate and process ingredients into regular consumer dairy products for distribution in local markets. For example, RSCM is used in the preparation of 'Teh tarik'—a frothed tea product that is popular in Asian markets (Clarke 1999).

A growing confectionery industry and increasing demand for dairy products is supporting the global growth for sweetened condensed milk market. The burden of SCM's relatively high calorie content is to some extent overshadowed by consumer sentiment for an indulgent experience of finished confectionary and beverages in which SCM is used. The long shelf life of SCM appeals to processors whose operational logistics centres around ample stocking of shelf-stable ingredients for subsequent food formulation and preparation.

Consumption of evaporated milk and sweetened condensed milk continues to grow and is expected to register an average annual growth of 2% from 2019 to 2024 (Cornal 2020). This growth is occurring in Latin America, the Middle East, and, to a lesser extent, in Asia. Major exporting countries of SCM are New Zealand, The Netherlands, USA, Germany, and France. US SCM exports are mainly to Mexico, Philippines, Indonesia, Vietnam and Colombia. Historically, large-scale military mobilisation during nineteenth and twentieth century wars created a major demand for the production of condensed milks. Many processing facilities were established in Great Britain, Scotland and Ireland during that time. The Condensed Milk Company of Ireland, established in Limerick in 1883 by Thomas Cleeve of Canada, was renowned also for its toffee production. Today, Meadow Foods claims to be the only independent manufacturer in the UK, producing 12,000 tonnes sweetened condensed milk (8% fat) and sweetened condensed skimmed milk (0.5% fat) packed in 5 and 13 kg bag-in box, 280 kg drums, 1 tonne or 1.3 tonne pallecons and bulk tankers (https://meadowfoods.co.uk/our-products/confectionery-ingredients/).

3.3.2 Processing Considerations

SCM is essentially evaporated milk that relies on the addition of high concentrations of sucrose to control water activity (a_w) and thus prevent bacterial spoilage. As there is no thermal-based sterilisation step involved, product preservation relies instead on achieving a critical concentration of added sucrose in order to achieve a sugar ratio value between 63.5 and 64.5. This expression is based on the amount of sucrose in the aqueous phase for an ambient stored product according to the equation:

$$\text{sugar ratio} = (\%\ \text{sugar in condensed milk} \times 100)/(100 - \text{total solids in condensed milk}).$$

According to Canadian food regulations (2020), a lower sugar ratio value (of 42) applies where whole milk-based SCM is stored in bulk under refrigerated conditions (Safe Food for Canadian Regulations, modified 2020).

Concentrated milk products are viscous by nature, and are exceptionally so following addition of large amounts of sugar in the case of SCM. Adherence to end-product specification may prove challenging due to seasonal variation in milk protein; the protein concentration of the starting milk has a direct effect on viscosity, particularly when higher levels occur towards the end of lactation. In-line preheat treatment of milk before entry to the evaporator is an initial step in the manufacturing process in keeping with its functional roles during the manufacture of most concentrated and dried milk products. In the case of SCM, some optimisation of the preheat temperature setting is usually advised in order to achieve the target end-product viscosity. The recommended application of UHT (ultra-high temperature) conditions of 135–140 °C for short holding times (up to 5 s) to the starting milk should be sufficient to inactivate bacteria and their heat-stable enzymes while limiting whey protein denaturation and viscosity increases. Lower temperatures with longer holding times may also be employed where higher viscosities are desired.

3.3.3 Manufacture of SCM

3.3.3.1 Preparatory Steps

From the outset, attention to hygienic conditions both in terms of milk selection and process plant preparation is essential in order to prevent spoilage by any contaminating osmotolerant microorganisms. As per the traditional British standard for SCM, milk is standardised according to the fat:milk solids non-fat ratio of 9:22 (Table 3.2). Sugar, in the form of sucrose crystals, is added in sufficient amounts to the fat-standardised milk (i.e. before preheating) in order to achieve the sugar ratio of 63.5–6.45 as outlined above. Thus, the sugar is simultaneously pasteurised using this process route; one caveat is that there is a risk of triggering the initial stages of Maillard browning reactions using this approach. Alternatively, the sugar may be dosed in syrup form at the end of the milk evaporation step (Fig. 3.7). Attention needs to be paid both to the syrup's concentration and heating temperature in order to eliminate the risk of osmophilic yeast contamination.

Table 3.2 Typical composition of sweetened condensed milk

Constituent	% (w/w)
Fat	9
Milk solids-non-fat (MSNF)	22
Lactose	11.4
Sucrose	43.5
Water	25.5

Adapted from Walstra et al. (1999a)

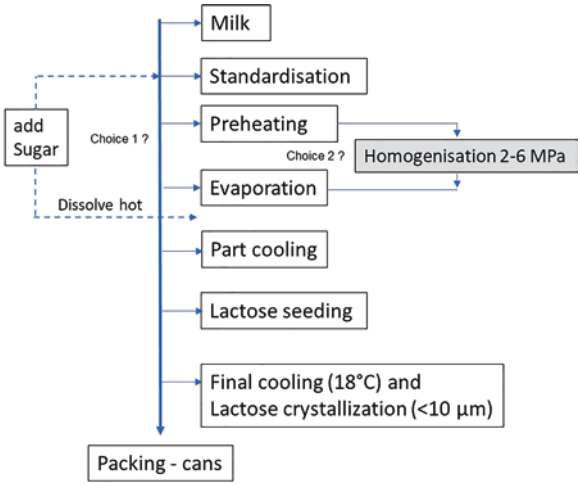

Fig. 3.7 Process schematic for the manufacture of sweetened condensed milk. Note that two process options are indicated (1) the point of sugar addition—either during milk standardisation or as a hot syrup following evaporation of milk solids, and (2) the inclusion of low pressure homogenisation following preheating

3.3.3.2 Preheat Treatment

The rationale for selecting either UHT-type conditions or a lower-temperature longer holding time heat treatment has already been outlined above.

3.3.3.3 Concentration

Traditionally, a two-step process which combines falling- and rising-film evaporators is a preferable approach to handling highly viscous SCM concentrate. A rising-film evaporator during the heavy concentration phase is more suited to overcoming tube wetting challenges normally associated with falling-film effects. Heating the sugar-containing milk concentrate to a higher temperature of 80 °C during this second-stage evaporation step mitigates against escalating viscosity towards the end of the process.

3.3.3.4 Homogenisation

SCM's dense concentrate matrix restricts creaming, and so use of a homogenisation step is not entirely necessary. When used, then low pressure (2–6 MPa) homogenisation is advised in order to avoid excessive viscosity increases. Increasingly, SCM manufacturers include homogenisation as a support tool to modulate end-product viscosity.

3.3.3.5 Lactose Seeding to Promote Nucleation

Lactose crystallisation begins with a nucleation step—a molecular aggregation/clustering phenomenon that relies on the extent to which the concentration of lactose in the product is supersaturated. In the event that the thermodynamics of this process is undermined by lower levels of supersaturation and temperature, addition of lactose seed crystals to the SCM concentrate on discharge from the evaporator as a means of heterogenous nucleation is advised during the course of cooling. With the ultimate objective of generating small lactose crystals (<10 μm), careful control of concentrate cooling is necessary to avoid lactose supersaturation and coarse crystal formation when too low a temperature is reached.

3.3.3.6 Packaging

Bulk packaging is usually employed where SCM is supplied as an ingredient for industrial scale confectionary manufacture. SCM intended for retail markets is packaged in cans under 'clean-fill' conditions which include pre-sterilisation of cans and lids along with a high-grade air filtration surround at the filling point. Accurate filling of cans to within 1 g of the set value is recommended in order to limit the amount of headspace air which otherwise could promote the growth of moulds and micrococci (Walstra et al. 1999a).

3.3.4 Quality

Both physico-chemical and microbiological monitoring of SCM is required given its status as a long shelf life product.

3.3.4.1 Physico-Chemical Aspects

Processors aim to achieve a consistent SCM viscosity of 0.3-0.4 Pa s (30 - 40 poise) in the final canned product. Higher viscosities outside of this target range can lead to an age-thickening defect over the course of storage, in which the SCM attains a gel-like consistency.

Milk salts play a key role in this regard; Samel and Muers (1962a) speculated that added Ca reduces the rate of age thickening by removing milk-serum anions such as phosphate and citrate as insoluble or undissociated salts. Curiously, the same authors found that, with the exception of added phosphate, the effect of both anions and cations added after concentration is almost the opposite of that produced by the same ion added before. Using another investigative approach, the high viscosity of recombined sweetened condensed milk prepared using low calcium reconstituted skim milk was further increased when Ca was added to the sweetened

condensed milk after condensing (Noda et al. 1986). However, the same authors found that, when calcium was added before the preheating step, the viscosity was similar to that of ordinary sweetened condensed milk. Increasing formation of insoluble calcium salt content over the course of SCM storage appears to be associated with the progression of age thickening. The latter two studies support the earlier hypothesis of Samel and Muers (1962b) that the thermal effects associated with preheating and condensing at higher temperature during manufacture followed by cool storage after concentration culminate in the precipitation of insoluble salts such as calcium phosphate and stabilise SCM.

Sandiness due to formation of crystals >10 μm is a physical defect that can arise due to lactose crystallisation and also that of sucrose when the sugar ratio value is exceeded. Optical microscopy in conjunction with ImageJ software for digital processing/analysis of images was investigated by Schumacher et al. (2015) for the measurement of crystal size in SCM samples. This technique proved useful for establishing representative values for mean crystal size but underestimated the total number of crystals present in samples. Non-enzymatic browning of SCM during storage has been studied and an Arrhenius relationship, taking into consideration negative correlations between SCM Maillard activity and absorbance at different storage temperature ranges, formed the basis of a mathematical model capable of predicting the product's shelf-life (Patel et al. 1996).

Autoxidation of fat may arise if sufficient oxygen is available in cans, particularly if low preheating temperatures were used during processing; high preheat temperatures generally enhance milk protein's antioxidant potential through the generation of Maillard reaction products (MRPs) in the presence of added sucrose.

3.3.4.2 Microbiological Quality

With an inherent composition that favours self-limiting bacteriostatic control throughout its shelf life, it has to be kept in mind that SCM is non-sterile; hence, vigilance is required to monitor the growth of osmotically tolerant microorganisms such as osmophilic yeasts (genus *Torulopsis*), some micrococci and particular strains of moulds if sufficient oxygen is available (e.g. *Aspergillus repens* and *Aspergillus glaucus*). Accelerated testing of SCM cans sampled from production batches and placed in warm storage (e.g. 35 °C) for a number of weeks is traditionally used as an early indicator of physical and microbiological defects. Bulging of cans due to gas formation arises due to the growth of yeasts, which also can cause fruity flavours and alcohol production. Defects associated with the growth of micrococci to colony counts $>10^5$ mL^{-1} include aggregate formation and off-flavour development (Walstra et al. 1999a).

Renhe et al. (2018) found that microbial analysis of nine sweetened condensed milk brands produced in Brazil revealed contamination levels by yeasts and coagulase-negative staphylococcus in 80% and 70% of samples, respectively. The levels of coagulase-negative staphylococcus, in particular, ranged from <3.1 to 5.7 log CFU g^{-1}. The fact that the same authors (Renhe et al. 2018) found significant

differences in the chemical composition, physico-chemical and sensory characteristics may provide clues as to the bacteriostatic status of each SCM product, but will not provide insights into the hygienic conditions under which the products were processed. A year previously, Siddique et al. (2017) reported on a survey of four different SCM brands undertaken in Bangladesh; the authors were motivated to monitor the quality of imported milk powder following local product manufacture using a recombination process. All four brands graded satisfactorily in terms of physical quality, though significant compositional differences occurred in the case of total solids, carbohydrates, protein and ash contents. The authors found no significant difference (range 12–14 × 10^2 CFU g^{-1}) between the total viable counts of all four commercial SCM samples, which were deemed satisfactory, in line with previous reports. As an indicator of good hygienic practices during processing, all samples were found to be coliform-negative. However, no specific measurements of pathogenic microorganisms and yeasts were undertaken. The survivability of different strains of *Listeria monocytogenes* (strains Scott A, California and V7) in SCM during 42-day storage was monitored by Farrag et al. (1990) following inoculation of between 10^3 and 10^7 cells/mL. There was evidence of greater lethality during storage at 21 °C compared to 7 °C depending on the listeria strain; the California strain appeared to be more susceptible according to its order of magnitude reduction (3.4) compared to the other two strains (1.6–1.7). Attention to plant cleanliness and hygienic practices is warranted as a result.

During the past 20 years, the Bangladesh government rescinded manufacturing licences from four SCM manufacturing plants for a period for breaching dairy product standards when it was discovered that cheaper vegetable oils were being used to replace the more expensive milk fat (Anon 2003). The practice of 'fat-filling' SCM with substitute oils during manufacture in Bangladesh was recognised in an FAO regional report (Haque 2009) as business-model-motivated to the benefit of local consumers, given the lower cost of raw material commodity imports (milk proteins and sugar), along with local availability of cheaper vegetable oil. It was speculated that an impending rise in global commodity prices in the following years would be expected to alter the long-term sustainability of this regionally adopted business model.

3.3.5 Recombined Sweetened Condensed Milk (RSCM)

Recombination processes in the dairy industry have been designed to use preserved milk ingredients such as skim milk powder (SMP) and anhydrous milk fat (AMF) as starting materials for the manufacture of conventional dairy products, particularly in countries where local milk production is either insufficient or not practised. The long storage life of SMP and AMF also suits food processor importers who prefer to work with approved vendors capable of fulfilling functional specifications. The logistics of handling both perishable supplies of fresh milk of unpredictable functional quality along with other dry ingredients is much more challenging. This is particularly the case with confectionary processors who prepare RSCM in-house as feedstock for

their production lines. The main contrasting difference between SCM and RSCM is the process starting point; the former relies on prior concentration of milk, while the latter commences with partial rehydration of highly concentrated/dehydrated SMP, mix recombination (AMF and sugar addition) and intensive shear mixing.

The selected SMP is chosen according to the whey protein nitrogen index (WPNI) heat classification, which is a measure of residual undenatured whey protein content. By increasing skim milk preheat temperatures in the evaporator feed, it is possible to alter the SMP heat classification from low- to medium- and finally high-heat, with the latter possessing the lowest concentration of undenatured whey protein. High preheat temperatures also increase the viscosity of the resulting reconstituted SMP which, in turn, also has a direct effect by increasing RSCM viscosity. Usually, medium-heat classified SMP is selected for RSCM manufacture subject to careful sample screening before approval for production.

One shortcoming with this approach is that medium-heat SMP classification is represented by a rather wide band of levels of undenatured whey protein (1.51–5.99 mg undenatured whey protein nitrogen per gram-SMP) which, in turn, reflects a wide range of preheat temperature and holding time options that can be chosen against a background effect of seasonal variance in milk/whey protein nitrogen composition. Many milk powder manufacturers claim to have built up custom and practice knowledge on the configuration of their own processes to produce SMP for RSCM application. Since there is no accurate measure of predicting SMP suitability, processors rely on the use of a laboratory benchtop RSCM process to simulate production scale. Two laboratory protocols exist, one developed by Australian and another by Dutch (Sjollema 1990) researchers to assist with process simulation. Lawrence et al. (2001) found that neither method accurately predicted the viscosity of RSCM manufactured on an industrial scale, but did establish a significant correlation ($p < 0.05$) between the apparent viscosities, as a result of the Australian method generating higher values than the Dutch. Although Lawrence et al. (2001) found that increasing mix temperature and holding time along with higher homogenisation pressures generally increased RSCM apparent viscosity, the effects were variable and difficult to predict precisely.

3.3.6 Nutritional Considerations

A critical review (Juffrie et al. 2020) of the diet of young Indonesian children recommends limiting SCM consumption within a supervised, actively monitored programme with oversight of product advertisements, product labelling, enforcement of regulations, and provision of effective customer education in a manner analogous to sugar-sweetened beverages. The authors were clear in their recommendations that the SCM should not be fed to young children (1–3 years old) because of its limited nutritional value and high sugar content. A lack of background scientific studies regarding the health risks of long-term SCM consumption by young Indonesian children is leading to much public confusion among consumers.

3.3.7 Future Perspective

The continuing growth in local Asian consumer markets for beverages formulated using SCM and RSCM as ingredients provides assurance that demand for these forms of preserved milks should hold firm in the medium term. There is an increasing sense that local public health authorities have a concern that vulnerable age groups in certain socio-economic classes may be consuming excess amounts of these carbohydrate-loaded beverages which otherwise offer limited nutrition. In addition, manufacturers of SCM and RSCM are faced with considerable commercial pressures due to one or more issues such as changes in formulation, manufacturing procedures and demand for consistent performance of raw ingredients. Skim milk powder, anhydrous milk fat and sucrose are being partially substituted by alternate ingredient sources for functional or economic reasons.

Manufacturing procedures have also been modified to produce more consistent products at lower cost and to tighter specifications, including specific functionality in some instances. This, in turn, places pressure on the suppliers of raw materials, particularly milk powders, to produce functional and cost-effective ingredients to help recombiners succeed in their business-to-business relationships.

3.4 Significance of Lactose in Milk Powders

P. M. Kelly and S. A. Hogan
Teagasc, Moorepark Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

3.4.1 Introduction

The term ‘milk powders’ is generally understood to embrace common, commodity-traded dairy products such as skim milk powder, whole milk powder and whey powder, as well as an ever-increasing range of dried ingredients derived from milk such as demineralised whey powders, de-lactosed whey powders, whey protein concentrate (WPC), milk protein concentrates (MPC) and permeate powders. In addition, fat-filled variations of many of these ingredients are also produced.

Lactose in isolated form is also harvested by crystallisation from either whey or permeate and dried after washing into forms suitable for food use (edible-grade lactose) or further purified for pharmaceutical application as a drug-carrying incipient during tablet or capsule production.

Lactose occurs in an amorphous state in skim and whole milk powders; the rapid rate of concentration during spray-drying does not allow sufficient time for lactose to crystallise. Hence, it is ‘trapped’ in a metastable glassy state, which for the most part does not affect the free-flowing nature of spray-dried milks provided that

appropriate environmental and packing conditions are adhered to. Otherwise, because of the hygroscopic nature of lactose in its amorphous state, such powders tend to absorb moisture and set in train a series of physical changes that ultimately impair product quality. Occasionally, problems may present at an earlier stage during drying when 'stickiness' is evident as powder particles adhere to the side walls of the drying chamber. Generally, the manufacture of whole and skim milk powders is not a problem in terms of significant powder deposit formation for lactose concentrations in the range 35–48%, w/w. However, the extensive range of spray-dried dairy ingredients now being manufactured from milk frequently include higher levels of lactose and, possibly, other carbohydrates which may present greater challenges during processing.

The chapter will examine the physical behaviour of lactose during spray-drying, how processing is affected by lactose state transitions, and how such behaviour contributes, both positively and negatively, to milk powder quality. Key behavioural properties of lactose, such as sticking and caking, are discussed, along with remedial strategies to control such phenomena during processing, storage and distribution. The chapter also explores the functional properties of lactose-containing powders and discusses ingredient applications in which lactose confers significant functional attributes.

3.4.2 Behaviour of Lactose During Spray-Drying

One of the attractions of spray-drying as a preservation process for a flavour-sensitive product such as milk is the rapidity of mass transfer (evaporation of water) that is facilitated by the greatly extended surface area of finely sprayed milk droplets projected into a hot air stream. Even allowing for droplet shrinkage during the transition from liquid feed to powder particles, the physico-chemical properties of the resulting powder will be influenced for the most part by material behaviour primarily at the surface of powder particles, and to a lesser extent by its bulk composition.

Dairy-based spray-dried powders tend to be hygroscopic to varying degrees, primarily due to the material behaviour of lactose. Skim milk powder is a major commodity dairy ingredient that is used as a benchmark with which other milk powders are compared. It is not possible to crystallise lactose in skim milk concentrates before drying, primarily because the degree of concentration falls below the level of super-saturation needed to trigger spontaneous crystallisation. The rapidity of the subsequent spray-drying process is insufficient to allow lactose to crystallise, with the result that it is trapped in an unstable, amorphous (glassy) state in skim milk powder particles. Hence, both drier design and operating conditions need to be optimised in order to reduce the impact of powder stickiness and prevent excessive powder adherence to the contact surfaces of the drying chamber. Maintaining lactose in this amorphous condition prevents sticking between particles and to equipment surfaces. Furthermore, the powder flow properties should be sufficient to

facilitate movement at all stages of the drying process, i.e. in a modern three-stage drier, this includes transfer from the primary drying chamber to an internal, integrated static bed, followed by discharge to an external fluidised bed for final drying and conditioning. All of these factors are accentuated when it comes to drying whey powder because of its higher compositional loading of lactose, ~70% in dry matter, compared to ~52% in the case of skim milk powder. Furthermore, higher mineral contents and lactic acid levels (compared to other milk-based ingredients) resulting from the cheese making process from which the whey is recovered present added complications. Up to 70% of lactose crystallisation is achievable by flash cooling of the whey concentrate post-evaporation, which includes additional controlled overnight chilling and stirring. However, this pre-crystallisation step alone is insufficient to generate a non-hygroscopic whey (or permeate) powder, and so further crystallisation is required post-drying through means of a slow-moving crystallisation belt.

Early success in the mid-1900s with the introduction of spray-drying as a mainstream unit process in dairy plants for skim and whole milk powder production saw this technology later extended to whey drying. However, greater stickiness encountered with whey drying was attributed to the impact of higher lactose levels. Pallansch (1972) established a direct relationship between the temperature of sticking and the degree of crystallisation of lactose, so that, by achieving up to 80% crystallisation, the temperature in the spray-drying chamber can be operated at normal levels without causing problems. Islam and Langrish (2010) reported that spray-drying of lactose at a higher inlet temperature (200 °C vs. 170 °C) resulted in an increase in crystallisation and a greater proportion of the β-lactose anomer produced. In a further development, the acid content of whey was also found to have a negative effect on sticking temperature, which presents additional challenges when drying whey obtained from low pH cheeses (Cottage, Quarg, etc.) and acid casein production.

Today, whey powder is usually offered on the market in either standard or non-hygroscopic specifications. Extensive lactose pre-crystallisation steps are implemented during the final stages of whey concentration and after subsequent discharge from the evaporator where non-hygroscopic powder is intended. The latent heat of crystallisation of lactose during whey powder manufacture is considerable, 10.63 kcal/kg, and needs to be factored into the cooling calculations during the design of crystallisation tanks (Písecký 1997). Additional post-drying crystallisation may also be factored into the process especially when handling acid whey. In this case, powder is removed from the drying chamber onto a drying belt in order to allow time for further crystallisation to take place (Knipschildt 1986).

3.4.2.1 Measures to Alleviate Hygroscopicity During Processing

Since the rapidity of the spray-drying process limits the opportunity for lactose to crystallise during concentration, the resulting amorphous lactose in powders is hygroscopic and will sorb available water. A sharp increase in moisture content is observed in amorphous milk powders during storage at relative humidity >50% RH (Thomas et al. 2004). This is, by and large, manageable during the manufacture and

subsequent storage of skim and whole milk powders. In the case of whey powders, in which lactose accounts for as much as 70% of dry matter content, hygroscopicity manifests itself immediately during spray-drying by excessive levels of stickiness of powder occurring on the chamber walls.

Successful remedial measures that may be incorporated into the manufacturing process include lactose pre-crystallisation of whey concentrates before drying, and post-crystallisation of the spray-dried powder as it emerges from the drying chamber (Roetman 1979). Lactose-rich powder, containing 12–14% moisture, is discharged from the primary drying chamber onto a slow-moving belt, where it resides for up to 10 min in order to increase the degree of post-drying crystallisation. The powder is then discharged from the crystallisation belt into a fluidised bed drier where conditioning and cooling brings the final moisture content within specification, while enabling an overall degree of lactose crystallisation of 85–95%. Powder hygroscopicity and caking are, thus, brought under control by lowering the content of amorphous lactose. Up to 50–75% degree of crystallinity (i.e. extent of lactose crystallisation) may be achieved in whey powder by pre-crystallisation, and this may be pushed closer to 95% by combining pre- and post-crystallisation treatments. This technological approach has enabled 'non-hygroscopic' forms of whey powders to be marketed commercially. There are suggestions that powders may be labelled as 'non-caking' at >75% crystallinity; however, there is no guarantee that lactose and milk powders that are free of amorphous lactose will not cake.

3.4.2.2 Phase Transitions During Drying of Milk

After years of technological innovation, food material science is now increasingly applied to improve our understanding of what happens during spray-drying. The material in this instance is dominated by the behaviour of the amorphous glass structure of rapidly solidified lactose (β:α ratio of 1.2–1.4) during the conversion of milk concentrate into powder. Amorphous lactose, which can be likened to a so-called 'solid solution', coats the protein and fat globules in spray-dried milk powder particles and acts as the continuous phase material. Thus, by considering physicochemical changes during drying in the light of underlying material state transitions, a more effective approach to optimising concentration and spray-drying of dairy ingredients is envisaged. The phase diagram of milk may now be augmented by consideration of a physical phenomenon critical to the functional behaviour of lactose during powder production, storage and application, i.e. glass transition.

Glass Transition

The glass transition is a reversible, second-order transition that represents a change between the solid- and liquid-like states of an amorphous phase, and hence is useful to describe the relative physical changes taking place in lactose and lactose-containing powders, especially given the time-dependent, non-equilibrium nature of

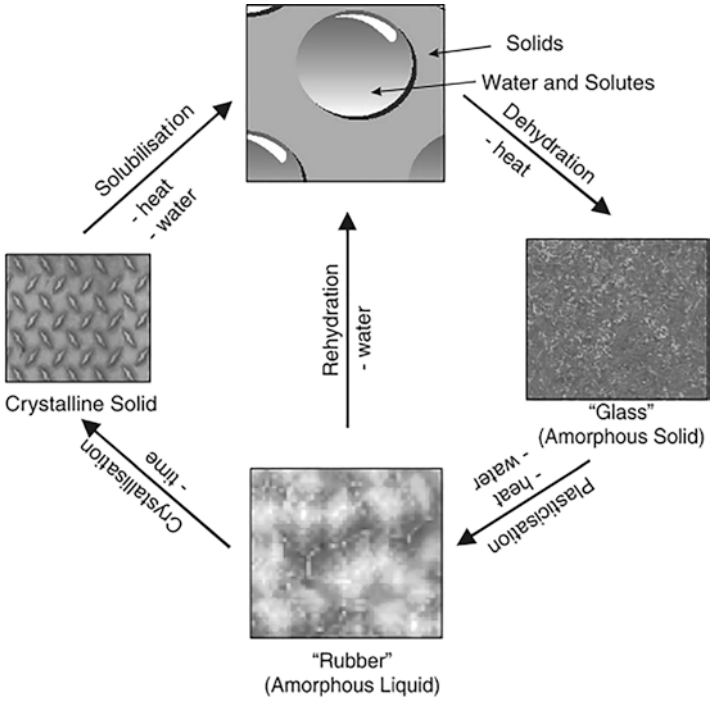

Fig. 3.8 Effects of concentration, temperature and time on material properties of ingredients during spray-drying and storage. (From Roos 2002)

amorphous lactose. The temperature at which the glass transition occurs (T_g) is identified typically by a characteristic change in heat capacity (ΔC_p) as measured by differential scanning calorimetry (DSC) following equilibration of powders under defined humidity conditions. Other manifestations of T_g include changes to dielectric, mechanical and thermal expansion properties of the material. The transition from amorphous (glassy) to a lower viscosity 'rubbery' state is due to heat and moisture-induced plasticisation of lactose molecules as they attain lower free energy states. Such molecular reconfiguration results ultimately (given enough time) in crystallisation (Fig. 3.8). For example (Fig. 3.9), the T_g of amorphous lactose, at room temperature, occurs at a water content of 6.8 g $(g \times 100)^{-1}$ solids, which corresponds to an equilibrium relative humidity of 37% (a_w 0.37) (Jouppila and Roos 1994). Increasing temperature or available moisture (or both) will result in a decrease in T_g—with subsequent effects on stickiness and caking. Storage below T_g results in a product with essentially indefinite physical stability.

A graphical representation by Roos (2002) of hypothetical powder particle temperature during spray-drying on a plot of T_g against water content provides guidance in relation to the identification of appropriate conditions for optimal operation, e.g. the use of integrated static beds within the primary drying chamber and promotion of agglomeration. Vuatez (2002) studied sorption isotherms in conjunction with

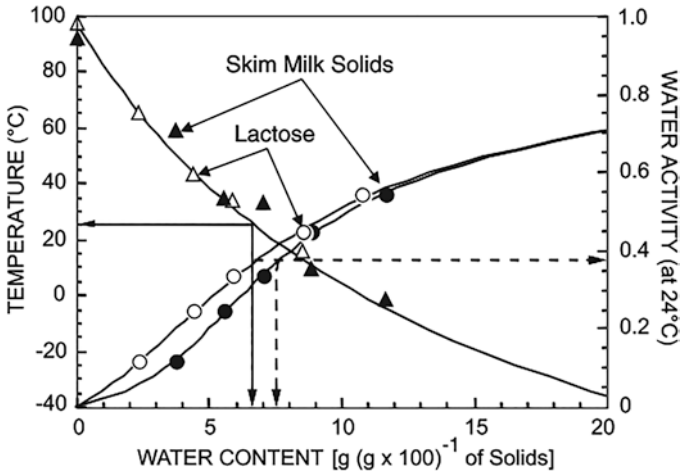

Fig. 3.9 Effects of moisture sorption on glass transition temperature of amorphous lactose and skim milk solids. (Data from Jouppila and Roos 1994)

glass transition as a function of concentration for both whole milk and skim milk and proposed a universal relationship between glass transition temperature and water activity.

The physical state of skim milk powders is effectively determined by lactose; Jouppila and Roos (1991) showed that the T_g of such powders was almost equal to that of pure lactose. Stickiness in milk powers is also recognised as a surface phenomenon governed predominantly by the T_g behaviour of lactose. Hence, current research aims to produce 'sticky curves' in conjunction with $T - T_g$ plots in order to identify sticky and non-sticky conditions during drying (Hogan and O'Callaghan 2010; O'Donoghue et al. 2020).

Spray-drying involves the creation of a very fine dispersal of liquid feedstock followed by rapid dehydration and conversion of fine particles into glassy structures (Roos 2010). The rapidly drying semi-wet particles are guided away from contact with the hot metal surfaces of the drying chamber by the trajectory of the atomisation spray and the heated air stream. When the drier is configured to operate in powder agglomeration mode (straight-through agglomeration), powder fines are recovered from other parts of the process (cyclones and/or fluidised beds) and recycled back to the wet zone close to the atomiser in order to promote adhesion between 'almost dry' fine particles and partially dried (sticky) droplets. Thus, agglomeration uses a controlled plasticisation (sticking) of particle surfaces at or above T_g by promoting aggregation through formation of inter-particle liquid bridges (Roos 2010). Similarly, the process of encapsulation by spray-drying exploits the ability of a glass-forming, solute such as lactose, to form a solid continuous phase that can entrap volatiles and flavours as well as disperse phase components, such as milk fat droplets in dairy powders (Roos 2010; Vega and Roos 2006). Progressive solidification of particle surfaces following initial mass transfer in the drying tower should

result in free-flowing behaviour as the semi-dry powder is hurled towards the drier outlet.

During spray-drying, the T_g of atomised powder increases as the water content is reduced towards the end of the drying operation during which particle temperature rises in the advanced stage of dehydration and approaches that of the dryer outlet air if residence time permits. Hence, 2- and 3-stage spray-drying interrupts this process by separating the slower stages of dehydration (at higher solids levels) from that which occurs in the primary drying zone (spray drier chamber), and thus facilitates secondary drying of particles at a slower pace and lower temperatures in either a static, integrated bed or external fluidised bed. High temperatures or residual water contents at the later stages of drying may cause stickiness, caking, browning and adhesion of powder particles to the drying chamber (Roos 2002). Roos (2002) reported that stickiness and caking tends to occur at approx. 10 °C above T_g in pure lactose systems as measured by DSC. A general rule of thumb is that stickiness occurs at about 20 °C above T_g, an approximation that accounts for the contribution of proteins in increasing sticking temperatures relative to lactose alone.

The effect of water content on T_g may be predicted using the Gordon–Taylor equation:

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2}$$

where T_g is the glass transition temperature of a mixture of solids with a weight fraction of w_1 and anhydrous T_{g1}, water with a weight fraction of w_2 and glass transition temperature of T_{g2} for pure water (frequently assumed to be −135 °C) and k is a constant.

When T_g data are combined with that of water sorption properties, insights into the extent of water plasticisation of milk powders are generated for various storage conditions (Jouppila and Roos 2002). Such information may be used to distinguish between the critical water contents of lactose and skim milk solids as 6.8 g/100 g and 7.6 g/100 g solids (Fig. 3.9), respectively (where critical a_w is defined as that at which water plasticisation depresses a measured T_g to ambient temperature; Roos 2002).

The effect of non-lactose solids on the T_g of powder may be predicted using the extended Couchman–Karasz equation, i.e. where three or more constituents (water, amorphous lactose, glucose and galactose, casein and whey proteins) are considered. The Couchman–Karasz equation for tertiary mixtures (casein, carbohydrates and water) takes the form:

$$T_g = \frac{W_1 \Delta C_{p1} T_{g1} + W_2 \Delta C_{p2} T_{g2} + W_3 \Delta C_{p3} T_{g3}}{W_1 \Delta C_{p1} + W_2 \Delta C_{p2} + W_3 \Delta C_{p3}}$$

where T_{gi} is the glass transition temperature of component i (K), ΔC_{pi} is the change in heat capacity of this component at T_{gi} (J/kg/°C) and W_i is its weight fraction.

Hogan and O'Callaghan (2010) explored the reasons as to why powders with higher protein/lactose ratios were less susceptible to sticking and concluded that, with increasing protein content, preferential sorption of water by non-amorphous constituents delayed the rate at which lactose underwent the requisite change from the 'glassy' to the 'rubbery' form in order that powder particles became sticky. In this way, $T - T_g$ (the temperature above particle T_g at which sticking occurs) increased with protein/lactose ratio.

3.4.3 Milk Powder Microstructure

The surface of skim and whole milk powder particles is typically characterised by an irregular shape, shrunken appearance and shrivelled-looking surface. Older, single-stage spray driers relied on high outlet temperatures in order to achieve the desired powder moisture which tended to form puffed-looking skim milk powder particles. Such conditions were usually associated with high levels of occluded air, the expansion of which at higher exhaust temperatures gave rise to such an appearance. During mechanical handling, these fragile powder particles were easily ruptured causing the release of smaller particles, so-called 'fines', which adversely affected bulk density and other physico-chemical properties. The progression to multi-stage spray driers brought some benefits to powder quality, especially in relation to reducing the amount of air entrapment in particles during drying. Chief among these is the lowering of particle temperature at the exit from the primary drying chamber in order to minimise expansion by hot occluded air. Thus, spray-dried skim milk powder particles produced by modern spray driers tend to have a slightly collapsed looking appearance with a wrinkled surface (Fig. 3.10). However, lactose content would seem to be prominent in affecting the surface morphology of spray-dried particles as their protein content increases, e.g. during ultrafiltration (UF) of skim milk for the production of milk protein concentrates (MPC). The surfaces of the resulting powder particles after spray-drying are smoother compared to the wrinkled surfaces of higher-lactose content skim milk powder particles (Mistry 2002). High-protein MPCs, with low lactose levels, appear to share similar powder morphology with those of commercial spray-dried caseinates.

3.4.3.1 Behaviour of Lactose in Milk Powders

The behaviour of lactose in milk powders has important consequences for other physical and functional properties. Firstly, powder composition and storage conditions influence crystallisation. Secondly, it is now widely accepted that the surface composition and morphology of powder particles, more so than internal microstructure, are major factors which dictate inter-particle interaction and behaviour. Thirdly, manipulation of surface properties during microencapsulation and spray-drying is desirable when attempting to protect sensitive ingredients during subsequent storage and delivery.

Fig. 3.10 Representative scanning electron micrograph of spray dried skim milk powder particles showing characteristic surface wrinkling

Because of their hydration behaviour, the presence of milk proteins in powders tends to delay lactose crystallisation because of competition for available water. Crystallisation in pure lactose and milk powders starts at about 40% and 50% RH, respectively (Thomas et al. 2004). In the case of whole milk powder, lactose crystallisation does not occur until ≥66% RH, due to the role of milk fat, which is believed to act as a hydrophobic barrier that limits the diffusion of hydrophilic molecules and growth of lactose crystals (Thomas et al. 2004).

The migration of internal fat to the surface of particles during the storage of milk powders is facilitated when lactose crystallisation creates an internal network of capillary interstices. Morphological changes, such as surface deformation, also occur due to the build-up of lactose crystals (Thomas et al. 2004). The fat content of powder has a positive influence on the surface fat coverage of powder particles, with the most dramatic effect evident for powders in the 0–5% fat range (skim milk powder category) giving rise to a surface fat of 0–35% (Nijdam and Langrish 2006; Gaiani et al. 2009). Nijdam and Langrish (2006) also include protein along with fat as the main components that migrate preferentially to the surface of particles during drying (in a laboratory scale dryer at an air inlet temperature of 120 °C). The migration of lactose increased when the experimental drying was conducted at an industrially more realistic air inlet temperature of 200 °C. However, release of water during crystallisation is likely to increase viscous flow on particle surfaces, induce lactose migration and further crystallisation to the point that particle bridging and agglomeration occurs. Deformation of the surface of particles is related to uneven shrinkage of atomised

droplets during the early stages of spray-drying. The surface properties of high-protein powders prepared from skim milk are known to be dependent on lactose content: the higher the lactose content, the more wrinkled the surface (Mistry et al. 1992). Rosenberg and Young (1993) found that the structure of WPI-based, spray-dried microcapsules differed from that of other milk-derived powders. Deformation was evident in WPC-based particles, but not when WPI was used, which suggests that lactose (present only in the former ingredient) was also a contributory factor. The physical effects appear to be due to solidification of particle wall solids content before completion of expansion during droplet/particle dehydration. Higher drying temperatures eliminated the tendency towards the formation of shallow deformations. Rosenberg and Young (1993) proposed that there is a critical viscosity determining the tendency for dents to occur, below which surface tension-driven effects are sufficient to smooth out morphological irregularities. Hence, high concentration of whey proteins and fat in product formulations play roles in limiting surface folds.

3.4.3.2 Caking

When a low-moisture, free-flowing powder shows signs of lump formation, followed by progressive agglomeration into a solid before finally transitioning into a sticky mass, the resulting problematic situation is described as 'caking'. These physical and microstructural changes take place during caking of skim milk powder while equilibrated at 43–94% RH (Aguilera et al. 1995). Initial changes are evident when powder reaches >54% RH while, ≥74% RH, lactose crystallisation, as well as bridging between particles, occurs (Listiohadi et al. 2005). The caking of milk powder is preceded by viscous flow on the surface of particles as amorphous lactose becomes sticky on exposure to humid air. Viscous flow was measured as an increase in density of lactose plugs within a cylindrical compaction apparatus after incubation under defined temperature/time conditions (Lloyd et al. 1996). The onset temperature of viscous flow decreased with increasing a_w and corresponded to the onset temperature of T_g (Lloyd et al. 1996). Viscous flow at $T > T_g$ increases the potential for caking to occur. Paterson et al. (2015) determined the viscosity of amorphous lactose as 1.1×10^{14} Pa s at T_g and reported that this decreased by one order of magnitude at ~T_g + 6 °C. It is speculated that other forms of lactose (α-lactose monohydrate, α-lactose anhydrous and the compound crystals of β/α-lactose), in addition to van der Waals and electrostatic forces between particles, also play significant roles in caking. The presence of mineral salts and proteins, surface wetting followed by water equilibration or cooling, and pressure are believed to also contribute (Hartmann and Palzer 2011; Carpin et al. 2016).

In the case of fat-containing powders, fat-induced caking becomes a problem when total or surface fat exceeds 41% and 12.6%, respectively (Foster et al. 2005). The caking mechanism in this instance is attributed to fat crystallisation in the liquid bridges between particles due to cooling of the powder.

Aguilera et al. (1995) developed a 'caking index' in order to quantify time-dependent, physical changes in food powders, defined as the state of a powder

system relative to its initial condition. Two morphological indicators of changes associated with caking were used: the ratio of system porosity to initial system porosity (p(t)/po); and the ratio of inter-particle bridge diameter to particle diameter ($D_{bridge}/D_{particle}$). They proposed that bridging occurs at the onset of caking as a result of surface deformation and sticking at contact points between particles, without a measurable decrease in system porosity. During this early phase, small inter-particle bridges may disintegrate under even mild shaking. Agglomeration follows and involves an irreversible consolidation of bridges, but the high porosity of the particulate system is maintained. The compaction that occurs at an advanced stage of caking is associated with a pronounced loss of system integrity as a result of thickening of inter-particle bridges, reduction of inter-particle spaces, and deformation of particle clumps under pressure.

Humidity, temperature, the presence of amorphous lactose fines, lactose impurities and logistical handling/storage are among the associated factors that contribute to powder caking (Carpin et al. 2016). Capillary condensation can occur in lactose at RH > 80% (Bronlund and Paterson 2004). Such surface moisture gives rise to the question of deliquescence (D), a form of dissolution which occurs when a crystalline solid takes up water vapour and turns into an aqueous solution. However, such an event is unlikely (Carpin et al. 2016) given that the high RH (95% DRH at 25 °C) and a_w values (0.99 for α-lactose monohydrate) required for deliquescence are outside the normal incident range for lactose caking. DRH may be lowered, however, as the solubility of a compound increases (e.g. due to increasing temperature). Lowering of deliquescence may also contribute to caking of lactose, particularly if impurities such as sulphated ash are present.

The extended specific surface area caused by fines in un-milled lactose becomes available for the adsorption of moisture and increased capillary condensation due to the creation of more contact points. Smaller capillaries allow liquid bridging at lower %RH. Fine crystals in a powder bed can increase the water sorption of sugars and contribute to caking by acting as binders between crystals (Rogé and Mathlouthi 2003). Amorphous lactose levels added at low concentration (0.5% w/w) considerably modified the shape of the sorption isotherm of α-lactose monohydrate at low water activities (Bronlund and Paterson 2004). Mechanical-induced caking results from powder consolidation due to mechanical pressure that typically occurs under warehouse storage conditions, where flexible intermediate bulk containers and loaded pallets are stacked on top of each other.

3.4.3.3 Stickiness and Caking of Milk Powders

Excess stickiness gives rise to product loss, fouling of process surfaces, risk of compromising product quality because of increased residence of deposits, which may be dislodged and re-entrained with product flow, along with the dreaded risk of spontaneous combustion within the drying chamber (Ozmen and Langrish 2002). The study of stickiness in spray-drying has followed two lines of investigation: (1) use of empirical-based methods for measuring stickiness and (2) understanding

stickiness in the context of the science of soft matter (particularly, with reference to glass transition phenomena) (O'Callaghan and Hogan 2013; Paterson et al. 2005). X-ray photoelectron spectroscopy, energy-dispersive X-ray spectroscopy and atomic force microscopy are among the list of modern advanced analytical tools used to study the influence of product formulation on stickiness using empirical approaches which have the potential to validate mechanistic models (Murrieta-Pazos et al. 2012a, b; Foerster et al. 2016).

Stickiness of powders arises from plasticisation of particle surfaces. Adsorbed water in contact with the surface of powder particles has a plasticising effect by lowering T_g and ultimately reducing viscosity. Direct methods for measuring stickiness are based on the use of physical indices such as resistance to shear, viscosity and optical properties (Boonyai et al. 2004; Schuck et al. 2006). T_g can be correlated indirectly with stickiness. In fact, Ozmen and Langrish (2002) suggested that T_g determined by DSC is virtually the same as the sticky point temperature measured using a thermo-mechanical test. Using a blow tester-based method, Paterson et al. (2005) applied the parameter $(T - T_g)$ to characterise the rate of stickiness development for a range of conditions (37–67 °C and 0.15–0.35 a_w) and found that, at a given $T - T_g$ value, the level of stickiness increased linearly with time. Paterson et al. (2007) also showed that stickiness curves for powders lie above and parallel to the T_g curve of amorphous lactose, In this way, the authors established a clear relationship between powder stickiness and T_g of lactose.

Fluidisation of partially crystallised whey powder at temperatures above T_g has been shown to have potential industrial applications especially when a processing aid such as partial blending of fully crystallised whey powder can help to support continued fluidisation, i.e. further fluidised bed drying, without sticking (Nijdam et al. 2008). The extra time needed for this process adaptation to achieve more complete crystallisation may prove challenging within the confines of a continuous drying process. In any case, instant properties of final powders may also be promoted by inducing lactose plasticisation at temperatures exceeding T_g, in conjunction with the utilisation of fines (Nijdam et al. 2008).

Increasing the proportion of protein to lactose in powders decreased their susceptibility to sticking, due to the combined influence on both T_g and $T - T_g$ (Hogan and O'Callaghan 2010). Using skim milk as a base, the authors used permeate addition and ultrafiltration to generate powders (low protein SMP and MPCs) ranging in protein content from 15% to 85%. Increased protein content in the powders had a relatively minor effect on T_g, but significantly increased $T - T_g$. That work showed that the rate at which amorphous lactose becomes sticky is delayed by the presence of proteins, which compete for available moisture (Hogan and O'Callaghan 2010). Shrestha et al. (2007) showed that dilution of the protein content of skim milk from 34% to 8.5% protein did not affect T_g after sorption by the powders at different humidities, thus confirming that lactose had the dominant effect on T_g of all powders.

Powders containing hydrolysed whey proteins were more susceptible to sticking compared to those containing intact proteins, largely because the surface coverage of powder particles by proteins or peptides was lower relative to lactose in powders containing hydrolysed WP (Hogan and O'Callaghan 2013). A greater degree of

whey protein hydrolysis was shown to further delay the time-dependent onset of lactose crystallisation, which also affected the relaxation behaviour of whey protein/lactose powders during glass-rubber transition (T_{gr}) analysis and altered the rate at which lactose underwent viscous flow behaviour (Hogan and O'Callaghan 2013).

Caking follows when inter-particle liquid bridges provide an environment for dissolution of milk components and the resulting lactose crystallisation transforms these interfaces into solid bridges (Thomas et al. 2004). Sticking is typically a problem encountered during drying, while caking is more prevalent during subsequent storage of powder (Schuck et al. 2005). Stickiness and caking phenomena in whole and skim milk powders differ due primarily to dissimilarities in their surface compositions (Özkan et al. 2002). The melting behaviour and high fat surface coverage of whole milk powders suggest that stickiness is induced by fat and occurs at a lower temperature than for skim milk powders. The higher temperatures at which caking occurs in skim milk powder is influenced by $T > T_g$, i.e. a lactose-based mechanism. The transition to the crystalline phase of lactose facilitated the formation of strong junctions between skim milk powder particles (Özkan et al. 2002).

A close relationship would appear to exist between the measured sticking point temperature of skim milk powder and that of lactose (Boonyai et al. 2004). However, the approaches used to correlate the sticky point and T_g are not very precise. The sticking point temperature may be taken at 20 °C above T_g for simple estimations, but this may not be sufficient when spray-drying some products at high temperatures where precise process control is generally required. A shift in temperature of a few degrees or small increases in RH may result in sticking.

A device developed by Hennigs et al. (2001) was used to measure sticking point temperature of SMP. This technique measured electric resistance of a stirrer in contact with powder, which was then translated into stickiness behaviour. At the sticking point temperature, the voltage increased sharply and the stirrer consequently stopped. It was found that the sticking point temperature of SMP could be predicted precisely from the T_g of lactose, using suitable curvature parameters. A limitation of the method is the risk of void formation during movement of the powder by the stirrer.

The caking temperature of powders (T_c) may be linked to variations in T_g. Both models share the same curvature index for lactose, emphasising that lactose is mainly responsible for the stickiness of milk powders. The T_c model is distinguished from the T_g model by inclusion of a coefficient of temperature difference (d) (Hennigs et al. 2001).

$$T_g = \frac{W_L \cdot T_g(l) + k \cdot W_w \cdot T_g(w)}{W_L + k \cdot W_w},$$

$$T_c = \frac{T_{g(mp)} + k \cdot X \cdot T_{g(w)}}{1 + k \cdot X} + d$$

where T_g is the glass transition temperature of lactose; W_L and W_w are the weight fractions of lactose and water; k is the curvature index (= 7.4 for pure lactose); $T_{g(l)}$

is the glass transition temperature of anhydrous lactose; $T_{g(w)}$ is the glass transition temperature of water; T_c is the caking temperature; $T_{g(mp)}$ is the glass transition temperature of anhydrous skim milk powder; X is the ratio of water to dry powder; and d is a coefficient (= 23.3 K for skim milk powder).

Schuck et al. (2005) described a sticking and caking sensitivity index (SCSI) ranging in values from 0 to 10, which they claim may be used to anticipate powder behaviour during drying and storage—the more favourable situation being an SCSI ≤ 4 (i.e. no sticking and/or no caking), while SCSI ≥ 6 suggests a high to very high risk of sticking or caking. Computation of the SCSI takes into consideration powder characteristics such as powder temperature (T), a_w, $T - T_g$, and changes in heat capacity during glass transition (ΔC_p). An increase in $T - T_g$ reflects an increase in the rate of physico-chemical changes such as thermoplasticity, crystallisation and Maillard reaction in the product. A points system is allocated to these physical characteristics according to Table 3.3.

Stickiness behaviour is also proving to be an important parameter in the latest efforts to model particle dehydration during spray-drying. Simulation of spray-drying processes is being used increasingly to facilitate more efficient dryer operation and implementation of advanced control systems. A particular challenge occurs when considering the drying profile of a range of particles. One of the difficulties is to be able to predict surface moisture content and temperature, which are important variables for determining whether the T_g of surface lactose has been exceeded and the likelihood of the particles becoming sticky (Nijdam and Langrish 2006). Hogan et al. (2009) developed a fluidised bed apparatus capable of differentiating stickiness behaviour as a function of powder composition. The fluidising apparatus was used to suspend powders in an air stream at constant temperature, but with increasing humidity up to the point where particle stickiness prevented continued fluidisation. Powder stickiness proved to be related to that proportion of lactose content in the amorphous state and occurred at a specific temperature above T_g, i.e. $T - T_g$.

The largely amorphous state of lactose in skim milk powder (SMP) helps to explain its greater stickiness compared to that of semi-crystallised whey permeate (WPP) and demineralised whey powders (DWP) (O'Donoghue et al. 2019). Even the relatively small amount of bulk protein (~11%) present in the latter (DWP) appears to contribute to reduced stickiness in contrast to that of whey permeate. It is

Table 3.3 Calculation of Stickiness and Caking Stability Index (SCSI)[a] according to Schuck et al. (2005)

$T - T_g$ (°C)	Number of points	ΔC_p (J/g/°C)	Number of points
≤5	0	<0.1	0
>5; ≤10	1	≥0.1; <0.2	1
>10; ≤15	2	≥0.2; <0.3	2
>15; ≤20	3	≥0.3; <0.4	3
>20; ≤30	4	≥0.4; <0.5	4
>30	5	≥0.5	5

[a] SCSI = Number of points for $[T - T_g]$ + Number of points for $[\Delta C_p]$

probable that competition for water, driven by the proteins' preferential sorption behaviour in DWP, makes water less available for plasticisation of amorphous lactose. In any case, a distinction needs to be made between powder surface and bulk compositions, as the two rarely coincide.

X-ray photoelectron spectroscopy (XPS) analysis has put the spotlight on the role of particle surface composition and its influence on the physico-chemical functionality of dairy ingredient powders (Gaiani et al. 2006; Kelly et al. 2015). One important insight resulting from XPS analysis is the apparent over-expression of fat on the surface of powder particles relative to that of the bulk. This is particularly surprising given that a bulk powder containing only residual lipid concentrations can have a relatively high concentration of surface fat when measured by XPS. Previously, Kim et al. (2003) suggested that some sort of solid/solute segregation must occur during spray-drying, in particular in the period leading up to the formation of a solid crust. Drying SMP with lower protein contents (range: 34–8.5%) caused preferential migration of proteins to the surface of powder particles while lactose remained in the bulk phase (Shrestha et al. 2007); the lower protein powders demonstrated increased water adsorption during sorption studies and lower a_w necessary for lactose crystallisation. As a general rule, lipids and proteins are preferentially represented at the surface of powder particle, while lactose is concentrated more towards the core (Gaiani et al. 2010). However, less lactose is typically observed at the surface of powders spray-dried at lower outlet air temperatures. Whey-protein-containing powders also had a higher representation of surface lipids than equivalent casein-containing powders (Gaiani et al. 2010).

Microscopic observations showed that WPP and DWP contained both larger lactose crystals and smaller amorphous particles. The bulk composition of SMP did not vary with particle size. The surface composition of the smallest SMP fraction (<75 μm) had lower protein (9%) and higher fat (5%) coverage compared to the non-fractionated powders. Smaller particles were more susceptible to sticking in all powders. Sieve fractionation (O'Donoghue et al. 2019) showed that smaller particles, in the size range 75–150 μm, were more susceptible to sticking in all three powders (SMP, DWP and WPP) tested, irrespective of the background degree of crystallinity for DWP and WPP powders. This is consistent with the occurrence of a higher proportion of amorphous lactose at the surface of smaller particles.

Surface fat also contributes to powder stickiness, as shown by the significant improvements in stickiness behaviour for all size fractions of DWP and SMP powders following organic solvent washing (O'Donoghue et al. 2019). As an example, the DWP 75–150 μm particle size fraction containing ~26% surface fat benefited most from solvent washing, in terms of stickiness reduction, compared to the equivalent SMP fraction with 11% surface fat.

As a complementary technique to other stickiness measurement methods, dynamic mechanical analysis (DMA) provides more detailed information on viscoelastic changes occurring during stickiness development (O'Donoghue et al. 2020). For lower protein powders (WPC 20 and 35), the mechanical Tα (α-relaxation temperature) determined from the storage modulus of the DMA (Tα onset) were in good agreement with the results obtained using the fluidisation method of Hogan

et al. (2009), whereas, for higher protein powders (WPC 50 and 65), the fluidisation results compared better to the loss modulus results generated by the DMA (Tα peak). In the case of the lower protein (<45% w/w) whey powders, stickiness occurred following a reduction in powder stiffness while, for whey protein concentrates of >45% protein (w/w), the initial reduction in powder stiffness is followed by an increase in viscosity (O'Donoghue et al. 2020).

3.4.4 Process-Based Functionalisation of Lactose-Containing Powders

3.4.4.1 Instantisation/Agglomeration

Regular spray-dried milk powders do not disperse readily or completely when poured onto water. In order to improve reconstitution properties, agglomeration was developed as a process whereby larger powder particles (particle clusters) are created by the adhesion of smaller to larger particles—the smaller particles limit the instant dispersion of powders in water. The resulting more open powder structure allows greater penetration of water in the course of subsequent wetting and dispersing.

The key to agglomeration in practice is to create an environment for adequate mixing of 'wet' (partially dried, sticky) powder particles with recycled dried fines (i.e. fine powder particles collected by cyclonic and/or fluidised bed separation) so that the smaller particles adhere to the larger ones and form clusters around them. The surface properties of partially dried powder in the wet zone of the spray-drying chamber influence the nature of particle bridges that may be formed. If the starting material is a dried powder, then partial rewetting and heating in excess of T_g will provide a viscous surface for adhesion of adjacent particles and formation of strong bridges (Bhandari and Howes 1999). There is a dependence on the presence of fat and amorphous lactose to provide the necessary stickiness to facilitate agglomeration. Nijdam and Langrish (2006) expressed a concern that inadequate amounts of lactose may be present because of competitive displacement by protein. However, since most agglomeration now takes place in the primary drying chamber (*Straight-through drying/agglomeration process*), it is likely that most agglomerating particles in this zone of the drier are sufficiently wet and have a surface composition more typical of the bulk matrix before component over-expression occurs during the final stages of particle dehydration.

Later, surface properties will also prevail during reconstitution along with other factors, such as the interplay between liquid (water), gaseous (air between particles) and solid phases (powder particles) during powder wetting. Efficient wettability and dispersibility are essential in order to prevent a viscous layer forming at the interface around grouped particles, which stalls dissolution of the powder in water.

Powder agglomeration may be regarded as intentional caking as a result of forced interaction and compaction under controlled conditions (Listiohadi et al. 2005).

Table 3.4 Influence of spray atomisation/fines return-mediated agglomerate structures on the physical properties of agglomerated particles (adapted from Písecký 1997)

	Agglomerate structure			
	'Onion-'	'Raspberry-'	'*Compact grape-*'[a]	'Loose grape-'
Physical property	- - - - - ▶	- - - - - ▶	- - - - - ▶	- - - - - ▶
Particle moisture at collision	High		Low	
Mechanical stability	High		Low	
Bulk density (no attrition)	High		Low	
Bulk density (after attrition)	High	Low	High	
Slowly dispersible particles	Many		*Few*	
Dispersibility (after attrition)	Poor	Good	Poor	

[a] Optimum agglomerate structure

In this way, particles are assembled into larger aggregates in which the original particles can still be distinguished (Cuq et al. 2013). Some insights into the effects of force during the recycling of fines when spray-drying can be gleaned from the descriptions of Písecký (1997). When fines return is positioned close to the spray atomiser, considerable penetration of wet primary particles occurs, which in turn, become covered by concentrate from the incoming spray. These newly formed agglomerates possess high moisture, plasticity and stickiness. On the other hand, if the fines return is positioned at a distance from the atomiser, less compact agglomerates displaying 'raspberry-' and 'grape-like' microstructures, are formed. The objective is to strive as far as possible to achieve a 'compact grape' structure as an optimal process condition where the powder has simultaneously good instant properties and sufficient mechanical strength to withstand the rigours of subsequent handling and packaging (Table 3.4). 'Onion-structured' agglomerates have also been described. These have high mechanical strength, bulk density and appear as slowly dispersible particles on reconstitution.

Two distinct processes for agglomerating milk powders are used in practice (Wulff 1980), the straight-through method (accomplished during spray-drying) and the rewet method (using powder that has already been prepared). Both processes exploit similar principles of instantisation—(1) wetting of particle surfaces (by steam, water or a mixture in the case of the rewet method), (2) agglomerating, (3) drying or redrying (rewet), (4) cooling and (5) classifying according to particle size in order to remove particles that are too large or too small. In a laboratory-based simulation of a basic rewet agglomeration of skim milk powder in which water was sprayed onto a batch conical fluidised bed, Turchiuli et al. (2013) observed that fluidising air flow rate was a key factor in promoting particle agglomerate formation up to the desired maximum size by which time a more uniform distribution of powder moisture was obtained throughout the fluidised bed. Given the interdependence between product properties and spray drier design/operating conditions, simulation tools are now increasingly used in order to predict the regions for coalescence and agglomeration within a drier chamber (Malafronte et al. 2015). By combining a validated distributed-parameter model for predicting single particle drying with a

CFD simulation model of an 8-mre pilot dryer to investigate drying kinetics of skim milk powders, the authors (Malafronte et al. 2015) identified the need to determine accurate water diffusivity values and sticky conditions during skim milk spray drier modelling in an effort to establish preconditions for coalescence and agglomeration.

3.4.4.2 Flow Properties

Powder transport systems in a modern dairy plant rely on both pneumatic and mechanical conveying techniques to move powder from the moment it is discharged from the spray drier to intermediate storage devices such as feed hoppers and eventually to powder silos. Such bulk handing systems comprise of a ducting network and valves, which link process plant, powder storage and packaging/filling machinery.

Shear cell techniques have been traditionally used to characterise powder flow properties. The effect of particle size and free-fat content on the flowability of experimentally produced skim milk powder (SMP), whole milk powder (WMP) and high fat milk powder (HFP, 73% fat) showed that both WMP and HFP powders were cohesive (Fitzpatrick et al. 2004). This cohesiveness increased when the particle sizes of WMP and SMP were decreased. Varying free-fat content in 26% fat milk powders was shown to have had no major effect on cohesion at 20 °C.

Both particle size and shape significantly affected the flow characteristics of pharmaceutical grade lactose over a wide range of stress conditions as measured by the Freeman FT4 powder rheometer—an instrument which is designed to measure functional attributes such as flow energy, aerated flow energy, shear properties, precision bulk density, compressibility and permeability (Fu et al. 2020). Pharma grade lactose is a highly pure form of lactose that is produced in crystal form before it is finely milled.

Olaleye et al. (2019) studied the flow properties of fat-filled milk powder (FFMP) fines with an average particle size of 94 μm in a pneumatic conveying rig by measuring pressure drop, powder deposition and by using an optical technique to measure the dynamics (probability densities) of local particle volume fractions as a function of operating conditions. At low air velocities, cohesive dairy powders such as FFMP re-agglomerated readily after 90° bends in the powder pipeline and then deposited at the bottom of the horizontal pipe. A higher powder loading ratio could be tolerated by operating at higher gas velocities with intermittent dispersion of particles and less particle deposition occurring (Olaleye et al. 2019).

3.4.4.3 Maillard Reactions

Non-enzymatic browning (NEB) of milk powders during prolonged storage has been associated with reactions taking place between proteins and lactose functioning as a reducing sugar (Thomas et al. 2004). Early Maillard reactions (EMR) develop more readily in WPCs with higher lactose contents (WPC-35 to WPC-50) than those lower in lactose (Morgan et al. 2005). A comparison of skim milk powder

and WPC with similar protein and lactose contents (35% and 51%, respectively) showed that the development of EMR was similar for both, except at prolonged heating times where a break in the progress of the amino-sugar reactions was observed for SMP (Morgan et al. 2005). These studies were undertaken under accelerated storage conditions at 60 °C and it is thought that differences in molecular mobility between WPC and SMP may occur at this temperature.

Miao and Roos (2006) reported that NEB kinetics in freeze- and spray-dried powders were affected by both composition and drying method and that crystallisation of lactose had a direct effect on NEB reactions through the release of sorbed moisture.

3.4.4.4 An Innovative Process for the Production of Permeate Powders

Tanguy et al. (2017) proposed a novel approach for the production of lactose-dominant permeate powders without the use of energy-intensive spray driers. The key features include an 'over-concentration step' using scraped-surface evaporators to cope with the increased viscosity as total solids increase from 60% to 80% w/w dry matter (DM), followed by granulation of the over-concentrate with recycled (pre-dried) powder to further increase DM to as high as 97%. The key parameters relating to T_g suggest the permeate powders produced by this process will store better and be less sticky (Tanguy et al. 2017).

3.4.5 Ingredient Applications Where the Role of Lactose Is Emphasised

3.4.5.1 Milk Protein Standardisation

The adoption in 1999 of a new Codex Standard for Milk Powders and Cream Powder, *CODEX STAN 207-1999*, provides for the protein content of milk intended for powder processing to be standardised to a minimum prescribed limit. In practical terms, this allows lactose to be added to manufacturing milk as a means of adjusting the protein in non-fat solids to not less than 34%. The ingredients permitted for this purpose are restricted to edible-grade lactose, milk permeate, i.e. permeate produced by ultrafiltration of milk, or mixtures of these.

The figure of 34% was reached after the International Dairy Federation (IDF) conducted surveys of protein and solids non-fat (SNF) levels of milks in individual countries (Higgins et al. 1995). With some countries experiencing variations in protein as a percentage of SNF in the range 34–42%, *CODEX STAN 207-1999* avoids instances where milk powders with a low level of protein would not be recognised in international trade. On the other hand, considerable quantities of lactose are required by those countries whose milk supplies contain higher milk protein levels than their competitors and, thus, have an opportunity to standardise protein content

in line with the terms of *the standard*. Thus, in virtually all instances, downward adjustment of milk protein content is required for standardisation using permitted lactose-containing ingredients in order to produce skim milk powder containing 34% m/m protein (minimum milk protein in milk solids-non-fat).

The consequences of milk protein standardisation on milk quality were judged by IDF experts at the time of submission to Codex to be minimal. It was felt that the practice of standardising the fat content of milk had already been in place since the early 1900s without any negative consequence. However, it is to be expected that, in the course of time, the diversity of functional applications encountered in the market for milk powders is certain to encounter some subtle change in functionality as a result of protein standardisation. Interesting differences in heat stability characteristics were observed following the blending of high-protein milk protein concentrate powder (MPC) [80% protein (w/w)] with either lactose or milk permeate to match the protein equivalent of skim milk powder (~35%) (Aydogdu et al. 2021). It would appear that the milk permeate containing soluble milk salts was superior to lactose in improving the heat stability of the protein-standardised MPC ingredient by restoring the colloidal/soluble salt milk balance typically present in milk. The considerably higher Ca^{2+} concentration of the milk permeate-based reformulated MPC, compared to that of the lactose, might have suggested a greater destabilising effect during exposure to the high temperature of the heat stability assay. However, the integrity of the colloidal/soluble salt system in the presence of casein would appear to have had an overriding effect.

3.4.5.2 Infant Milk Formula

As the lactose content of human milk is significantly higher than that of bovine milk, infant milk formula manufacturers use edible-grade lactose and whey powders as ingredients during processing in order to 'humanise' their product formulations. Lactose loading of formulations in this instance is driven by the necessity of nutritional demand, and in processing terms is facilitated by sourcing demineralised and partially demineralised whey powders (containing typically 70%, w/w, lactose). Such dairy ingredients provide, in addition to lactose, minerals and other desired key minor constituents. However, a greater challenge is faced when drying powders of such high lactose content.

During an investigation of the effects of temperature on the causes of surface caking (T_{sc}) and advanced caking (T_{ac}) of several dairy-based infant formula powders, Chuy and Labuza (1994) identified that differences in viscous flow time constants were responsible for T_{ac} and T_{sc} being greater than T_g and that the presence of increasing amounts of low-molecular-weight carbohydrate (corn syrup solids or maltodextrin DE 10) helped to reduce sticking. As expected, stability towards collapse and sticking decreased in the presence of increasing amounts of low-molecular-weight carbohydrate. The water activity at which $T_{storage} = T_g$ was determined to be a good predictor of the %RH at which caking collapse commences (Chuy and Labuza 1994).

When the protein content of infant formulae was increased from 6.68 to 11.88 g protein 100 g^{-1} powder particle surface composition became altered as a function of storage RH (McCarthy et al. 2013). The lowest protein content reduced T_g and resulted in increased free fat levels while high %RH promoted time-dependent lactose crystallisation. Powder surface composition was largely unaffected during spray-drying of IMF concentrates at different drier inlet and outlet temperatures. However, differences in powder physical characteristics including T_g were apparent in freshly produced powders (Masum et al. 2020a). These differences, arising from the initial moisture contents, were amplified when the powders were subsequently stored at 22 or 40 °C at 54% RH. Increased moisture uptake was linked to lactose crystallisation, greater surface fat formation, poorer solubility and more extensive caking (Masum et al. 2020b). When lactose was substituted with maltodextrin, at levels up to a maximum of 30%, the resulting IMF powders were mainly stable throughout storage for 180 days at 23% RH—the stabilising effect of the maltodextrin resulted from an increase in T_g and a decrease in the impetus towards lactose crystallisation (Masum et al. 2020b) without compromising emulsion stability of the pre-mix apart from some increase in apparent viscosity after evaporation (Masum et al. 2019). Substitution of lactose in infant formula mixes with 18–24% pre-crystallised lactose (PCL) successfully lowered surface free fat, particle size and colour changes under all storage temperatures and RH tested. The presence of maltodextrin appeared to work in synergy with PCL in order to maintain powder stability throughout the storage period (Saxena et al. 2021). A further observation by the same research group highlights a negative correlation between surface fat and lactose crystallisation along with what appears to be a preferential migration of unsaturated C18:1 and C18:2 fatty acids in the surface fat of those powders with higher levels of lactose crystallisation (Saxena et al. 2019).

The selection of drying temperatures appropriate for production of IMF powder may now be predicted by direct analysis of a fresh sample of concentrate using a desorption drying assay to determine T_g according to its total solid content, viscosity and average evaporation rate (Zhu et al. 2011). The assay which is supported by the SD2P® software enables the concentrate's behaviour during spray-drying to be predicted on the basis of T_g.

A quality defect known as 'white fleck' formation occasionally appears during reconstitution of IMF. The term 'white fleck' refers to the appearance of what may seem like undissolved particles or, alternatively, aggregates of destabilised colloidal constituents. Their appearance on baby feeding bottles is unsightly and may cause unnecessary concern. The scientific reason for the formation of 'white flecks' is not fully understood apart from tentatively conceding that some form of protein instability may be at play. Consequently, much focus has been on the functional status of the ingredients used and the effects of unit process treatments applied during preparation of the concentrates before drying. A recent research paper which has probed this phenomenon sheds some light on the role of lactose. Toikkanen et al. (2018) scrutinised the incidence of 'white fleck' formation in spray-dried infant milk formula and traced the origin of two particular fleck shapes, i.e. round and sharp-edged, back to the drying process and powder storage, respectively. The occurrence of sharp-edged flecks reflected the progression of lactose crystallisation during IMF

storage under unfavourable humidity conditions. Addition of a low molecular weight emulsifier along with a reduction in fat to protein ratio was advised by the authors in order to minimise fat clustering.

Kondor and Hogan (2017) evaluated inverse gas chromatography (IGC) as a tool to analyse surface energy of IMF powders in conjunction with other methods of functional characterisation. A slightly higher T_g in IMF powders, as determined by IGC with reference to pure lactose, suggests a surface dominated by lactose even in the presence of 30% bulk fat. Surface heterogeneity appeared to be a better indicator of functional behaviour than total surface energy. IGC proved to be a useful complementary technique for chemical and structural analysis of milk powders and allows improved insight into the contribution of surface and bulk factors to functionality (Kondor and Hogan 2017).

Early stage infant milk formula simulates the gross composition of human milk which means that higher concentrations of lactose are incorporated during formulation and processing. Murphy et al. (2014) decoupled such a model formulation in order to evaluate key processing effects starting out at a binary level involving lactose with other individual ingredients. Lactose added post-heat treatment had a dramatic effect in lowering the viscosity of WPI. An 8.0% w/w WPI solution had twice the viscosity of the same protein solution when combined with added lactose to increase its dry matter to 47.9% w/w, the effect being attributed to delayed protein conformational changes.

3.4.5.3 Chocolate

Lactose may be generally regarded as having a secondary role in milk chocolate, its presence being largely opportunistic by virtue of being a constituent of the milk powder used. However, attempts at explaining variations in chocolate properties, especially viscosity, have led to greater scrutiny of the functional quality of such dairy ingredients. When Aguilar and Ziegler (1995) partially substituted sucrose in milk chocolate with lactose provided by whole milk powder, the physical state of the lactose had important effects on chocolate properties; higher concentrations of amorphous lactose decreased chocolate viscosity, increased particle size of chocolate mass post-refining and reduced the requirement for surface active agents to achieve the desired Casson yield value, while increasing content of crystalline lactose had the opposite effect.

Spray-dried whole milk powder (WMP) with its relatively low levels of free fat is sub-optimal as a dairy ingredient for use in chocolate production compared to roller-dried WMP. However, adapting our knowledge of lactose behaviour may now be the basis of a technological approach to circumventing this problem. Baechler et al. (2005) showed that the phase diagram of whole milk powder may be exploited to achieve greater functionality of whole milk powder (WMP) for use in chocolate. They demonstrated that careful control of the a_w of WMP before heating at 90 °C for 70 min could bring about a desired release of free fat (>70%), while maintaining WMP in powder form and avoiding the induction of browning. This release of free fat onto the surface of WMP was correlated with increased lactose crystallisation in the β-form.

3.4.5.4 Role of Lactose as Wall Material During Ingredient Microencapsulation by Spray-Drying

Microencapsulation involves the optimisation of ingredient formulation and process technology (homogenisation, spray-drying) to produce stable emulsions that may be spray-dried to yield powder particles with defined characteristics. The protection of an active ingredient (e.g. oils/fats susceptible to oxidation, probiotics, bioactives, vitamins, etc.) either during processing, delivery or subsequent storage is usually the objective (Maciel et al. 2014; Maher et al. 2014; Mohammed et al. 2020). The process differs from conventional whole milk powder production in that emulsion composition and formation are finely tuned to take advantage of structural changes that occur in powder particles during spray-drying. The selection of 'wall' materials and the manner in which they solidify during powder particle dehydration is key to determining the amount of free fat formed and/or the encapsulation efficiency of functional ingredients. The porosity of such 'wall' materials may be critical when protecting sensitive materials such as fish oils (Keogh et al. 2001; Hogan et al. 2003). While protein is the principal emulsifying agent at the concentrate preparation stage, the presence of carbohydrate is additionally important during the subsequent spray-drying of whey protein-based emulsion. Amorphous lactose is recognised as the main encapsulant of milk fat in WMP and spray-dried dairy like emulsions made with WPC and WPI (Buma 1971; Young et al. 1993; Oliver and Augustin 2009; Li et al. 2017). Lactose is proving useful in microencapsulation studies aimed at optimisation of ingredient formulation and process technology (homogenisation; spray-drying) to yield spray-dried powder particles with defined characteristics in terms of active ingredient protection (Kelly 2007). In its amorphous state, lactose acts as a hydrophilic filler or sealant that significantly limits diffusion of solvent (an indicator of encapsulation efficiency) through the powder wall (Young et al. 1993). Crystalline lactose, on the other hand, reduces microencapsulation efficiency by facilitating greater solvent diffusion.

The presence of lactose is also important in obtaining complete encapsulation of fat during spray-drying of milk protein-stabilised emulsions (Fäldt and Bergansthål 1995). The normally hydrated protein-based interfacial surface film in an emulsion is believed to shrink because of the loss of water during drying (Fäldt and Bergansthål 1995). However, the presence of lactose may replace water to some extent and keep the protein solubilised after drying to reduce shrinkage. Thus, the stability of a sodium caseinate film on powder surfaces is increased, and less fat leaks out onto the powder surface during the drying process (Fäldt and Bergansthål 1995). Encapsulation systems using mixtures of lactose, maltodextrin and WPI mixtures as wall materials during spray-drying of ethyl butyrate as core materials showed that a 4:1 mixture of lactose:WPI gave optimum encapsulation efficiency while the inclusion of maltodextrin restricted lactose crystallisation and limited flavour release during storage (Li et al. 2017).

The effect of fat globule size (FGS) on lactose behaviour during the spray-drying of nanoemulsions has shown that the resulting powders crystallised more quickly than those prepared from conventional emulsions (Maher et al. 2015). It is believed

that reduced protein in the continuous phase with lactose contributed to the observed increase in crystallisation rate. Elongated lactose crystals formed initially at the surface, but progressively developed within the particle matrix when stored at 55% RH (Maher et al. 2015).

3.4.6 Conclusion

Lactose is an important ingredient in a wide range of spray-dried milk powders and contributes to powder functionality in a number of ways. During the manufacture and storage of spray-dried powders, lactose can exist as solute, amorphous glass, so-called viscous 'rubber' and crystalline material. Its varied physical forms are determined by concentration, temperature and time, and comprise a mix of both stable and unstable forms—with wide-ranging implications for powder production. Lactose influences liquid dispersions (viscosity and solids content), atomisation behaviour (agglomeration), drying efficiency (sticking), powder quality (structure, size, flow, redispersion behaviour) and storage/distribution stability (moisture sorption, crystallisation, caking). Its association with water is essential to its behaviour and determines both its kinetic and thermodynamic fates. Lactose acts as viscosity regulator, inert filler, encapsulating agent, protein stabiliser and standardising agent and, although of lower economic value compared to proteins and milk fat, its physical properties make it a key determinant of powder stability and quality.

The state transitions associated with lactose mean that an adequate understanding of its material properties are necessary to maintain lactose in the physical state required and to ensure that effective strategies are implemented to account for its many contributions to processing and thereby maximise efficiencies during production of spray-dried milk powders.

3.5 Lactose-Free Milk Products

P. M. Kelly and S. A. Hogan
Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

3.5.1 Introduction

Lactose is the main carbohydrate source in milk and has the specific function of providing energy for the newborn during the early stages of its life. Its nutritional efficacy for humans is reliant on the presence of lactase (β-galactosidase) enzyme in the lining of the human gut wall in order to cleave the disaccharide into its

constituent monosaccharide components, i.e. glucose and galactose, to facilitate subsequent digestion and absorption. Once the weaning stage is reached, an infant's capacity to produce lactase diminishes, which raises a question as to how humans subsequently cope with continuing ingestion of milk since both lactase persistence (LP) and non-persistence (LNP) are common phenotypes of healthy humans (Misselwitz et al. 2019). Anthropological studies suggest that human digestive systems adapted over time as early hunter-gatherers consumed milk drawn from domesticated lactating mammals.

The lactase genetic region in the human genome is among the strongest that has been shaped by human evolution within the last 10,000 years, with LP providing a selective advantage of up to 4–5% per generation. However, Silanikove et al. (2015) regard the demarcation around weaning as significant in defining the extent of potential intolerance prevailing among the world's human population relative to the ingestion of dietary lactose. Lactose intolerance (LI) is defined as the onset of abdominal symptoms such as abdominal pain, bloating and diarrhoea after milk ingestion by a lactose mal-absorbing (LM) individual (Misselwitz et al. 2019). Based on such a broad range of gastrointestinal symptoms, it is possible that 'self-diagnosing' consumers may mistakenly classify themselves as LI and either opt out of milk consumption or, alternatively, commit to a lactose-free dairy food-based diet. Misselwitz et al. (2019) noted that the association between self-reported LI, objective findings and clinical outcomes of dietary intervention is variable, taking into account such options as a low-lactose consumption regime, lactase supplementation and potential colonic adaptation by prebiotics. The authors speculated that these modest effects may have been due to conflation with background factors such as ingestion of other poorly absorbed carbohydrates. Previously, Szilagyi (2015) reported that regular consumption of dairy foods by LNP individuals over a prolonged period of time not alone improves aspects of LI symptoms, but that LNPs may naturally reach the stage of becoming asymptomatic. Furthermore, Szilagyi (2015) speculated that colonic adaptation in the form of a prebiotic effect by the microbiome may be taking place as a result of lactose ingestion, thus allowing LNPs to consume more dairy foods. With scientific hindsight, it appears that early agrarian practices that tapped into potential of 'wild' fermentation by native lactic acid producing bacteria not alone facilitated milk preservation by souring, but very likely moderated lactose digestibility through a reduction in its content, as well as modulation of the composition and functional behaviour of the human intestinal microflora following consumption.

Traditionally, lactose-intolerant individuals had little choice but to reduce milk intake or avoid its consumption altogether. Some dairy products could be tolerated such as those produced by fermentation and/or reduced in lactose content by virtue of their manufacturing processes, e.g. cheese. Earlier research suggests that acidophilus milk produced by fermentation using *Lactobacillus acidophilus*, a lactic acid bacterium (LAB) that is capable of fermenting milk to <pH 5.0, is better digested by the lactose intolerant. The bacterial conversion of lactose to lactic acid provides part explanation for this digestibility improvement. Lactic acid conversion levels of 1–2% are achievable which make acidophilus milk quite sharp to taste.

Considerably higher levels of lactose cleavage into its constituent monosaccharides occur in yogurt by its specific fermenting cultures, except that these same cultures metabolise galactose poorly (Anbukkarasi et al. 2014). Previously, Alm (1982) observed a 50% reduction in lactose content in 11-day-old stored yogurt and a concomitant increase in galactose levels from trace to 1.3 g/100 g. Smaller decreases in lactose, of the order to 20–30%, occurred in the case of other fermented milks such as buttermilk, kefir, and ropy milk. Ingestion of fermented milks also contributes additional mitigating factors to support easier digestion of lactose, e.g. the inherent lactase activity of yogurt bacteria and the stimulation of lactase activity of the intestinal mucosa by yogurt itself. The rate of emptying of the stomach contents into the duodenum is also delayed following intake of fermented milks, so that extra substrate contact time favours greater lactose breakdown (Walstra et al. 1999b). Alm (1982) found that eight lactose-intolerant individuals did not experience any symptoms of abdominal distress following consumption of 500 mL of yogurt or acidophilus milk compared to the clinical symptoms following intake of an equivalent volume of low fat milk.

Misselwitz et al. (2019) emphasised the significance of the human microbiome and the capacity of the intestinal microbiota to adapt to a regular consumption of dairy. While not involving lactase upregulation, this adaptation in response to the regular intake of lactose is reflected in a reduction of physiological markers such as breath hydrogen levels and other symptoms of lactose intolerance. In Japan, where the population is 90–100% LNP, a study of healthy individuals showed a high correlation between an abundance of Bifidobacteria and dietary intake of dairy products (Kato et al. 2018). With the production of physiologically beneficial modulating compounds such as short chain fatty acids (SCFA) by the microbiota's fermentation of lactose in the large intestine, Misselwitz et al. (2019) suggest that lactose malabsorbing individuals could still benefit in health terms from the consumption of low levels of lactose where tolerated.

As a low lactose-containing dairy product, Silanikove et al. (2015) highlighted that historical developments in cheesemaking can be traced back to the Middle East and Southern Europe, where lactose intolerance was widespread at the time. Milk clotting resulting from the use of animal stomachs to store milks was an opportunistic discovery that was later explained by the action of gastrointestinal tract enzymes present in the stomach walls. The whey separation step in cheesemaking facilitates the removal of large amounts of lactose, so that the resulting cheese is substantially depleted of lactose. The make procedure of some cheese varieties, the so-called washed curd cheeses such as Gouda, includes an in-vat washing of the cheese curd following whey drainage which helps to reduce residual lactose levels further. From a fermentation perspective, the remaining starter culture activity in the cheese matrix during extended ripening of semi- and hard cheeses, in particular, will breakdown residual lactose. Thus, when considered within the context of 10,000 years of human development that coincided initially with high incidence of lactose intolerance basic innovation among different dairy herding cultures in the development of hundreds of varieties of cheeses and fermented products forged a way so that people could benefit nutritionally from dairy consumption without undue restriction of

lactose malabsorption (Silanikove et al. 2015). It is possible to speculate that this prolonged period of low level lactose exposure also afforded an opportunity to build up greater lactose tolerance amongst LNP individuals.

3.5.2 *Pioneering Market Launch of Low-Lactose Dairy Products: A Case Study*

A 17–18% incidence of lactose intolerance among Finnish people has led to much innovation by their major dairy company, Valio Ltd, in the development of low-lactose and lactose-free dairy products. Finland also has a high milk per capita consumption, so that the promotion of low-lactose based dairy product enabled consumers to maintain their high milk intake without triggering intolerance to lactose. In 1981, Valio successfully introduced *hy*drolysed *lac*tose skim milk powder (SMP) under the brand name HYLA® on the Finnish market, based on the simple addition of lactase to skim milk at the outset of the SMP manufacturing process. This was followed shortly after by HYLA UHT milk and other hydrolysed lactose-containing dairy derivatives. One shortcoming is that milks hydrolysed by incubation with added soluble β-galactosidase are slightly sweeter due to the effect of glucose release. This is tolerable in the case of milk powders where flavour following powder reconstitution does not resemble that of the original milk. By 2001, the company has succeeded with innovative processes that surmounted the flavour distortion and launched a lactose-free milk with the taste attributes of normal milk and a residual lactose level <0.01% (Harju et al. 2012). An ad-hoc decision appears to have been subsequently taken by authorities in the adjoining Scandinavian countries to adopt this limit when defining 'lactose-free' milk. The key to achieving this lactose-free claim lay with the effectiveness of the separation technologies summarised below.

The company first proved the lactose-free milk concept by means of industrial chromatographic separation using a strong cation-exchange resin in the sodium form. An advantage of this approach is that milk minerals are retained. The process required careful supervision in order to avoid excess calcium binding that could culminate in micellar casein destruction. However, Valio researchers subsequently developed a lower cost processing approach based on membrane separation technologies already familiar within dairy processing plants. Tossavainen and Sahlstein (2003) patented a cascade-type membrane filtration system starting out with ultrafiltration (UF) of skim milk to remove lactose and serum salts in the UF permeate. Further processing by nanofiltration (NF) separates the milk salts into the permeate, from which they may then be concentrated by reverse osmosis (RO) before recombining with the original milk. As a final lactose 'polishing' step, a small amount of lactase enzyme is added to the filtered milk in order to hydrolyse residual lactose. The patent authors claim that the taste of the original milk is not altered by this process, since the small amount of glucose released does not affect flavour.

3.5.3 Technological Processes for the Production of Lactose-Free Dairy

Enzymatic cleavage by lactase (β-galactosidase) and a non-enzymatic approach using acid hydrolysis of lactose are the main process options. In the case of the former, both soluble and immobilised forms of the enzyme may be used. The reaction conditions necessary to carry out acid hydrolysis are chemically severe and that process is more suited to the use of concentrated lactose as substrate.

Production of β-galactosidase (lactase enzyme) from the dairy yeast *Kluyveromyces lactis* on a commercial scale dating back to the 1970s presented an opportunity to add the enzyme to milk and other dairy substrates in order to facilitate the enzymatic cleavage of lactose to glucose and galactose. Dekker et al. (2019b) distinguished between neutral lactases that are currently used in the production of lactose-free dairy products on an industrial scale and acid lactase suited for use as a nutritional supplement—the terms 'neutral' and 'acid' being used to denote the pH optima of the respective lactases. Dekker et al. (2019b) also highlight that the choice of available lactase enzyme is dependent on one European producer (Maxilact® by DSM) and three Japanese companies (Godo, Amano and Nagase). The Japanese companies rely on a network of US and European resellers to distribute their respective lactases in local markets. One other distinction (Dekker et al. 2019b) is that some commercial β-galactosidases harvested from fermentations using different microorganisms e.g. *Bacillus circulans* or *Aspergillus oryzae* are more suited for galacto-oligosaccharide (GOS) production than for producing lactose-free products.

In general, a maximum hydrolysis of approximately 80% of the lactose content in milk is achievable under optimum conditions. In addition, heat treatment is required following hydrolysis in order to inactivate the enzyme, thus potentially triggering the initial stages of Maillard browning.

3.5.3.1 Enzymology

Enzymatic hydrolysis of lactose has evolved in a number of directions, e.g. simple addition of β-galactosidase to milk and other lactose-containing substrates in the form of a soluble enzyme, or immobilisation of β-galactosidase onto an appropriate support as a means of lowering the costs of producing lactose-hydrolysed syrups from whey and exploiting lactose hydrolysis for the production of prebiotics such as galacto-oligosaccharides.

3.5.3.2 Hydrolysed Lactose Syrups

Hydrolysed lactose-containing syrups were produced industrially during the 1980s from whey and UF permeate using immobilised enzyme systems. Dairy Crest Ltd., UK employed technology developed by Corning Glass in which lactase was carried

on porous glass with crosslinking by glutaraldehyde. Valio Ltd developed its own proprietary technology using an adsorption resin as carrier and glutaraldehyde for crosslinking (Harju 1987). The investment in lactase immobilisation was an attempt to reduce manufacturing costs given the relatively high cost of the soluble enzyme. However, the hydrolysed lactose syrups had to compete with other cheap carbohydrate sources in food ingredient markets where it was challenging to establish a unique functional selling point so that manufacture was eventually discontinued. Meanwhile, the market for demineralised whey expanded since both its high lactose and whey protein contents were particularly sought after for the standardisation of infant milk formula.

Additional technological approaches to those described above for the production and formulation of reduced- and low-lactose dairy products include the use of improved separation processes and innovative low-lactose dairy ingredients.

3.5.3.3 Low-Lactose Dairy Ingredients–Low-Lactose Milk Re-formulation

High protein dairy ingredients are naturally low in lactose having been produced using UF or microfiltration (in the case of casein) in conjunction with extensive water-based diafiltration. The higher the protein contents in milk protein concentrates (MPC), milk protein isolates (MPI) and micellar casein (MC) the lower will be the lactose content. For example, an 85% protein containing MPC (MPC-85) on a dry matter (DM) basis contains 8.0% lactose compared to 5.0% lactose for MPI-90 containing a minimum protein level in DM of 89.5% (ADPI, n.d.). A simulation of the reconstitution of the MPC-85 and MPI-90 powders to approximate a typical protein content (3.5%) of fresh milk would yield residual lactose levels of 0.38% and 0.22%, respectively. Recombination with other ingredients such as fat will bring about additional marginal reductions in these lactose levels. However, should more extensive lactose reductions be required, then further treatment with lactase enzyme may need to be considered.

Even lower lactose levels <1.0% occur in whey protein isolate (WPI) (90–95% protein); this much valued protein is recovered by membrane filtration from whey in a manner similar to the MPC processes described above. WPI is the principal ingredient used in the formulation of performance nutrition products; hence, its almost negligible lactose content will be appreciated by those engaged in sporting and physical activities without fear of triggering LI.

Acid and rennet casein are casein isolates prepared by isoelectric acid precipitation at pH 4.6 and enzyme-induced skim milk clotting processes, respectively. Both protein curds undergo multi-stage counter-current washing so that only negligible amounts of lactose remain in the finished dried caseins. Rennet casein is utilised solely for the formulation of cheese analogue products, while acid casein becomes highly functional following solubilisation in the sodium form (as sodium caseinate). Sodium caseinate is utilised in a wide range of processed foods for protein enrichment and its excellent emulsification and whipping properties.

3.5.3.4 Galacto-Oligosaccharides (GOS)

GOS are non-digestible oligosaccharide with prebiotic properties that are derived during targeted hydrolysis of lactose or lactose-containing substrate. Specific β-galactosidases from fungi of the genus Aspergillus and yeasts of the genus Kluyveromyces are selected on the basis of their glycoside hydrolase activity as biocatalysts for the industrial synthesis of GOS (Martins et al. 2019). A targeted degree of polymerisation (DP) of the cleaved monosaccharides (3–6) optimises the prebiotic potential of the resulting GOS. GOS are promoted for a wide range of food formulation applications and is increasingly included as a prebiotic in infant milk formula.

3.5.3.5 Market Developments for Lactose-Free Dairy

The rapidly growing market for lactose-free and reduced lactose dairy products in Western European markets that were, hitherto, not overly concerned about the prevalence of lactose intolerance is a relatively modern phenomenon. Starting from a base of addressing a niche market opportunity, lactose-free dairy is figuring prominently in the chilled cabinet displays of retail outlets. The quality and relatively unaltered taste profile of lactose-free milk appears to be making it an easy decision to switch over from regular milk at a time when consumers are also presented with the option of selecting alternatives to dairy-based beverages, particularly from those formulated using plant-based ingredients. A preoccupation with healthier living and dietary intake is encouraging consumers to choose from the increasing array of 'Free-From …' dairy products and other foods. Dekker et al. (2019b) states that lactose-free retail milk (also referred to as 'Liquid' and 'Market' milks) accounts for the largest percentage of the category followed by lactose-free yogurt which is expected to reach a €1 billion turnover by 2020. This is followed by lactose-free cheese which, at an expanding rate of 8.3% (based on compound annual growth rate over the period 2017–2022), is outpacing that of lactose-free milk at 7.3%. So far, the market is offering little choice other than 'lactose-free' with few, if any, 'lactose-reduced' options.

Meanwhile, biotechnology research is shining a light on the mechanisms by which the human microbiome and its microbiota within the gastrointestinal tract react to the ingestion of prebiotics and fermented milk-based probiotics. Arnold et al. (2018) used physiological and transcriptomics analyses to show distinct differences in carbohydrate metabolism profiles and galacto-oligosaccharide (GOS) utilisation between different intestinal *Lactobacillus rhamnosus* strains. A putative operon responsible for GOS utilisation was identified and characterised by genetic disruption of the 6-phospho-β-galactosidase. These results point to the importance of synbiotic optimisation in the course of alleviating gastrointestinal upset attributable to LI. It is suggested that even low levels of lactose can function as fermentable substrate for the benefit of human bowel health, while at the same time being tolerated by lactose mal-absorbers.

3.5.3.6 Regulatory and Food Safety Aspects

According to the European Food Safety Authority's (EFSA 2010) panel on dietetic products, nutrition and allergies (NDA), the scientific evidence suggests that most people with lactose intolerance can cope with up to 12 g in a single dose, and 20–24 g lactose once the overall intake is distributed throughout a daily diet. Given such expert guidance, it should be possible for consumer dairy markets to develop 'reduced lactose' variants of regular milk products in addition to the 'lactose-free' only products currently on display. Particular attention to product labelling is required so that LI consumers can keep track of their daily lactose intake from all food sources (Silanikova et al. 2019). Many processed foods such as soups, sauces and confectionery mixes utilise whey powders and lactose ingredients for functional purposes during the course of formulation. Greater transparency regarding the amounts of lactose incorporated is advised via improved food labelling declarations. At a clinical level, infants diagnosed with galactosemia require special care, as enzymatically hydrolysed lactose-based milk beverages from which the released galactose is not removed are not suitable irrespective of the residual lactose content.

3.5.3.7 Determination of Residual Levels of Lactose in Lactose-Free Milk

Churakova et al. (2019) examined a wide range of chemical and advanced analytical bio-assays to determine those most suited to the monitoring to the apparent voluntary threshold for lactose-free milk of 0.01%. High performance anion exchange chromatography coupled to a pulsed amperometric detector (HPAEC-PAD) stood out as a putative reference test compared to the other advanced analytical techniques, e.g. HPLC-RI, NMR, enzymatic kits, cryoscopy and lactose biosensors. The performance of a Biomilk^{300}, amperometric-type lactose biosensor compared favourably with analysis by HPAEC-PAD in terms of accuracy, precision and sensitivity for the detection of low levels of lactose in milk (Churakova et al. 2019) and provides industry with a rapid analytical tool for compositional quality control of lactose-free milks.

3.5.4 Conclusion

Mankind, with the aid of evolutionary forces, has wrestled with the issue of lactose malabsorption from drinking milk for over 10,000 years due predominantly to the cessation of lactase activity in the gut from around the time of weaning as a child. Early basic preservation initiatives pointed to the value of milk souring and fermentation, while milk clotting made possible the separation of lactose-rich whey from curd; both of these developments most likely led to a gradual accustomisation to dairy intake by LNPs. However, a quantum leap during the past 40 years was the development of technologies to delactose milk to <0.01% without alteration of

flavour. While aiming to address niche market opportunities on behalf of LI individuals, sales of lactose-free milk now appear to have gone mainstream at retail level. At a time when an increasing number of consumers are embracing 'free-from' dairy options in the course of transitioning to veganism, the dairy industry is benefiting from an added value product opportunity as health-conscious individuals switch their allegiance to 'lactose-free' dairy options. Widespread global industrial manufacture of high protein dairy ingredients such as MPC-80, MPI, MC, WPC-80+, WPI means that the food and beverage processing sector has access to an extensive base of functional low-lactose dairy ingredients for use in food and beverage formulations. The low-lactose nature of hard and semi-hard cheeses, along with their complex biologically active matrices that aid subsequent digestion, also provides LI consumers with greater dairy product choice.

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Chapter 4
Production and Uses of Lactose

Anthony H. J. Paterson

4.1 Theoretical Approach to Production

Lactose has been produced industrially for over 100 years (Dryden 1992). The objectives of the lactose manufacturer were summed up by Herrington (1934): "In the manufacture of lactose, it is desirable to secure a maximum yield of crystals in a minimum time, and to secure crystals which may be readily washed with a minimum of loss". These objectives are still valid for the modern lactose manufacturer and this chapter will examine how these objectives might be met.

The production of lactose from solution generally follows a standard crystallisation process involving concentration, nucleation, growth and harvesting/washing. The overall yield is determined by the conditions used in all four phases of production. Figure 4.1 shows the lactose solubility data in water as gleaned from the literature. The equation for the fitted line in the figure is:

$$C_{LS} = 10.9109 \exp^{0.02804T} \tag{4.1}$$

C_{LS} = concentration of anhydrous lactose (g lactose/100 g water)
T = temperature (°C)

This equation is the same as that given by Butler (1998) and can be used to demonstrate how various processing conditions impact on the overall yield of a process although actual solubility data for any particular whey will vary from the pure water data depending on what other impurities are present (Bhargava and Jelen 1996).

A. H. J. Paterson (✉)
School of Food and Advanced Technology, Massey University,
Palmerston North, New Zealand
e-mail: A.Paterson@massey.ac.nz

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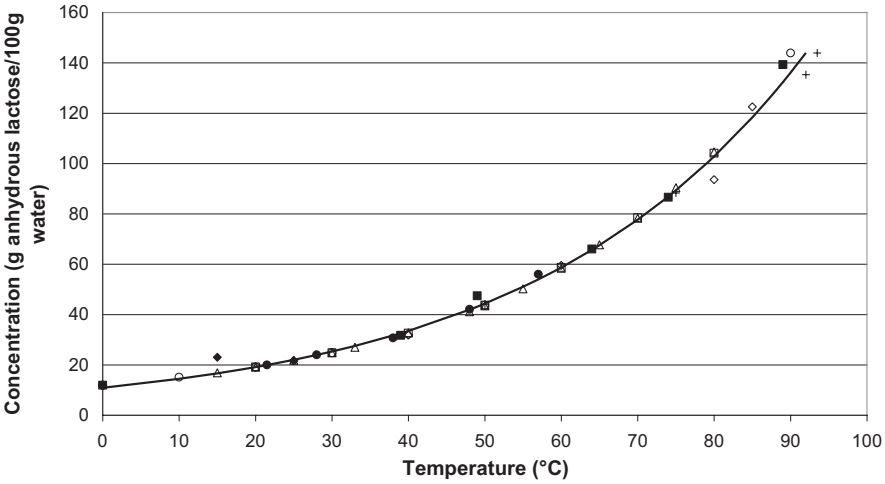

Fig. 4.1 Solubility data for lactose from the literature Mcleod (2007). (Foremost-Foods 1970; Gillis 1920; Haase and Nickerson 1966a, b; Herrington 1934; Hudson 1904, 1908; Kendrew and Moelwyn-Hughes 1940; Rozanov 1962; Saillard 1919; van Kreveld 1969; Visser 1982)

Whey arriving at a factory needs to be concentrated, usually by reverse osmosis and evaporation, until the lactose concentration is typically around 110 g lactose per 100 g water (58% TS for whey permeates). It is then cooled over up to 48 h (but times as short as 12 h have been used) to allow first nucleation, and then growth, of lactose crystals from solution. In many industrial plants, the final temperature will be between 15 and 25 °C. If it is assumed that the concentrate is cooled to 20 °C, that sufficient time is allowed for the solution to come to equilibrium with the crystals, that all crystals are of sufficient size, that there is 100% recovery through the recovery and washing zone, and ignoring the losses incurred by recycled lactose from the washing stages, then the theoretical yield will be given by:

> In the concentrate, 100 g of water will have 138 g of lactose associated with it, while at 20 °C, 100 g of water will contain 19.1 g of lactose in solution at equilibrium, therefore, the lactose removed as crystals will be 118.9 g, and the theoretical yield is 118.9 g/138 g or 86.2%.

Normal yields will be less than this because of losses due to dissolved lactose and fine crystals that are not recovered during the recovery/washing operations being lost in the mother liquor and to recycled lactose from the washing operation increasing the water load through the plant.

To increase this yield, operating conditions can be manipulated and several options present themselves for improving the yield:

1. Increase the concentration of whey/permeate exiting the evaporators. The limit to this, without crystallisation occurring in the evaporators, is the solubility limit at the final temperature of the evaporators. If the concentration can be increased to 163 g lactose/100 g water (62% TS for whey permeates), the potential yield would increase to 88.3%.

2. Cool the crystallisation batch to a lower temperature. If it could be cooled to 5 °C, then the concentration of the mother liquor at equilibrium would be 12.6 g lactose/100 g water, resulting in a theoretical maximum yield of 90.9% with an evaporator concentration of 138 g lactose/100 g water and yield of 92.3% if the evaporator concentration is 163 g lactose/100 g water.
3. By allowing crystallisation to occur in the evaporator, even higher concentrations could conceivably be achieved, and this enables even higher yields to be obtained. If the concentration can reach 200 g lactose/100 g water (66.6% TS for whey permeates) and that the batch is cooled to 5 °C, then the yield would be 93.7%.

These examples have not considered other practical limitations that might occur, such as calcium deposits in the evaporators or crystalline concentrates being too viscous to pump. The actual limits that can be achieved will vary between factories depending on whey purity, plant cleanliness and the specifics of the plant design. The precipitation of calcium phosphate on the evaporator tubes limits the run time of the evaporators. Steps can be taken to minimise this effect, such as the addition of agents to sequester calcium ions or to use ion exchange or ion chromatography to remove the calcium phosphate from the whey before it reaches the evaporator.

Actual plant data are commercially sensitive and hence the figures given here are indicative to show how plant yield can be influenced by changing plant conditions. Actual plant yields are usually considerably below the theoretically possible yields due to inefficiencies in the harvesting and washing cycles of the plants. According to APV (2007), 65% yield is typical, but as indicated above, it should be possible to improve on this. Plants have reported yields as low as 50%, which is completely unacceptable in my opinion. It should be possible to improve yield to the range of 60–75% by altering the operating conditions of a plant. Many of the contributing factors to such low yield values can often be traced to the generation of fines within the system, which end up partitioning in the mother liquor or causing increased loads on the evaporators through recycling which leads to more water going through the process, and hence larger losses. Control of the nucleation event within the plant is the only way this challenge can be addressed as it is the nucleation process that determines the number of crystals in the brew and hence the final particle size distribution. The production of fines also leads to further problems during downstream processing as the cake coming off the centrifuges will be high in moisture content, making it hard to dry and also leading to excessive circulation of fines in the fluid bed dryers.

Another cause of nucleation problems is the quality of the feed stream, especially if the whey or whey permeate has had to be stored or transported for more than 12 h at ambient temperature. Storage under these conditions allows time for bacteria to grow, which produce oligosaccharides and possibly other substances which have the effect of retarding nucleation and slowing the growth of the lactose crystals while also modifying the habit of the crystals (Ihli and Paterson 2015). Microbial fermentation of dilute streams causes losses both directly and indirectly as a result of effects of the by-products on downstream processing. Dryden (1992)

reported the production of fine flat lactose crystals in several New Zealand plants, resulting in considerable loss of yield due to the fines being lost with the mother liquor and on investigation, the problem was traced to whey which had been held too long and which had fermented. Other impurities that come with some whey streams can also result in the same challenges, and lactose crystals that are not the usual tomahawk shape are an indication of impurities in the feed. These impurities can also lead to very high supersaturations developing in the crystallisers by inhibiting the nucleation of the lactose crystals, resulting in a showering event and hence very small crystals at the end of the crystallisation run, leading to further losses in the washing stages.

Incorrect cooling procedures can also lead to local nucleation events occurring at the walls within the crystallisers, or even to induce a second major nucleation event, creating a batch with bimodal particle size distribution. These events lead to batches with too many fines, leading to significant losses. Recycling of lactose through the plant leads to further losses, as every kilogram of lactose that is recycled from the washing stage to the evaporator leads to losses of 6.3–17.4% of the recycled lactose (depending on the conditions under which the plant is run). Hence, any change in the process that leads to increased recycling of lactose within the plant leads to increased losses. Another area where unwanted fermentation of lactose within the plant can cause problems is that the fermentation can produce endotoxins and/or bacteria such as enterobacteria. The carry-over of these into the product is worse when small lactose crystals are produced, as small crystals have a much larger surface area than larger crystals, and hence the level of these contaminants is greater. These problems will carry over into the production of pharmaceutical-grade lactose if these smaller edible-grade lactose crystals are used as the starting material for production.

4.2 Edible-Grade Lactose

The CODEX definition of lactose is: “Lactose: A natural constituent of milk normally obtained from whey with an anhydrous lactose content of not less than 99.0%, w/w, on a dry basis. It may be anhydrous or contain one molecule of water of crystallisation or be a mixture of both forms” (FAO 2022).

Some producers detail the specifications of their products on their websites, with examples including Biolac GmbH & Co. Biolac GmbH & Co., Bayerische Milchindustrie and Lactose India Ltd.

The process for making edible-grade lactose is relatively straightforward, in that the basic procedure is to concentrate whey or whey permeate, and then allow it to cool, so that the lactose crystallises out of the mother liquor. The crystals are then separated from the mother liquor, washed, dried and packed. A description of this

process was given by Weisberg (1954) and an excellent review of the science and its implications has been given by Wong and Hartel (2014). Figure 4.2 is a process flow diagram showing a typical lactose production process. Most manufacturers follow this method, with some site-to-site variations. Some producers like to include various steps either before or after concentration and different concentration techniques can be used, including reverse osmosis before a final concentration using evaporation. All the processes the author knows of use a final concentration step of evaporation to achieve the desired solids level.

The critical steps in the process are:

1. *Removing as much water as possible in the final stages of evaporation.*
2. *Transfer of the high-solids concentrate from the evaporator to the crystalliser without uncontrolled nucleation.* Where a process uses higher concentrations than it was originally designed for, it may require an appropriate increase in temperature before transfer to the crystalliser to limit supersaturation. The key here is to maintain the same absolute level of α-lactose supersaturation as it enters the crystalliser, thus maintaining similar conditions to those that occurred in the original process at the time of nucleation. This is because the rate of nucleation is a strong function of the absolute level of α-lactose supersaturation, as well as the fluid dynamics of the crystalliser.
3. *Cooling to as low a temperature as is economically feasible.* A lower temperature means lower lactose solubility, allowing a greater mass of lactose to grow onto the crystals. Again, there are limits, as the rate of crystal growth is quite slow at these low levels of supersaturation and this requires long crystallisation times; this in turn requires greater capital expenditure for larger crystallisers.

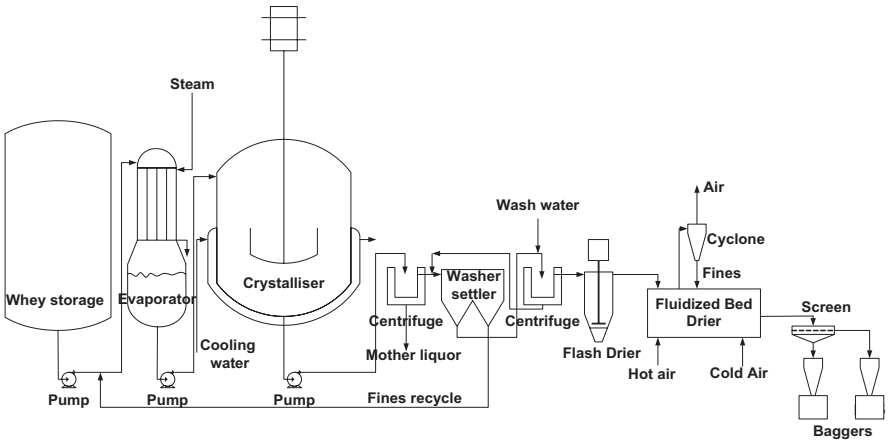

Fig. 4.2 Process flow diagram for the manufacture of edible-grade α-lactose monohydrate

4. *Cooling at the appropriate rate is critical.* Aggressive early cooling may result in too many crystals being formed during the nucleation event, leading to crystals that are too small for convenient downstream washing and processing. If early cooling is too slow, not enough nuclei form, rendering it highly likely that a secondary nucleation event will occur, giving a bimodal distribution with a large tail of fines in the particle distribution. Traditionally, these problems have led to the mystique that lactose crystallisation is an art rather than a science. Recent research has started to unravel some of this mystique (Agrawal et al. 2011, 2012, 2015, 2016, 2017; Agrawal and Paterson 2014; Butler 1998; Haase and Nickerson 1966b; Kauter 2003; Kendrew and Moelwyn-Hughes 1940; McLeod et al. 2011, 2010, 2016; Mcleod 2007; Shaffer et al. 2016; van Kreveld 1969; Wong and Hartel 2014). Even with all this research, the nucleation processes remain almost impossible to predict, with the local hydrodynamic conditions, combined with the supersaturation and local impurities, having a large effect on the nucleation that occurs. The review by Wong and Hartel (2014) is highly recommended as a support in understanding the lactose crystallisation process.
5. *Separating the crystals from the mother liquor.* This is usually completed with a decanter centrifuge and subsequent washing stages. The washing plant can consist of a series of hydro-cyclones or a series of counter-current mixing and settling separators, but other designs have been used also. There is very little information in the literature on the performance of the alternative washing regimes and this is an area identified as having potential for further research (Keller 1982; Nickerson 1970; Weisberg 1954; Whittier 1944).
6. *Drying of the crystals.* The final operation involves separating the washed crystals in a centrifuge from which the lactose cake at 5% and 12% (w/w) free moisture is discharged into a dryer. Today, most lactose plants use a flash dryer with an inlet air temperature of 120–180 °C. This process flashes off the water and produces a thin layer of amorphous lactose on the surface of the lactose crystals. This amorphous lactose may be crystallised in the fluid bed dryer following the flash dryer.

   At this point, I would like to digress to discuss recent work on the crystallisation of amorphous lactose and its role in the storage of crystalline lactose. The background to the problem was the hearsay reports from industry about how dryers are run and the downstream problems that manufacturers have encountered. The fluid bed dryer is usually run in two compartments, with the first compartment running with hot air (110 °C) to remove the final moisture and/or to crystallise the amorphous lactose layer from the flash dryer. The second compartment uses cold air to cool the product before it goes to the sieves and then the packaging lines via pneumatic conveying. This process is similar in many plants, but some plants can pack off without problems while other plants report an increase in water activity of their product over time. One solution manufacturers have tried is to store the lactose crystals in silos while passing low humidity air

through the bed to take away the moisture as it is released. The question arises as to why this phenomenon happens and what can be done to address the issue. The first question to be answered was: does the amorphous lactose formed during the flash drying stage crystallise or not under the typical conditions in the fluid bed dryer? Looking at the literature, Roos and Karel (1992) showed that the rate of amorphous lactose crystallisation at lower temperatures, but higher water activities, was related to the extent to which the glass transition temperature (T_g) was exceeded. Kedward et al. (2000) showed that at lower water activities the amorphous lactose crystallised at peak temperatures similar to what would be expected at the hot end of a fluidised bed lactose dryer (i.e. 93, 112, and 129 °C at a_w of 0.33, 0.22, and 0.11, respectively). This data is similar to that found by Fan and Roos (2017); it was not clear from this data whether the amorphous lactose would crystallise or not in the fluid bed as it would depend whether or not the time, temperature and moisture content profiles during drying would allow the amorphous lactose to crystallise. Assuming it did, the question remained as to why some dryers had problems and some did not.

Taking a different approach, whereby the amorphous lactose was equilibrated at 0.33 water activity and then held at various temperatures greater than T_g for various lengths of time, Clark et al. (2016) showed that the crystallisation was an all or nothing event, in that once it started it went to completion very quickly. This was shown in two sets of experiments: the first experiment was run for 1.6 h and it was found that at a $T - T_g$ of 31.5 °C two runs did not start crystallisation while one run had completely crystallised. In the second set, the samples were held at a $T - T_g$ of 23.3 °C for various lengths of time and it was found that anything over 16 h had completely crystallised, while some at 15 h had not started, while others had completely crystallised and a few were only partly crystallised. The authors hypothesised that the difference between dryers might be because of the different ways in which the amorphous lactose crystallised in the fluid bed. If drying was not complete in the flash stage, such that moisture remained in the amorphous lactose forming on the surface of the lactose crystals, then the crystallisation could start at the surface of the amorphous lactose and work its way in, thus trapping water within the matrix of lactose crystals formed by the crystallisation of the amorphous lactose, causing the subsequent problems noted by industry. On the other hand, if the drying was quick enough then a moisture content gradient might form, meaning that as the particle heated up the amorphous lactose would start crystallising from the inside out, enabling the water released by the crystallisation to escape. Experiments were designed to test this hypothesis and it was found that both inside out and outside in crystallisation could be instigated, and both mechanisms resulted in the slow release of moisture after crystallisation was complete, as shown in Figs. 4.3 and 4.4.

It was concluded that when there is a major increase in water activity of the dried lactose during storage as encountered by some in the industry it is a result of amorphous lactose crystallisation occurring in the fluid bed. It was concluded that the reason for some manufacturers not experiencing this problem was

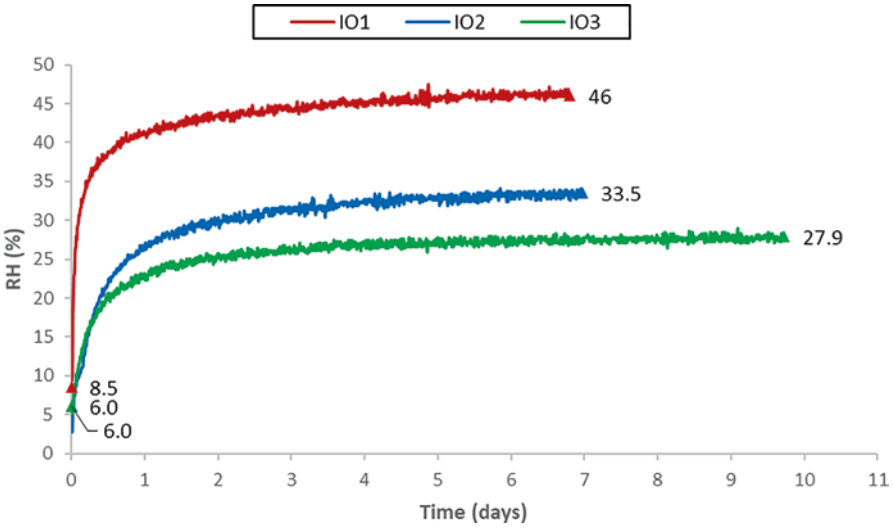

Fig. 4.3 Moisture release from samples of amorphous lactose that were exposed to Inside Out crystallisation conditions (Ibell-Pasley 2018)

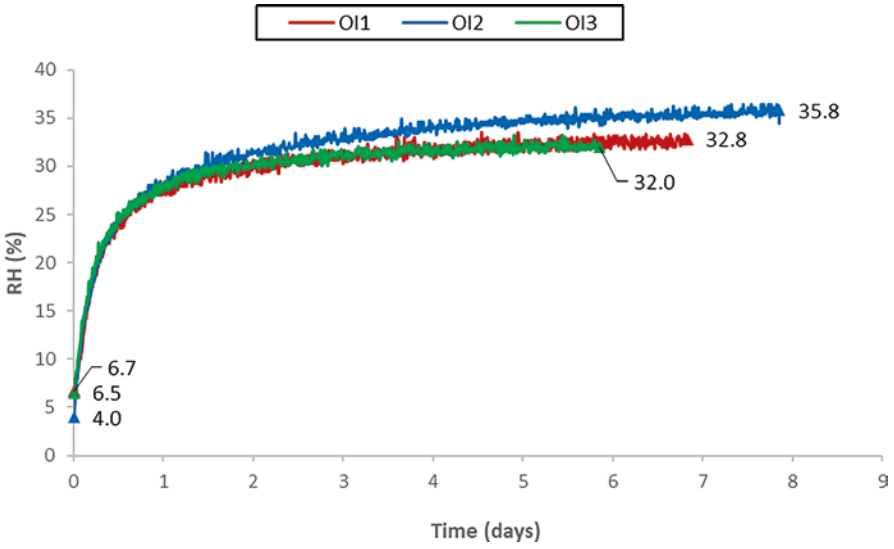

Fig. 4.4 Moisture release from samples of amorphous lactose that were exposed to Outside In crystallisation conditions (Ibell-Pasley 2018)

because they did not have the amorphous lactose crystallising in the dryer and they ensured that the crystallisation did not happen afterwards either (Ibell-Pasley 2018). This work will be published in a peer-reviewed scientific journal soon.

If the increase is only slight and occurs quickly then stabilises, then it is the result of the moisture gradient that exists in the amorphous lactose layer on the surface of the lactose crystals at the end of the drying.

7. *The temperature of packing.* It is important that the product is cooled to below 40 °C before it is packed, because temperature gradients drive moisture movement within the bags during storage (Bronlund and Paterson 2008; Paterson and Bronlund 2009). This is the major cause of caking of bulk lactose. It has been demonstrated that for caking to occur, the relative humidity in the air spaces within the product must rise above 80% so that significant amounts of capillary condensation can occur.

A temperature gradient within the product will cause moisture to move from the hot area to the cold area (Paterson and Bronlund 2009). It is this moisture movement, caused by temperature gradients imposed on the product by the day-night temperature cycles as the product is transported about the world, that can cause free-flowing product to arrive at its destination as solid 900 kg bricks (Bronlund and Paterson 2008; Paterson and Bronlund 2009). In order to prevent caking during transport, it is vital that the moisture level be reduced to below a critical moisture content. This water content can be determined most easily by measuring the water activity and relating this to the moisture content via the isotherm for the lactose crystals. Figure 4.5 shows the isotherm for α-lactose monohydrate (Bronlund and Paterson

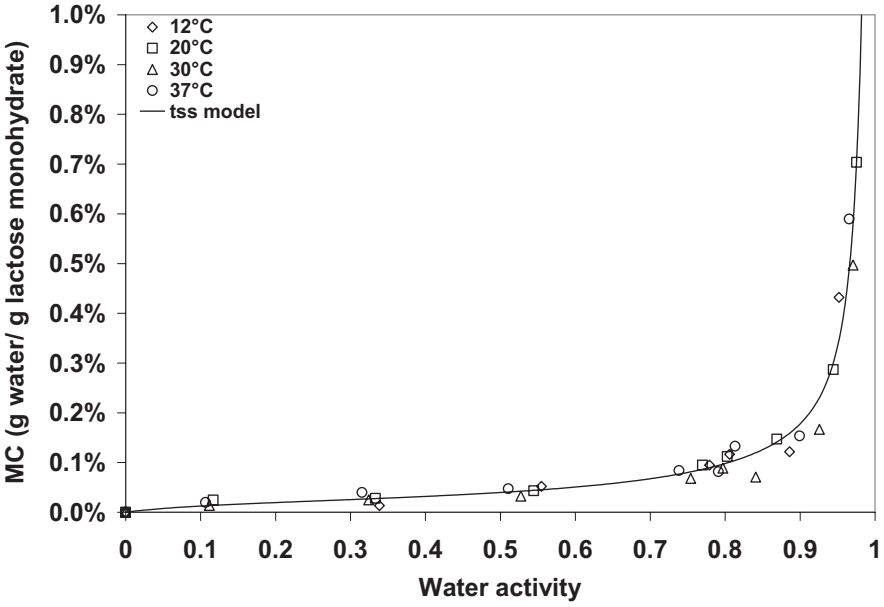

Fig. 4.5 Effect of temperature on the adsorption isotherm of α-lactose monohydrate, with the *t*hird *s*tage *s*orption (tss) model fitted to the data. (Reproduced from Bronlund and Paterson (2004) with permission)

2004). It is obvious from this figure that the water activity is a much more sensitive measurement than the moisture content and it is also quicker and easier to measure. Hence, it is recommended that water activity should be the preferred quality control method of determining whether a dried product is suitable for shipping or long-term storage.

Figure 4.6 shows a graph that has been produced based on the model presented by Paterson and Bronlund (2009). The figure shows that if a product is packaged at 40 °C and then placed in a warehouse at 10 °C for storage, in order to avoid caking, the water activity of the powder must be below 0.32. The figure can also be used as a guideline for the target water activity to be achieved in the dryer in order to prevent caking during transport. To use Fig. 4.4 in this way, one assumes that the entire bag is heated to the maximum temperature and then has the outside of the bag subjected to a cold environment. Looking at the figure, it is obvious that provided the final product has a water activity below 0.3, no caking should occur under most conditions that are likely to be encountered during transport from cold to hot climates, such as when product is shipped across the equator.

The KELLER™ edible lactose process is the most common turn-key lactose plant. It is marketed by RELCO of the USA and follows the traditional path, with concentrations leaving the evaporator of about 58% TS. The cooling curve with time is considered proprietary property and is a key part of its success. By controlling the cooling curve in the standard Keller design crystallisers, it is possible to

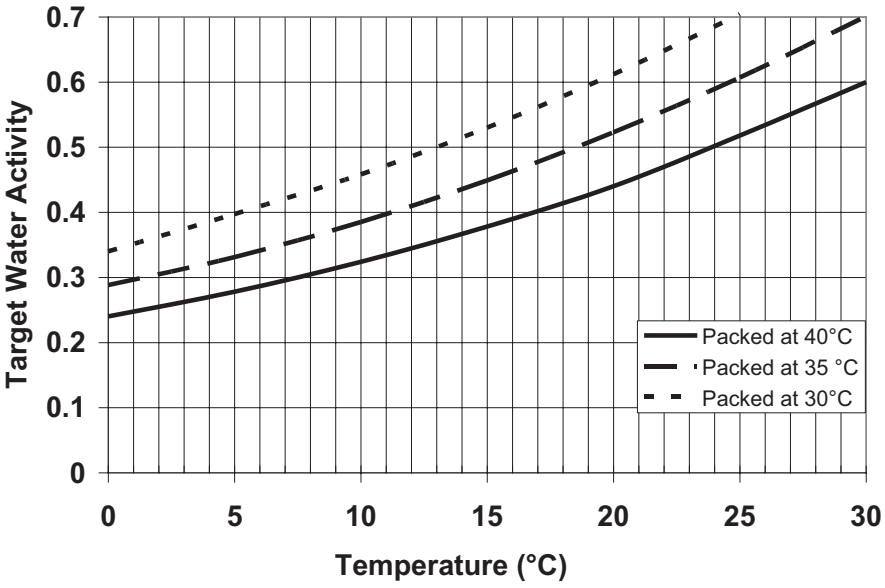

Fig. 4.6 Graph showing the hot packed temperature, the cold storage temperature and water activity combinations that lead to relative humidity conditions in the fluidised bed exceeding 80%, which precedes caking. (Reproduced from Paterson and Bronlund (2009) with permission)

control nucleation to produce the right number of crystals, allowing growth of optimally sized crystals for the washing and recovery section of the plant.

Typical lactose recoveries achieved by many manufacturers are around 65%, although some very well-run plants that control their nucleation events and their supersaturation levels going to the crystallisers have achieved close to the theoretical maximums of about 80% when using the evaporator followed by cooling crystallisers. As shown above, the way to achieve higher yields is to have higher lactose concentrations going to the crystallisers. An alternative technology is to use an evaporating crystalliser such as the CrystaLac™ which is marketed by RELCO. These produce crystals while at the same time they evaporate water from the mother liquor enabling very high total solids levels to be achieved before the stream is sent to a cooling crystalliser to mop up most of the remaining lactose still in solution. Yields greater than 80% should routinely be obtainable with this technology, provided the feed to it is of a good quality. To summarise, the main objective of plants producing edible-grade lactose is to achieve the maximum yield. The main parameters are to have the solids concentration coming from the evaporator as high as possible and to cool the batch to as low a temperature as can be achieved. But it is always critical to control α-lactose supersaturation at all points during the crystallisation process, so that the final crystal size distribution is optimal. Maximising the plant yield is a matter of finding the optimum balance.

4.3 Pharmaceutical-Grade Lactose

Pharmaceutical-grade lactose must meet the standards for contaminants laid out in Anonymous (1993) and USP-25 (2001). These two standards are almost identical and the test procedures that must be followed are prescribed. As a general guideline, heavy metals must be less than 5 μg/g, the microbial count must be less than 100/g, with no *Escherichia coli* present and with a combined mould and yeast count below 50/g. There are criteria for ash, clarity and light-absorbing tests which must also be satisfied. In general, the process for the manufacture of edible-grade lactose described above produces a product that does not meet the ash, protein and light absorption requirements for pharmaceutical-grade lactose. The impurities usually consist of riboflavin, a variety of proteins, lactose phosphate and lactic acid. The process of producing USP-grade lactose from edible-grade lactose is to re-dissolve the lactose in clean water and then to remove the impurities by a mixture of adsorption and filtration processes, followed by re-crystallisation (Kellam 2007). This process is shown in Fig. 4.7.

A recent method published by Durham et al. (2007) claims to produce USP lactose directly from whey permeates using ion exchange, nanofiltration, chromatography, evaporation and crystallisation, without the need for a second crystallisation step, with a yield approaching 95%.

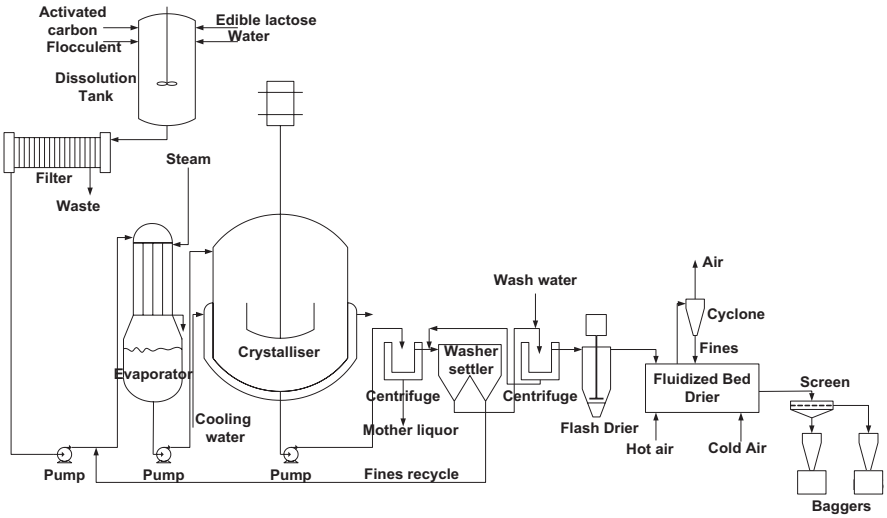

Fig. 4.7 Process flow diagram for the production of pharmaceutical-grade α-lactose monohydrate

Pharmaceutical-grade lactose is generally sold by mesh size, the different products being milled to different degrees, possibly in conjunction with air or sieve classification. In addition to the traditional α-lactose monohydrate, two other forms are sold to the pharmaceutical industry, they are anhydrous lactose (β-lactose) and spray-dried lactose.

4.3.1 Anhydrous Lactose

This has the same heavy metal, microbial and colour specifications as USP-grade α-lactose monohydrate. Anhydrous lactose is usually made by roller drying, at a temperature greater than 93 °C, a solution of USP-grade lactose which produces a flaked-type product made up of very fine crystals of β-lactose caked together. The flaked cake is then milled to the required size distribution (Whittier 1944).

4.3.2 Spray-Dried Lactose

Fine pharmaceutical-grade α-lactose monohydrate crystals are partially dissolved in clean water and then spray dried. This produces a product that has crystals of monohydrate lactose joined together by amorphous lactose into roughly spherical agglomerates. Because most of the amorphous lactose is in the centre of the agglomerates, the resultant powder is free-flowing without becoming too sticky (Darcy and

Buckton 1998). The amount of amorphous lactose present can be controlled by controlling the temperature of the water or lactose solution in which the α-lactose monohydrate crystals are suspended.

4.4 Uses of Lactose

There are three major reports that are available for anyone who needs an in-depth analysis of the uses and markets for lactose (ADPI 2018; Affertsholt and Pedersen 2016; GIRA 2019). I have only scratched the surface in a brief summary below for the more casual reader. Lactose has many uses in the food and pharmaceutical industries and a breakdown of those uses of lactose both in Europe and in the USA are provided in Figs. 4.8 and 4.9.

In the food industry, its uses are based around its relative sweetness and as a source of energy. It is less sweet than sucrose, with up to 3.3 times the concentration of lactose being required to give the same level of sweetness as sucrose (Parrish et al. 1981). This means that more lactose can be used without making the product too sweet. Lactose is a crystallisable sugar; thus it maintains the crystallised sugar texture without causing food to become too sweet (Burrington 2007).

Lactose is used in the confectionary industry to produce caramel flavours through the Maillard reactions, usually with milk proteins, often added with the lactose in the form of sweetened condensed milk (Weisberg 1954; Anonymous 2007b). The Maillard reaction is also important for its use in the baking industry where it is used to promote crust browning as the yeast used during the rising process cannot utilise lactose, leaving it as a reducing sugar available to undergo the browning reaction.

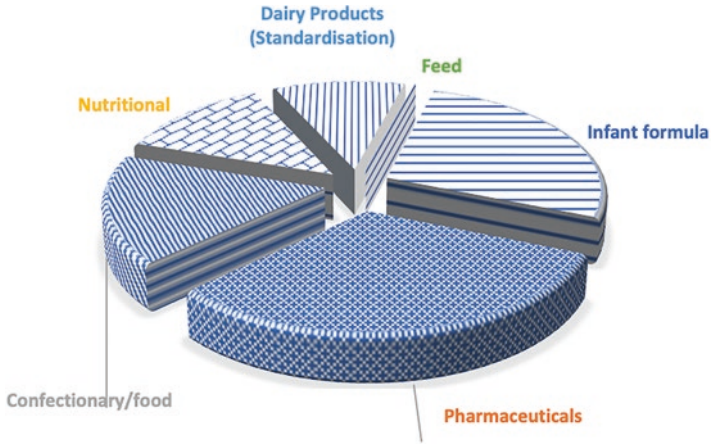

Fig. 4.8 Usage of 130,000 MT of lactose in the EU in 2018. (Reproduced from GIRA (2019) with permission)

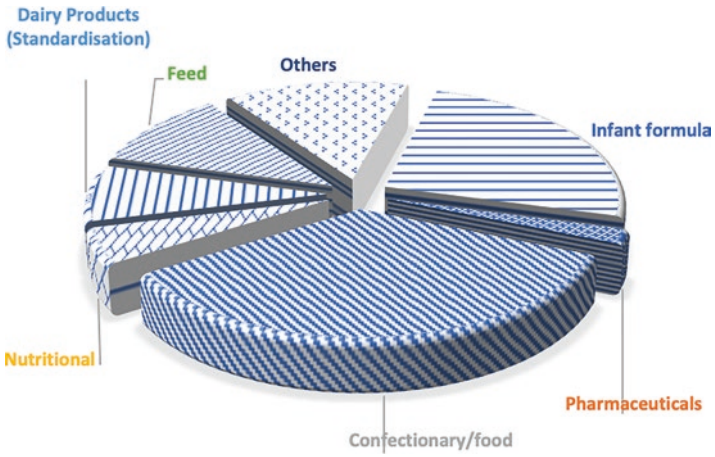

Fig. 4.9 Usage of 155,000 MT of lactose in the USA in 2018. (Reproduced from GIRA (2019) with permission)

Lactose can also adsorb food dyes and flavours and it finds uses in confectionery where this property is utilised.

Lactose is a primary energy source in mammalian milk, including that of humans. The level of lactose in human milk is about 7%, compared to 4.7% in cows' milk. This means that cows' milk does not naturally contain enough energy for babies when compared to human mothers' milk. This was recognised by Henri Nestlé who enriched milk powder made from cows' milk with lactose to produce the first infant formulae in 1867 (Anonymous 2007a) and this has been one of the major uses of lactose since.

The price of lactose since 2000 is shown in Fig. 4.10. The period from 2004 to 2007 saw a dramatic rise in the price of lactose from around US$ 0.5 to a high of US$ 2/kg and decreasing later to US$ 1.1/kg (Affertsholt-Allen 2007). This price increase is likely to result in changes in the uses of lactose as it moves from being a low-cost energy source to a relatively expensive source. For comparison, the cost of sucrose in 2007 fluctuated around US$ 0.175/kg. The reason for the increase in the price of lactose can be traced back to the market forces of supply and demand. In this case, the demand has been stimulated by the standardisation of milk powders. In countries, such as New Zealand, where the lactation of the national herd is largely synchronised to match seasonal grass availability, the innate protein content of milk powder fluctuates markedly through the year. The addition of lactose to standardise protein levels is now permitted, provided that the adjustment does not alter the whey protein to casein ratio of the milk being adjusted. Standardisation of milks for protein as well as fat levels is being introduced within Europe also and the result has been a great demand for lactose, causing a worldwide shortage. This effectively means that lactose has an inherent value approaching that of skim milk powder, rather than being a substance that has to be disposed of so that it does not cause problems in the environment due to its high biological oxygen demand. The price of

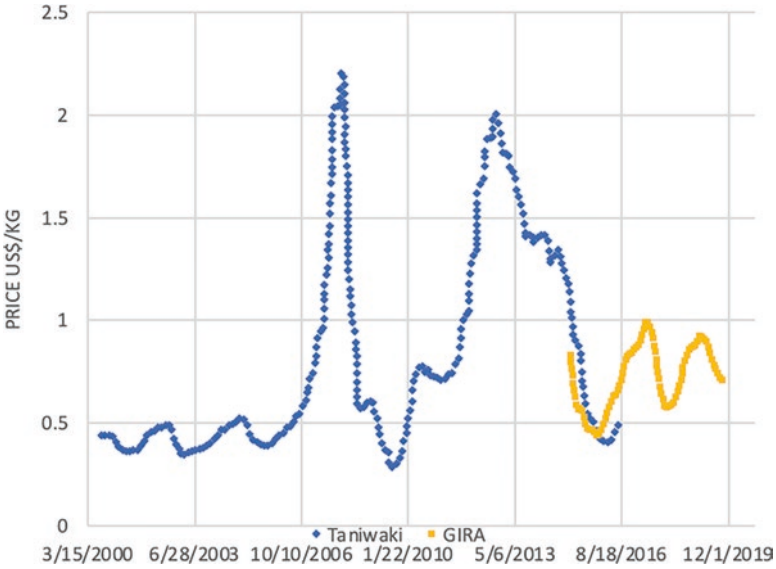

Fig. 4.10 Price of lactose in the period from 2000 to 2019. (Reproduced with permission from GIRA 2019 and Taniwaki 2016)

lactose fell again when new supply matched the increased demand, and this had happened by March 2008 followed by another peak price in 2012. At any point in history, there are usually multiple lactose production projects waiting to be placed on the boardroom tables of cheese-producing companies.

Lactose is limited as a food ingredient in that many people around the world are lactose intolerant (hypolactasia), meaning that their bodies do not produce lactase, the enzyme which breaks the lactose down in the gut into glucose and galactose, which can then be absorbed. Consequently, the lactose passes into the lower intestine where it provides a ready source of energy for anaerobic bacteria to grow. These bacteria produce gas as a by-product and this can cause cramping, flatulence and perhaps diarrhoea.

In the pharmaceutical industry, lactose is used as the main carrier (about 70% of tablets contain lactose) for drugs because it is not sweet, it is safe, it is available in highly refined form, and it makes good quality tablets. It has found uses within the industry in several different product forms. The main one is α-lactose monohydrate which can be used as a tablet excipient, but it can also be finely milled to produce inhaler grade lactose. Here, the lactose acts as a carrier for micronised drug materials to reach the lungs. Both anhydrous lactose (beta lactose) and spray-dried lactose are also used to make tablets. The form of the lactose is critical for consistent tabletting formulations, and much emphasis is placed on the reproducibility between batches of the particle properties that are required to produce consistent tablets, with an even spread of the active drug dispersed within the lactose powder being used as an excipient.

For some of the uses of lactose, it is essential that the customer is able to readily dissolve lactose into water. It is important, therefore, to understand how lactose dissolves. Since α-lactose monohydrate is the cheapest form of lactose available commercially, it is the product that is generally dissolved. Hodges et al. (1993) has shown that the mechanism that determines the rate of dissolution depends mainly on the desired concentration in solution. At levels above the solubility of α-lactose, the rate of dissolution is governed largely by the rate of mutarotation of α-lactose to β-lactose (Haase and Nickerson 1966a). Below the α-lactose solubility limit, the rate has been shown to be modelled by the mass transfer rate of moving the α-lactose into solution (Lowe and Paterson 1998). These mechanisms have been combined in a mathematical model and then summarised into a series of graphs (Figs. 4.11 and 4.12) for different particle sizes (Lowe 1993). The graphs have been rearranged to emphasise the effect of particle size. These graphs are the dissolution times expected for producing a desired concentration of lactose (expressed as kg anhydrous lactose per m^3 of solution) from mono-sized α-lactose monohydrate crystals of either 50, 150, or 400 μm size.

Figure 4.11 covers the concentration range from 40 to 200 kg/m^3 and temperatures of 10–40 °C. They can be used as follows: if it was desired to produce a solution with a concentration of 90 kg per m^3 then it can be seen that at 10 °C the dissolution process takes 600 min and is totally governed by the mutarotation kinetics and particle size has very little effect. This is still largely true at 20 °C, where the increased temperature reduces the dissolution time to 60 min. At 30 °C, the mutarotation reaction is sufficiently fast that the mechanism governing the dissolution has moved to mass transfer controlled, and the particle size has a large effect, with the

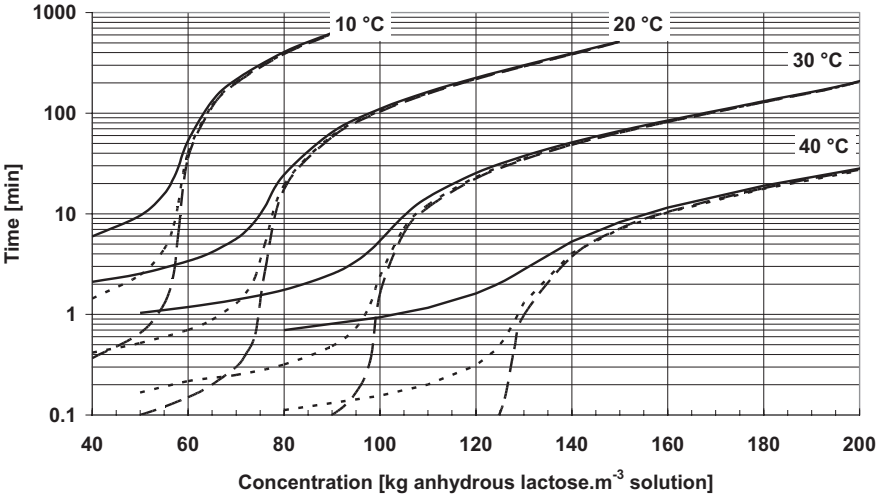

Fig. 4.11 Dissolution times for producing solutions with lactose concentrations of 40–200 kg/m^3 [— — 50 μm, - - - 150 μm, —— 400 μm] . (Reproduced with permission from Hodges et al. 1993 and Lowe and Paterson 1998)

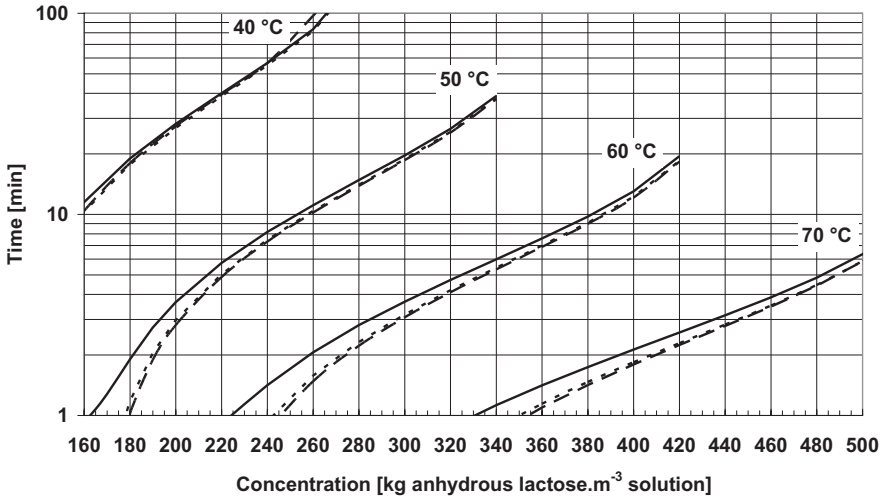

Fig. 4.12 Dissolution times for producing solutions with lactose concentrations of 160–500 kg/m^3 [— — 50 µm, - - - 150 µm, —— 400 µm]. (Reproduced with permission from Hodges et al. 1993 and Lowe and Paterson 1998)

time required being 2.5 min for the 400 µm crystals, 0.5 min for the 150 µm size and 0.1 min for the 50 µm size. Figure 4.12 covers the range of concentrations from 160 to 500 kg anhydrous lactose per m^3 of solution. If the lactose powder contains a range of particle sizes, then the dissolution time for the largest particle size is the best one to use, but it will underestimate the time required, especially if near-saturated solutions are being generated. The curves have been stopped short of the saturated limits; however, within these limitations, the graphs can be used to estimate the approximate dissolution times required to achieve a given concentration when dissolving α-lactose monohydrate in water at a given temperature.

4.5 The Future for Lactose

Lactose has moved over the last 30 years from being a problem biological oxygen demand (BOD) component in dairy wastewater to being a valuable by-product of the dairy industry. Lactose will continue to have a market as a required supplicant in increasing the energy value of bovine milk to match that of human milk in infant food formulations. The increased use of standardisation of milk powders for both fat and protein levels will continue to keep the price of lactose higher than it has been over the last 20 years, although some corrections in the market place will occur as more cheese manufacturers start to realise what a valuable asset they have in their lactose. A lot of current research is being carried out in the area of lactose derivatives, with the aim of producing high value nutraceuticals. This is an area in which

more commercial activity is likely to be seen over the next 10–15 years and if it works, then it will be another driver in increasing the price of lactose. On the pharmaceutical side, there is research on alternatives to lactose as an excipient, but it is expected that it will be some years before these are serious replacement threats to the position pharmaceutical-grade lactose currently holds.

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Chapter 5
Galacto-Oligosaccharides and Other Products Derived from Lactose

D. E. Otter, S. Wu, and D. N. De. S. Jayasinghe

5.1 Introduction

Lactose is the most prevalent component in milk and is present as an energy source for the newly born offspring. It has always been considered to be the poor cousin to the milk fat and protein fractions in milk with respect to its value as a dairy ingredient, and considerable research has been undertaken around how excess lactose in the dairy processing industry can be valorised. As a disaccharide composed of the monosaccharides glucose and galactose, lactose can provide the backbone building block for numerous sugar-derived synthetic compounds that are becoming increasingly significant in our food and health industries. The six lactose derivatives discussed in this chapter include galacto-oligosaccharides (GOS), lactulose, lactosucrose, lactitol, lactobionic acid, and tagatose. These compounds are only observed in trace amounts in natural cow's milk, if present at all, but all can be produced either chemically or enzymatically.

GOS and lactulose are the two most important commercially produced lactose derivatives and are principally used as prebiotic ingredients in functional foods, beverages, dietary supplements and pharmaceuticals. Tagatose, an isomer of galactose, is also gaining prominence as an artificial, low calorific sweetener. Commercial uses for the other compounds are emerging and lactobionic acid, lactitol, and

D. E. Otter (✉)
Auckland University of Technology, Auckland, New Zealand
e-mail: don.otter@aut.ac.nz

S. Wu
Massey University, Auckland, New Zealand
e-mail: sinong.wu.1@uni.massey.ac.nz

D. N. D. S. Jayasinghe
Produce Delivered Ltd., Auckland, New Zealand
e-mail: devin@producedelivered.co.nz

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lactosucrose are also currently commercially produced for a variety of food and pharmaceutical applications.

The aim of this chapter is to build on the excellent work of Playne and Crittenden (2009) in the previous edition of this book. It is very pertinent that a number of issues raised in their conclusions are now considered facts and current dogma. The chapter attempts to summarise previous knowledge, capture the current state of play and will specifically highlight research advances over the last decade in the areas of the synthesis, applications and health benefits of the various lactose derivatives. Excitement in the economic and scientific potential of lactose-derived products can be seen in the annual number of scientific publications which have doubled since the second edition (Fig. 5.1). It is fair to say that the relative importance of the different compounds is mirrored in their publication numbers over the past 12 years, especially with respect to the increased demand for GOS and lactulose as the potential of these products has been realised. With the proliferation of interest in the area, this chapter can only ever be a 'once over lightly' summary of the most important aspects underpinning the future importance of lactose-derived compounds and some areas, such as epilactose, have not been covered.

In the last decade, a large number of new players have entered the manufacturing space such that it was not feasible to list all the manufacturers. Both China and India have emerged as significant manufacturers of a number of these compounds. It has also not been possible to glean production volumes, production processes and prices from the main producers. The commercial production methods have raised more interest as enzymatic synthesis processes are becoming more economically and environmentally feasible. Potential uses for the various lactose-derived products have also increased and have been accompanied by robust scientific evaluations. A

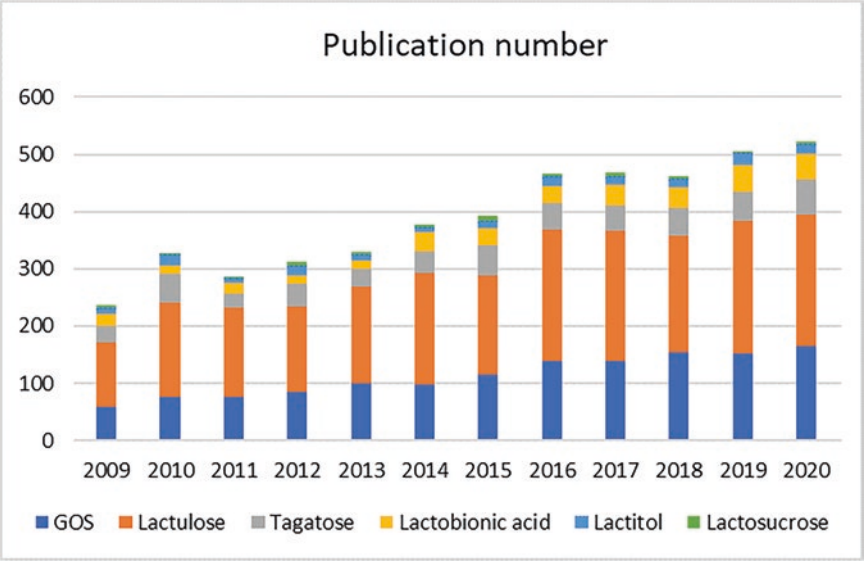

Fig. 5.1 Publication statistics for the lactose-derived compounds (as per Google Scholar)

number of countries have also made the appropriate regulatory changes to allow these compounds to be ingredients in food and pharmaceutical products. The patent literature has expanded in unison with the publication rate, and readers are advised to consult the many portals that access these resources. Within each section, the reader is also directed to numerous excellent detailed recent reviews that cover all aspects of the production, applications and health effects of these lactose derivatives.

5.1.1 Overview of Lactose-Derived Compounds and Their Synthesis from Lactose

The structures of lactose and the six compounds are shown in Fig. 5.2. GOS is an oligosaccharide composed of differing numbers (2–7) of β(1 → 6) linked galactopyranosyl (galactose) monomers linked to a terminal glucopyranosyl residue (glucose) via an α(1 → 4) glycosidic bond by a transgalactosylation reaction. Lactulose is an isomer of lactose where the lactose moiety is converted to fructose. Tagatose is an isomer of galactose and can occur naturally. Lactobionic acid is a direct oxidation product of lactose. Lactitol is a sugar alcohol produced by the catalytic hydrogenation of lactose, and lactosucrose is a trisaccharide from by the enzymatic transglycosylation of lactose and sucrose.

Lactose derivatives can be obtained from lactose by chemical, enzymatic, or microbial methods. Traditionally, classical carbohydrate chemistry was used to synthesise the different lactose derivatives. While the chemical synthesis of each compound will be discussed where appropriate, the increasing use of enzymes to produce more specific linkages between the monomers, together with the desire for 'cleaner' production methods, means that the enzymatic methods are gradually displacing the chemical methods. Figure 5.3 highlights the various reactions required

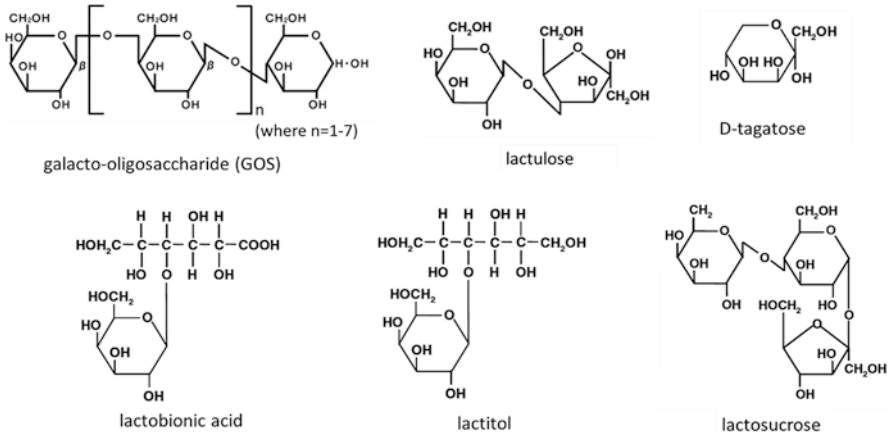

Fig. 5.2 Chemical structures (Haworth) of lactose-derived products

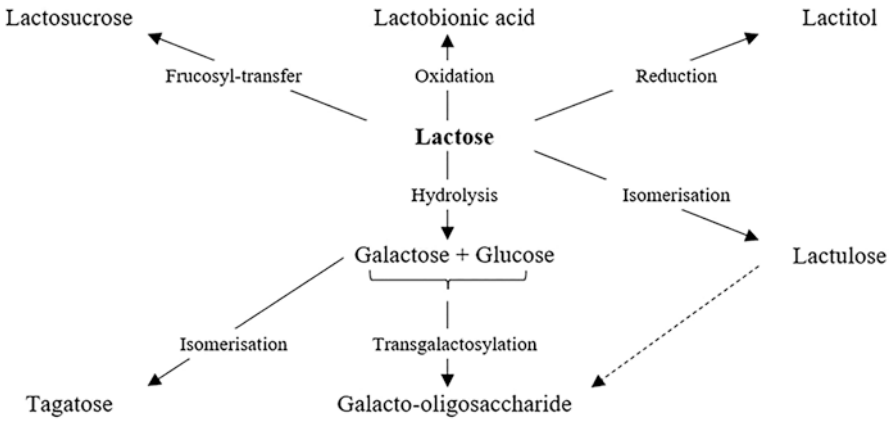

Fig. 5.3 The preparation of GOS, lactulose, tagatose, lactobionic acid, lactitol and lactosucrose from lactose

to produce the different lactose derivatives. All but lactosucrose can be produced via chemical and/or enzymatic action, although not necessarily in a linear fashion. Lactosucrose requires the presence of sucrose for the transfructosylation reaction. The specifics for each compound will be described in greater detail in the following sections.

One of the drivers for the increased interest in lactose-derived compounds is the recognition of their prebiotic and health properties, especially the importance of prebiotics in the diet and their ability to help maintain a healthy gut microbiota. The enzyme β-galactosidase features widely in any discussion on lactose-derived products. It is a ubiquitous enzyme in nature, the activity of which has been described in a diverse range of organisms, although only a few have been considered as sources for technological applications. Most of the β-galactosidases currently used come from yeasts of the genus *Kluyveromyces* and filamentous fungi from the genus *Aspergillus*. These enzymes are readily available and have a generally recognised as safe (GRAS) (or equivalent) status, allowing their unrestricted use in foods and pharmaceuticals. The following sections will detail the synthesis, applications and health effects of the lactose-derived compounds.

5.2 Galacto-Oligosaccharides

Galacto-oligosaccharides (GOS) are sugars consisting of between approximately two and eight saccharide units that are either found naturally in foods like banana, artichoke, onion, garlic, and honey, or can be synthesised from industrial dairy by-products (e.g., cheese whey and whey permeate) through the enzymatic transgalactosylation of lactose by β-galactosidase (EC 3.2.1.23). GOS are non-digestible prebiotics used for the dietary modulation of the gut microflora to improve health by

stimulating the numbers and/or activities of the beneficial intestinal bifidobacterial and lactobacilli populations. This selective metabolism to create an 'optimal' gut microflora can help increase the body's resistance to pathogenic bacteria, lower blood ammonia, increase stimulation of the immune response and reduce the risk of cancer. For further background information, readers are directed to a number of excellent reviews on different aspects of GOS by Torres et al. (2010), Vera et al. (2016) and Panesar et al. (2018).

5.2.1 Chemistry

GOS are prebiotic oligosaccharides derived from lactose and consist of a number of galactose monomers linked to a glucose monomer (Fig. 5.2). They predominately have a degree of polymerisation of between two and eight with the galactose terminating at the reducing end via an α-(1 → 4) linkage to a glucose residue. The galactose monomers are typically connected with β-(1 → 6) and β-(1 → 4) linkages, but β-(1 → 2) and β-(1 → 3) linkages have also been reported. The enzymatic production of GOS typically results in a mixture of carbohydrates consisting of GOS with various degrees of polymerisation, glucose as a by-product, substantial amounts of lactose and small amounts of galactose remaining in the reaction mixture. The GOS structure depends on the source of the β-galactosidase used for their synthesis and the conditions of the transgalactosylation reaction, and oligosaccharides within the polymerisation fractions can differ in glycosidic linkages (Torres et al. 2010). They are not limited in their molecular structure and can branch three-dimensionally. The number of combinations of structural linkages between monomers is high. For example, a galactose moiety can take two anomeric configurations and can also occur in both the furanose and pyranose forms. As the number of linkages expands, so does the number of possible combinations. The nomenclature of carbohydrates has been described fully in a series of publications authorised by the International Union of Pure and Applied Chemistry (IUPAC), with detailed information on currently accepted nomenclature and its historical development available online www.iupac.org/publications/pac/1996/pdf/6810x1919.pdf.

5.2.2 Synthesis of Oligosaccharides

Chemical and enzymatic methods are available for the synthesis of GOS. Chemical synthesis is not used commercially because of the lack of specificity, the number of reaction steps and the environmental impact of the chemical reactants (mineral acids) used in the formation of disaccharides and trisaccharide products by the reversion process. Enzymatically, the ability of members of the glycosidase family of enzymes (including β-glucosidase, β-glycosidase and β-galactosidase) to carry out synthetic reactions by reversing the equilibrium conditions has been known

since the late 1800s. The enzymatic synthesis of GOS from lactose by transgalactosylation via the transfer of the galactosyl moiety to another carbohydrate molecule acting as an acceptor was first studied in detail in the 1950s, and a number of papers published in the 1970s and 1980s examined the production of oligosaccharides from lactose using enzymes derived from various sources (Torres et al. 2010). While transgalactosylation with H_2O as the acceptor molecule results in lactose hydrolysis, the presence of lactose, glucose or galactose as alternative galactosyl acceptors results in GOS formation, and transgalactosylation of other acceptor carbohydrates generates hetero-oligosaccharides. The enzymes β-glucosidase and β-glycosidase are naturally present in a number of microorganisms, plants and animals, and are both able to transfer glycosyl moieties from a donor saccharide to an acceptor. However, in the presence of water as a competing nucleophile, the transglycosylation yields are less impressive. They are highly regioselective, stereo-selective and efficient but are not used for the manufacture of GOS as a result of their inaccessibility, the prohibitive cost of commercial preparations and the need for specific sugar nucleotides as substrates.

GOS are commonly produced from lactose using β-galactosidase (for a representative flow schematic, see Fig. 5.4). β-Galactosidases are categorised as GH1, GH2, GH35 and GH42 where enzymes in the families GH1 and GH2 predominantly use lactose as their substrate and have been characterised as belonging to *Enterobacteriaceae*, lactic acid bacteria and bifidobacteria. GH35 and GH42 enzymes prefer β-(1-3)- or β-(1-4)-linked galactans or GOS over lactose, and most enzymes characterised to date have been of bifidobacteria origin and bacteria associated with habitats that do not contain lactose. During the transgalactosylation reaction, lactose serves as both a galactosyl donor and an acceptor to form disaccharide, trisaccharide or higher GOS units. The reaction mechanism involves two sequential steps. The first step is the irreversible hydrolysis of lactose via the formation of an enzyme-galactosyl intermediate complex and the release of the glucose molecule. The second step then involves the reaction of the transition galactose complex with a nucleophile, usually another lactose molecule, to form a galactose-galactose-glucose trisaccharide (GOS-3) via β-(1-6), β-(1-3) or β-(1-4) glycosidic bonds, depending on the origin of the enzyme. This in turn can then act as an acceptor of the enzyme-galactosyl complex to form a galactose-galactose-galactose-glucose tetrasaccharide (GOS-4), and up to GOS-8. Alternatively, the nucleophile water can act as the acceptor of the enzyme-galactosyl intermediate complex, resulting in the straight hydrolysis of the lactose to glucose and galactose. The overall

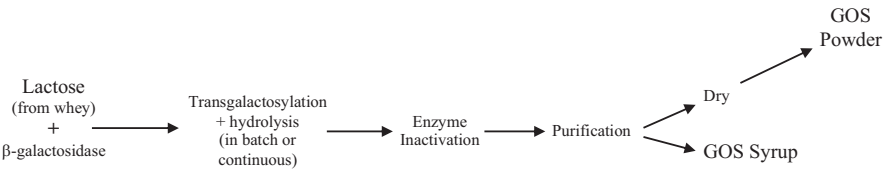

Fig. 5.4 Schematic flow diagram of GOS production

reaction is kinetically controlled with both possible reactions, hydrolysis and transgalactosylation, competing with each other and with the terminal galactosyl acceptor playing an important role. In the reaction vessel, both the reactions occur simultaneously, and any mono/oligosaccharides produced by either reaction are not excluded from subsequent hydrolysis or transgalactosylation.

The transgalactosylation reaction is favoured over lactose hydrolysis at high lactose concentrations, and the main products are disaccharides and trisaccharides that can then also act as galactosyl acceptors to form oligosaccharides with degrees of polymerisation (DP) of up to 8. This results in increasing concentrations of GOS up until a point when the rate of hydrolysis equals the rate of transgalactosylation, after which the hydrolysis reaction will start breaking down the newly formed GOS (as well as any lactose still present). During any GOS synthesis, therefore, it is important to find the 'sweet point' of the competing reactions and stop the reaction at the time of maximal GOS yield.

As well as the amount of GOS produced, the composition and configuration of the GOS moieties is also becoming increasingly important to researchers and consumers. The glycoside linkages, saccharide composition and DP all depend to differing degrees upon the source of the β-galactosidase enzyme, the reaction pH, temperature and time, and the initial lactose concentration (Moreno et al. 2014). The interplay between these variables has attracted a lot of study as, for example, the rate of β-galactosidase activity will increase with temperature until such time that the enzyme becomes unstable, while the initial lactose concentration is in turn limited by its solubility and this increases with the reaction temperature (Otieno 2010). The source of the β-galactosidase enzyme is very important in determining the final conversion percentage of lactose to GOS, the rate of the reaction and the GOS composition. Yin et al. (2017) has used ^{13}C6 labelled galactose and glucose to show that both monosaccharides can act as acceptor sugars during the transgalactosylation reactions. For more in-depth information on β-galactosidase enzyme from a wide variety of sources, readers are directed to reviews by Playne and Crittenden (2009), Saqib et al. (2017), Xavier et al. (2018) and Martins et al. (2019).

Galacto-oligosaccharide yield is increased with higher initial lactose concentration, but solubility is limited (between 20% and 30% w/w) at temperatures tolerated by most β-galactosidase enzymes (20–60 °C). In supersaturated lactose solutions (up to 40% w/w), the GOS yield can double, but above this concentration (up to 60% w/w), lactose precipitation reduces the yield (Vera et al. 2012). The proportion of GOS with DP values between 3 and 5 is also influenced by the initial lactose concentration. Also, most β-galactosidase enzymes are inhibited competitively by galactose, so high lactose concentrations may also reduce this inhibition. The use of β-galactosidases sourced from thermophilic organisms is an attractive way to circumvent the problem of low enzyme operational stability, and GOS yields greater than 50% have been attained (Torres et al. 2010). However, there are currently no commercially available enzymes that have also attained food and pharmaceutical safety status, although there are increasingly numerous reports detailing the potential of thermostable enzymes in the literature. The origin of the β-galactosidase, its pH and temperature profiles, together with its kinetic properties, all determine the

final GOS yield, composition and range of β-glycosidic bonds. Commercially, GOS is produced using β-galactosidases sourced from *Aspergillus oryzae*, *Bacillus circulans* and *Kluyveromyces lactis* as these enzymes possess high transgalactosylation activity at favourable pH optima and temperatures (Warmerdam et al. 2013). Their GOS yields range between 30% and 40%, they have different product profiles (e.g., *K. lactis* produces a high content of disaccharides) and lactose hydrolysis is the preferred pathway with the yeast enzymes. Enzymes from the probiotic bacteria *Bifidobacteria* (*B. infantis* CCRC 14633, *B. longum* CCRC 15708 and B6) and *Lactobacillus* also have the potential to produce GOS products with profiles that may produce a stronger stimulatory effect on the healthy intestinal microbiota (Schwab et al. 2011).

Whole cells have been used to produce GOS as they are readily available, enzyme purification is not required, and they are cheap to produce with relatively high stability. However, there is the disadvantage of the permeability of lactose into, and GOS and galactose out of, the cell, and this can lead to lower reaction rates. One method to remedy this is through cell membrane permeabilisation. A 44% yield of GOS was achieved when *K. lactis* yeast cells were permeabilised with ethanol and then lyophilised to facilitate the passage of substrate and products (Rodriguez-Colinas et al. 2011). Similarly, toluene-treated *Bifidobacterium bifidum* cells were able to be used in batch mode for eight cycles and produced yields of between 36% and 43% GOS (Goulas et al. 2007). Whole cells (*Yarrowia lipolytica*) have also been used in an immobilising surface display technology to anchor a β-galactosidase from *A. oryzae*. The β-galactosidase gene was encoded into a cell wall protein gene from *Y. lipolytica*, and the resulting cells could produce 160 g/L from 500 g/L lactose (32% yield) (An et al. 2016). Lastly, in a feasibility study, Fischer et al. (2021) synthesised GOS from casein whey, traditional Greek yoghurt whey and concentrated acid whey permeate using *Cryptococcus laurentii* as a whole cell biocatalyst. Yields of approximately 36% GOS were obtained, twice the concentration attained with *A. oryzae*. The maximum yield was also achieved in considerably less time than with *A. oryzae*. The type of whey used as the substrate influenced the final GOS composition, because the enzyme from *C. laurentii* was able to use galactose, which is present in Greek yoghurt whey, as an acceptor substrate resulting in higher DP3 and DP4 levels and greater structural diversity.

Free β-galactosidases have been reported from a vast array of microorganisms, either as crude cell-free extracts or as purified enzymes. They display a wide range of transgalactosylation activities, and only a few will be discussed here. Lactose conversion rates are generally between 80% and 100% within 24–48 h, depending on the enzyme source, substrate concentration and enzyme:substrate ratio, and GOS yields of approximately 100 g/L are typical due to the kinetics of GOS hydrolysis to monosaccharides at higher concentrations (Splechtna et al. 2006). GOS yields are typically lower when whey or milk products are used as the lactose source. This is because the lactose concentration is also lower, influencing the reaction kinetics (Frenzel et al. 2015). When an ultrafiltration-membrane reactor was used for the continuous production of GOS (Córdova et al. 2016), extended run times were

possible with a yield of 135 g/L GOS, increased substrate conversion and the continuous removal of inhibitory by-products. Although this process was limited by membrane fouling, advances in membrane technology and operation suggest that this method has potential for the large-scale production of GOS. Continuous systems using a β-galactosidase from the moderately thermophilic fungus *Talaromyces thermophilus* as both a free and an immobilised enzyme produce yields of 100 g/L in 50% GOS solutions (Nakkharat et al. 2006).

Multiple enzyme systems using combinations of *A. oryzae* and *C. laurentii* or *A. oryzae* and *K. lactis* have been evaluated for any synergistic properties (Fischer and Kleinschmidt 2018). While the structural diversity of the final GOS product could be increased due to different preferences of the various β-galactosidases in terms of types of glycosidic linkages, neither consecutive nor simultaneous synthesis with the different combinations led to increased GOS yields. When glucose oxidase and catalase were included with *A. oryzae* to facilitate depletion of the glucose inhibition there was only a small increase in GOS yield (5%) but the synthesis of tri- and higher oligosaccharides was increased (Fischer and Kleinschmidt 2019). Recombinant technologies offer the ability to produce β-galactosidase enzymes that have either high productivity or the desired GOS composition as over-expressed proteins in food-grade organisms. Geiger et al. (2016) cloned the β-galactosidase gene from *Streptococcus lactis* into the food-grade *Lactobacillus plantarum* WCFS1, producing an enzyme that converted the lactose from whey permeate into an approximately 100 g/L GOS product in 5 h. Recombinant enzymes from both *Halothermothrix orenii* and *Sulfolobus solfataricus* P2, which were then subjected to site directed mutagenesis, resulted in β-galactosidases that were more thermostable and had higher transgalactosylation activity than the original enzymes. GOS yields of approximately 60% were achieved with these enzymes (Wu et al. 2013; Hassan et al. 2016). Similar GOS yields, and in shorter reaction times, were also obtained using protein engineering and an intelligent double-hydrophobic amino acid scanning strategy to target the nine residues forming the glycon-binding site of a cloned β-galactosidase from a marine metagenomic library (Qin et al. 2019). The mutant enzyme reached higher GOS yields in a shorter time (59.1% at 10 h and 51.5% at 2 h compared with 45.3% at 16 h in the original enzyme). When skim milk was the substrate, the GOS concentration was doubled (19.9 g/L compared with 10.3 g/L) at a lactose conversion of 90%. Gosling et al. (2009) have used facile heat treatment of the β-galactosidase, elevated at 60 °C for 20 min, to selectively inactivate β-galactosidases I and III, reducing the net hydrolytic activity, and effectively enhancing the enzyme-transfer activity. GOS yields increased from 38% to 45% and 23–28% for lactose and skim milk, respectively. A recent novel GOS production system included the addition of β-galactosidase from *K. lactis* during the production of soft cheese to produce a cheese containing GOS (Vénica et al. 2020). The cheese make was extended due to a delay in reaching the target pH, and there were only low levels of GOS produced, but the proof of concept was successfully demonstrated.

Enzyme immobilisation has the advantages over free enzymes of increased enzyme stability, the ease of enzyme recycling and the removal of the enzyme from

the product after the reaction is complete. This in turn reduces the enzyme consumption costs and allows more flexibility for reactor design. Immobilisation has been achieved by enzyme adsorption, entrapment, covalent binding and cross-linking, and immobilisation media include self-supported cross-linked aggregates (Gaur et al. 2006), activated agarose, activated chitosan, magnetic polysiloxane–polyvinyl alcohol beads (Neri et al. 2009a), functionalised polymer nanofibers (Misson et al. 2015), methacrylic polymer carriers (Carevic et al. 2018) and whole permeabilised cells containing β-galactosidase (Sun et al. 2016). Comprehensive reviews on β-galactosidase immobilisation have been written by Benjamins et al. (2014), Osman (2016) and Basso and Serban (2019).

While the specific enzyme activity can be compromised through the immobilisation step, this is often compensated for by the increased enzyme stability, allowing longer production run times with decreased loss of enzyme activity. Huerta et al. (2011) immobilised β-galactosidase from *A. oryzae* onto gyloxyl-agarose and achieved almost double the GOS production in a ten sequential batch run when compared with the free enzyme. Similarly, when β-galactosidase from *B. circulans* was immobilised onto Eupergit C250L, both enzymatic and volumetric productivity was increased compared to the free enzyme due to shorter reaction times, a higher E/S ratio and decreased enzyme usage, resulting in yields of 57–65% over 15 cycles (Benjamins et al. 2014). β-Galactosidase has also been immobilised by entrapment in polyvinyl alcohol (PVA) lenses and used for continuous production of GOS in a packed-bed reactor (Jovanovic-Malinovska et al. 2012). High productivity rates were achieved with both lactose and whey and GOS production corresponded to 30% of total sugars. Shin and Wang (1998) used covalent binding to immobilise the β-galactosidase from *Bullera singularis* to chitosan beads (Chitopearl BCW 3510). Over 15 days, a packed bed reactor produced 55% yields of GOS with a productivity 6.5 g/L/h. Glutaraldehyde has been used to cross-link β-galactosidase from *B. circulans* to a microporous polyvinylidenefluoride (PVDF) membrane, thus allowing the membrane-based purification of GOS from the reaction mixture during and after the reaction (Palai and Bhattacharya 2013). The reactor was run for 30 days with a GOS yield of 30% and an enzyme loss of 50% (Palai et al. 2014). In a comparison of different immobilisation matrices and binding techniques, Osman et al. (2014) obtained maximum GOS yields of 49–53% and six consecutive batches of 24 h with a bifidobacterial β-galactosidase.

Another route for the synthesis of GOS is via lactulose. These GOS are considered to be distinct to lactose-derived GOS as the end group is fructose instead of glucose, but they have a strong resistance to digestion, have been shown to be good carbon sources for the development of probiotics and have demonstrated bifidogenic effects (Yin et al. 2018). Lactulose-derived GOS have been produced using a range of β-galactosidases including from *Propionibacteria*, *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL450 and *B. circulans* and vary in their ratio of GOS and lactulose-derived GOS depending on the enzyme source and the synthesis conditions (Sabater et al. 2019; Fara et al. 2020). Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) and nuclear magnetic resonance (NMR) spectroscopy analysis has identified the trisaccharide

β-D-Gal*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-D-Fru as the major structures, and they have been purified from reaction mixtures using fresh *Saccharomyces cerevisiae* yeast and activated charcoal (Julio-González et al. 2018). Either on their own, or most probably together with GOS, they have the potential to become a new range of functional food ingredients.

As noted above, a number of different reactor configurations have been used successfully for the production of GOS. These include membrane reactors, sequential batch operation in stirred tank reactors, continuous operation in packed-bed reactors (PBR), and to a lesser extent, continuously operated stirred-tank reactors (CSTR). Reactors have the advantage over batch operations of enzyme retention and extended run times, although bed clogging and membrane fouling can be problems with prolonged use; this may limit the substrate to pure lactose solutions or to lower concentrations of lactose and milk/whey permeate solutions. Another method to 'push' the reaction towards transgalactosylation and depress the hydrolysis of lactose and GOS is to synthesise GOS in low water activity solvents. This favours GOS production although, as some water is required for the initial hydrolysis reaction to produce the enzyme-galactosyl intermediate, aqueous/organic solvent biphasic systems containing, for example, 95:5 cyclohexane to water have been used (Shin and Yang 1994). A lot of the β-galactosidases studied by researchers are currently not approved for food use, are costly and many are not available or not available in sufficient quantities for industrial application. Therefore, selection of microorganisms which are safe for human use, and are capable of producing high levels of β-galactosidase, are significant factors in the production of GOS. In summary, a thermostable β-galactosidase that has been subject to site-directed mutagenesis to optimise the transgalactosylation kinetics and, if possible, eliminate the GOS product hydrolysis, which is cloned and then overexpressed in a food grade bacteria such as *Lactobacillus*, and finally immobilised onto a membrane and used in a continuous membrane reactor, may be the 'ideal' production scenario (Table 5.1).

5.2.3 Purification

As the synthesis of GOS from lactose is usually incomplete and can also result in lactulose as a by-product, a number of strategies have been investigated to purify the GOS stream for use as a final syrup or for subsequent drying to a powder product. Nanofiltration does not have the selectivity necessary to separate mono-, di- and oligosaccharides. Yeast treatment can remove monosaccharides, allowing a high recovery of GOS and disaccharides (Aburto et al. 2019). Activated charcoal treatment in an 8% ethanolic solution gives a high recovery of GOS (~90%), but 20% of disaccharides were also recovered, while with 10% ethanol, there was almost complete removal of disaccharides, but only ~53% of GOS tri-saccharides were recovered. Size exclusion chromatography gives the purest GOS fractions (DP up to 8) but is limited by processing volume (Hernádez et al. 2009).

Table 5.1 Enzymatic synthesis of GOS using various β-galactosidase sources and reaction formats

β-Galactosidase origin	Form	pH	Temp (°C)	Lactose (g/L)	Yield GOS (%)	Productivity GOS (g/L/h)	Time (h)	Reference
Free enzyme								
A. oryzae	Purified	4.5	55	190	24.3	24		Fischer and Kleinschmidt (2015)
A. oryzae		4.5	40	400	21	14		Frenzel et al. (2015)
A. oryzae		4.5	40	427	26	130		Neri et al. (2009a)
A. oryzae		4.5	47.5	500	29	150		Vera et al. (2012)
B. circulans		7.0	40	400	41	38		Frenzel et al. (2015)
B. circulans	Immobilised—glyoxyl agarose	6.0	60	500	39.4		10 cycles	Urrutia et al. (2013)
B. bifidum		6.5	65	430	53.1	36		Osman et al. (2014)
B. longum BCRC 15708	Crude	6.8	45	400	50	13		Hsu et al. (2007)
K. lactis		6.5	45	190	32	32		Fischer and Kleinschmidt (2015)
K. lactis		6.5	40	230	26	33		Martinez-Villaluenga et al. (2008)
L. sakei LB790		6.5	37	200	41	29		Iqbal et al. (2011)
L. bulgaricus L3		7.6	45	352	49	157		Lu et al. (2012)
S. solfataricus		6.0	80	500	53	636		Park et al. (2008)
S. solfataricus		6.5	75	500	61.7	10		Wu et al. (2013)
C. laurenti	Acid whey		55	35–173	36.1			Fischer and Kleinschmidt (2021)
Immobilised enzyme								
A. oryzae	Covalent, cotton cloth, RBR	4.5	40	352	26.6	106	330	Albayrak and Yang (2002a)
A. oryzae	Covalent, chitosan, batch	4.0	40	200	25.5	4.3	4 cycles	Gaur et al. (2006)
A. oryzae	Covalent, magnetic polysiloxane-PVA, batch	4.5	40	500	26	130	–	Neri et al. (2009a)

(continued)

Table 5.1 (continued)

β-Galactosidase origin	Form	pH	Temp (°C)	Lactose (g/L)	Yield GOS (%)	Productivity GOS (g/L/h)	Time (h)	Reference
B. circulans	Covalent, microporous beads, batch	6.3	58	550	64	193	45	Benjamins et al. (2014)
B. circulans	Covalent, microporous beads, CPBR	6.0	50	337	39	1347	-	Warmerdam et al. (2014)
B. bifidum		6.5	55	430	51.9	143.5	20	Osman et al. (2010)
K. lactis	Entrapment, permeabilised cells, batch	8.0	40	352	35	93.3	21	Sun et al. (2016)
K. lactis	Entrapment, permeabilised cells, batch	6.5	40	190	36	24	–	Srivastava et al. (2015)
L. reuteri	Adsorption, microcrystalline cellulose, batch	7.6	45	352	49	156.8	25	Lu et al. (2012)
L. bulgaricus	Entrapment, membrane reactor CSTR	6.0	37	192	30	33	140	Splechtna et al. (2006)
T. *thermophilus*	Covalent, microporous beads, batch	6.5	40	190	40	3.3	96	Nakkharat and Haltrich (2007)
Recombinant enzyme								
B. circulans	95:5 cyclohexane:water	6.0	60	550	66.8			Shin and Yang (1994)
Streptococcus thermophilus	Recombinant—in *L. plantarum*	6.5	50	205	50			Geiger et al. (2016)
H. orenii	Thermostable, recombinant, site directed mutagenesis	6.0	70	300	57.4			Hassan et al. (2016)
S. solfataricus P2	Thermostable, recombinant, site directed mutagenesis	6.5	70	600	61.7			Wu et al. (2013)

Nanofiltration, where the mono- and di-saccharides permeate the filtration membranes while GOS is retained, is one purification method that is very easy to scale up for commercial production (Córdova et al. 2016). However, it is limited by the difficulty of separating the highly concentrated feed streams used to increase GOS yield in the enzymatic transgalactosylation of lactose, as high lactose concentrations favour transgalactosylation over hydrolysis (Vera et al. 2012; Huerta et al. 2011). One way to circumvent this dilemma, in terms of economy and operational

simplicity, is to process the raw GOS stream as close as possible after leaving the bioreactor (Michelon et al. 2014). The evaluation of a number of commercially available membranes has shown that trade-offs between purity and efficiency are always required, but that good selectivity can be attained with polyethersulphone membranes (Montesdeoca et al. 2019; Schmidt et al. 2017).

5.2.4 Properties

Galacto-oligosaccharide products have a wide range of compositions, which in turn determines their physicochemical properties. GOS are water-soluble, translucent or colourless, and their relative stability at high temperatures and in acidic environments (160 °C for 10 min at pH 7 and 100 °C for 10 min at pH 2) make them of particular interest for the food and drink industry, for both their prebiotic properties and their use as sweeteners, especially in beverages, confectionery and fermented dairy products. The physicochemical properties of GOS depend on their purity and chemical composition; for example, the viscosity of GOS increases as their molecular weight increases, which may be significant in modifying texture and mouthfeel in foods. GOS also do not bind minerals, which is advantageous when incorporating into foods.

The chemical structures of the individual GOS components also greatly influence their hydrolysis by the human digestive enzymes and their potential prebiotic characteristics. Trimeric or higher GOS compounds are non-digestible in vitro by human intestinal enzymes, which enables them to exert their prebiotic effects in the large intestine. Disaccharide GOS fractions were heterogeneous in this respect, as some were partially digested under the same conditions. Vera et al. (2016) have written a detailed review of GOS structure. An overview of a number of physicochemical and nutritional properties of GOS are listed in Table 5.2.

Table 5.2 Overview of a number of physicochemical and nutritional properties of GOS

Parameter	Value	Method
Solubility	80%	Stability
Viscosity	2700 mPa s	Rheometer
Appearance	Colourless	Visual
Sweetness	Typically 0.3–0.6	Sensory (sucrose = 1)
Water activity	0.74	Standard
Heat capacity	2.5 J/g/°C	Evaporation
Freezing point	50	Osmometer
Boiling point	106 °C	Evaporation
Density	1.37–1.38	Pycnometer

5.2.5 *Analysis*

There are a number of aspects to the analysis of GOS. Firstly, during synthesis and industrial production, the total amount and yield of GOS need to be determined. Secondly, the amount of GOS, in particular for food or pharmaceutical applications, has to be quantified and reported. In addition, GOS products often need to be characterised for their individual GOS constituents. The AOAC 2001.02 official method is the only validated method for the determination of GOS in raw materials and food samples (AOAC International 2006). In this method, GOS is converted to galactose and glucose via the hydrolysis reaction, then the monosaccharides released from the reaction are quantified to enable the calculation of the total GOS content. However, this method cannot be applied to products that contain high levels of lactose, or either glucose or galactose (e.g., infant formula; Yang and Bednarcik 2001) as other sugars will interfere with the analysis.

A wide array of analytical methods currently employed for the analysis of GOS have been reviewed by Catenza and Donkor (2021). Early analysis of oligosaccharides by gas chromatography (GC) after derivatisation to enable detection by ultraviolet (UV) or laser-induced fluorescence has been mostly superseded by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). HPLC analysis in different operational modes, and coupled to various detectors, and where the carbohydrates do not have to be derivatised beforehand, offer more detailed, quicker analysis. High-performance anion-exchange chromatography (HPAEC) is the most widely used HPLC method, although the particular method will usually depend on the amount of information required about the product. For example, GOS production can be monitored using HPAEC-PAD (pulsed amperometric detection) to simultaneous quantify galactose, glucose, lactose and GOS (Lin et al. 2018). Capillary electrophoresis with laser-induced fluorescence (CE-LIF) has also been used for the determination of GOS, but CE-LIF is not a common method in food analysis with the shortcoming of limited method validation.

NMR, MS and methylated GC-MS are the preferred methods for the structural identification of individual GOS constituents. Determination of the chemical structure is often critical to both acquire a basic knowledge of the GOS synthesis profile and increase the understanding of the mechanisms for their metabolic effects. Thus, the identification of their constituents (qualitative) and the determination of their concentrations (quantitative) are important. The complexity of GOS mixtures is compounded by the number of different saccharides and isomers, requiring structural analysis to determine the position of glycosidic linkages, monomeric composition and anomericity. Although di- and tri-saccharides have been well characterised, the chemical structures of higher molecular weight oligosaccharides have not been studied in detail. In addition, since the transglycosylation in lactose solutions may be performed under various conditions and with a vast array of β-galactosidase enzymes, new derivatives from lactose are continuously being isolated and characterised. The utility of LCMS has been demonstrated by Chen and Liu (2021) using targeted selected ion monitoring, data-dependent tandem mass spectrometry, with

additional in-source collision-induced dissociation in a HPAEC-MS to characterise the structures of individual GOS isomers with a degree of polymerisation up to 6. An MS-based method, logically derived sequence (LODES) tandem mass spectrometry (MS^n), uses dissociation mechanisms and logical sequencing to structurally characterise galactose tri-saccharides and tetra-saccharides (Huang et al. 2021), and by using a combination of HPLC-size exclusion chromatography (SEC) and ^1H NMR (to determine the DP) and MALDI-TOF-MS, HPAEC-PAD and one dimension or two dimension (1D/2D) $^1H/^{13}C$ NMR (to analyse the individual moieties). van Leeuwen et al. (2014, 2016) characterised seven commercial GOS products and could identify over 40 different sugar structures. These techniques were also used to structurally characterise glucosylated GOS products to mimic human milk oligosaccharides that were synthesised with glucansucrases from *L. reuteri* (Pham et al. 2018).

5.2.6 Commercial Producers and Products

GOS are well-established as prebiotics in an increasingly large number of pharmaceuticals and functional foods, especially in infant formulae. The original, traditional companies involved in the manufacture and marketing of GOS included Yakult Honsha Co Ltd. (Oligomate®), Nissin Sugar Manufacturing Co. Ltd. (Cup Oligo), Snow Brand, Ingredion and Friesland Foods Domo (Vivinal®). Over the last decade, however, the demand for GOS has increased markedly as the use of GOS in nutritional applications has grown significantly, and now the number of large-scale producers also include numerous manufacturers from India and China. GOS production has increased from 15,000 tons in 1995 to a forecast figure of 175,000 tons for 2020. While most of the early production came out of Japan, more is being produced in Asia, Europe, and America. The global GOS market size was calculated to be between US$ 570 and US$ 881 million in 2021 and is projected to reach US$ 850–1500 million by 2027, growing at a compound annual growth rate (CAGR) of 6–8.3% over the period. The US is currently the largest market, with demand from China projected to increase in the coming years. The other main markets include Japan, Germany and Canada. Some of the newer producers manufacture GOS products for incorporation into their own products as well as for sale in the ingredient market. The range of products available includes concentrated syrups and powders containing between 32% and 72% and 55–99% GOS, respectively.

The commercial, large-scale production of GOS generally uses β-galactosidase enzymes of either bacterial or fungal (*A. oryzae*) origin, for example, *B. circulans* for 'Vivinal®' GOS, *B. bifidum* for 'Bimuno' GOS and *S. thermophilus* for 'Oligomate 55' GOS (Kaneko et al. 2014). Crude cell extracts or purified enzymes are preferred and in the future purified recombinant enzymes, designed with enhanced transgalactosylation activity and selective linkage types and DP distributions, may become available. GOS production using a batch system is most extensively employed commercially although continuous systems have been described.

5.2.7 *Uses and Applications*

GOS are non-digestible oligosaccharides (NDO) that possess prebiotic activity and are considered to be favourable to health. They are not digested in the upper intestinal tract, can enhance the growth and activity of beneficial bacteria in the intestine and help to modulate the immune system. As stated by the International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus panel, a prebiotic is 'a substrate that is selectively utilised by host micro-organisms conferring a health benefit' (Gibson et al. 2017). The initial application of GOS was principally as beverages and in infant milk formula, follow-on formula and infant foods, although in recent years they are also increasingly being used as functional food ingredients in a variety of food products. Nowadays, infant formulae are routinely supplemented with GOS to mimic the biological effects of human milk oligosaccharides (HMO), although recently a number of more predominant HMO moieties have been produced by microbial, fermentative methods using engineered microorganisms. Galactose-containing hetero-oligosaccharides, such as GOS, have also attracted an increasing amount of attention recently because they are structurally more closely related to HMO than some other oligosaccharides. The synthesis of these novel oligosaccharides, which resemble the core of HMO, is of great interest for applications in the food industry (Hernández-Hernández et al. 2012). They have been shown to mimic the metabolic and microbial effects of HMO when added to infant formula (Bakker-Zierikzee et al. 2005). GOS has been demonstrated to be the best substitute for HMO, giving it a unique property among other prebiotics. GOS:FOS (fructo-oligosaccharide) mixtures in the ratio 9:1 have gained wide acceptance as a prebiotic supplement in infant formulas (Bode 2009) since such prebiotic mixtures, comprising short-chain oligosaccharides from GOS and medium to long-chain oligosaccharides from FOS, result in a molecular size distribution similar to HMO (Moro et al. 2002).

Prebiotics are rapidly rising in popularity within the functional food market segment (Table 5.3). Probiotics and prebiotics are fundamental ingredients in fermented milks and yoghurts, the most important segment of the overall market for functional foods. Other major applications of probiotic and prebiotic ingredients include health

Table 5.3 Physicochemical attributes of GOS used to modify foodstuffs

Attribute	Examples
Low sweetness	Low glycaemic index, low calorie foods
Taste and transparency	
Prevention of hygroscopicity	Confectionary, sweets, chocolates
Prevention of colouration	Fruits and jams
Reinforcement agent or thickener	Sauces, creams, jelly
Glazing agent	Rice crackers, hard lollies/boilings
Regulation of freezing point	Ice cream, frozen foods
Humectant	Cakes, pastries
Powderising material	Coffee whitener, soups

drinks, nutrition bars, breakfast cereals, beverages, bakery products, meat products, mineral supplements, weight loss products, green foods, infant food and pet food (Austin et al. 2014). When added to sheep milk ice cream, prebiotic dietary oligosaccharides, including GOS, could replace the fat in the ice cream formulation, improved the whiteness and lightness of the ice cream, decreased its caloric value and improved the stability in relation to the physicochemical parameters and sensory perception (Balthazar et al. 2015, 2017a, b). Recently, most prebiotic ingredient applications have been in breakfast cereal, bread, baked goods and snacks, including energy bars, pet foods and athletic drinks. However, much of this growth is being driven by an interest in sugar, fat and calorie reduction rather than an interest in the bifidogenic effects of prebiotics. A rise in obesity and other health concerns related to digestive health, bones and joints is fast encouraging consumers to focus on low-carbohydrate, well-balanced, healthy diets, which in turn are driving the demand for prebiotics. Based on these trends, the future outlook for the prebiotics markets seems very encouraging. GOS is also being added to mineral supplements to aid in mineral adsorption. One interesting observation from the trend for lactose-free milk is the presence of small amounts of GOS in commercial milk products such as UHT milk after β-galactosidase treatment to remove the lactose (Ruiz-Matute et al. 2012). A more controlled lactose hydrolysis step may increase the GOS content high enough to confer a beneficial prebiotic effect.

Functional foods such as GOS and other prebiotics principally target gut health. GOS and other prebiotics have been suggested as ingredients for a wide variety of human food products such as baked goods, sweeteners, yoghurts, nutrition bars and meal replacement shakes, as well as pet foods. GOS can be added for either its nutritional advantages or its functional properties, and it is often applied to offer a double benefit, for example, an improved organoleptic quality and a better-balanced nutritional approach. The major physicochemical properties of GOS also make them useful in various food systems such as to improve the taste and texture in food, along with health benefits. In yoghurt and dessert applications, GOS can be used for sugar replacement, texture and mouthfeel, and fibre delivery, whereas in beverage applications, foam stabilisation also becomes important. In bakery and cake applications, moisture retention is the important property imparted by GOS, along with sugar replacement and fibre delivery. Other applications of GOS are for mouthfeel, fibre and sugar replacement attributes in foods including baby food, fillings, confectionery and sauces. In bakery and dairy products, they can be used as partial fat and sugar replacers and as bulking agents, and they can improve nutritional functionality by acting as fortifying agents in foods such as infant formulae, ice cream and cereal products (Nobre et al. 2015; Ibrahim 2018a, b; Zhao et al. 2017).

There is also interest in producing synbiotic food products composed of prebiotic GOS and probiotic bacteria and yeast. Lactic acid bacteria in milk permeate have been added with GOS to an apple by-product to produce a fermented beverage with high GOS content (up to 0.268 g/L) and good antimicrobial activity (Zokaityte et al. 2020). Beverages have also been made with the synbiotic probiotic yeast *Saccharomyces boulardii* and cheese whey permeate (dos Passos et al. 2020), and *Lactobacillus paracasei* PB9 and *L. plantarum* 2108 have been grown on residue

lactose to give a beverage high in lactic and citric acid that also contains high-purity GOS (Pázmándi et al. 2021). A yoghurt containing low lactose plus GOS and Lactobacilli probiotics has also been shown to improve calcium absorption and bone retention in a rat model (Seijo et al. 2021).

Due to the prebiotic properties of GOS, they can also be used in nutritional enhancers, livestock and aquaculture feed and companion animals' food. Their ingestion can help improve bowel consistency and provide firmer and less malodorous faeces. In controlled animal studies, GOS has been associated with improved growth performance of broiler chickens and dietary supplementation with GOS has also been linked with improvements in the transition to a mature intestinal microbiota in broiler chickens and in suckling piglets (Flaujac Lafontaine et al. 2020). The addition of GOS in feed to improve animal health and minimise antibiotic use in the chicken, pig and calf industries is therefore increasing, and GOS also has the potential for further uses as a possible agent to help suppress methane production in ruminants.

5.2.8 Health Benefits

As described in the previous section, GOS is well established as a prebiotic ingredient in a wide range of functional foods and as a human milk oligosaccharide alternative in the infant formula market. In vitro and in vivo experiments have demonstrated the indigestibility and stability of GOS to hydrolysis by digestive enzymes. Tri- and tetra-GOS saccharides are unable to be hydrolysed in vitro by human salivary α-amylase, artificial succus gastricus, α-amylase of hog pancreas and rat intestinal acetone powder, although disaccharides can be partially digested by the intestinal enzymes. The prebiotic health benefits from GOS therefore occur indirectly via their stimulation of the growth and activity of beneficial microorganisms such as lactobacilli and bifidobacteria in the large intestine. The bifidobacteria population can then provide resistance against colonisation of the intestine by pathogens, thereby reducing exogenous and endogenous intestinal infections and leading to a wide number of well-recognised health benefits. These include stimulation of the immune system, inhibition of the growth of pathogenic intestinal microorganisms, an increase in the production of a number of B vitamins, reduction of blood ammonia and cholesterol and aiding in restoring a healthy intestinal microbiota after antibiotic treatment (Gibson and Roberfroid 2008; Roberfroid 2007). Readers are directed to two comprehensive reviews on the biological activities of GOS and the symbiosis with probiotics in relation to the GI system, osteoporosis, blood lipid and glucose levers by Nath et al. (2018a, b).

The prebiotic activity of GOS is dependent on the carbohydrate glycosidic linkages, monosaccharide composition and degree of polymerisation (DP). In a structure-function study on the selectivity of fermentation of different oligosaccharides by mixed microbial cultures, different GOS and FOS moieties were purified and characterised using MS and NMR spectroscopy. A 'prebiotic index (PI)'

(Palframan et al. 2003) value for GOS, which is based on the comparative relationship of the effect on growth of faecal beneficial (bifidobacterial and lactobacilli) and harmful bacteria (bacteroides and clostridia), was calculated for each oligosaccharide (Li et al. 2015a, b, c). GOS showed a high selective stimulation towards bifidobacterial, giving a higher PI value (11.66) compared with FOS (5.05), and GOS with a β-(1 → 6)-linkage had a relatively higher PI value than GOS with β-(1 → 4)-linkage. Their respective prebiotic properties were investigated via in vitro fermentation by human intestinal microbiota in mixed cultures and verified the stimulation findings. These values were indicative of the selective metabolism of GOS by beneficial bifidobacterial and lactobacilli and to a lesser extent by pathogenic bacteria, like clostridia.

While breast-feeding is overwhelmingly recommended for all babies, when this is not possible, infant formula based on bovine milk is usually used. An excellent review on GOS as an infant prebiotic has been written by Ambrogi et al. (2021). GOS-fortified infant formula is becoming more prevalent and has been conclusively proven to promote the growth of bifidobacterial and lactobacilli, resulting in an infant intestinal microbiome similar to that reported for infants fed breast milk (where bifidobacter are the dominant species with 60–80% of the total microbiota) (Sierra et al. 2015). Bifidobacter prefer to grow with GOS over the simple sugars such as lactose and glucose as their principal carbon source. GOS has been shown to provide cell protection and inhibit pathogenic cell adhesion, thus reducing potentially harmful bacteria such as *Clostridium* and reducing the incidence of diarrhoea. GOS-containing milk formula has also been reported to lower the frequency of colic symptoms, result in stools with a softer consistency, decrease the incidence of atopic dermatitis and respiratory infections and increase colonic iron absorption in infants. When GOS was fermented in vitro by infant faecal microbiota (using infant faecal inoculum of 2- and 8-week-old infants) the degradation of GOS coincided with an increase in Bifidobacterium and the production of acetate and lactate. GOS fermentation digesta also attenuated the cytokine profiles in immature dendritic cells, with the extent dependent on the infants' age and GOS structure (Logtenberg et al. 2021).

After weaning, the intestinal microbiota becomes more established and stable with 30–40 species dominating a population of more than 500 microbial species. GOS has demonstrated some prebiotic effects in adults but the bifidogenic impact is not as consistent as for infants. GOS ingestion in adults can also promote an increase in the concentration of lactobacilli, which in turn favours lactose digestion in lactose intolerant individuals. It can also reduce constipation by improving stool frequency and consistency in infants and adults, though it is used more in functional foods as opposed to pharmaceutical applications (Wang 2009). The beneficial effects of GOS on gut health also help in preventing infections from, for example, Salmonella species.

GOS has been shown to have a clear role in colorectal cancer prevention by modulating the intestinal microbiota and impacting the host physiology and immune system (Bruno-Barcena and Azcarate-Peril 2015). GOS increases the intestinal lactate, short chain fatty acids, and stool frequency and weight, and decreases the faecal concentration of lithocholic acid, faecal pH, and nitroreductase and

β-glucuronidase activities. The improved colonic environment helps to depress toxigenic microbial metabolism and reduce the levels of mutagenic enzyme activity (e.g., β-glucuronidase and azoreductase) and bacterial metabolites (e.g., secondary bile acids, phenols and indoles) that are purportedly associated with the risk of colon cancer. In a rat model, oral administration of prebiotic lactulose-derived GOS preparation resulted in a reduction in the number of colon tumours in the treated animals. Metagenomics sequencing of colon microbiota populations revealed significant reductions in populations of pro-inflammatory bacteria families and species, and significant increases in interesting beneficial populations, such as Bifidobacterium, suggesting that lactulose-derived GOS may be an effective strategy for preventing colorectal cancer (Fernández et al. 2018). The risk reduction of colon cancer has, however, yet to be quantitatively established.

The consumption of GOS is known to increase the absorption of Ca, Mg and Fe, with two in vivo human studies having demonstrated that GOS supplementation can increase iron absorption. When ferrous fumarate and sodium iron EDTA in a GOS-containing micronutrient powder and ferrous fumurate-GOS powder were given to Kenyan infants and in iron-depleted women, respectively, there were significant increases in iron absorption in both the groups (Paganini et al. 2017; Jeroense et al. 2019). GOS also has a possible role in weight management and controlling metabolic syndrome. In animal studies, dietary supplementation with the short-chain fatty acid butyrate (a prebiotic fermentation product) has been found to prevent diet-induced obesity and improve insulin sensitivity. There was also a concomitant increase in energy expenditure and fatty acid oxidation and an increase in mitochondrial respiration. In mice studies, the selective growth of certain Lactobacillus species in the colon can cause a reduction in body fat storage through the upregulation of fasting-induced adipose factor (FIAF) gene expression and the inhibition of lipoprotein lipase. Also, several animal studies have demonstrated the protective effects of prebiotics on the development of obesity and insulin resistance. A mouse study used GOS supplementation of a Western-type diet to demonstrate improvements in body weight gain, dyslipidaemia and insulin sensitivity, thus supporting the therapeutic potential of GOS for individuals at risk of developing metabolic syndrome (Mistry et al. 2020). However, more robust human studies are required to confirm the protective effects of prebiotics on these pathways in human physiology.

The administration of GOS has been demonstrated in a double-blind, placebo-controlled, randomised human study to reduce the incidence of travellers' diarrhoea (Drakoularakou et al. 2009) and was effective in alleviating the symptoms. In a similar manner, GOS has potential as a therapeutic agent for irritable bowel syndrome (IBS). In a clinical trial, it was observed to specifically stimulate gut bifidobacteria in IBS patients and to alter the faecal bacterial flora, as well as increase the number of bifidobacteria in a dose-dependent manner, leading to an improved overall quality of life in the patients (Silk et al. 2009). It thus has potential as a therapeutic agent in IBS treatment. Other possible health indications for GOS include reducing the risk of hypercholesterolaemia, and the potential control of several physiological processes such as mucosal proliferation, inflammation, the elimination of nitrogen compounds and treatments for diseases such as cardiovascular disease, cancer and type 2 diabetes (Catenza and Donkor 2021).

5.2.9 Product Safety, Dosage Rates, Regulating Issues

Several studies have been carried out to demonstrate the safety of GOS. Results showed no significant adverse toxicological effects attributable to the treatment. Galacto-oligosaccharides are considered Generally Recognised As Safe (GRAS) by the US Food and Drug Administration (FDA) for different intended uses, for example, infant formulae, dairy products, fruit drinks, waters and cereals (GRAS file Vivinal GOS GRN 236, 2007; GRAS file Oligomate, GRN 334, 2010; GRAS file GTC Nutrition, GRN 285, 2009). As a result, GOS can be used in the USA as an ingredient in a broad range of food categories (FDA 2010, 2014a, b, 2015, 2016, 2017a, b, 2018a, b). In the EU, GOS was used as a food ingredient before the Novel Foods Regulation (258/97/EC) went into effect in May 1997. In 1996, the Dutch Ministry of Health, Welfare and Sport approved GOS for use in food products. Based on the approval and use of GOS before 1997, GOS can be used as a non-Novel Foods ingredient in food products in all EU member states. GOS have also received official approval from a number of other authorities for the use in food products in China, Brazil, Canada and Japan.

Studies have shown that consumption of GOS by infants in amounts up to 0.9 g/100 mL have no influence on the incidence of side effects such as crying, regurgitation and vomiting, and 10–20 g/day is the recommended dosage for adult humans. A minimum dose of 5 g of GOS per day can induce significant alterations in the gut microbiota in healthy human adults, mainly by increasing the number of bifidobacteria. When there is already a relatively high bifidobacterial population in the colon a bifidogenic response is not always elicited by additional GOS in the diet. Sawatzki et al. (2005) demonstrated that GOS have no negative effect on the water balance and growth parameters and can be considered as safe.

Different regulations are applicable to food and nutritional products containing GOS. In several countries around the world, including Austria, Finland, Italy, Belgium, the Netherlands and Japan, GOS is used as a food ingredient, for example, in dairy products, beverages and confectionery. Wide-ranging tests have been performed in order to substantiate the safety of β-galactosidase, which is used for the production of GOS, including tests to prove the absence of mycotoxins, antibacterial activity, toxicity and potential mutagenicity during the enzyme preparation. Based on this, many countries have approved the use of the β-galactosidase. A safety evaluation ruling by the European Food Safety Authority (EFSA) on a β-galactosidase from *Bacillus* sp. (strain M3-1) has stated that the enzyme preparation does not give rise to safety concerns under the intended conditions of use (EFSA 2019).

5.3 Lactulose

Lactulose, a disaccharide of galactose and fructose, does not occur naturally in mammalian milk, but small amounts can be observed in heat-treated milk, e.g., ultra-heat treatment (UHT) milk, and thus its presence has been used as an indicator of milk heat abuse or severity of heat treatment. Lactulose was initially considered to be a milk component oddity until potential prebiotic applications for lactulose in both adults and bottle-fed infants were reported by Petuely (1957). A few short years later, in 1960, Morinaga began adding lactulose to infant milk, and it was the first lactose-derived product marketed and sold as a laxative for the treatment of acute and chronic constipation. Lactulose can be produced by the isomerisation of lactose, and the primary use of lactulose for the first 50 years of its production has been pharmaceutical. However, in the last decade, it has been used more as a functional food ingredient, such that approximately three quarters of current lactulose production are used in the food industry. The reader is directed to two recent reviews by Nooshkam et al. (2018) and Ruszkowski and Witkowski (2019). Also, the Illanes group in Chile have been very active in all aspects of lactose-derived products and have published a considerable amount of research and review articles, together with books and book chapters on the subject.

5.3.1 Chemistry

Lactulose (CAS name 4-*O*-β-D-galactopyranosyl-D-fructofuranose; CAS Registry Number: 4618-18-2; additional names: 4-D-galactopyranosyl-4-D-fructofuranose; 4-*O*-β-D-galactosyl-D-fructose; 4-β-D-galactosido-D-fructose) is a synthetic, non-digestible disaccharide product of lactose isomerisation and is composed of the two saccharides galactose and fructose. It has the same empirical formula and molecular mass as lactose ($C_{12}H_{22}O_{11}$ and 342.30 g/mol, respectively; percent composition: C 42.11%, H 6.48%, O 51.41%).

5.3.2 Synthesis

The ‘natural’ formation of lactulose during thermal treatment of dairy products occurs when the lactosyl-amine complex formed from the Maillard reaction of lactose with amines and ammonia subsequently undergoes an Amadori rearrangement and hydrolysis to form lactulose. Only small amounts of lactulose are produced, even at high temperatures, as the catalysis is favoured by hot alkaline conditions, depends on the lactose concentration, the time and temperature of heating, and follows pseudo-zero-order kinetics with an activation energy of 90.2 kJ/mol (Claeys et al. 2001). At the normal pH of milk (~6.7–6.8), lactulose levels of 0.3 g/L and

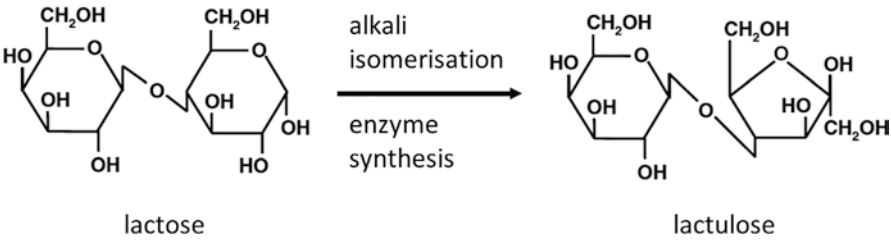

Fig. 5.5 Chemical structures (Haworth) of lactulose and its conversion from lactose

1.6 g/L for UHT and sterilised milk, respectively, have been reported by Schuster-Wolff-Bühring et al. (2010).

Lactulose can be produced either by chemical synthesis or by enzymatic processes (Fig. 5.5 and Table 5.4). Chemically, lactulose is formed by the alkaline isomerisation of lactose, which is characterised by the transformation of the glucose moiety of lactose to fructose. The first reported synthesis was by Montgomery and Hudson in 1930, using the alkaline isomerisation of lactose via an enolisation step, followed by β-elimination in a dilute $Ca(OH)_2$ solution (the Lobry deBruyn-Alberda van Ekenstein reaction). Many other mechanisms for this transformation have been proposed, but the Lobry de Bruyne-Alberda van Ekenstein reaction is the most accepted. While more environmentally friendly alternatives, such as electro-isomerisation and biochemical enzymatic processes, have also been investigated, these have yet to be used industrially (Guerrero and Wilson 2016), but they have the potential to replace existing chemical approaches in the future (Xiao et al. 2019a, b). Additionally, although most synthesis processes are initially developed using lactose, they also need to operate using a cheaper, dairy stream such as cheese whey for commercial viability.

The chemical synthesis of lactulose via the Lobry de Bruyn-Alberda van Ekenstein transformation uses catalysts such as borates and aluminates, hydroxides and carbonates, and proceeds via the formation of an intermediate 1,2-endiol, which is then isomerised into lactulose. The synthesis is optimal at high-temperature and high-alkaline conditions and typically results in a low (20–35%) product yield. High amounts of inorganic catalysts are usually required, and the overall process remains relatively expensive. Other disadvantages of this method include the low specificity of the reaction, numerous side reactions resulting in low product yields and a number of contaminant by-products. These by-products include galactose, iso-saccharinic acid and coloured products, and result from the degradation of either the original lactose or the newly formed lactulose. Their removal necessitates major purification or clean-up using costly and cumbersome downstream processing (Villamiel et al. 2002). In the quest to develop an ideal catalyst that is more economic, eco-friendly, safe and non-toxic, three types of catalyst have been evaluated.

Alkaline catalysts A large number of alkaline catalysts, including sodium hydroxide, potassium hydroxide, potassium carbonate and tertiary amine, have been evaluated for their ability to convert lactose to lactulose. At reaction temperatures between

Table 5.4 Synthesis of lactulose using chemical and enzymatic methods

Chemical catalyst/microorganism	Form	pH	Temp (°C)	Substrate (g/L) (lactose or lactose/fructose)	Yield (g/L)	Conversion (%)	Productivity (g/L/h)	Time (h)	Reference
NaOH		11.0	70		27			0.25	Zokaee et al. (2002)
NaOH ultrasound-assisted; 70% intensity		10.6	60			24		1	Corzo-Martínez et al. (2014)
Na_2CO_3	Fed batch system		90	Cheese whey		29.6	15.8	0.35	Seo et al. (2015)
Complex reagent, recycle 5 times	Na aluminate $NaAlO_2$	12.0	60	350		85.5		0.83	Wang et al. (2017)
Recycle 5 times	NaOH/Na tetraborate	11.0	70	100		86		4	Pazourek (2019)
Eggshell, oyster shell, limestone	Natural catalysts		96	Milk permeate		18–21		2	Paseephol et al. (2008)
Alkaline substituted sepiolites				Milk permeate		20	12	2.5	Villamiel et al. (2002)
Egg shell			98	Milk permeate		25		1	Montilla et al. (2005)
Electro-isomerisation (200 mA current)		14.0	23	Lactose		25		1	Aïder and Gimenez-Vidal (2012)
		12.5	23	Whey		10			
Electro-activation (200 mA current)	Na_2SO_4 as electrolyte	11.0	10	Lactose		30		0.5	Aït-Aissa and Aïder (2013)
Electro-activation (400 mA current)	Na_2SO_4 as electrolyte	11.0	10	Cheese whey		35		0.67	Kareb et al. (2016)
Electro-activation (330 mA current)				6% whey permeate		40		0.6	Djouab et al. (2019)
Electro-activation (900 mA current)				6% whey permeate		37		0.83	Karim and Aïder (2020a)

(continued)

Table 5.4 (continued)

Chemical catalyst/microorganism	Form	pH	Temp (°C)	Substrate (g/L) (lactose or lactose/fructose)	Yield (g/L)	Conversion (%)	Productivity (g/L/h)	Time (h)	Reference
Electro-activation (900 mA current)				10% whey permeate		38		0.7	Karim and Aïder (2020b)
Hyperthermostable recomb	*P. furiosus*	5.0	75	2.62/20.7		44		2	Mayer et al. (2004)
Immobilised recomb	*P. furiosus*	5.0	75			43	52	3.5	Mayer et al. (2010)
β-Galactosidase	*A. oryzae*			150/200	65	19			Adamczak et al. (2009)
Free β-galactosidase	*K. lactis*	6.7	40	9–12/25–30		20			Khatami et al. (2014)
Recombinant in *E. coli*	*L. plantarum*	7.0	40	400/200	18.4			6	Liao et al. (2016)
β-Galactosidase	*K. lactis*		37	Whey permeate		50.1		3	Zimmer et al. (2017)
β-Galactosidase	*A. oryzae/K. lactis*			Acid/sweet whey	9.7			2	Schmidt et al. (2020)
β-Galactosidase	*B. circulans*	6.5	50	25/475		55			Aburto et al. (2020a, b)
Free immobilised	*A. oryzae*	4.5	50			31			Guerrero et al. (2015)
						25.4			
β-Galactosidase + glucose isomerase, immobilised	*K. lactis*	8.0	30	800/100	151	19	75.5	2	Hua et al. (2010)
	Streptomyces murinus								
β-Galactosidase immobilised chitosan l:f 1:2 free vs. immobilised	*K. lactis*	7.0	50	Cheese whey 133/67	15.3	23.6	24.6	2	de Albuquerque et al. (2018)
					17.3	26.7	31.5		

Cellobiose 2-epimerase, recomb. *E. coli*	*C. saccharolyticus*	7.5	80	700	408	74	204	2	Kim and Oh (2012)
Cellobiose 2-epimerase	*Borate cofactor*			Whey permeate	614	88	205		Kim et al. (2013)
Cellobiose 2-epimerase, recomb. *B. subtilis*	*C. saccharolyticus*	7.0	80	Whey permeate 200	117	58.5		2	Wu et al. (2017)
E. coli, Ni^{2+}-charged iminodiacetic acid column IMAC	*C. saccharolyticus*		50	UHT milk 48.5	28.0	57.7		24	Rentschler et al. (2015)
			8		27.5	56.7		72	
	Single site mutant, heat stability	7.5	80	50		76			Shen et al. (2016)
Recomb. in *E. coli*, immobilised on *Bacillus* spores	*C. saccharolyticus*	7.0	80	700		395	99		Gu et al. (2015)
	7 epi enzymes, only one good	7.5	20	50		56.8		14	Kuschel et al. (2017)
Recomb. in *E. coli*, ethanol permeabilised + borate	*C. saccharolyticus*	7.5	80	600	391	65	195	2	Wang et al. (2015)
	D. thermophilum		80	200		50.7		4	Xiao et al. (2019a, b)
				400		46.7			

30 and 70 °C, a natural maximal conversion rate to lactulose of approximately 30% has been observed due to the subsequent degradation of lactulose into galactose and other secondary products such as formic and iso-saccharinic acid. Some of these by-products are coloured pigments and require extensive removal steps. Additionally, lactulose is difficult to separate from the catalyst and other by-products as they are all mostly soluble, and this not only reduces the yield of lactulose but also makes subsequent purification and crystallisation of lactulose more difficult (Hashemi and Ashtiani 2010). When a fed-batch reactor was used to suppress galactose production, lactulose could be produced at a rate of 15.8 g/L/h from dried cheese whey (Seo et al. 2015) using a sodium carbonate catalyst at 90 °C. Performing the reaction in subcritical aqueous ethanol (60% w/w) at 200 °C also increased the maximum yield of lactulose (34% in 5–8 min) by promoting and suppressing the isomerisation and hydrolytic reactions of lactose, respectively (Soisangwan et al. 2017); the use of higher concentrations of ethanol was restricted due to the low solubility of lactose.

Complexing catalysts The low lactose to lactulose conversion rates observed with alkaline catalysts can be increased by the addition of complexing reagents such as borates and aluminates that form insoluble and stable complexes with lactulose at alkaline pH values. The equilibrium of the reaction moves towards the direction of lactulose production, minimising the secondary reactions and degradation products, resulting in only a small fraction of the lactulose being converted into by-products. The reaction temperatures are generally low (60–70 °C), with reaction times of less than 1 h, resulting in conversion rates of lactulose of between 75% and 86% (Wang et al. 2017; Pazourek 2019). On completion of the reaction, the lactulose-borate/aluminate complex can be disrupted by lowering the reaction system pH to an acidic pH (pH 4.5–6.5). This also precipitates the catalyst, aiding in the lactulose recovery. After recovery, a sodium aluminate catalyst was able to be recycled five times while retaining its initial catalytic ability (Wang et al. 2017). Removal of at least 95% of the catalyst was achieved by pH precipitation, and any remaining borate or aluminate can be removed using ion-exchange chromatography or nanofiltration. This is also the main method for the industrial production of lactulose.

Natural catalysts Some heterogeneous catalysts, including zeolite, sepiolite, eggshell powder and oyster shell powder, have been used in the production of lactulose. Although the conversion rate of lactulose is not as high as with alkaline and complexing reagents, these catalysts are natural, readily accessible and usually used in the powdered form. As such, they remain in the solid state and are easily removed by filtration after the completion of the reaction. At temperatures close to boiling (90–98 °C) optimum lactulose yields of 16–25% can be attained within 3 h using milk permeate as the lactose substrate. Final lactulose concentration of 12 mg/mL has been attained, and the short reaction times can result in lactulose streams with lower levels of coloured and undesirable products (Villamiel et al. 2002; Montilla et al. 2005; Paseephol et al. 2008). Therefore, the preparation of lactulose with non-homogeneous catalyst shows good potential for development.

Electro-activation isomerisation Another method to affect the isomerisation of lactose to lactulose is through the use of electro-activation to self-generate the high-alkaline conditions required to drive the isomerisation process. Aïder's group (Université Laval, Quebec) have developed this approach and published extensively since 2012. The technology is free of reagents, safe, clean and green, utilising an electric field across two electrodes in an electrolysis reactor. Initial research with this method gave yields of lactulose of between 10% and 35% at ambient temperatures and with the production of only small amounts of the impurities galactose and fructose, and no epilactose (Aït-Aissa and Aïder 2013; Kareb et al. 2016). More recent studies (Djouab and Aïder 2019; Karim and Aïder 2020a, b), using both lactose and whey permeate streams, showed that this method could be operated in situ, was lactose-concentration-, electric-current-, and electro-activtaion (EA)-time-dependent and reached the highest lactulose yield of 37–40% in just 35–50 min. When permeate was used as substrate, the resulting electro-activated whey was shown to contain high antioxidant capacity. The outcome of these studies suggest that electro-activation is a promising technique for the enhanced production of lactulose from lactose, dependent on the optimal size of the electro-activation reactor.

The limitations and challenges associated with the chemical synthesis of lactulose has resulted in a growing need for the development of an environmentally friendly method for the production of lactulose for human consumption. Enzymatic production of lactulose is a potential alternative process as it offers benefits with respect to waste management, product purification and classification as a 'natural product' in the food and pharmaceutical industry. There are three possible pathways for the enzymatic conversion of lactose to lactulose. Transgalactosylation of lactose by either of the glycosyl hydrolases β-glycosidase (EC 3.2.1.21) or β-galactosidase (EC 3.2.1.23), or isomerisation of lactose by an isomerase. The molecular rearrangement of lactose via direct isomerisation to lactulose by a specific isomerase is the 'ideal' biochemical choice, although the reaction was only first reported in 2012 (Kim and Oh 2012). Unfortunately, no isomerase enzyme is yet available for industrial use, nor have any been approved for use in the food industry. β-Glycosidases and β-galactosidases are commonly used as hydrolytic enzymes, but under appropriate conditions, they can also catalyse transgalactosylation reactions, leading to the synthesis of lactulose, prebiotic galacto-oligosaccharides (GOS) and other galactosyl derivatives. Transgalactosylation was first reported in the 1970s and consists of the reversible hydrolysis of lactose to galactose and glucose, with the galactosyl moiety then being reacted with fructose to form lactulose. The reaction is dependent on the enzyme, to facilitate both the hydrolysis of lactose, and the transfer of the galactosyl–enzyme complex to galactosyl acceptors. Transgalactosylation can result a number of different products and the main factors directing the enzymatic synthesis to lactulose include the source of enzyme, the immobilisation protocol, the lactose/fructose ratio, the total substrate concentration, temperature, pH, enzymatic loading and the addition of ions.

A recombinant hyperthermostable β-glycosidase from *Pyrococcus furiosus*, expressed in *E. coli* and partially purified after cell disruption by heat treatment and

dialysis, produced lactulose at yields of up to approximately 45% for the free and immobilised enzyme in enzyme membrane reactor (EMR) and PBR modes (Mayer et al. 2004, 2010). There were a number of possible end products from the hydrolysis of lactose, including galactooligosaccharides, lactose or isomers, di-galactoside, and higher galactooligosaccharides. A high fructose concentration, together with a high fructose to lactose ratio (20:1), was required to depress the hydrolytic potential of the enzyme and drive the transgalactosylation reaction towards lactulose production. When immobilised, the enzyme was stable in a packed bed reactor for at least 14 days, compared with only 1.5 days for the free enzyme in an enzyme membrane reactor.

The production of lactulose using β-galactosidases isolated from a wide number of different sources, including animals, plants and microorganisms, has been the subject of intensive research dating back to the 1970s. The readers are directed to a relevant review by Silvério et al. (2016). β-Galactosidases are multi-functional enzymes with the ability to hydrolyse lactose to glucose and galactose and also to produce lactose-derived sugars such as galactooligosaccharides and lactulose. The latter is achieved via a rapid trans-galactosylation mechanism that transfers the galactosyl moiety from the galactosyl-β-galactosidase complex to fructose as an acceptor to generate lactulose. When the enzyme catalyses the reaction between lactose and fructose to form lactulose via transgalactosylation, it is kinetically controlled, while the reverse hydrolysis reaction between galactose and fructose is equilibrium controlled. Also, the reverse hydrolysis of D-galactose and D-fructose with β-galactosidases from *A. oryzae* and *Escherichia coli* were shown to result in a mixture of four differently linked disaccharides, with the major product being 1-lactulose (Schmidt et al. 2020).

In a similar fashion to β-glycosidase, the major disadvantage of using β-galactosidase is the requirement to 'push' the reaction towards the transgalactosylation pathway, while at the same time recognising that if either the lactulose concentration or the amount of enzyme are too high, this works in favour of lactulose hydrolysis. β-Galactosidase enzymes also generally need a cofactor, which increases the cost of the reaction. The most significant factors that can affect the selectivity to lactulose production are the fructose:lactose ratio and the initial lactose concentration. As with the enzymatic synthesis of other lactose-derived products, increasing the enzyme selectivity and yield are the major stumbling blocks to large scale industrial use. Strategies to overcome these obstacles include:

1. The use of permeabilised whole cells,
2. Thermostable and hyperthermostable enzymes,
3. The recombinant expression of enzymes in hosts that have GRAS status,
4. Immobilisation of the purified enzymes on supports to increase the stability and longevity of their activity,
5. Reactor design (enzymatic membrane and packed bed reactors),
6. The addition of glucose isomerase to isomerise the glucose produced from lactose hydrolysis to fructose, and
7. Site-directed mutagenesis to produce more specific or stable enzymes.

There is also the economic driver to use whey or whey permeate as the lactose source, with the associated disadvantage of the low lactose concentration pushing the reaction in the wrong direction unless the whey source has been subject to prior processing (Schmidt et al. 2020). Lactulose was initially synthesised enzymatically using either whole cells or free enzymes. Lactulose conversion rates from lactose are typically between 20% and 30%, approximately half that achieved by chemical synthesis. Two strategies to increase enzymatic lactulose production include protein engineering and the use of nonconventional media. When the β-galactosidase gene from a hyperthermophilic bacteria was expressed in *E. coli* and then purified (Kim et al. 2006), the resulting thermostable enzyme was able to result in higher lactulose concentrations (50 g/L) than the free enzymes. Recombinant technology has also been used to produce a purified β-galactosidase from *L. plantarum* FMNP01, a probiotic and GRAS organism (Liao et al. 2016) with high activity. As an aqueous environment favours the hydrolysis of lactose ahead of transgalactosylation, the lactulose yield can also be enhanced by using a two-phase media to depress hydrolysis activity by lowering the water activity (a_w) (Hua et al. 2010). However, a small percentage of water was required to drive the initial lactose hydrolysis. Similarly, the inclusion of triethyl phosphate up to a concentration of 30% (w/w) increased lactulose production by up to 20% while acetone had a negative effect (Khatami et al. 2014).

While the primary hydrolysis of lactose is required to produce galactose, secondary hydrolysis also occurs as soon as lactulose is produced, consequently, there is always a balance between the hydrolysis and transgalactosylation reactions. Excessive amounts of enzyme are unfavourable as it leads to the acceleration of both primary and secondary hydrolysis, but after lactulose is produced, it is very susceptible to secondary hydrolysis. Large amounts of fructose seem to be advantageous for lactulose synthesis, as this gives a higher probability for fructose to react with the galactosyl–enzyme complex. The secondary hydrolysis could be circumvented by the continuous removal of lactulose, either by the addition of borate to complex the lactulose or, during continuous operation, by the physical removal of the lactulose using, for example, an enzymatic membrane reactor (Sitanggang et al. 2014). Reactors also permit the continuous production of lactulose at a constant flux, yielded significantly higher specific productivities under ‘steady state’ conditions for longer operating times. Shorter hydraulic retention times (flux) can also be used in constant flux reactors for sustained production.

Immobilisation of β-galactosidase can enhance productivity by increasing the enzyme stability and thermal tolerance although the enzyme activity is often affected by the immobilisation step. When both β-galactosidase and glucose isomerase were immobilised, high lactulose yields and productivities were achieved with lactose as the substrate although some fructose had to be added as the glucose isomerase could not supply all the fructose required for the transgalactosylation reaction (Hua et al. 2010). Inhibition by galactose, glucose and fructose was confirmed for both the hydrolysis and transgalactosylation reactions. Lactulose synthesis in a continuous packed bed system was higher than that in a batch system when β-galactosidase from *K. lactis* was immobilised on activated silica gels, probably due to the different

kinetic properties of immobilised β-galactosidase resulting from decreased accumulation of inhibitors during the reaction (Song et al. 2013a, b). Similarly, de Albuquerque et al. (2018) achieved extended lactulose synthesis over a number of hours using an enzyme immobilised onto glutaraldehyde-chitosan. Commercial feedstocks such as cheese whey, whey powder and reconstituted whey ultrafiltration permeate have increasingly been used as substrates (de Albuquerque et al. 2018; Song et al. 2013a, b; Schmidt et al. 2020). High final lactulose concentrations and yields have been obtained together with acceptable productivity rates.

The Illanes group (Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile) have published extensively since 2011 on the production of lactulose and GOS using β-galactosidase. Early research centred on evaluating various microbial β-galactosidases from commercial sources of *B. circulans*, *K. lactis* and *A. oryzae* and optimising the reaction conditions for the kinetically controlled synthesis of lactulose by transgalactosylation. The lactose to fructose ratio strongly affected product composition with a low lactose:fructose ratio (1:8) and high initial total sugars (50% w/w) resulting in maximal lactulose yields (Guerrero et al. 2011). Galactose was determined to be a competitive inhibitor of transgalactosylation, while glucose had a smaller, but more complex, mechanism of action (Vera et al. 2011). Immobilised β-galactosidase from *A. oryzae*, either by cross-linking and aggregation with glutaraldehyde or on an amino-glyoxyl-agarose support, produced a more stable, active enzyme able to withstand repeated batch operations and with a lactulose yield of approximately 30% (Guerrero et al. 2015, 2017). Aggregated cross-linked β-galactosidases were formed by the precipitation of the enzyme under non-denaturing conditions followed by crosslinking of the precipitated enzyme. Glyoxyl-agarose immobilised β-galactosidase was also used in a continuous PBR to convert lactose (28%) and fructose to lactulose (60 g/g yield) and transgalactosylated oligosaccharides at a molar ratio of 5.4:1 (Guerrero et al. 2019). Propanol precipitation of β-galactosidase produced aggregated, cross-linked enzyme with high lactulose yields and could be utilised for up to 90 repeated batches before the threshold of 50% residual activity was reached (Guerrero et al. 2020). The β-galactosidase from *B. circulans* has also been evaluated for lactulose production (Aburto et al. 2020a, b; Guerrero et al. 2020). At a high molar fructose:lactose ratio (20), and with 70% conversion of lactose, a yield of 54% lactulose was achieved (Aburto et al. 2020a, b), and after immobilisation with propanol aggregation and glutaraldehyde cross-linking, a 42% yield of lactulose was obtained at a fructose:lactose molar ratio of 8:1 (Guerrero et al. 2020).

Alternatively, an epimerase enzyme (cellobiose 2-epimerase) can directly catalyse the isomerisation of lactose to form lactulose (Chen et al. 2018). The catalytically preferred specificity of the reaction is the epimerisation of lactose to form epilactose, but this specificity is temperature dependent, with higher reaction temperatures leading to higher isomerisation rates. Thermostable cellobiose 2-epimerases have been shown to be useful catalysts for industrial lactulose production processes, not only because they can avoid any contaminating microbial growth and can increase the solubility of the reactants, but also because of their higher

isomerisation activities at relatively high temperatures. Epimerase enzymes from the thermophilic organisms *Caldicellulosiruptor saccharolyticus* and *Dictyoglomus thermophilum*, have been cloned and purified from *E. coli* and optimised for pH, temperature, and substrate and enzyme concentration (Kim and Oh 2012; Shen et al. 2016; Wang et al. 2018). They do not require co-substrates such as fructose, are more stable against chemical denaturation, and typically produce lactulose and epilactose at ratios of approximately 80:20 at temperatures of 80 °C and above, and with a final lactulose, epilactose and lactose ratio of approximately 60:15:25 (Kuschel et al. 2017). Cloned epimerase enzyme from *C. saccharolyticus* has retained high levels of activity with high lactulose production and high productivity when immobilised on *Bacillus* spores (Gu et al. 2015). Rentschler et al. (2015) have also produced lactulose directly in UHT milk in situ at temperatures in the range 8–50 °C, amenable to industrial processes and at a concentration (a maximum of 28 g of lactulose per litre of milk) such that the final milk product may be directly used as a prebiotic drink (doses of 2–10 g of lactulose per day).

Site-directed mutagenesis and molecular dynamics simulation techniques have been employed to generate isomerase enzymes with enhanced thermostability, maximum activity and isomerase selectivity (Shen et al. 2015; Xiao et al. 2017; Feng et al. 2020). Lactulose yields approaching those obtained using chemical synthesis have been achieved without the chemical catalyst, and purification and chemical drawbacks of the chemical methods. The addition of borate to alter the reaction equilibrium has also been shown to push the selectivity of the enzyme towards lactulose production (Kim et al. 2013) although, as for the β-galactosidase enzymes, borate is difficult and uneconomic to remove from the reaction products. Cellobiose 2-epimerase genes from *C. saccharolyticus* have also been expressed in food grade *Bacillus subtilis*, resulting in 4.5-fold higher activity when compared to the crude enzyme (Wu et al. 2017). The crude enzyme was concentrated using ultrafiltration, could convert 58.5% of the lactose (200 g/L) in cheese whey to lactulose, and in an enzymatic membrane reactor with a 2 h reaction time retained over 70% of its original activity after ten cycles. The success of the epimerase enzymes suggests that these may soon be viable alternatives to chemical methods for the economic production of lactulose.

5.3.3 Purification

Chemical synthesis of lactulose, due to its complexity, low specificity, high catalyst concentrations and hot alkaline conditions, can lead to undesirable by-products such as galactose, tagatose, epilactose, iso-saccharinic acid and coloured compounds. Wang et al. (2013) have detailed a typical six-step process involving acidification, decolourisation, desalinisation, separation steps to remove monosaccharaides and residue lactose, followed by final concentration and drying steps to obtain a purified lactulose product. Lactulose has been purified on an industrial scale using ion exchange chromatography with a sodium-type strong acid ion

exchange resin column, and using water to elute the lactose, followed by the lactulose and finally the galactose (Tamura et al. 1993). In 1973, Morinaga Milk Industry Co., Ltd. received a patent for a lactulose powder containing more than 55% lactulose and stated that it had to 'overcome major difficulties before succeeding in the production of powdered lactulose'. These difficulties arise in the drying stage because of the hygroscopicity of the resulting powder. The monosaccharides produced during the enzymatic synthesis of lactulose have been selectively removed using either fresh *S. cerevisiae* yeast (Julio-González et al. 2018; Young et al. 2019) or activated charcoal with water or ethanol/water solutions (Julio-Gonzalez et al. 2019).

5.3.4 Properties

Lactulose is produced as either a syrup or a dried powder, and some of its properties are given in Table 5.5. The syrup is a transparent yellowish solution with no odour and a sweet taste, and, depending on purity, it generally also contains lactose along with minor quantities of fructose, galactose, tagatose and epilactose. In the crystalline form lactose occurs as either the anhydride or trihydrate with commercial products having the anhydride structure due to processing conditions. The anhydride crystal form has a melting point of 168–171 °C. The trihydrate form crystalises easily from water, contains up to 14% water, is stable at 30 °C and 81% relative humidity, and must be stored at or below ambient temperature (Tamura et al. 1993). Lactulose displays mutarotation, and different isomeric types of lactulose, including α- or β-furanose, α- or β-pyranose and acyclic, have been recorded (Aït-Aissa and Aider 2014).

Lactulose is sweeter (0.6–0.8 c.f. sucrose) than lactose (0.17–0.2) and has excellent technical and technological properties, such as high solubility, wettability, good

Table 5.5 Properties of lactulose

Parameter	Lactulose (anhydrate)
Empirical formula	$C_{12}H_{22}O_{11}$
Molecular weight (g/mol)	342.3
Melting point (°C)	168.5–170
Heat of solution (J/g)	–11.68
Specific rotation [α] D20 at 589 nm	–51.5°
Sweetness (relative to sucrose)	0.6–0.8
Water solubility at 20 °C (g/L)	2060
Methanol solubility at 30 °C (g/L)	25.4
Glass transition temperature (T_g, °C)	–47

dispersibility and resistance to high temperatures and acid pH levels. It is highly soluble in water and acid hydrolysis yields galactose and fructose. Lactulose is partly soluble in methanol, and insoluble in ether, and its high thermal-acid stability makes it a great prebiotic additive in acidic food products.

5.3.5 Analysis

A wide array of methods have been reported for the analysis of lactulose, for use either to determine the presence and amount in various dairy products or during the production of lactulose (Zhang et al. 2010). These include colorimetry (Adachi 1965), spectrophotometry (Amine et al. 2000), HPLC (Nelofar et al. 2010), GC-MS (Rodriguez et al. 2009), CE (Paroni et al. 2006), differential pH (Luzzana et al. 2003; Hashemi and Ashtiani 2010), NMR (Mayer et al. 2004; Jayalakshmi et al. 2009) and flow analysis (Marconi et al. 1999, 2004) methods. As lactulose has been considered a principal chemical compound from heat damage in dairy products its quantification can provide information about the degree of heat exposure or abuse (Elliott et al. 2003; Olano and Calvo 1989). When detecting lactulose in dairy products, the analytical methods must be able to operate in high lactose solutions, and when analysing production streams lactulose is often just one component in a complex mixture containing many other by-product sugars. The official International Dairy Federation method (IDF 1995) measures lactulose in the presence of varying amounts of lactose and is based on an enzymatic hydrolysis assay of lactulose followed by the detection of the resulting sugars. Commercial kits using a similar enzymatic format are available. An enzymatic kit based on a cloned and expressed β-galactosidase gene from *Exiguobacterium acetylicum* that is specific for lactulose, and not lactose, has been proposed as a way to avoid the need to eliminate any background glucose (Aburto et al. 2019).

A large number of HPLC methods have been reported (Aït-Aissa and Aider 2014) that employ either anion/cation exchange, amino-modified silica phases or hydrophilic interaction liquid chromatography (HILIC) resins and different detection methods, including PAD (Pazourek 2019), refractive index detectors (RID) (Silveira et al. 2015), ELSD (Schmidt et al. 2019) and MS-MS detectors (Lee et al. 2014). Separation of lactulose from lactose under isocratic conditions has been achieved within 5 min with a resolution of 1.5, allowing the rapid quantification of lactulose in complex sugar solutions, with baseline separation (Pazourek 2019). A green chemistry HPLC-RID method has also been developed where only water and ultracentrifugation was used for the sample preparation, and water was also used as the mobile phase (Gonzaga et al. 2019). Thin layer chromatography (TLC) allows the separation of epilactose, a by-product of the enzymatic synthesis of lactulose (Kuschel et al. 2017). The different oligosaccharide structures and purity obtained during the enzymatic synthesis of lactulose have been identified using MALDI-TOF-MS and NMR spectroscopy analysis (Yin et al. 2018), and

differential scanning calorimetry coupled with optical microscope (DSC-thermomicroscopy) (Bisinella et al. 2017).

Capillary electrophoretic methods have been developed for measuring the formation and presence of lactulose in UHT and hydrolysed UHT milk (Neves et al. 2018; Neves and de Oliveira 2020a, b). Capillary zone electrophoresis with indirect UV detection resulted in lactulose limits of quantification of 100 mg/L and a single-point standard addition method circumvented matrix and high lactose effects. Biosensors have also been produced for lactulose, based on the two enzymes fructose dehydrogenase (FDH) and β-galactosidase, with the resulting fructose measured in vitro by either a tetrathiafulvalene-tetracyanoquinodimetane (TTF-TCNQ) salt on a ring electrode (Sekine and Hall 1998), or via $K_3[Fe(CN)_6]$ as a mediator and a platinum based electrochemical transducer (Moscone et al. 1999). In a third biosensor, the *E. coli* lac operon repressor, LacI, was bioengineered to respond to lactulose, and not to the other disaccharide lactose, epilactose, maltose, sucrose, cellobiose and melibiose (Wu et al. 2017). A whole-cell in vivo lactulose biosensor was then developed around this repressor mutant for the high-throughput screening of lactulose hyper-producing strains and in engineering cellobiose 2-epimerase for enhanced lactulose synthesis efficiency.

5.3.6 Commercial Producers and Products

The lactulose market is segmented into liquid (normal purity) and crystalline (high purity) products destined for the pharmaceutical, human nutrition and animal feed industries. Liquid lactulose is a colourless to dark yellow transparent liquid with low-calorie content and sweetness, and a lactulose content of 50–75%, and the crystalline product is a powder of 75–98% lactulose. The uses of lactulose have grown and diversified in all the healthcare products, medical and food ingredients sectors, and the market size is predicted to maintain a CAGR of 1.5–4.5% over the next 5 years, reaching production volumes in excess of 50,000 MT p.a. and US$ 159–219 m by 2025. The current price is approximately US$ 100/kg. More than 75% of overall lactulose production is destined for the food industry, and geographically, the North American and European regions dominate the lactulose consumer market.

Solvay Pharmaceuticals (now Solactis Food and Feed Ingredients) (medical applications), and Morinaga Milk Industry Co., Ltd. (food ingredients) were the two original lactulose producers, with Morinaga Milk Industry Co., Ltd. producing an infant formula containing lactulose in the 1960s. While lactulose production is mainly concentrated in Europe and Canada, in recent years India, Africa and China have also become high volume manufacturers.

All current commercial lactulose production is assumed to be via chemical synthesis. Julio-Gonzalez et al. (2019) analysed 12 commercial lactulose preparations and noted that the presence of epilactose is indicative of chemical synthesis. This synthesis is a technology intensive industry although one producer, Illovo, claims

the product is manufactured by the isomerisation of lactose utilising no solvents other than water. While the majority of lactulose is provided as a bulk ingredient product, Morinaga Milk Industry Co., Ltd. has a probiotic supplement co-formulated with lactulose, and a number of producers package liquid products in single-dose sachets and bottles of sizes from 10 g/15 to 1000 mL. The growth in the lactulose market is partially driven by increased food product development, e.g., high protein yoghurts, cereals bars and healthy snacking, where lactulose offers alternatives to 'standard' fibre or other non-digestible carbohydrates. Claims that lactulose may help modulate glucose absorption, blood sugar management, and promote fat storage, leading to improved energy management and weight control concepts all resonate with consumers. There is also increasing consumer awareness of the importance of digestive comfort and maintaining a healthy gut microbiota, both for infants and later in life.

5.3.7 Uses and Applications

Lactulose is a non-absorbable form of sugar that has two complimentary applications in the food and pharmaceutical industries. At low doses, it can act as a prebiotic carbohydrate, and at high doses, it is primarily used as a pharmaceutical oral osmotic laxative to treat constipation. Thus, in food products, lactulose is added as a bifidogenic agent or as a functional additive for intestinal health, while pharmaceutically it is used in the treatment of constipation and hepatic encephalopathy, for tumour prevention and to maintain insulin and blood glucose levels. Lactulose is also an animal feed ingredient offering digestive health benefits.

5.3.7.1 Food

As a 'bifidus factor' lactulose acts, and is recognised, as a prebiotic on the colonic microflora, increasing the number of bifidobacteria and optimising intestinal tract function by regulating intestinal transit. When ingested, lactulose is not metabolised in the stomach or small intestine but can be used as an energy source by the *Bifidobacteria* and *Lactobacilli* in the colon, promoting their growth. It has also been shown to positively influence the growth, acidification profile and viable counts of the probiotics *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis* in co-culture with *S. thermophilus* in fermented skim milk (de Souza Oliveira et al. 2011). The digestive qualities of lactulose are recognised by the EFSA (Sadler 2018) and the Ministry of Food and Drug Safety of Korea (MFDS) and it is approved for use as a prebiotic in Italy, the Netherlands and Japan. Lactulose also inhibits the growth of pathogenic bacteria, and both in vivo and in vitro studies have shown that the effects of lactulose on human microbiota composition are patient- and dose-dependent (Bouhnik et al. 2004; Ruszkowski and Witkowski 2019). The importance of dose levels has been

demonstrated in infant formula; when lactulose was incorporated at a level of 0.5%, it stimulated bifidobacterial flora, whereas at 1% it acted as a partial laxative (Olano and Corzo 2009). The addition of lactulose to yoghurt has also been used to treat childhood constipation.

The key advantages of including lactulose as a food ingredient include its good heat and acid stability and its ability to promote the adsorption of calcium and magnesium. Lactulose has a low calorific value and sweetness and does not participate in the glycaemic response. Oral ingestion of 2 g/day lactulose has a prebiotic effect, increasing the number and percentage of bifidobacteria in faeces with a concomitant decrease in *Clostridia*, softening the faeces, and increasing defaecation frequency, but without increasing flatulence (Sakai et al. 2019a, b).

It has been included in a large variety of foods, including a number of fruit-based preparations, food supplements, dietary resistance products, dairy products and yoghurts, clinical foods, cereals, biscuits and rusks, beverages and drinking milk, baby food and kefir. For example, Morinaga Milk Industry Co., Ltd. has developed a number of dairy products incorporating lactulose, including yoghurt, drinks, ice cream and infant formula. The addition of lactulose to cake, cookies, yoghurt and chocolate can improve their sensory and browning attributes. In confectionary, lactulose is used as a replacement for sucrose, providing sweetness, while lactulose decreases the effective viscosity when replacing sugar in pectin gels. In foods containing added probiotics, lactulose acted as a protectant, increasing the survival rate of these beneficial bacteria (Adebola et al. 2014). As a sweetener, lactulose is non-cariogenic and does not cause tooth decay and dental plaque, because it is not metabolised by the bacteria in the mouth. Lactulose has also been used as a food grade colon-targeted delivery system (CODES) and as a filler for capsules and tablets in the medicinal/pharmaceutical industry. Due to its good tolerability, lactulose is added as a food ingredient to animal feed as a prebiotic to aid animal digestive health and minimise antibiotic usage.

5.3.7.2 Pharmaceutical

Lactulose has been a part of physiological and laxative therapy since the 1960s. It has a more than 40 years' long safety record and is used as a nutraceutical and pharmaceutical for successfully treating chronic constipation. It is not digested in the small intestine, transiting the digestive tract to the colon where it is metabolised by the colonic bacteria, producing short-chain fatty acids that reduce the luminal pH, and cause an increase in bacterial mass and osmotic pressure. This in turn results in water retention in the colon that softens the stool and extends the intestinal volume in the colon, leading to increased stool volume and peristalsis enhancement and indirectly stimulating bowel movement. Its efficacy and safety profile, comprising prebiotic, osmotic and peristalsis-activating properties, allows lactulose to be used in all age groups, from infants to elderly patients, where a high-roughage diet or other general measures are ineffective. The efficacy of lactulose has been compared with a low-dose polyethylene glycol electrolyte solution, another common laxative,

in several randomised multicentre chronic constipation studies (Lee-Robichaud et al. 2010). Both treatments were affective and displayed similar adverse reactions including liquid stools, abdominal pain, bloating, and rumbling were similar in the two groups. A lot of the commercial medicinal products also contain small amounts of digestible carbohydrates, e.g., fructose, galactose and lactose. Only a small amount thereof is absorbed from the intestine into the body and thus has only minor nutritive value.

Lactulose is widely used as a detoxifying agent in the treatment of hepatic (or portal systemic) encephalopathy and the associated cerebral dysfunction caused by chronic liver diseases, in particular liver cirrhosis (Gluud et al. 2016). These diseases impair liver function allowing toxic substances, including ammonia, to accumulate in the bloodstream (hyperammonaemia). Elevated blood ammonia can interfere with brain functions, causing cognitive dysfunction and psychiatric disorders. High doses of lactulose get degraded by the colon bacterial flora into lactic acid and small amounts of formic and acetic acid. This acidic environment ionises ammonia in the colon to the ammonium ion which cannot diffuse across the colon membrane and is ultimately excreted in the stool. This process reduces blood ammonia levels by 25–50% and usually results in a beneficial effect on the patient's mental status, helping to restore normal neurological function. Lactulose can also inhibit nonbacterial, glutamine-dependent ammonia production in the intestinal wall.

Lactulose ingestion causes increased production of low molecular weight organic acids in the colon, lowers the faecal pH and favours the growth of *L. acidophilus* while inhibiting the growth of *coliforms*, *Bacteroides*, *Salmonella* and *Shigella* (Panesar and Kumari 2011). It can therefore act as an indirect antioxidant that mobilises endogenous hydrogen production which in turn can reduce oxidative stress (Chen et al. 2011). All these factors help alleviate inflammatory bowel disease symptoms by reducing the growth of potential pathogens, reducing the risk of urinary and respiratory tract infections, and lowering the production of gut endotoxins. Lactulose treatment has also reduced the incidence of endotoxaemia in patients undergoing surgery for obstructive jaundice treatment and prevented endotoxin-dependent complications such as renal dysfunction.

When lactulose was used to symptomatically treat constipation in normal and type 2 diabetic patients, blood glucose levels were not observed to increase and were not affected by the carbohydrate impurities contained in crystal or liquid lactulose formulations (Pieber et al. 2021). Lactulose also showed an anti-diabetic effect, positively affecting blood sugar management, with possibilities for energy management and weight control concepts. Colon cancer occurs in the lumen, mucosa and adjacent tissues of the large intestine. Prebiotics such as lactulose help prevent cancer by reducing the colonic and caecal pH, leading to lowered 7-α-dehydroxylase activity and lower carcinogenic secondary bile acids. In rats, lactulose has been shown to suppress pre-cancer DNA damage in the colon mucosa (Verma and Shukla 2013), and there was a reduction in the number of colon tumours in a rat model after lactulose treatment, with significant reductions in populations of pro-inflammatory

bacteria families and species, and significant increases in more beneficial populations, such as *Bifidobacterium* (Fernández et al. 2018).

Stable isotope ratios using ^{44}Ca and ^{25}Mg were fed to adult men with lactulose to demonstrate that lactulose enhances the adsorption of Ca and Mg (Seki et al. 2007). Dog and rat studies also showed that Ca, Mg, Zn, Cu and Fe adsorption and retention were increased by lactulose ingestion with subsequent increases in both osteoporosis and bone strength. Lactulose has been shown to ameliorate the cognitive deficiencies associated with Alzheimer's disease in a mouse model, with the neuroprotective effects being attributed to anti-inflammation and autophagy mechanisms (Lee et al. 2021). These findings may pave the way for the development of lactulose-based preventive and/or therapeutic treatments for Alzheimer's disease.

5.3.8 Product Safety, Dosage Rates, Regulatory Issues

Lactulose has come to prominence mainly due to its prebiotic properties, and health claims have been approved on transit regulation from the EFSA ('lactulose contributes to an acceleration of intestinal transit', for food that contains 10 g lactulose/single quantified portion/day, Regulation EU 432/2012, EFSA), and on its prebiotic effects from the Korean MSDF (Sadler 2018). While it can be safely used by most population groups including pregnant women, elderly patients, nursing mothers, children and adults with chronic liver disease, and post-surgical patients, care must be taken as current chemical synthesis processes may result in residual, but substantial, levels of lactose, galactose and epi-lactose remaining in the product. In the USA, lactulose is classified as an FDA pregnancy risk category B drug. The benefits of lactulose include that it maintains the healthy good bacteria in the colon, is non-toxic and non-habit forming, and people do not develop a tolerance. When used as a laxative the normal dosage is 20 g lactulose, and although gas, bloating, burping, stomach rumbling/pain, nausea and cramps may occur, these are usually only transitory.

For food uses, lactulose gained FOSHU (Foods for Specified Health Uses) status in Japan in 1992 and meets the EFSA description of a non-digestible carbohydrate (NDC). In 2016, crystalline lactulose obtained a self-affirmed GRAS status in the USA and, after a comprehensive review on the safety aspects of lactulose crystal intake under the conditions of use as a food ingredient, an independent panel of experts has declared it safe for various dairy and beverage applications, including meal supplement drinks, nutritional bars and food supplement products. Finally, lactulose has been approved as an animal feed ingredient in the EC (Quality and Safety of Feeds and Food for Europe; European Commission regulation (EC) No. 575/2011, 2011).

5.4 Tagatose

Tagatose is a natural, low-calorie sugar with EFSA-approved health claims that is currently prohibitively expensive for use as an everyday food ingredient. It has a high sweetness power (90%) combined with a low caloric value compared with sucrose. The wide range of benefits associated with the use of D-tagatose include that it is only partially digested by the body with the rest acting as a fibre in the intestine. Ingestion also does not raise the glycaemic index, suggesting that tagatose may also be of benefit in the treatment of metabolic syndrome. Furthermore, tagatose exhibits antidiabetic, antioxidants, prebiotics and non-cariogenic properties.

The reader is directed to a number of recent reviews for more details (Khuwijitjaru et al. 2018; Mogha et al. 2016; Oh 2007; Ravikumar et al. 2021).

5.4.1 Chemistry

D-Tagatose is a rare, natural hexoketose, a stereoisomer of D-galactose and fructose, that is found in nature, mostly in gums and lichens. The cyclic form of D-tagatose consists of α-D-tagato-2,6-pyranose (79%), β-D-tagato-2,6-pyranose (14%), α-D-tagato-2,5-furanose (2%), and β-D-tagato-2,6-furanose (5%) (Köpper and Freimund 2003). Unless stated otherwise, tagatose will be used in the place of D-tagatose in the text.

5.4.2 Synthesis

Tagatose can be produced either by chemical or biological methods (Fig. 5.6). Chemically, lactose must first be hydrolysed, either with acid or enzymatically, to glucose and galactose. This is followed by the isomerisation of galactose into tagatose using a soluble alkali metal salt, alkaline earth metal salt or potassium aluminate as a catalyst (Beadle et al. 1992). The metal hydroxide is commonly calcium hydroxide ($Ca(OH)_2$) which forms an intermediate metal hydroxide-tagatose complex in the presence of an inorganic salt catalyst. Acid is then used to neutralise the intermediate complex to yield tagatose (Roy et al. 2018; Zhang et al. 2020a, b, c). A major limitation of this method is that a mixture of the initial galactose substrate, together with the different by-products and intermediate products, and tagatose are all formed during the isomerisation step. Thus, complex purification procedures are necessary to remove the other compounds and obtain a purified tagatose product (Oh 2007).

The enzymatic synthesis of tagatose from lactose also proceeds via galactose and typically involves either isomerisation or reduction followed by dehydrogenation, to produce the final tagatose. This method can overcome the problems of chemical

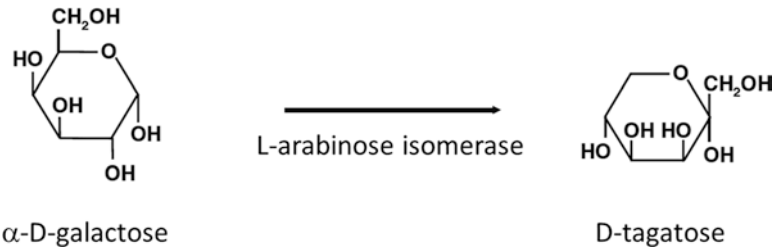

Fig. 5.6 The chemical (Haworth) structures and isomerisation of galactose to tagatose

waste and low efficiency of chemical tagatose generation (Ibrahim 2018a, b; Khuwijitjaru et al. 2018). The enzyme L-arabinose isomerase (EC 5.3.1.4) has been the most extensively applied biocatalyst for tagatose production. In nature, L-arabinose isomerase catalyses the isomerisation of the pentose-sugar L-arabinose to the keto sugar L-ribulose, and thus D-galactose is not an optimal substrate. Various strategies, including using either whole-cell systems or recombinant or purified enzymes, have been devised to optimise the enzyme productivity. A large number of different bacteria, including mesophilic, thermophilic and hyperthermophilic strains, have been screened for their natural ability to produce L-arabinose isomerase, including screening for a greater specificity for galactose as a substrate. Bacteria have also been permeabilised and immobilised to enhance their accessibility and stability. Table 5.6 shows the diversity of technologies used to expand the capability of the different enzymes and the key performance indicators, i.e., lactose/galactose conversion, time, substrate concentration and productivity.

Whole-cell systems are not routinely used to produce tagatose due to their low enzyme productivity, although approximately 50% conversion of lactose or whey permeate lactose to tagatose has been achieved with permeabilised and/or immobilised *Lactobacillus* cells (Xu et al. 2012; Jayamuthunagai et al. 2017). While *E. coli* is the microorganism of choice when producing recombinant enzyme, it does not have GRAS approval and cannot be used directly in a food system. *Corynebacterium glutamicum*, *L. plantarum* and *B. subtilis*, which all have GRAS status, have been used as vehicles to express the genes for either thermostable galactose isomerase or L-arabinose isomerase enzymes, and the resulting microorganisms have produced lactose to tagatose conversion rates of between 50% and 80% (Shin et al. 2016; Bober and Nair 2019; Liu et al. 2014; Guo et al. 2018; Zhang et al. 2021). The conversion can be enhanced and sustained by cell permeabilisation and enzyme and cell immobilisation. A yeast system containing genes for the enzymes xylose reductase (from *Scheffersomyces stipitis XYL1*) and galactitol-2-dehydrogeanse (from *Rhizobium leguminosarum*) has resulted in a lactose to tagatose pathway via galactitol (Liu et al. 2019). The galactose kinase gene was also blocked to stop the metabolism of galactose, and a conversion rate of 62.5% was achieved in a continuous bioreactor at 30 °C.

When recombinant technology has been used to express L-arabinose isomerase genes in *E. coli*, the resulting enzymes are usually purified by either His-tag or Ni^{2+}

Table 5.6 Enzymatic synthesis of tagatose using different enzyme sources and reaction formats

Form	Origin of L-arabinose isomerase (or D-galactose isomerase)	pH	Temp (°C)	Galactose (g/L) (or lactose)	Yield (g/L)	Conversion (%)	Productivity (g/L/h)	Time (h)	Reference
Whole cells									
Immobilised cells	*Lactobacillus fermentum*	6.5	65	100 (La)		55	11.1	24	Xu et al. (2012)
Permeabilised/immobilised cells	*L. plantarum*	7.0	50	Permeate		48		48	Jayamuthunagai et al. (2017)
Whole cells, recombinant enzyme									
Host *E. coli*, also recomb β-galactosidase	*E. coli*	6.5	34			95 La, 43 Ga			Zhan et al. (2014)
Host *C. glutamicum* permeabilised : perm + borate : perm + immobilised	*Geobacillus thermodenitrificans* (D-galactose isomerase)		50	300	144	48	48	3	Shin et al. (2016)
					180	60.6	61	3	
					165	55	55	3	
Host *L. plantarum*	*Lactobacillus sakei*	7.4	50	54		50	6.7	4	Bober and Nair (2019)
Host *E. coli*, SSB, 5 mM Mn^{2+}	*L. plantarum (CY.6)*	6.5	50	Whey	51.5	73.6 (37 La)	0.54	96	Zhang et al. (2020b)
Host *B. subtilis*, spore surface	*L. fermentum*			100		75	3.1	24	Liu et al. (2014)
Host *B. subtilis*, spore surface,	*Lactococcus brevis*			125		79.7	4.3	28	Guo et al. (2018)
Host *Lactiplantibacillus plantarum*	*L. casei*	7.5	65	150		33 (La)		56	Zhang et al. (2021)
Galactitol enzyme pathway									
Host yeast, via galactose and galactitol	*S. cerevisiae*	5.6	30	50 (La)	37.7	62.5		300	Liu et al. (2019)
Host *E. coli*, galactitol substrate	*Rhodobacter sphaeroides D*	9.0	30	50		90.2	7.61	12	Su et al. (2021)

(continued)

Table 5.6 (continued)

Form	Origin of L-arabinose isomerase (or D-galactose isomerase)	pH	Temp (°C)	Galactose (g/L) (or lactose)	Yield (g/L)	Conversion (%)	Productivity (g/L/h)	Time (h)	Reference
Purified recombinant enzyme									
Host *E. coli*, purified, Mn^{2+}, Co^{2+}, purified, heat, IEX, SEC, hyperthermophile	*Thermotoga neapolitana*	7.0	80	1.8		68		20	Kim et al. (2002)
Host *E. coli*, His-tag, +borate	*G. thermodenitrificans*	8.5	60	500	370	74	15.4	24	Lim et al. (2007)
Host *E. coli*, purified Ni^{2+}, + borate	*Anoxybacillus flavithermus*	9.5	95	0.9		60		1	Li et al. (2011)
Host *E. coli* affinity His-tag	*Enterococcus faecium*	5.6	55	72		26		24	de Sousa et al. (2017)
Host *E. coli*, Ni^{2+} purified, mesophile	*E. faecium*	5.5	50	90		45		48	Manzo et al. (2019)
Host *E. coli* affinity His-tag single step, highly D-galactose specific	*Bifidobacterium adolescentis*	6.5	55	18		56.7		10	Zhang et al. (2020a)
Modified enzyme									
Rational design, molecular modelling and docking; recomb *E. coli*, His-tag	*Bacillus coagulans*	8.0	50	150	67.5	35.4	4.5	15	Zheng et al. (2017)
				250	88.4	45.0	5.9		
Polyol dehydrogenase (EC 1.1.1.-)			30	100 (Gl)	90	>99	160 (24 h)	15	Sha et al. (2018)
Mesophilic—rational design	*Shewanella* sp.	7.0	55	0.9		38		5	Jayaraman et al. (2021)

La lactose, *Ga* galactose, *Gl* galactitol

affinity chromatography (Kim et al. 2002; Lim et al. 2007; Li et al. 2011; de Sousa et al. 2017; Manzo et al. 2019; Zhang et al. 2020a). These enzymes often require a metal ion, e.g., Mn^{2+} or Co^{2+}, for maximal activity, and a large selection of thermophiles or hyperthermophiles have been screened for their higher catalytic efficiency, higher thermostability at temperatures >40 °C and greater equilibrium conversion. Enzymes have also been modified or selected for enhanced galactose specificity and increased tagatose synthesis efficiency using in silico docking modelling, rational design and site-directed mutagenesis (Zheng et al. 2017; Jayaraman et al. 2021). To date, these methods have not been as successful as the recombinant route. A computationally guided enzyme screening approach identified a possible pathway for tagatose synthesis from galactitol using a polyol dehygrogenase (Sha et al. 2018). This system, which required coupling to a water-forming NADH oxidase, resulted in almost complete conversion of galactitol to tagatose. Borate has also been shown to increase the production of tagatose by binding to the newly formed tagatose and shifting the reaction equilibrium; however, boric acid is not suitable for the production of food-grade D-tagatose because it can be toxic in humans. *E. coli* expressing a *L. plantarum* L-arabinose isomerase have been added along with a β-galactosidase to whey powder in a simultaneous saccharification and biotransformation process with high conversion efficiency (73.6%) and good tagatose yields (Zhang et al. 2020b).

In an ideal scenario, whey streams rich in lactose would be sustainably used for D-tagatose production. After hydrolysis of the lactose into D-galactose and D-glucose using a β-galactosidase (EC 3.2.1.23), there are a number of pathways to convert the D-galactose to tagatose. The enzymatic synthesis of tagatose is the most economic production method, providing the advantages of improved specificity, stereoselectivity and high conversion yields under mild temperature and pH conditions in comparison with chemical methods. The large-scale production of tagatose remains costly however, as the process often requires multiple purification steps, including simulated moving bed chromatography using a cation exchanger, vacuum evaporation steps, continuous crystallisation and drying, to produce the final pure powder. The galactose fraction separated from the tagatose/galactose mixture can be recycled for further processing to tagatose. Also, when borate is used to increase tagatose production, it can be separated from the final syrup using Ca^{2+} cation-exchange chromatography (Zhan et al. 2014). Different excipients which affect the crystallinity of tagatose but not sweet taste can be used to facilitate spray drying (Campbell et al. 2020).

5.4.3 Properties

A number of the properties of tagatose are shown in Table 5.7. The melting temperature of tagatose is 134 °C, and it is stable at pH 2–7. Tagatose has high solubility [58% (w/w) at 21 °C], which can be utilised as a flavour enhancer or fibre in soft drinks and yoghurts.

Table 5.7 Chemical and biological properties of D-tagatose as a sweetener (from Kim 2004)

Property	Value
Chemical family	Carbohydrate ketose, isomer of D-galactose
Molecular formula	$C_6H_{12}O_6$
Molecular weight	180 Da
Physical form	White crystalline solid
Odour	None
Melting point	134 °C
Optical rotation	$\alpha^{20}_D = -5°$ (c = 1 in water)
Solubility	58% w/w at 21 °C
Stable pH range	2–7
Relative sweetness	92% of sucrose in 10% (w/w) solution
Sweetness quality	Similar to sucrose, faster onset like fructose
Cooling effect	None
Caloric value	1.5 kcal/g
Maillard reaction and caramelisation	Yes

5.4.4 Analysis

The synthesis of tagatose from lactose via galactose, either chemically or enzymatically, can result in a number of by-products and sugars, and it is necessary for any analytical method to be able to resolve tagatose from these other impurities. Refractive index, ELSD and electrochemical detectors have been used to detect the various compounds after separation by standard carbohydrate ion-exchange HPLC chromatography for both the laboratory (Shin et al. 2016: Bober and Nair 2019) and large-scale production of tagatose (Xu et al. 2012). A rapid green capillary electrophoresis method using a fused silica capillary and UV detection at 265 nm has been developed for the high resolution, high throughput analysis of tagatose product streams (Surapureddi et al. 2020). The method was fast (20 min), utilised a background electrolyte and did not require any derivatisation. Infrared (IR) spectroscopy and proton nuclear magnetic resonance (^{1}H NMR) were used to characterise D-tagatose production (Zhan et al. 2014).

The hydrophilic nature and low glass transition temperature (T_g) of tagatose created processing difficulties when spray drying tagatose to produce a powder. Differential scanning calorimetry (DSC), attenuated total reflectance Fourier transform infrared spectrometry (ATR-FTIR), powder X-ray diffraction (PXRD), and scanning electron microscopy (SEM) have been used to characterise the resulting powders after drying in the presence of different excipients to determine any functional changes (Campbell et al. 2020).

5.4.5 Commercial Producers and Products

The high cost of tagatose, retailing in 2020 for about US$ 26/kg compared to just 50 cents for sucrose, has been an obstacle to the successful uptake of this sugar. Tagatose is predominantly produced using the biotransformation method with L-arabinose isomerase as the biocatalyst and D-galactose as the substrate, although some (Wuxi Jcantek and Arla) are using chemical synthesis from galactose under alkaline conditions in the presence of calcium. The market has grown substantially since 2011, with South Asia and East Asia anticipated to experience considerable growth, and an estimated CAGR of 4.7% throughout the period 2019–2029. The lead producers in the global tagatose market include Damhert Nutrition NV, Ltd. and CJ Cheiljedang Corporation. While the tagatose market is consolidated in nature, and primarily dominated by a small number of manufacturers, they are increasingly being joined by a large number of other producers.

Tagatose powder is the dominant product in the current market (compared with liquid) with greater utilisation of tagatose being expected in the foodservice and as a functional ingredient for beverage and confectionary products. Tagatose is gaining traction as a low-calorie sweetener and is perceived as a healthy option to sugar, as consumers desire to reduce their risk of diabetes, obesity and heart diseases.

5.4.6 Uses and Applications

D-Tagatose is a naturally occurring simple reducing sugar that is 90% as sweet as sugar but contains only 1.5 cal/g. It has similar sensory, bulking and baking properties as sucrose, making it an ideal replacement for sugar in foods and beverages. It has been used as a low-calorie sweetener in a wide variety of foods, beverages, yoghurt, health foods, bakery, sweets, confections and dietary supplements. In the beverage industry, D-tagatose is added in synergy with strong sweeteners such as sodium cyclamate, aspartame, acesulfame and stevioside. With no aftertaste, tagatose is mainly used to complement and eliminate the bad aftertaste, such as metal taste, bitterness and astringency, produced by strong sweeteners, and to improve the taste of the beverage (Mogha et al. 2016). Tagatose is added to dairy products, especially chocolate-flavoured products, to obtain a rich and mellow toffee flavour. It can also be used in yoghurt-making to provide a sweet taste, increase the number of viable bacteria, improve the nutritional value, and make the yoghurt flavour more rich and mellow. As a reducing sugar, tagatose is susceptible to Maillard browning on cooking and is easy to caramelise at relatively low temperatures, which makes it easier to produce ideal colour and mellow flavour than sucrose. It can be used in bakery products and has been shown to react with amino acids to produce volatile flavour compounds such as 2-acetylfuran, 2-ethylpyrazine and 2-acetylthiazole.

Tagatose also has favourable texturiser, stabiliser, humectant and formulation aid properties resulting in a wide number of applications as an additive in detergent,

cosmetic and pharmaceutical formulations. These include health products such as nonchronic drugs, toothpaste and mouthwash, and in pharmaceuticals preparations, including diabetes-specific foods, diet foods, cough syrups, anti-adhesives for fixed dentures and oral disinfectants (Ibrahim and Spradlin 2000; Kim 2004; Marylane et al. 2017). Lastly, D-tagatose can also be used as an intermediate for the synthesis of other optically active compounds, and the biotransformation of D-tagatose has been produced using biocatalyst sources (Oh 2007).

5.4.7 Health Benefits

The current health benefits of tagatose include prebiotic effects on gut microflora, reduced blood glucose levels and prevention of tooth decay. Tagatose is considered to be a prebiotic, as typically only approximately 20% of ingested tagatose is absorbed in small intestine with the unabsorbed fraction (75–80%) passing through to the lower intestine. There, it can be fermented by indigenous bacteria to produce short-chain fatty acids and carbon dioxide, resulting in the promotion of more favourable microbial flora in the colon (Laerke and Jensen 1999; Roy et al. 2018). Tagatose has also been partnered with the probiotic lactobacilli *L. rhamnosus* GG, *L. casei*, *L. acidophilus* and *L. fermentum*, to symbiotically inhibit the growth of the enteric pathogens *E. coli* and *S. typhimurium* (Roy et al. 2021).

Tagatose does not contribute to calorie production, which makes it an ideal low-calorie sweetener. It is processed in the body using the same pathways as other sugars, although at a slower rate, thus slowing down the pathways and preventing the stimulation of insulin secretion, resulting in a lowering of blood glucose levels (Guo et al. 2018; Guerrero-Wyss et al. 2018). It has also been tested successfully as an oral treatment for glycaemic control in patients with type 2 diabetes and other indications. Factors that may contribute to its effectiveness include the pH of dental plaque after consumption, its caloric value, and the glycaemic and insulinemic response (Wong 2000; Hasibul et al. 2018; Nagamine et al. 2020).

5.4.8 Product Safety, Dose Rates and Regulatory Issues

D-Tagatose has been approved as a food ingredient in many countries and is recognised by the WHO/FAO and the international Codex Alimentarius Commission. In the USA, the FDA has granted it GRAS status for use in food and beverages since 2001 (dietary ingredient (GRN No. 78 for Arla Foods Ingredients, Denmark). Another 30 countries, including New Zealand/Australia (Novel Food—Food Standards Australia New Zealand (FSANZ) 2004), the EU (Novel Food Ingredient for marketing—UK Food Standards Agency 2005), Korea (health food (Functional grade II) by Korean Food and Drug Administration (KFDA) (GRN No. 352 for CJ Cheiljedang) 2011) and China (novel food ingredient—National Health and Family

Planning Commission of the People's Republic of China (Announcement (2014) No. 10th)), have also approved its use.

Tagatose has two approved EU health claims, for contributing to tooth mineralisation and inducing a lower blood glucose spike than table sugar. Also, although it possesses prebiotic properties, tagatose does not meet the current FDA definition of a dietary fibre as it has fewer than three carbohydrate monomers. Currently, tagatose has an ADI (acceptable daily intake) of 'no specified', as no maximum allowable daily dosage ingested by humans or animals that may result in any known adverse health effects has been established.

5.5 Lactobionic Acid

5.5.1 Chemistry

Lactobionic acid (also 4-*O*-β-galactopyranosyl-D-gluconic acid) is a very rare, natural, biodegradable sugar polyol acid consisting of a galactose moiety bound by an ether-like linkage to a gluconic acid molecule. It has been observed to occur naturally at low concentrations in 'Caspian Sea yoghurt' (Kiryu et al. 2009) and is used in both high value pharmaceutical products and functional food ingredients.

More detailed reviews of lactobionic acid production and applications include those of Goderska et al. (2014), Alonso et al. (2018), Sarenkova and Ciprovica (2018) and Cardoso et al. (2019).

5.5.2 Synthesis

The synthesis of lactobionic acid from lactose can be achieved via four pathways and has been comprehensively reviewed by Nath et al. (2016). All these methods centre around the oxidation of lactose, using biochemical, chemical, electrochemical and catalytic mechanisms, and in an ideal system would use whey instead of lactose as a cheap substrate source (Fig. 5.7). Table 5.8 contains a summary of the various methods of synthesis of lactobionic acid. Fisher and Meyer (1889) first reported the synthesis of lactobionic acid through the chemical oxidation of the lactose-free aldehyde group. Currently, lactobionic acid is manufactured on an industrial scale by chemical synthesis using refined lactose as the feedstock (Gutiérrez et al. 2011; Gutiérrez et al. 2012a; Maki-Arvela et al. 2011; Belkacemi and Hamoudi 2010). This process is expensive due to the energy demand, and the use of costly immobilised supported catalysts, such as gold, bismuth and platinum (Vlad-Cristea 2007; Kuusisto et al. 2007; Regenhardt et al. 2020). A number of alternative methods have been developed, but no manufacturers have stated that they are using these methods on an industrial scale. Catalytic wet oxidation and

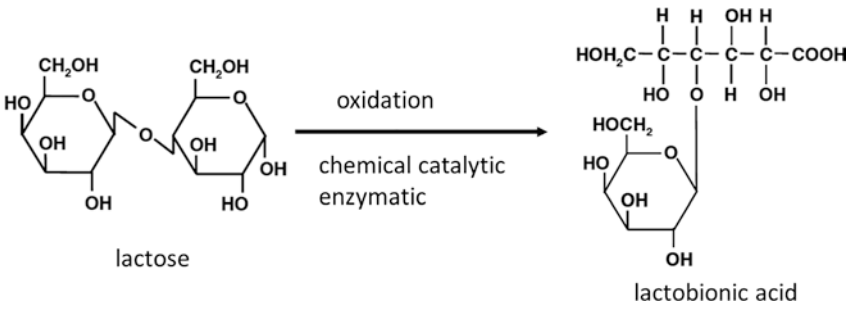

Fig. 5.7 The chemical structures and oxidation of lactose to form lactobionic acid

electrochemical lactose catalysis have also been explored. The electrochemical oxidation of lactose yields lactone, which is further hydrolysed to lactobionic acid. All these chemical methods require expensive, often environmentally unfriendly, catalysts and generate unwanted side-reaction materials (Chia et al. 2008; Murzina et al. 2008), leading to the development of more sustainable bio-production of lactobionic acid using microbial and enzymatic processes.

Microbial synthesis, using lactose as a waste or renewable source material instead of a synthetic media, may become an environmentally friendly and cost-effective production method in the future. To be economically feasible, lactobionic acid production needs to be greater than 1 g/L/h, with a final product concentration of at least 50 g/L (Sarenkova and Ciprovica 2018). The *Pseudomonas* species naturally produces lactobionic acid via the lactose oxidation pathway and lactobiono-δ-lactone with flavin adenine dinucleotide (FAD) as an electron acceptor (Alonso et al. 2013c, 2015). While conversion rates are high (80–100%), the productivity of this system is limiting (typically less than 5 g/L/h lactobionic acid) (Kiryu et al. 2012; Kim et al. 2020). Productivity rates of up to 10 g/L/h and almost 20 g/L/h lactobionic acid have been achieved, however, using other microbial systems such as *Burkholderia cepacia* (Murakami et al. 2006) and *Zymomonas mobilis* (Pedruzzi et al. 2011; Malvessi et al. 2013), or by using recombinant methods to increase the catalytic activity of the quinoprotein glucose dehydrogenase enzyme, respectively (Oh et al. 2020a, b). Recent advances in permeabilising and immobilising bacterial cells, together with different production processes, have increased the productivity of whole-cell systems (Carra et al. 2020). An engineered strain of *Neurospora crass* has also been reported that is non-pathogenic, is not constricted by stationary phase or resting cell conditions, and produces lactobionic acid from cheese whey, making it suitable for industrial applications (Fan et al. 2016).

Another production method with both high productivity and lactobionic acid yields is enzymatic synthesis. A number of enzymes from the lactose-oxidase group have been used; these include cellobiose dehydrogenase, glucose/fructose dehydrogenase and carbohydrate oxidase. These enzymes all require a redox mediator or

Table 5.8 Synthesis of lactobionic acid using chemical and enzymatic methods

Chemical/ microorganism	Enzyme(s)/cell system	Production system/ feed	Yield (g/L)	Conversion (%)	Productivity (g/L/ h)	Time (h)	Reference
Chemical							
Au/SiO_2		Semi-batch, batch		100		120–200	Gutiérrez et al. (2011)
Au/SiO_2-CeO_2		Batch		80–100		100	Gutiérrez et al. (2012a)
Pt/Al_2O_3		Catalytic flow reactor		67–100		150–300	Maki-Arvela et al. (2011)
Pd-Ti/SiO_2		Semi-batch, batch		40–100		120–180	Belkacemi and Hamoudi (2010)
Microbial—fungi							
Pycnoporus sp. SYBC-L10	Cellobiose dehydrogenase	Batch	37.2	96	3.1		Tian et al. (2018)
Microbial—bacteria							
Z. mobilis	Permeabilised cells	Batch, lactose	125	100	5.8		Pedruzzi et al. (2011)
Z. mobilis	Permeabilised cells	Batch, lactose/ fructose	182	78	7.6		Malvessi et al. (2013)
B. cepacia No. 24	Mutant strain, resting cells	Fed batch; batch	150	100	5.6; 10	27	Murakami et al. (2006)
Pseudomonas taetrolens		Batch in shake-flask and bioreactor	180	90.2	2.51	72	Kim et al. (2020)
			178	88.5	4.93	48	
P. taetrolens		Fed batch, conc cheese whey	164	82	2.05		Alonso et al. (2013b)
P. taetrolens		Fed batch, conc cheese whey, lactose	100	100	2.05		Alonso et al. (2015)

(continued)

Table 5.8 (continued)

Chemical/ microorganism	Enzyme(s)/cell system	Production system/ feed	Yield (g/L)	Conversion (%)	Productivity (g/L/ h)	Time (h)	Reference
P. taetrolens	Recombinant quinoprotein glucose dehydrogenase	Batch in bioreactor; lactose	200	100	8.70		Oh et al. (2020b)
		Batch in bioreactor; whey	200	100	2.11		Oh et al. (2020a)
		Whole-cell biocatalysis	200	95.6	16.7		
Acetobacter orientalis		Batch, synthetic media	45	98	0.54		Kiryu et al. (2012)
Z. mobilis	Ca-alginate immobilised, permeabilised, periplasm enzyme	Batch, large scale	183	76	6.8		Carra et al. (2020)
Enzymatic—fungi							
Sclerotium rolfsii CBS 191.62	Cellobiose dehydrogenase	Enzymatic catalysis	72	100	18		Baminger et al. (2001)
Microdochium nivale	Carbohydrate oxidase	Enzymatic catalysis	49	98	4.9		van der Werf et al. (1995)
S. rolfsii CBS 191.62	Cellobiose dehydrogenase	Acceptor: ABTS	72	100	19.3		Ludwig et al. (2004)
Paraconiothyrium sp. KD-3	Lactose-oxidising enzyme	Batch	100	100	14		Kiryu et al. (2008)
P. sp. KD-3 + *A. niger*	Lactose-oxidising enzyme + catalase	Batch, immobilised	185	100	9–11	24	Murakami et al. (2008)
Enzymatic—bacteria							
A. fumigatus	Cellobiose dehydrogenase/laccase	Acceptor: ABTS	100	100	7.14		Yang et al. (2021)
S. solfataricus	Recombinant β-glycosidase + cellobiose dehydrogenase	Acceptor: DCIP	40	96	16		Splechtna et al. (2001)

co-factor such as nicotinamide adenine dinucleotide (NAD) or laccase and may result in the co-production of hydrogen peroxide due to the use of oxygen as the electron acceptor. As hydrogen peroxide can cause the deactivation of the oxidase, a catalase is often added as a reducing agent (Murakami et al. 2008). Enzyme systems from both fungal (van der Werf et al. 1995; Kiryu et al. 2008) and microbial (Yang et al. 2021) sources produce lactobionic acid yields of up to 200 g/L with high substrate conversion (>95%) and productivity rates approaching 20 g/L/h (Baminger et al. 2001; Ludwig et al. 2004; Splechtna et al. 2001). The increasing use of recombinant enzymes, integrated enzyme systems, immobilisation technology, enzyme recycling and improved reactor design suggest that the enzymatic approach will become a more favourable production pathway. Indeed, in 2009, Chr. Hansen and Novozymes marketed an enzyme product for converting lactose to lactobionic acid for both food- and non-food-based applications (Novozymes 2009).

In summary, both the microbial and enzymatic approaches to the production of lactobionic acid may overcome the major drawbacks associated with the chemical processes. In addition, advances in metabolic and protein engineering may help overcome the rate-limiting steps associated with both of these processes, and, together with the introduction of new functions into the host strain, and improving lactobionic acid production by wild-type strains through enzyme overexpression, may result in economically favourable production strategies.

5.5.3 Purification

The various production processes all result in a mixture of products together with lactobionic acid. As a charged molecule, lactobionic acid can be easily removed using ion-exchange chromatography with 100% yields, followed by filtration, concentration and drying (Alonso et al. 2013a). Electrodialysis has also been successfully used to remove other end products, sugars and ions (Pedruzzi et al. 2011), as have simulated moving bed technology, ethanol precipitation, evaporation and crystallisation (Sarenkova and Ciprovica 2018).

5.5.4 Properties

Chemically, lactobionic acid is a natural polyol acid that consists of galactose bound to gluconic acid and is distinguished by containing eight hydroxyl groups and one carboxyl group. Its multifunctional groups allow it to act as a metal ion chelator and calcium sequester, with the calcium salt of lactobionic acid being 40,000 times more soluble in water than calcium carbonate and 10 times more than calcium lactate (Alonso et al. 2013a). Lactobionic acid is highly soluble in water, poorly soluble in organic solvents such as ethanol, glacial acetic acid and methanol, and has a sweet taste despite being a weak acid (Gutiérrez et al. 2012b). Being an acid,

Table 5.9 General properties of lactobionic acid

Property	Value
pH (10% solution)	2.37
pKa	3.6
Melting point (°C)	113–118
Water solubility (mg/mL)	100
Molecular weight	358.30
Moisture (%)	4.68
Ash (%)	0.08
Apparent density (g/mL)	0.66
Specific rotation	25.6

lactobionic acid can produce salts with positively charged mineral cations, i.e., potassium, calcium, zinc and sodium lactobionate. Some chemical properties of lactobionic acid are summarised in Table 5.9. Its composition and physicochemical characteristics, including high biocompatibility, biodegradability and non-toxicity, as well as its chelating, amphiphilic and antioxidant effects, mean that this organic acid possesses attributes suitable for a large number of therapeutic and cosmetic applications. Similarly, lactobionic acid has acidification capacity, antioxidation, emulsifying and water solubility properties that make it a possible food ingredient (Cardoso et al. 2019).

5.5.5 Analysis

As with the purification of lactobionic acid, ion exclusion with a cation exchange polymer in the hydrogen ionic form is a common method of analysis (García et al. 2019). Mass spectrometry (negative mode), ^{1}H-nuclear magnetic resonance (NMR; 300 MHz) and ^{13}C-NMR (75 MHz) spectra HPAEC-PAD can provide more detailed analysis (Kiryu et al. 2009). The physicochemical effects of lactobionic acid, e.g., as a cryoprotectant agent, have been followed using circular dichroism spectra and isothermal titration calorimetric profiles (Misugi et al. 2017), while thermogravimetry/derivative thermogravimetry (TG/DTG), DSC-thermomicroscopy, infrared spectroscopy (FTIR) and X-ray diffractometry (XRD) can provide information on thermal decomposition, purity and melting point (Bisinella et al. 2017).

5.5.6 Commercial Producers and Products

The production and application of lactobionic acid has been subject to intensive worldwide patents since 1927. More than 18,000 patents have been lodged for numerous production processes and medical applications, while food-related applications account for approximately 1700 patents (Alonso et al. 2015; Gutiérrez et al. 2012b). When compared to other organic acids, the industrial-level production of lactobionic acid from lactose has low economical value due to the high production costs and raw material price, even though production volumes are predicted to increase from approximately 30 MT in 2021 to 40 MT in 2026 (Pais-Chanfrau et al. 2020). Similarly, the market for lactobionic acid is predicted to have a 5.2–12.1% compound annual growth rate (CAGR) in terms of revenue, from US$ 23 to 36 million p.a. When its price becomes competitive with other essential food-grade acids, such as citric, tartaric, acetic and lactic acids, its chemical and enzymatic production in large quantities may become more feasible. The principal industrial producers currently include Solvay (Germany), FrieslandCampina Domo (Netherlands), Sandoz (Germany), Reliable Biopharmaceutical Company (USA), with an increased amount of production also coming out of India and China, although volumes are not known (Alonso et al. 2013a).

5.5.7 Uses and Applications

The major drivers for the expansion of the lactobionic acid market are its diverse range of bioactivities, which include antioxidant, chelating, amphiphilic and self-assembling abilities, together with its biodegradability, biocompatibility and non-toxicity. These properties have resulted in the development of a wide variety of applications in the chemical, food, cosmetic, and pharmaceutical/medicine industries, which are outlined below.

In the chemical industry, lactobionic acid is used as a sugar-based surfactant, or as a co-builder, in biodegradable detergents where its iron-chelating and emulsifying properties result in enhanced surface and efficiency properties and decreased environmental effects (Bize et al. 2010). It can also be reacted with primary fatty acid mixtures to produce detergents and cleaning formulations with strong foam stabilising, drying, emulsifying and softening properties. Similarly, eco-friendly formulations of lactobionic acid *N*-alkylamides have been suggested as corrosion prevention agents for use in certain metal-working operations (Alonso et al. 2013a). Lactobionic acid has also specifically been used as an antibacterial agent that has excellent preservation stability, and as a building block for the biocatalytic synthesis of novel polymers (Kakasi-Zsurka et al. 2011), as a functionalisation agent for the synthesis of carbon nanotubes with lactobionic acid amide amphiphiles (Feng et al. 2011), and in direct electrochemistry films to create an unmediated third-generation hydrogen peroxide biosensor (Zhou et al. 2006).

Lactobionic acid was first found in a Caucasian fermented milk product popularly known as 'Caspian Sea yoghurt' in Japan, from which Kiryu et al. (2016) identified a lactobionic acid-producing acetic acid bacterium (*Acetobacter orientalis*). This bacterium has been used to make a number of commercially available lactobionic acid-containing food products. Chr. Hansen has also produced strong proofs of concept for the use of lactobionic acid in different dairy-based products to produce various physicochemical properties including increased adhesive gelling, reduced water loss, as a replacement for skim milk powder, and to provide dry matter for pizza cheese without affecting the overall properties of the ingredient cheese in pizzas. Lactobionic acid has also been incorporated as an acidifier agent in fermented milk products (Faergemand et al. 2012) and is also an excellent source for calcium supplementation of dairy products, providing higher solubility and stability properties without the off-taste resulting from other calcium sources. The inclusion of lactobionic acid, or its mineral salts, into foods can stimulate the absorption of intestinal Ca^{2+} or minerals, thereby exerting a strong health-promoting effect (Alonso et al. 2013a). In other foods, lactobionic acid has received FDA approval as an antioxidant, a stabiliser and a gelling agent in dessert products (FDA 2011). It can act as an ageing inhibitor for bread (Oe and Kimura 2011), a lipid oxidation retardant (Baldwin et al. 2004), and its thermal stability and high solubility open up a wide number of possible applications in food. In meat and meat products, the water-holding capacity of lactobionic acid can result in a higher industrial yield due to decreased water losses through the thawing or cooking processes (Nielsen and Hoeier 2009). It has also shown significant potential for the preservation of colour and bioactive compounds in commercial refrigerated juices from the yacon plant (Marques et al. 2020) and as a flavour enhancer for foods or beverages (Walter and Begli 2011).

Lactobionic acid has prebiotic properties as it is resistant to digestive enzymes, is poorly absorbed in the small intestine and is then subsequently fermented by the gastrointestinal microflora. It has been incorporated into a number of food products including a microencapsulated symbiotic preparation (Goderska and Kozłowski 2021) and a synbiotic coating with the probiotic *L. plantarum* CECT 9567 to produce a functional cottage cheese (Saez-Orviz et al. 2020). A symbiotic dairy food containing the probiotic *L. casei* and the food grade bacterium *P. taetrolens* has been used to directly produce lactobionic acid via a sequential fermentation system (García et al. 2019). The antimicrobial properties of lactobionic acid are also ideal for inclusion in sustainable and bioactive packaging material, and to assist attenuation of the antimicrobial spectrum of the food preservatives nisin and thymol (Chen and Zhong 2017). The mechanism of action of the antibacterial activity of lactobionic acid is by breaking down the structure of the bacterial cell wall and membrane, thereby releasing the cellular contents as well as inhibiting protein synthesis, which ultimately lead to cell death (Cao et al. 2019; Kang et al. 2021). The salt calcium lactobionate (E-399) is also classified as a preservative. Lastly, lactobionic acid has been proposed as a technical feed additive for laying hens, with the objective of optimising eggshell characteristics by increasing calcium absorption (Kimura 2012).

Lactobionic acid has become established as a key active component of novel anti-ageing and regenerative skin-care cosmetic products. It is added as a keratinisation agent to promote the biosynthesis of glycosaminoglycans or collagen, to improve skin thickness and firmness, and has multiple potential benefits for the therapeutic treatment of dermatological pathologies such as atopic dermatitis and rosacea (Algiert-Zielińska et al. 2018; Tasić-Kostov et al. 2019). It also exhibits strong moisturising, antioxidant, exfoliative and humectant properties. A principal medicinal attribute of lactobionic acid is its high liver specificity. This has led to novel therapies in the treatment of hepatic diseases, especially the target-specific, sustained release, delivery of anticancer drugs (Jain and Jain 2010). The synthesis of potentially biocompatible, bio-functionalised nanoparticles and targetable drug delivery vehicles, from DNA to bioactive molecules, for the treatment of recalcitrant diseases such as liver cancer is reviewed by Alonso (2018). It is also a major constituent of organ preservation solutions prior to transplantation such as the University of Wisconsin solution (UWsolution), also known as ViaSpan (commercial name) or Belzer solution, which was developed in the late 1980s. This is due to the ability of lactobionic acid to chelate ferric ions, to render osmotic support and to eliminate the risk of cell swelling. In addition, lactobionic acid has the potential to act as a platform for biomaterials or scaffolds in tissue engineering or be employed in the pharmaceutical industry as an excipient (Delagustin et al. 2019), while the lactobionate salt can also stabilise antibiotics such as erythromycin during intravenous delivery. More recently, Olivieri et al. (2018) showed that the highly hygroscopic and powerful antioxidant properties of lactobionic acid, together with it being an iron chelator and matrix metalloprotease inhibitor, make it an ideal supplement in artificial tears to treat ocular surface dysfunction such as dry eye.

5.5.8 Health Benefits

Although lactobionic acid is not naturally present in nature, Kiryu et al. (2009) estimated the annual amount unknowingly ingested from a Caucasian yoghurt to be very small at 760 mg and with no apparent side effects. At the other extreme, a trial administered 24 g of lactobionic acid daily to healthy male subjects and observed signs of bloating and flatulence, and symptoms similar to those for lactose intolerance (Van Dokkum et al. 1994). Lactobionic acid possesses a number of unique health-promoting functions including to promote calcium absorption in the intestine. An important physiological effect of lactobionic acid is the possible anticoagulant and antithrombotic action of its sulphate derivative bis-lactobionic acid amides, and, as a potent humectant, it exhibits anti-ageing effects, including skin plumping and surface topography smoothing (Tasic-Kostov et al. 2010). In addition, lactobionic acid has been reported to suppress oxygen-induced tissue damage, is used to aid wound healing and has also demonstrated antioxidant effects in tissues by inhibiting the development of hydroxyl radicals due to its iron-chelating properties. Lactobionic acid has also been shown to act as a special high-affinity inhibitor

compound of carbohydrate-binding proteins that promote vaccine stimulated immune responses against breast tumours that may lead to tumour regression, as well as an improved survival outcome (Stannard et al. 2010).

5.5.9 Product Safety, Dose Rates and Regulatory Issues

Lactobionic acid salt (or more specifically, calcium lactobionate) is considered safe for human use by the U.S. Food and Drug Administration (FDA 2011, 2018a, b), and the lactobionic acid salt can be used specifically as a firming agent in food items such as pudding mixtures and as an inactive ingredient for medical use in organ transplantation solutions. The European Union (European Commission 2009) does not allow the inclusion of lactobionic acid in food, principally because of its use as a chelator in tissues of the body and organs during transplants. With high doses of lactobionic acid (24 g/day), there can also be side effects that closely mimic those of lactose intolerance. Overall, however, there are still no definitive findings on the toxicological aspects of lactobionic acid (or lactobionate salts), and safety data sheets of manufacturers or vendors commonly contain standardised warnings, such as 'May cause digestive/respiratory tract irritation'. Japan has no known regulatory restrictions, and the consumer product Caspian Sea yoghurt contains naturally occurring lactobionic acid (Kiryu et al. 2009, 2012). Also, there are two Japanese patents, one using lactobionic acid as a flavour enhancer in fruit preparations (Kimura et al. 2007), and the other producing lactobionic acid in cheese preparations by including lactose oxidase in the cheese mix (Sato 2016).

5.6 Lactitol

5.6.1 Chemistry

Lactitol (4-*O*-β-D-Galactopyranosyl-D-glucitol; $C_{12}H_{24}O_{11}$; FW 344.32 Da)

- A sugar alcohol derived from lactose by catalytic hydrogenation
- Synonyms are lactit, lactositol, lactobiosit

Lactitol, a sugar alcohol, has developed a well-established reputation as a sucrose replacement sweetener in low-calorie foods, and there is renewed interest in lactitol as a prebiotic carbohydrate. Lactitol is the primary sugar alcohol formed by the hydrogenation of lactose using a reducing agent and a Raney-nickel catalyst. For the past century, this basic synthesis process has been the mainstay of lactitol production at both the laboratory and industrial scale.

A number of comprehensive reviews on lactitol have been published recently by Martínez-Monteagudo et al. (2019), Cheng and Martínez-Monteagudo (2019) and Zhang et al. (2020a, b, c).

5.6.2 Synthesis

Lactitol was first chemically synthesised in the early twentieth century when Ipatiew (1912) produced a hydrogenated disaccharide lactitol syrup via the reduction of lactose. It was almost a decade later before lactitol was first crystallised by Senderens (1920), and even then, this sugar alcohol would not be used in foods until the 1980s.

Lactitol synthesis is a complex chemical process involving catalytic hydrogenation where hydrogen is added to the carbonyl group of the lactose (Fig. 5.8). It is characterised by the formation of small amounts of numerous side products that include lactulose, lactulitol and lactobionic acid, as well as sorbitol and galactitol. Typical food-grade lactitol is more than 97.5% pure, with less than 2.5% other polyols and 0.1% reducing sugars. Therefore, it is a purer food-grade product than galacto-oligosaccharides, lactosucrose and lactulose equivalents. Furthermore, the food grades of these other products contain substantial amounts of other reactant and product carbohydrates.

The catalytic hydrogenation reaction occurs via Langmuir-Hinshelwood-Hougen-Watson kinetics (Salmi et al. 2011; Cheng et al. 2019) where the reactants are initially absorbed onto the catalyst, facilitating a surface reaction, followed by the desorption of the reaction products. Lactose and hydrogen are adsorbed through chemisorption and a non-competitive mechanism where the affinity of the reaction products is much less than the reactants. The surface reaction of the two adsorbed molecules results in the hydrogenation of lactose (Meyer et al. 2015, 2016). Lastly, the products are resorbed from the catalyst in a rapid step that is independent of pressure (Doluda et al. 2013).

The efficiency of the reaction is dependent on the type of catalyst, the temperature, time, and pressure of the reaction and the accessibility of the reactants to each

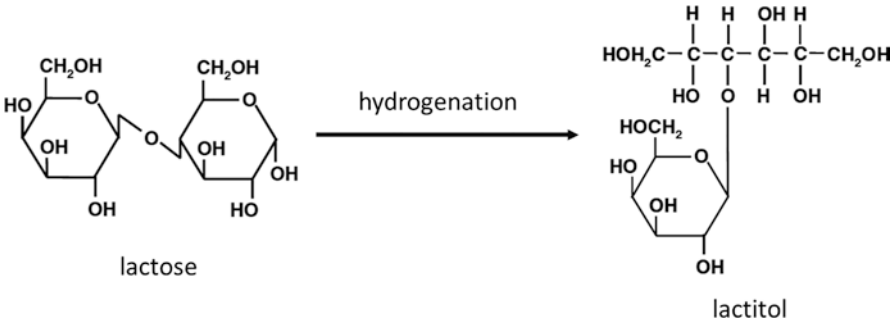

Fig. 5.8 The chemical structures (Haworth) and hydrogenation of lactose to form lactitol

other. The production of lactitol is generally performed in a continuous stirred-tank reactor designed to bring together the hydrogen (gas), the lactose (solution), with the catalyst (solid or slurry), with the hydrogen solubility in the lactose solution being the rate limiting factor. Typically, a number of different lactitol end products can be produced, including syrups, dihydrate or monohydrate crystals, or lactitol in the anhydrous form. It is also important to note that the lactitol product can undergo further dehydrogenation and hydrolysis to galactitol and sorbitol if the reaction time is extended (Martínez-Monteagudo et al. 2019).

Lactitol was initially prepared by reducing lactose using $NaBH_4$ in an autoclave at over 40 bar, over 100 °C and using a lactose solution of 30–40% (Saijonmaa et al. 1978). On an industrial scale, a catalytic hydrogenation process is used to increase the lactitol selectivity and reduce the production of other by-products (Cheng and Martínez-Monteagudo 2019). Raney-nickel catalysts, such as sponge nickel, were first used industrially, although they were limited by a deactivation problem caused by fast nickel leaching and weak catalyst sintering (Van Velthuijsen 1979). In addition, although selectivity for lactitol as high as 99% can be achieved in reasonably fast reaction times using sponge nickel catalysts, the well-established toxicity of Ni suggests that it is not necessarily suitable when the final product is used in food formulations (Kuusisto et al. 2006, 2007).

Nickel-based catalysts have now been superseded by modified ruthenium-based catalysts, with ruthenium on carbon (Ru/C) and ruthenium-nickel bimetallic nanohybrids on TiO_2 (Ru-NiO/TiO_2) being the most active and selective catalysts. These catalysts result in almost complete conversion of lactose to lactitol (>98% and 99.4% yields, respectively), have high specificity in an environmentally friendly aqueous solution and, in the case of Ru-NiO/TiO_2, can be reused four times with no loss of activity (Kuusisto et al. 2008; Mishra et al. 2018). A green synthesis reaction has also been described using direct hydrogenation by an amorphous copper on silica catalyst in ethanol solvent (instead of water) (Zaccheria et al. 2017). The different catalysts used in the production of lactitol from lactose, and their reaction conditions, are shown in Table 5.10.

After hydrogenation is completed, the spent catalyst can be removed by different combinations of sedimentation, filtration, ion-exchange resin purification and activated carbon, after which the purified slurry is concentrated by evaporation under vacuum to produce a lactitol syrup. This is followed by lactitol crystallisation with a carefully prescribed protocol of temperature and time to achieve the required crystal form. Unlike lactose crystallisation, the nucleation of lactitol has not been well studied, although there are some general guidelines for cooling temperatures and times, and seeding concentrations, to produce specific crystalline forms (Nurmi et al. 2002). The lactitol crystals are then recovered from the mother liquor by centrifugation and the process repeated.

Table 5.10 Chemical synthesis of lactitol (from Cheng et al. 2019)

Catalyst	Catalyst (%)	Lactose (g/L)	Temperature (°C)	Pressure (bar)	Time (min)	Selectivity (%)	Reference
Mo-promoted sponge Ni	2.5–10	200–400	110–130	20–70	0–250	90–99	Kuusisto et al. (2006)
Al_2O_3, silica, TiO_2, cross-linked polystyrene, activated C-supported Ru	5	450	110–130	40–60	0–300	96.5–98.5	Kuusisto et al. (2008)
Nanoparticles of metallic Ru in hyper-cross-linked polystyrene	1.1–4.9	40–170	120–150	10–50	0–300	96–97	Doluda et al. (2013)
Boron-nitride-supported Pd	1–5	40	130	50	0–240	<30	Meyer et al. (2015)
Silica-supported Cu	8	24	150–200	30	360	68	Zaccheria et al. (2017)

Table 5.11 Properties of lactitol

Property	Value
CAS No.	585-86-4
Molecular formula	$C_{12}H_{24}O_{11}$
Molecular weight (g/mol)	344.3
Melting temperature (°C)	93–100 (monohydrate)
Water solubility	Very soluble
Relative sweetness	40% (sucrose = 100%)
Calorie value (kcal/g)	2.4

5.6.3 Properties

Lactitol is a stable sugar alcohol that is known for its mild and clean sweetness (Kadoya et al. 2010). It has a relative sweetness of 0.3–0.42, meaning it is only 30–35% as sweet as sucrose. It is odourless, colourless and non-hydroscopic, with good flowability (see Table 5.11). In the solid state, lactitol can exist in different crystalline forms (three hydrate, two anhydrate and one amorphous forms), each having different melting points (Yajima et al. 1997). The most common form is the monohydrate form, which is obtained after slow crystallisation of the lactitol slurry. It occurs in the dihydrate crystalline form, as well in pure products. Lactitol is stable

under both alkaline and acidic conditions and at higher temperatures and is therefore stable during most food production and processing conditions.

While the melting point of lactitol is generally stated as 146 °C, this value is presumed to be referring to the two anhydrate forms (A and B), which have melting points of 124 °C and 151 °C, respectively. The individual melting points depend on the crystalline form, and the most common lactitol monohydrate form has melting points between 93 and 100 °C, depending on how it was dried and milled during manufacture (Halttunen et al. 2001). All the crystalline forms of lactitol are very soluble in water, a feature that is utilised for their commercial preparation.

Unlike a lot of other sugars, lactitol is not hydrolysed or actively absorbed in the small intestine and passes through the colon where it can enhance the growth of certain groups of bacteria in individuals. It is therefore considered to have prebiotic functionality (Drakoularakou et al. 2007).

5.6.4 Analysis

There are two aspects to the analysis of lactitol, the first of which is the purity of lactitol after synthesis from lactose, together with the levels of the different contaminant co-products. The standard method for such analysis is based on HPLC on either a strong cation exchange column or a polymer-based matrix (polystyrene divinylbenzene) column that separates sugars using a combination of size exclusion and ligand exchange mechanisms, with the ion-moderated partition chromatography technique, normally coupled with a refractive index (RI) detector. These methods have the levels of detection and quantification required to measure the sugars and sugar alcohols at low levels. For the quantification of lactitol in complex food matrices such as teas, jelly, chocolates and tablets (ramune candy), Takemori et al. (2018) developed an LC-MS/MS method that uses an amino group-binding polymer-based column followed by MS/MS detection in the selected reaction monitoring (SRM) mode. It has been used to distinguish the sugar alcohols erythritol, maltitol and lactitol, together with the sugar trehalose.

Secondly, a number of complementary analytical methods are required when establishing the crystalline form of the final lactitol product. These include differential scanning calorimetry (melting points), X-ray powder diffraction peak analysis, thermogravimetry and IR spectra (Yajima et al. 1997). The six different forms of lactitol (mono-, di- and tri-hydrate, two anhydrate (A and B) and one amorphous form) can be identified and quantified, and the monohydrate crystalline state has been shown to be the most common form.

5.6.5 Commercial Producers and Products

Since its first production around 100 years ago, lactitol synthesis has grown into a highly efficient industrial process with an estimated output of 1.9 million metric tons by 2022 (Martínez-Monteagudo et al. 2020). The Hayashibara Seibutsu Kagaku Kenkyusho Kk company of Okayama, Japan took out an early patent on lactitol production as a sweetener in food and drinks in 1976 (Hayashibara 1976). Lactitol is commercially available as crystalline and milled-crystalline powders, with its properties and potential applications dependant on the crystal form (anhydrous and monohydrate). Industrial production is located in the USA, China and Europe (Germany, UK, Switzerland and Latvia), and it is priced at between US$ 6 and 10/kg. Currently, lactitol is produced and marketed by a large number of companies, including DuPont Nutrition and Health and Danisco. China and India have become increasingly prevalent in the production of lactitol; however, there is very little information available on the production figures.

5.6.6 Uses and Applications

The principal application for lactitol is as a bulk sweetener and a sucrose substitute in beverage, bakery, confectionary and dessert manufacture. Due to its low caloric density and moderate sweetness, lactitol is usually combined with high-intensity sweeteners, e.g., aspartame, sodium cyclamate, acesulfame-K, or saccharin sodium, in high-sweetness foods (Tennant 2014). Lactitol confers greater chemical stability to the final product than that of lactose and sucrose due to the absence of a carbonyl group. Thus, it is not a sugar reducer and does not take part in the Maillard reaction, resulting in stability over a broad pH range (3–9). The binding mechanism to the human sweet taste receptor has been elucidated and provides an in-depth understanding of the differences in the sweetness response of different artificial sweeteners (Mahalapbutr et al. 2020).

In bakery formulations, the low hygroscopicity of lactitol allows it to be added as a sugar substitute while maintaining the good taste, hardness and brittleness imparted by sugar (Psimouli and Oreopoulou 2012). Thus, in a cake batter or dough, the flow index and temperature of starch gelatinisation were consistent with the substituted sugar, resulting in a nearly equal batter. The sensory attributes of a regular layer cake were also retained when lactitol was used as a sweetener, resulting in a 45% decrease in the calorie content (Frye and Setser 1992). It is used in cookies, chocolates, biscuits and ice cream where other sugar alcohols, such as sorbitol, mannitol and xylitol, can compete with each other. Due to their hygroscopic properties, mixtures of lactitol, sorbitol and mannitol can provide the sticky texture in products such as hard-boiled sweets. It is used by diabetics in glycaemic diets and

is also used extensively in formulating chewing gum as it does not induce dental caries. The good stability, and low cooling effect of anhydrous lactitol, has also been used to retain the quality and flavour of sugar-free chocolate (Zhang et al. 2020a, b, c).

Lactitol has also been used as a surfactant and emulsifier in cleaning formulations (Wilson et al. 2009), and as a platform chemical and delivery agent for the formation of hydrogels for pharmaceutical purposes (Luo et al. 2016), and as a lactitol-based polyether polyol (LPEP) to control release rates of bioactive compounds in drug delivery systems (Han et al. 2000). As a polyol, lactitol has the ability to resist physical and chemical degradation of protein formulations during freezing and drying. The efficacy of lactitol as a cryoprotective factor has been shown in fish muscles (rainbow trout) where lactitol helped retain the structure of myofibrillar proteins (Kadoya et al. 2010). In addition, lactitol can form hydrogen bonds with the surrounding protein, helping them to retain enzyme activity during the drying of protein preparations, and thus, stabilising enzymes for use in biosensors (e.g., immobilised isoenzyme glutathione transferase, Karamitros and Labrou 2017). Other applications where lactitol is used include animal feed to improve feed utilisation (Piva et al. 2005), hair styling products due to its stability and hygroscopicity (Huynh et al. 2008) and as a structural stabiliser when drying waterlogged archaeological cultural relics (Babiński 2015; Majka et al. 2017).

5.6.7 Health Benefits

Lactitol is not only considered an important food ingredient, but a variety of health benefits have also been attributed to its intake. These have been reported extensively elsewhere (Nath et al. 2017) and include dental caries, constipation, diabetes (Olli et al. 2016), and viral hepatitis (Chen et al. 2013). For oral bacteria, lactitol provides a small source of energy, resulting in less acid production in comparison to sucrose (van Loveren 2004). By combining sucrose with sugar alcohols in chewing gum and candies, the occurrence of caries could be lowered (van Loveren 2004). This protective effect could be attributed to the activation of increased salivary flow, which creates a buffering ability that washes away soluble carbohydrates. While there is currently no consensus on the minimum dose necessary to combat caries, chewing sugar-free chewing gum at least three times a day may minimise the occurrence of caries. Lactitol is also known to maintain tooth mineralisation (EFSA 2011).

Lactitol is also prescribed for the prevention of chronic constipation by acting as an osmotic laxative agent that increases intestinal osmolality (Vanderdonckt and Ravelli 1993; Prasad and Abraham 2017). It is ingested minimally in the small intestine, and, as it enters the large intestine, it induces an osmotic gradient that enhances the preservation of water in the faeces, facilitating faecal movement. A meta-analysis on the effectiveness and responsiveness of lactitol for adult constipation observed that supplementation of lactitol was not only well tolerated, but also greatly relieved constipation symptoms (Miller et al. 2014). Li et al. (2020) also showed that lactitol supplementation altered the faecal flora composition in patients

with and without diabetes mellitus, producing an increasing trend of *Bifidobacterium* while relieving constipation symptoms.

Lactitol is not absorbed in the small intestine or hydrolysed by enzymes in the gastrointestinal tract but is metabolised rapidly by microorganisms in the gastrointestinal tract. It thus possesses prebiotic properties resulting in increased numbers of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria* (Chen et al. 2007), although additional research using molecular techniques is needed to confirm its status as a prebiotic (Drakoularakou et al. 2007). Results from a rat study indicate that inclusion of lactitol in the diet results in lower insulin levels and that lactitol supplementation may therefore be an additional method to regulate postprandial metabolism and weight management, and a protection against diabetes onset (Olli et al. 2016). Two human studies have also shown that in patients with chronic viral hepatitis, lactitol can decrease the levels of plasma endotoxin and gut-derived endotoxemia more effectively than standard medical treatment through improving regulation of intestinal microflora (Chen et al. 2007, 2013). The prebiotic fermentation products of lactitol also improved intestinal barrier repair in a mucus-secreting human cell model (Yue et al. 2021).

5.6.8 Product Safety, Dose Rates and Regulatory Issues

Lactitol is approved as a sweetener in over 30 countries (including China, Japan, Canada, Australia, Brazil and Argentina) and has been allowed as a food additive (sweetening agent E966) in EU countries since 2008. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) approved lactitol as a safe product in 1983, and as a permitted sweetener in 2008 after assessing a number of studies on its absorption, delivery and excretion in laboratory and animal models (EC Directive 96/83/EC). Toxicological tests included carcinogenicity, dermal irritation, eye irritation, mutagenicity, reproduction and teratogenicity studies (for further details, please see JECFA 2008; Inchem 2008). European authorities gave lactitol a blanket label caloric value of 2.4 kcal/g (van Es et al. 1986; Radeloff and Beck 2013), while the FDA allows a value of 2.0 kcal/g; these values correspond to a reduction of 48–40% compared with sucrose.

The US Food and Drug Administration (FDA) accepted lactitol for GRAS status in 1993, and it has also been approved (FDA 2020) as a treatment for chronic idiopathic constipation (CIC) in adults at a recommended oral dose of 20 g/day (FDA 1993, 2020). Blood glucose levels of healthy or diabetic people were observed to not change at this dose, although diarrhoea can occur with higher doses (50 g/day). It was concluded that after massive doses, lactitol still has very low general toxicity and does not pose a health threat at the usual rate of consumption likely to be observed. No numerical figure for the appropriate dietary intake for men (ADI) was deemed necessary (Prasad and Abraham 2017). Lactitol monohydrate is also used as an excipient, or pharmacologically inactive substance, in a number of medications (Pearson and Olinger 1996).

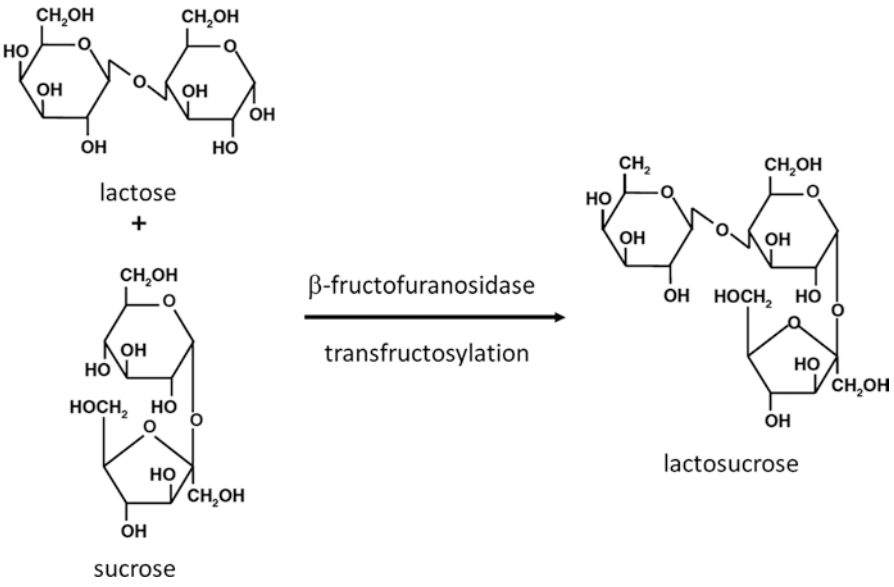

Fig. 5.9 Chemical structures (Haworth) of lactosucrose and its formation from lactose and sucrose

5.7 Lactosucrose

5.7.1 Chemistry

Lactosucrose (β-D-fructofuranosyl-4-*O*-β-D-galactopyranosyl-α-D-glucopyranoside; $C_{18}H_{34}O_{17}$; FW 522 Da)

The synthetic trisaccharide, lactosucrose, consists of galactose, glucose and fructose monosaccharides (Fig. 5.9). This compound is obtained using lactose and sucrose as the substrate by an enzymatic synthesis (transglycosylation). It is known to have many prebiotic and certain beneficial effects associated with its ingestion.

For a more detailed discussion of lactosucrose, readers are directed to very thorough recent reviews by Mu et al. (2013), Silvério et al. (2015) and Xiao et al. (2019a, b).

5.7.2 Synthesis

Lactosucrose is only present in very small amounts in nature and is difficult to synthesise chemically, hence commercial production is via an enzymatic process (Mu et al. 2013). This involves the transfer of the fructosyl moiety of sucrose to lactose (an acceptor molecule) using enzymes such as β-galactosidase (EC 3.2.1.23),

β-fructofuranosidase (EC 3.2.1.26), and levansucrase (EC 2.4.1.10). The reaction can be described as:

$$\text{Sucrose} + \text{Lactose} \leftrightarrow \text{Lactosucrose} + \text{Glucose} \quad (1)$$

Transgalactosylation using β-galactosidase is not considered to be commercially viable due to the formation of a wide range of other oligosaccharide by-products (Li et al. 2009). Duarte et al. (2017) attempted to overcome this obstacle by covalently immobilising β-galactosidase from *B. circulans* onto microspheres of chitosan and altering the processing conditions to favour the production of lactosucrose. A maximum lactosucrose concentration (79 g/L) was attained at pH 7 and 30 °C, although there were still high concentrations of both galacto-oligosaccharides (37 g/L) and total oligosaccharides (250 g/L).

The synthesis of lactosucrose, using either β-fructofuranosidase or levansucrase, is a transfructosylation reaction, and the enzymes can also hydrolyse the final lactosucrose product. As the yield and productivity of both of these enzymes are usually affected by the occurrence of the simultaneous hydrolysis of the newly formed lactosucrose, it is therefore important to find efficient strategies to avoid or minimise product degradation. β-Fructofuranosidase and levansucrase have been classified into the same family, glycoside hydrolase 68 (GH68), in the CAZy database (http://www.cazy.org/GH68.html; Xu et al. 2018), and there are suggestions that they may be one and the same enzyme (Playne and Crittenden 2009), although researchers have generally treated them as separate entities. The enzyme nomenclature used by individual researchers has been retained for the purposes of this chapter. Production conditions and yields for the three different enzymes, from various microbial sources, and with different degrees of purification, recombination and/or immobilisation are summarised in Table 5.12.

Avigad (1957) first reported the enzymatic synthesis of lactosucrose using a levansucrase from the soil bacterium *Arthrobacter*. The ratio of sucrose to lactose to enzyme was very important, with a 1:1 ratio of the substrates resulting in the highest conversion to lactosucrose. Avigad also recognised that too much enzyme can result in increased hydrolysis of the final lactosucrose product, and thus the need to continuously remove sugars from the system to prevent inhibition and the reversal of the levansucrase action. This was achieved by adding *Torulopsis glabrata* yeast cells to the reaction mixture. Another successful strategy in trying to overcome the enzyme inhibition caused by the reaction products was to add an invertase-deficient yeast to remove any excess monosaccharides (mainly glucose) (Arakawa et al. 2002).

Early commercial production of lactosucrose used the enzyme β-fructofuranosidases from the soil bacterium *Arthrobacter* sp. K-1 (reclassified in 2013 as *Microbacterium saccharophilum* K-1 (Ohta et al. 2013); GenBank/EMBL/DDBJ accession number: AB736273). This was originally isolated from soil at a sucrose refinery in Japan and is now thought to be used by the only two commercial lactosucrose producers from Japan; Ensuiko Sugar Refining Co., Ltd. and Hayashibara Seibutsu Kagaku Kenkyujo KK (Fujita et al. 1990, 1992a, b; Arakawa et al. 2002).

Table 5.12 Enzymatic production of lactosucrose

Microorganism	Form	pH	Temp (°C)	Substrate (g/L) (sucrose + lactose)	Yield (g/L)	Conversion (%) (sucrose:lactose)	Productivity (g/L/h)	Time (h)	Reference
β-Galactosidase									
B. circulans	Crude enzyme	6.0	40	300 + 300	56				Li et al. (2009)
B. circulans	Enzyme extract	7.0	30	300 + 300	79				Duarte et al. (2017)
β-Fructofuranosidase									
M. saccharophilum K-1	Crude enzyme	6.0	55	200 + 200	135				Fujita et al. (1990)
Arthrobacter sp. K-1 (Ensuiko)	Immobilised enzyme		55	200 + 200	120		Cont.	35	Mikuni et al. (2000)
Arthrobacter sp. K-1 (Ensuiko)	Purified enzyme		43	222 + 137	202	25:33	60.6		Pilgrim et al. (2001)
Arthrobacter sp. K-1 (Ensuiko)	Simulated moving bed reactor		50	171:181	133	70s			Kawase et al. (2001)
Arthrobacter sp. K-1 (Ensuiko)	Simulated moving bed reactor		50	342:342	348				Pilgrim et al. (2006)
Arthrobacter sp. 10137	Immobilised bi-enzymes		40	200 + 200	160.8				Long et al. (2019)
Arthrobacter sp. 10137	Recombinant thermostable	6.0	50	150 + 150	109				Chen et al. (2020)
Levansucrase									
Aerobacter levanicum/ *Torulopsis glabrata*	Purified enzyme/ washed cells	5.4	30	100 + 292	NR	NR	NR	NR	Avigad (1957)
Bacillus natto	Purified enzyme	6.2	35	85.5 + 85.5	53	54:42	26.5	2	Takahama et al. (1991)

Paenibacillus polymyxa	Free whole cells	6.0	40	225 + 225	170	42 l	28	6	Choi et al. (2004)
P. polymyxa	Crude free enzyme	6.0	40	225 + 225	140		210	0.7	Choi et al. (2004)
B. subtilis KCCM35411	Concentrated cell suspension	6.0	55	225 + 225	183	64:44	18.3	10	Park et al. (2005)
B. subtilis NCIMB 11871	Crude recombinant enzyme	6.0	NR	205 + 410	131	77 s	21.9	1	Seibel et al. (2006)
S. elviae mutant	Free whole cells	NR	50	250 + 250	184			15	Lee et al. (2007a)
S. elviae mutant	Immobilised mutant cell	6.0	50	250 + 250	192		180	For 48 h	Lee et al. (2007b)
Pseudomonas aurantiaca	Crude enzyme	4.0	45	510 + 360	285	NR	142.5	2	Han et al. (2007)
Z. mobilis	Crude recombinant enzyme	7.0	23	180 + 180	103	66:28	25.8		Han et al. (2009)
Z. mobilis + glucose oxidase	Crude recombinant enzyme	6.0	30	180 + 180	156	66:33	34.7		Han et al. (2009)
B. licheniformis 8-37-0-1	Purified recombinant enzyme	6.5	40	171 + 171	25.8	28 s	25.8	1	Lu et al. (2014)
L. mesenteroides B-512 FMC	Purified recombinant	6.5	50	270 + 270	224	41	224	1	Li et al. (2015)

(continued)

Table 5.12 (continued)

Microorganism	Form	pH	Temp (°C)	Substrate (g/L) (sucrose + lactose)	Yield (g/L)	Conversion (%) (sucrose:lactose)	Productivity (g/L/h)	Time (h)	Reference
B. methylotrophicus SK 21.002	Crude enzyme	6.5	37	180 + 180	143	36	7.15	20	Wu et al. (2015)
B. goodwinii	Purified recombinant enzyme	6.0	35	180 + 180	100	27	50	2	Xu et al. (2018)
B. subtilis CECT 39	Purified recombinant enzyme	~5.0	37	Tofu whey/cheese whey	80	61		2	Corzo-Martinez et al. (2015)

Notes:
Arthrobacter = *M. saccharophilum* K-1
Lee et al. (2007b)—continuous production for 48 h in a packed bed reactor

The mechanism and kinetics of commercial lactosucrose production using both free and immobilised β-fructofuranosidase have been studied (Mikuni et al. 2000; Pilgrim et al. 2001, 2006). On a pilot scale, in a column reactor, the immobilised enzyme was able to produce a lactosucrose stream of approximately 120 g/L for 35 days. A simulated moving bed reactor (SMBR) separated the lactosucrose and glucose after synthesis to minimise lactosucrose hydrolysis resulting in a predicted yield of 69% lactosucrose at 65 °C (Kawase et al. 2001). A more recent US patent filed by Hayashibara Seibutsu Kagaku Kenkyujo KK (Okabe et al. 2008) centres around a *Bacillus*-derived β-fructofuranosidase, together with a sucrose-unassimilable yeast, to produce a reaction mixture comprising 70% lactosucrose.

A variety of methods have been investigated to optimise the enzymatic production of lactosucrose. These have included using whole microbial cells from other microbial sources (*B. subtilis* KCCM32835, Park et al. 2005; *Paenibacillus polymyxa*, Choi et al. 2004; and *Sterigmatomyces elviae* ATCC 18894, Lee et al. 2007b), permeabilised cells, crude cell extracts (*Bacillus methylotrophicus* SK 21.002, Wu et al. 2015) and more purified forms of the enzyme (*Bacillus licheniformis* 8–37–0–1, Lu et al. 2014; *Bacillus natto*, Park et al. 2005; and *Leuconostoc mesenteroides* B-512 FMC, Li et al. 2015a, b, c).

Other methods to improve productivity include increasing the reaction temperature to increase the substrate solubility and improve the transfructosylation rate (Choi et al. 2004). The thermostability of a β-fructofuranosidase from *Microbacterium saccharophilum* K-1 has also been enhanced by random mutagenesis and saturation mutagenesis to produce a highly stable mutant with a 3 h half-life at 60 °C. This half-life was 16.5-fold longer than the wild-type enzyme (Ohta et al. 2014).

Other strategies include enzyme immobilisation and glucose oxidase addition for continuous lactosucrose production with higher productivity. For example, Long et al. (2019, 2020) showed that co-immobilisation of β-fructofuranosidase and glucose oxidase by sol-gel encapsulation resulted in a thermally enhanced enzyme system with a high yield of lactosucrose (40.2%), and good enzyme stability after eight consecutive recycles. Lee et al. (2007a) used mutated *S. elviae* immobilised whole, washed cells on sodium alginate beads to achieve continuous production of lactosucrose at a concentration of 180 g/L for up to 48 days, with a maximal concentration of 193 g/L. A simulated moving bed reactor has also been used to produce lactosucrose (Kawase et al. 2001), resulting in an increased yield of 56% compared to 48% typical in a batch fermentation. Similarly, Petzelbauer et al. (1999, 2000, 2002a, b) used thermostable purified enzymes from *S. solfataricus* and *P. furiosus* in a series of publications to produce lactosucrose in a continuous stirred-tank reactor at 70 °C, coupled with a cross-flow ultrafiltration module and using an immobilised enzyme system. While their data does not reveal if they were able to achieve the yields obtained in commercial systems, they successfully demonstrated the stability of these thermostable enzymes at high temperatures for a prolonged period under realistic bioprocessing conditions.

A number of groups have also used recombinant enzymes to increase productivity and specificity, including Lu et al. (2014) who expressed the *Bacillus licheniformis* 8-37-0-1 levansucrase gene in *E. coli*, followed by purification of the enzyme

using ion exchange chromatography. Similarly, Li et al. (2015a, b, c) and Xu et al. (2018) cloned levansucrase from *Leuconostoc mesenteroides* B-512 and *Brenneria goodwinii*, respectively, by expressing the target gene in *E. coli*, followed by purification to homogeneity using nickel affinity and gel filtration chromatography. Recombinant levansucrase and β-fructofuranosidase enzymes have also been developed to both increase their lactosucrose producing specificity, and to minimise their product hydrolysis capability. The efficient extracellular excretion of a recombinant thermostable β-fructofuranosidase from *Arthrobacter* sp. 10138 resulted in a maximum titre of lactosucrose after 10 min of 109 g/L at pH 6.0 and 50 °C. With a molar conversion ratio of 49.3%, there was still the suggestion that as the reaction progressed, there may be product or glucose inhibition (Chen et al. 2020).

While most research has used pure lactose and sucrose as the reaction substrate, Corzo-Martinez et al. (2015) showed that lactosucrose could be produced from two inexpensive agro-industrial by-products, tofu whey and cheese whey permeate. Levansucrase SacB from *B. subtilis* CECT 39 (overproduced in *E. coli* and purified) was able to transfructosylate lactose, using not only sucrose, but also raffinose and stachyose, which are present in considerable amounts in tofu whey. A yield of 80.1 g/L lactosucrose was obtained after a short reaction time of 120 min at 37 °C.

5.7.3 Purification

While the objective of any production process is to generate a product stream rich in lactosucrose, this is dependent on the enzyme(s) used, the reaction conditions, and the type of reactor. Lactosucrose yields can be limited by other carbohydrate products, such as glucose, and residual amounts of unreacted lactose and sucrose. An invertase-deficient yeast can be included in the reaction mix to remove unwanted monosaccharides (mainly glucose) (Arakawa et al. 2002), or ion exchange resin-filled reactors can separate the different saccharides during the enzyme synthesis (Kawase et al. 2001). Unreacted lactose can also be removed by crystallisation (Okabe et al. 2008).

If a liquid product is desired, syrups containing up to 55% of lactosucrose can be attained by the purification steps of decolouration, carbonation, filtration, desalination, ultrafiltration and concentration (Arakawa et al. 2002). Alternatively, column chromatography (using a strongly acidic cation-exchange resin) and fermentation have been used to remove monosaccharides like glucose (Hara et al. 1994). The two major producers sell lactosucrose as a powder with different purity levels (40%, 55% and up to 70% and 99% lactosucrose in the powder). These high levels are achieved using purification with DEAE ion exchange chromatography prior to spray drying.

5.7.4 Properties

Lactosucrose (*O*-β-D-galactopyranosyl-(1,4)-*O*-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside), a galactose, glucose and fructose synthetic trisaccharide, is often classified as 4^G-β-D-galactosyl sucrose and lactosylfrucoside (CAS No. 87419-56-5). It has the molecular formula and a molecular weight of $C_{18}H_{32}O_{16}$ and 504.44, respectively.

Produced as a white solid powder, it has a bland taste and can form tiny monoclinic platelets that are extremely hygroscopic with moderately high moisture-binding potential. Lactosucrose has a flavour comparable to sucrose, but with a relative sweetness of 30% compared to sucrose (Fujita et al. 2013). In water, lactosucrose has a higher solubility than sucrose (3600 g/L compared with 2000 g/L at 25 °C, respectively) (Torres et al. 2010). Stability experiments showed that lactosucrose powder was stable for 2 h at pH 7.0 and 80 °C, for 1 h at pH 4.5 and 120 °C, and was marginally stable under acidic pH conditions (pH 3.0, 80 °C), where any decomposition (less than 20%) was seen after 2 h (Hirota et al. 1993).

Lactosucrose was chemically non-reducing to the sugar analysis alkaline copper reagent of Somogyi (1945) and developed a green colour when reacted with diazouracil, a compound used primarily to classify sucrose or sucrose-containing oligosaccharides (Avigad 1957). It can be hydrolysed either enzymatically by the combined action of invertase and β-galactosidase (Avigad 1957), or in supercritical water using a continuous flow-type reactor and running at 10 MPa with high temperatures (200, 210 and 230 °C) (Khajavi et al. 2006).

5.7.5 Analysis

The analysis of lactosucrose follows standard sugar analysis using HPLC analysis followed by detection using refractive index (differential RI), electrochemical or a corona charged aerosol (CAD) detector. A number of column chemistries can be used, including amine groups bound to silica, polymer or C18 matrices (Wu et al. 2015), ion exchange (cation and anion) sugar or carbohydrate Ca^{2+} columns (Choi et al. 2004; Park et al. 2005; Seibel et al. 2006; Li et al. 2009). GC-MS can also separate the different sugars after derivatisation with MS trimethylsilyl oxime (TMSO) (Corzo-Martinez et al. 2015).

The structure of the final purified lactosucrose product can be elucidated through analysis of 1D and 2D NMR data, primarily from ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY (2D ^{1}H shift correlated spectroscopy), ^{1}H-^{13}C HSQC (^{1}H detected heteronuclear single-quantum coherence), ^{1}H-^{13}C HMBC (^{1}H detected heteronuclear multiple-bond correlation), and HSQC experiments (Li et al. 2015a, b, c; Wu et al. 2015). The functional groups have also been determined using FT-IR spectrum analysis (Xu et al. 2018), while Duarte et al. (2017) and Chen et al. (2020) identified the lactosucrose structure using electrospray ionisation (ESI) LC-MS analysis.

5.7.6 Producers and Commercial Products

The global production of lactosucrose currently appears to be limited to two Japanese companies, although there are a number of Chinese companies offering large quantities on their websites. The two Japanese companies, Ensuiko Sugar Refining Co. and Hayashibara Shoji Inc., with the brand names Newka-Oligo and Nyuka-Origo, respectively, hold a number of patents for the production of lactosucrose using the enzyme method (Japanese Patent Kokai No. 27.285/91; Japanese Patent Kokai No. 224,665/97; Japanese Patent Kokai No. 66,586/98 (recombinant enzyme); Japanese Patent Kokai No. 293,494/92; EP 1 772 461 A1 Ensuiko 2005; and US 2008/0027027 A1, Hayashibara).

Lactosucrose production is thought to be by a continuous process, using β-fructofuranosidase in a simulated moving bed reactor, with a conversion ratio of sucrose into lactosucrose of over 80% (Arakawa et al. 2002). The principal products are either solutions of between 40% and 70% on dry matter, or powders with 50–98% lactosucrose (LS-98, 99.2% lactosucrose, Ensuiko Sugar Refining Co., Ltd., Tokyo, Japan; Kishino et al. 2015). Current production totals or forecasts could not be sourced beyond 2010 (of up to 5 MT/year, US$ 40 million) (Paterson and Kellam 2009). The raw product is sold by a variety of other Japanese firms (e.g., Pearl Ace Corporation, ex-Maruha Corporation, now owned 100% by Ensuiko Sugar Refining Co., Ltd.) and new applications, e.g., concentrated liquid nutrition products for fluid replenishment and nutrition supply for medical and care facilities (Anon 2009). The price per kg is approximately US$ 2.00–5.00. While Japan remains the major market for lactosucrose, as an emerging prebiotic it is also being marketed in the USA and in Europe (Diez-Municio et al. 2014). The Chinese company Shandong Bailong Chuangyuan Biological Technology Co., Ltd. also has a large number of patents dealing with the enzymatic production of lactosucrose using a levansucrase from *Arthrobacter chlorophenolicus* SK33.001, but no information is currently available regarding whether it is producing lactosucrose, and if so, its production capability (including CN104480164A 214 Enzymic method for preparing lactosucrose, 2015).

5.7.7 Uses and Applications

Lactosucrose has been approved in Japan since 2005 as a FOSHU product and is currently used in over 30 food and beverage items as a low-digestive and low-cariogenic sweetener food ingredient (Mu et al. 2013; Li et al. 2015a). The different types of foods that have been launched onto the market in the last two decades include confectionaries, cakes, snacks, baking products, yoghurts, coffee and tea. Several patents make claims for its inclusion in chocolates, chewing gum, instant juice, instant broth and mineral water (Fujii et al. 2006; Okabe et al. 2008). Lactosucrose has also been added to pet food to simultaneously control intestinal

microflora and minimise the unwanted odour of faeces and urine, as well as to fish feed to improve nutritional absorption and decrease self-contamination by excretion (Silvério et al. 2015). Other uses of lactosucrose include as an excipient for spray dried powders, and to stabilise proteins or polyplexes (Schüle et al. 2008; Kasper et al. 2011). Lastly, lactosucrose has a high water holding capability that may be beneficial in the food processing industry for decreasing syneresis or serum separation during product storage (Krasaekoopt et al. 2003) in fermented milk products, such as yoghurts or cheese. It may also potentially be used as a fat replacer in products which are susceptible to surface serum or whey accumulation, leading to a decrease in syneresis and improving product texture and consistency.

5.7.8 Health Benefits

There have been many studies of the various physiological advantages associated with lactosucrose intake in both humans and animal models. Lactosucrose is rarely hydrolysed by humans and is a form of indigestible prebiotic oligosaccharide that is selectively used by and increases the concentration of, enteric intestinal *Bifidobacterium* (and *Lactobacillus*) (Fujita et al. 2009). It has a beneficial role in the maintenance and defence of intestinal microflora and has been reported to promote the proliferation of *Bifidobacterium* in vivo better than other oligosaccharides (Arakawa et al. 2002). An increase in *Bifidobacterium* results in increased production of short-chain fatty acids, reduced intestinal pH, and the inhibition of the growth of *Clostridium* and other intestinal pathogenic bacteria. This in turn reduces the formation of toxic products, such as ammonia, phenol, indole, skatol and ethylphenol (Fujita et al. 1995). In addition, following consumption of lactosucrose by healthy people, blood glucose or serum insulin levels have not substantially increased.

Although lactosucrose supplementation can result in short-chain fatty acid microbial fermentation products, reduced pH values and enhanced mineral bioavailability, Gibson and Roberfroid (2008) stated that there was still insufficient evidence to classify lactosucrose as a prebiotic. However, it has been shown to be beneficial to elderly patients with constipation (Kumemura et al. 1992) by increasing stool frequency, faecal weight and moisture content, together with improved outcomes for abdominal pain, distention, stomach rumble and nausea. Interestingly, Oku et al. (2002) have also reported that lactosucrose can help prevent the abdominal symptoms experienced by lactose-intolerant patients.

Lactosucrose has been suggested as a possible treatment for Crohn's disease and ulcerative colitis patients. In rat studies, lactosucrose has been shown to have a protective effect on intracolonic-indomethacin-induced small intestinal ulcers (Honda et al. 1999), and to also ameliorate 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. Chronic intestinal inflammation can contribute to an increased risk of colon cancer, which, in lactosucrose fed rats, is countered by increased IL-10 production, suppressed secretion of IL-12 in the colon, and decreased production of

TLR-2 protein and nuclear NF-κB p65 protein (Zhou et al. 2014, 2015a, b). A metabolomic study of lactosucrose supplemented colitic rats (Ruan et al. 2013) demonstrated a whole-body alteration of amino acid metabolism, resulting in increases in serum aspartate aminotransferase and glucose metabolites, together with enhanced production of short chain fatty acids in the intestinal lumen.

Indigestible oligosaccharides have been shown to enhance intestinal calcium absorption, leading to possible increased calcium transport, deposition and bone strength (Fujita et al. 1999). Human studies reported improved absorption of intestinal calcium in healthy men after a single or 2-week supplementation with lactosucrose (Kikuchi et al. 2003; Fujita et al. 2006), and long term enhanced intestinal calcium absorption and reduced bone resorption in healthy young women (Teramoto et al. 2006). Lactosucrose shows possible beneficial results in reducing body fat accumulation and avoiding obesity via a number of mechanisms. It can decrease the serum cholesterol levels in mice, block the absorption of 2-monoacylglycerol by rat small intestine brush boundary membrane, and lower the intestinal synthesis of dietary fat by inhibiting beta-monoglyceride absorption (Mu et al. 2013). Mizote et al. (2009) reported that, in rats, lactosucrose can directly interact with the symmetrical triglyceride triolein, resulting in a reduced elevation of serum triglycerides and free fatty acids (FFA). Furthermore, long-term consumption of LS by rats resulted in a significant decrease in the total abdominal fat mass. Therefore, lactosucrose has the potential to be an obesity preventing dietary supplement.

Lactosucrose can modify the intestinal microflora and indirectly strengthen the gut IgA, suppressing the type 2 helper T systemic immune response (Hino et al. 2007). In addition, in a mouse model, the IgE reaction caused by intraperitoneal immunisation with ovalbumin/alum lactosucrose intake can be suppressed, via a mechanism observed in IgE-mediated allergic diseases (Taniguchi et al. 2007). Kishino et al. (2015) also demonstrated that lactosucrose supplementation may suppress influenza A virus infection in the respiratory tract by augmenting innate immune responses and enhancing cellular and mucosal immunity.

5.7.9 Product Safety, Dose Rates and Regulatory Issues

The minimum effective dose to improve intestinal microflora faecal conditions and defecation is 1–3 g/day (Hara et al. 1994; Yoneyama et al. 1992). When taken in large amounts, lactosucrose may increase gastrointestinal osmotic pressure and induce diarrhoea, although it has a higher laxative threshold than other lactose-based prebiotics such as lactulose and galactooligosaccharide (Arakawa et al. 2002). Its maximum no-effect dose is 0.6 g/kg bodyweight, and an optimum dose is considered to be between 5 and 36 g/day for an adult human (Playne and Crittenden 2009). The threshold lactosucrose concentration for a laxative was calculated to be 0.802 g/kg body weight, which is significantly greater than that recorded for other non-digestible carbohydrates such as lactulose (0.26 g/kg body weight, Oku and

Okazaki 1999). Two or more doses daily decreased the risk of diarrhoea relative to the ingestion of a single dose of the same total volume. The avoidance of gastrointestinal symptoms in lactose-intolerant patients can also be accomplished by lactosucrose administration.

5.8 Conclusions

The saying 'the world is your oyster' is particularly relevant when looking at the future for lactose-derived products. Research into the health benefits of the different products is constantly unearthing new potential uses and is also providing robust scientific evidence to underpin these claims. While the prebiotic properties of GOS and lactulose are well recognised, there is increasing data to suggest that tagatose, lactobionic acid, lactitol and lactosucrose may also offer prebiotic benefit to our intestinal microbiota. As more is discovered about the complex role bacteria play in our physical and mental health, in addition to their bifidogenic activity, interest is growing in the ability of prebiotics to nurture bacterial populations that may counteract detrimental species. For GOS especially, compositional differences will become more important and the synthesis of novel (galacto- or lactulose-) oligosaccharides that better mimic HMO or have other beneficial functions may lead to higher returns. There will always be a drive for functional food ingredients with claims backed up by solid science, and the number of non-food applications will also become important. Co-products such as synbiotics are gaining traction and compounds with enhanced specificity towards targeted gut microbiota groups, that elicit beneficial effects for human infants, and can improve infant formula, will always be in demand.

In the drive for more efficient GOS production, purification is often neglected, but is an essential factor in the economic production of a number of lactose-derived products. When optimising enzymatic synthesis, the reaction is usually terminated before reaching completion leaving unreacted substrate (lactose). Often there are also a number of other by-products that may need to be removed, depending on the final use of the product. Filtration and chromatographic methods, or yeasts to selectively digest the monosaccharides all need further research to optimise their potential. Lastly, the economics of a number of these production scenarios are very dependent on the availability of low valued whey streams. There will always be competition for whey from other uses, and economics and market access are big factors in processing decisions.

Perhaps if we were offering up an ideal scenario, this would be a green process that is both technically and economically feasible at small and medium scales to take advantage of reasonably priced whey sources. It would use enzymes, perhaps genetically engineered from thermostable bacteria, yeasts or fungi. These recombinant technologies, together with site-directed mutagenesis and directed evolution, may become important tools to obtain and reconstruct enzymes with the desired specificity and activity to produce GOS and other products tailored to specific

needs. Immobilised enzyme techniques and bioreactor design, together with AI and advanced process control systems, could then be used to optimise production while minimising side reactions, product inhibition and the synthesis of unwanted by-products. Finally, simplified purification techniques, such as specifically tailored filtration membranes, continuous chromatographic separation methods or food grade co-cultures could be used to give products of the desired purity and containing minimal reactant and by-product contaminants.

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Chapter 6
Lactose Malabsorption

Catherine J. E. Ingram, Nicolás Montalva, and Dallas M. Swallow

6.1 The Small Intestine and Digestion of Lactose

Milk is the sole source of nutrition of neonatal mammals, and the disaccharide lactose is the major carbohydrate component and energy source in the milk of most mammalian species. Lactose is digested and absorbed in the small intestine. The surface of the small intestine has a specialised structure, composed of hundreds of thousands of 'villi' (tiny finger-like structures that protrude into the lumen). The absorptive epithelial cells (enterocytes) on the surface of the villi have additional extensions called microvilli which make up the apical 'brush border', and this maximises the surface area through which the body absorbs nutrients. The enzymes that facilitate digestion and absorption of carbohydrates are anchored to the surface of the brush border.

Small intestinal lactase activity was first demonstrated by Pautz and Vogel (1895). Lactase cleaves lactose into its constituent monosaccharides (galactose and glucose) which can then be transported across the epithelial cell membranes into the enterocytes via active transport by a sodium-dependent glucose and galactose transporter, and then into the blood stream (Wright et al. 2007). Intact lactose cannot cross the cell membrane, and hence lactase is essential for neonatal nutrition. In adult mammals however, lactase is no longer needed when lactose is no longer part

C. J. E. Ingram · D. M. Swallow (✉)
Research Department of Genetics, Evolution and Environment, University College London, London, UK
e-mail: d.swallow@ucl.ac.uk

N. Montalva
Society and Health Research Center, Faculty of Humanities, Universidad Mayor, Santiago, Chile

School of Public Health, Universidad Mayor, Santiago, Chile
e-mail: nicolas.montalva@umayor.cl

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of the diet and usually decreases significantly in quantity following weaning (Büller et al. 1990; Lacey et al. 1994; Pié et al. 2004; Sebastio et al. 1989).

Humans are different in this respect, and it has long been noted that some adults are able to digest large quantities of fresh milk, whilst others cannot, and show symptoms of lactose malabsorption after consuming lactose. This difference is most commonly due to normal genetically determined variation in the quantity of lactase present in the adult small intestine. Adult humans with low lactase activity (the usual situation in other mammals) are described as lactase non-persistent (LNP) or are said to have primary adult hypolactasia. Adults who have high lactase activity are said to be lactase persistent (LP). This normal variation in lactase activity is quite distinct from congenital alactasia (the absence of lactase from birth), which is an extremely rare and potentially fatal inborn error of metabolism caused by mutations in the coding region of the lactase gene, *LCT* (Kuokkanen et al. 2006).

Lactase activity may also be reduced if the brush border is damaged due to gastrointestinal disease, and this condition (which is usually reversible) is referred to as secondary or acquired hypolactasia (Villako and Maaroos 1994). People who have either primary or secondary lactase deficiency are lactose maldigesters as determined by a lactose tolerance test and may exhibit symptoms of lactose intolerance. This chapter focusses on lactose malabsorption in the context of genetically controlled variation of *LCT* expression in adults.

6.2 Lactase and Its Structural Gene, *LCT*

Lactase–phlorizin hydrolase is encoded by the gene *LCT*, located on chromosome 2q21. The nucleotide sequence spans 49.3 kb and exhibits fourfold internal homology, suggesting that two partial gene duplication events occurred during the evolution of the gene. Two of the homologous domains (I and II) occur in the pro-region of the molecule and the others (III and IV) are found in the mature polypeptide (Mantei et al. 1988).

The 5787 bp pre-pro-lactase-phlorizin hydrolase mRNA transcript is encoded by 17 exons (Boll et al. 1991). The pre-pro-protein, composed of 1927 amino acids, contains a putative signal sequence of 19 amino acids, and a large ‘pro’ portion of 847 amino acids, both of which are proteolytically removed before the protein assumes its mature form (Mantei et al. 1988). The signal sequence is removed in the ER by signal peptidase, yielding pro-LPH molecules, which become *N*-glycosylated and pair up to form homodimers (Grünberg and Sterchi 1995). This dimerisation is essential for the acquisition of transport competence and full enzymic activity of LPH (Naim and Naim 1996). Further (*O*-linked) glycosylation occurs once the pro-LPH-homodimer has been translocated to the Golgi apparatus, which is also the predominant site of proteolytic cleavage of the pro-sequence (Naim et al. 1987). The pro-sequence has been shown to play a vital role in the maturation of LPH, being involved in folding, targeting and dimerisation of the molecule (Panzer et al. 1998).

Residues Ala867 onwards comprise the 160 kDa glycoproteins found anchored to the brush border of the jejunum as mature LPH homodimers (Mantei et al. 1988). LPH is an amphiphilic molecule, consisting of a short cytoplasmic domain followed by a membrane-spanning hydrophobic domain (residues 1883–1901) at its C-terminus, orientating the molecule such that the bulky, hydrophilic N-terminal projects into the lumen (Skovbjerg et al. 1981; Wacker et al. 1992). It is within this N-terminal portion that both catalytic activities reside, and it has been demonstrated that the active site for phlorizin hydrolysis is distinct from that of lactose (Colombo et al. 1973; Leese and Semenza 1973; Skovbjerg et al. 1981). The active sites are situated within the homologous domains III (Glu1271) and IV (Glu1747), respectively (Arribas et al. 2000; Wacker et al. 1992).

The 'phlorizin-hydrolase site' is situated closest to the brush border and preferentially catalyses the hydrolysis of β-glycosides with large, hydrophobic aglycones (galactosyl and glucosyl β-ceramides, phlorizin and other aryl- or alkyl-β-glycosides). The 'lactase' catalytic site has been shown to have a preference for β-glycosides with hydrophilic aglycones (e.g., lactose, cellobiose and some β-1,4-linked small glucose oligomers) (Colombo et al. 1973; Leese and Semenza 1973; Skovbjerg et al. 1981). LPH is also capable of hydrolysing various flavanol and isoflavone glucosides (Day et al. 2000).

6.3 Lactose Intolerance

Lactose intolerance is a clinical syndrome caused by lactose malabsorption that can occur when milk is consumed by a LNP person. Lactose intolerance does not occur in all individuals who are diagnosed as lactose maldigesters, and the symptoms vary between individuals, but usually manifest themselves within 1–2 h of ingestion; they are caused when undigested lactose passes through the small intestine into the colon. Firstly, an osmotic gradient is created across the gut wall, which results in a large influx of water that causes diarrhoea. Secondly, colonic bacteria digest the lactose by fermentation, producing short-chain fatty acids and gasses (hydrogen, carbon dioxide and methane) that can cause pain, bloating and flatulence.

Most LNP individuals can tolerate a small amount of lactose (around 12 g, equivalent to a single glass of milk) and larger doses may be tolerated, particularly if consumed with food or spread over a whole day (Misselwitz et al. 2019). Inter-individual variation in the composition of the gut flora accounts for some of this variation (Goodrich et al. 2017; Hertzler et al. 1997; Hertzler and Savaiano 1996), as well as a psychosomatic component (Briet et al. 1997). In fact, self-diagnosed lactose intolerance is poorly correlated with results from lactose digestion tests (Jellema et al. 2010; Peuhkuri et al. 2000; Saltzman et al. 1999; Zheng et al. 2015). This effect is possibly due to a high public awareness of lactose intolerance, leading individuals to assign any symptoms of gastrointestinal discomfort to this cause (Szilagyi and Ishayek 2018).

The symptoms of lactose intolerance were first connected to decreased lactase activity following the observation that it was virtually absent in a proportion of intestinal tissue samples from healthy adults with histologically normal mucosa (Auricchio et al. 1963; Dahlqvist et al. 1968). The discovery of this enzyme deficiency or 'abnormality' (as it was viewed at the time) prompted researchers to examine the inter-individual differences in our capacity to tolerate milk and its derived dairy products. The worldwide data on LP frequencies that subsequently accumulated enabled a global picture to develop that challenged the perception of LNP as abnormal.

6.3.1 Diagnosis of Lactose Malabsorption

To collect information on the worldwide frequencies of LP, alternatives to direct quantification of lactase activity from biopsies of the small intestine are used. Biopsies are the most accurate method of establishing lactase activity; however, they are invasive and are not a preferred routine diagnostic for lactose malabsorption (normally being obtained only when a patient is undergoing endoscopy to exclude another gastro-intestinal complaint).

Several indirect methods have been developed to evaluate lactose digestion capacity, from which LP status is inferred. The general practise is to give a lactose load after an overnight fast. The two most widely used methods are described below:

1. **The Blood Glucose Test**
   A baseline measurement of blood glucose is taken before ingestion of a lactose load, and then at various time intervals (usually every 30 min) for the following 2 h. An increase in blood glucose indicates lactose digestion (as glucose is absorbed into the bloodstream), and no increase, or a flatline, is indicative of lactose malabsorption.
2. **The Breath Hydrogen Test**
   This test measures hydrogen production by colonic bacteria. A baseline measurement of breath hydrogen is taken prior to ingestion of a lactose load, and further readings are taken at 30-min intervals from the time of ingestion for the following 3 h (Peuhkuri et al. 2000). If the lactose is digested by lactase in the small intestine, no changes in breath hydrogen will be observed, and a diagnosis of lactose tolerance will be made. Conversely, if the lactose load passes through the small intestine and into the colon intact, it will be fermented by bacteria and hydrogen will be produced as a by-product. Some of the hydrogen is absorbed into the blood stream and released into the breath as the blood passes through the lungs, indicating lactose malabsorption.

For both methods, somewhat arbitrary cut-off points are set for distinguishing between lactose digesters and maldigesters, and because *LCT* expression is inferred from these indirect tests, there is an error rate in both directions.

Some of these errors can be attributed to test design, particularly in the quantity of lactose administered. Non-persistent individuals express a residual amount of lactase, approximately 10% of adult levels (Semenza et al. 1999), and so a low lactose dose may be insufficient to increase breath hydrogen by the standard >20 ppm increment required for classification as a maldigester. A low lactose dose can lead to the opposite misclassification in the blood glucose method: LP individuals may show an insufficient blood glucose rise to cross the nominated threshold (usually 1.1 mmol/L) for digester status. Some studies suggest that a proportion of consumed lactose goes undigested even in LP people, and therefore, an increase in breath hydrogen can sometimes occur in persistent subjects when a high-dose challenge (such as 50 g) is used (Bond and Levitt 1976).

Apart from dose, many other factors can impact upon the test result; gastric emptying and intestinal transit times can exert an effect both on blood glucose and breath hydrogen measurements. Diarrhoeal disease is known to reduce *LCT* expression temporarily as a result of villus flattening and loss of the cells which express lactase (Villako and Maaroos 1994), and this could cause a genetically LP individual to be misclassified as a maldigester. The use of antibiotics may disrupt the gut flora and result in erroneous results. Colonic adaptation to dairy products may affect breath hydrogen production by increasing bacterial populations that have increased metabolic activity for lactose (Goodrich et al. 2017; Hertzler and Savaiano 1996). Some individuals are 'hydrogen non-producers' (having few hydrogen-producing bacteria), and in this situation, the breath hydrogen test is uninformative.

In the clinical setting, there are ways of improving the quality of the test. These include retesting, giving a dose of lactulose to test for hydrogen production and investigating other causes of lactose intolerance.

Mulcare et al. (2004) attempted to estimate the error rates of both the blood glucose and the breath hydrogen test from published data. Results were pooled from papers that compared either indirect method with each other or with a verified phenotype based on direct enzyme assays from jejunal biopsy. Exact protocols varied between the pooled data set, but all included a minimum 50 g lactose load and measured a change in parameter one or more times between 30 min and 4 h after ingestion. The blood glucose method error rates were 7% false positive (i.e., LNP individuals classified as lactose digesters) and 9% false negative (i.e., LP individuals classified as lactose maldigesters). The breath hydrogen method was found to give a slightly more accurate assessment of LP status, with approximately 5% false-positive and 7% false-negative error rates. Thus, the evidence suggests that to obtain the most accurate indirect assessment of LP status, a breath hydrogen test should be undertaken. In population studies, the most accurate method (Ingram et al. 2007; Mulcare et al. 2004) requires a fast of 12 h to be observed prior to consumption of a 50 g lactose dose (equivalent to approximately 1 L of cow's milk), although in clinical practice, an intermediate lactose dosage of 20–25 g is suggested (Misselwitz et al. 2019; Rezaie et al. 2017). Test results for subjects with a H_2 baseline of zero (possible hydrogen non-producers), or greater than 20 ppm (suggestive of failure to fast, or bacterial overgrowth of the colon), should be interpreted with caution and followed up if possible.

6.4 Worldwide Distribution of Lactase Persistence

Several populations have been surveyed for lactose digester frequencies over the years, so that the global distribution of LP is now fairly well characterised (Fig. 6.1) (Anguita-Ruiz et al. 2020; Ingram et al. 2009a; Itan et al. 2010; Ségurel and Bon 2017). These surveys clearly show that lactose maldigestion/LNP is the most common phenotype in humans; LP being common only in those populations with a long history of pastoralism and where milking has been practised. Lactase persistence is at highest frequency in north-western Europe, with a decreasing cline to the south and east. In India, the frequency of LP is higher in the north than the south, and in the rest of the world, the frequency is generally low. In Africa, the distribution is patchy, with some pastoralist nomadic tribes having high frequencies of LP compared with the neighbouring groups that inhabit the same country (Bayoumi et al. 1981), with a similar pattern observed between Bedouin and neighbouring populations in the Middle East (Fig. 6.2) (Cook and al-Torki 1975; Dissanayake et al. 1990; Hijazi et al. 1983; Snook et al. 1976). The noted correlation of the LP phenotype with the cultural practise of milking engendered the hypothesis that this trait has been subject to strong positive selection (Holden and Mace 1997), and further evidence for this is presented in Sect. 6.8.

6.5 The Genetic Basis of Lactase Persistence

The mechanism by which *LCT* expression is downregulated is not yet fully characterised. Initially, it was proposed that lactase might be an inducible enzyme (Cook 1988; Gilat 1971), comparable with the β-galactosidase with lactase activity from *E. coli*, which is only expressed in the presence of lactose, under the control of the well-known Lac operon (Jacob and Monod 1961). However, experiments in animals

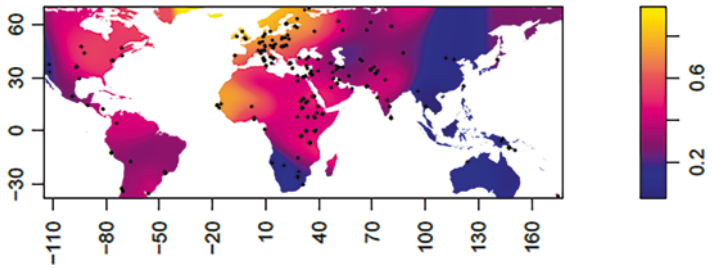

Fig. 6.1 Worldwide distribution of lactase persistence phenotype. Data points from the Americas include people of very varied ancestries, including recent immigrants from the Old World as well as native Americans. Colour gradients generated by interpolation of the data. Values on axes are degrees of latitude and longitude. The scale bar shows frequencies of lactase persistence. (Data updated from https://www.ucl.ac.uk/biosciences/departments/genetics-evolution-and-environment/research/molecular-and-cultural-evolution-lab/glad)

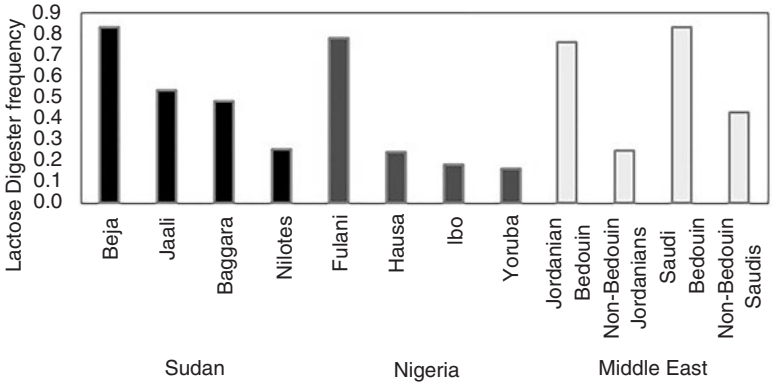

Fig. 6.2 Examples of countries/regions in which individual ethnic groups display large differences in lactose absorption capacity

have shown this is not the case (Gutiérrez et al. 2002; Leichter 1973; Plimmer 1906), and a study of adult Thai men who consumed lactose doses daily for a month confirmed that lactase is not inducible in humans (Keusch et al. 1969).

Lactase persistence was shown to have a genetic cause in the late 1960s and early 1970s using family studies, which also revealed an autosomal dominant mode of inheritance (Ferguson and Maxwell 1967; Sahi 1974). Monozygotic twins showed 100% concordance of the LP phenotype, and phenotype frequencies in dizygotic twins were consistent with expectations for autosomal dominant inheritance (Metneki et al. 1984). Ho et al. (1982) demonstrated that LP was likely caused by a *cis*-acting element. They measured lactase activity in autopsy material from the small intestine (retrieved from British individuals free from gastrointestinal disease). Sucrase activity provided an internal standard to correct for non-genetic variation. A trimodal distribution of sucrase:lactase ratios was observed, which represented individuals homozygous for LP (highest lactase activity), heterozygotes (mid-level activity) and non-persistent homozygotes (lowest lactase activity). The intermediate lactase activity observed in heterozygotes indicated that only one copy of *LCT* was being fully expressed, and concordant results were subsequently obtained in individuals of German ancestry (Flatz 1984). Confirmatory evidence that LP is caused by a *cis*-acting mechanism was obtained from mRNA studies. Allelic variants of exonic single-nucleotide polymorphisms (SNPs) within the gene *LCT* were used to identify individual transcripts and their expression levels. Europeans of the LP phenotype who were heterozygous for exonic polymorphisms were used to demonstrate monoallelic expression at the mRNA level (Wang et al. 1995).

Studies of the immediate promoter of *LCT* show that a ≈150 bp region drives low-level expression in an intestinal cell line (Troelsen et al. 1992) and is conserved in human, rat, pig and mouse, suggesting that key regulatory elements important for lactase expression are encoded within this small region in the proximal promoter (Troelsen 2005). Transgenic mouse experiments using rat and pig promoter

constructs of different sizes show that elements outside of the conserved 150 bp region are required for high and tissue-specific expression of *LCT* and upstream enhancer regions contribute to its spatial and temporal expression (Krasinski et al. 1997; Lee et al. 2002; Troelsen et al. 1994; Wang et al. 2006). The difference in promoter structure outside the proximal region in different species demonstrates the difficulty of finding a suitable model organism in which to replicate the LP phenotype.

Several transcription factors that are important for *LCT* expression have been identified (as reviewed by Troelsen 2005). These include Cdx-2 (Fang et al. 2000; Troelsen et al. 1997), which is involved in the regulation of many intestinally expressed genes (Beck 2004); HNF1 (Bosse et al. 2006; Krasinski et al. 2001; Spodsberg et al. 1999), which acts synergistically with Cdx-2 to activate the *LCT* promoter in vitro (Mitchelmore et al. 2000); and the GATA-4/5/6 transcription factors (Fang et al. 2001; Fitzgerald et al. 1998) that play a critical role in the development of endoderm-derived tissues such as the small intestine (reviewed in Burch 2005), and participate in the transcriptional regulation of a number of intestinally expressed genes (Boudreau et al. 2002; Gao et al. 1998; Krasinski et al. 2001; Oesterreicher and Henning 2004).

Whilst the recognition sites and corresponding transcription factors of the proximal *LCT* promoter are undoubtedly important in the regulation of lactase expression, they are not sufficient for the correct temporal and spatial expression of the gene, and hence, this region is not thought to be involved in causing LP. Indeed, sequencing of *LCT* and the immediate promoter region in Europeans showed no nucleotide changes that were absolutely associated with LP/LNP (Boll et al. 1991; Lloyd et al. 1992; Poulter et al. 2003).

Subsequent research therefore focused more intensely on the upstream regions of *LCT*, looking for regulatory elements that influence the LP phenotype. Interestingly, many enhancer motifs occur upstream of the proximal promoter, which differ across species, and are recognised by various transcription regulators (including Cdx-2, HNF-1α and GATA factors) (Lewinsky et al. 2005; Troelsen 2005). One such highly variable region was identified ~900 bp upstream of the *LCT* start site, and a nucleotide change, C-958T (Harvey et al. 1995), was found to greatly affect interaction with an unidentified DNA-binding protein (Chitkara et al. 2001; Hollox et al. 1999). However, this polymorphism was not associated with LP in Europeans and, if functional in vivo, may affect the timing of down-regulation or spatial expression along the length of the intestine or modulate the effect of other nucleotide changes.

In fact, several polymorphisms exist across the 50 kb *LCT* gene and association studies revealed that very few haplotypes (i.e., a particular combination of alleles at each SNP) occur in most of the human populations tested, although greater diversity was observed in African populations (Hollox et al. 2001). One combination of alleles, designated the ‘A’ haplotype, was shown to be particularly common in northern Europe and is found to associate with LP (Harvey et al. 1998).

6.5.1 *Identification of Causal Variants*

In 2002, a putative causative single-nucleotide polymorphism (*–13910C>T*) was identified that was completely associated with LP (ascertained from jejunal biopsy samples) in a cohort of Finnish individuals (Enattah et al. 2002). *–13910C>T* is located 13.9 kb upstream of the *LCT* initiation site in an intron of an adjacent gene, *MCM6*, and occurs on the A haplotype. Subsequent studies in populations of northern European ancestry confirmed that the association between *–13910*T* and LP was very strong (e.g., Poulter et al. 2003). The A haplotype extends far beyond the 50 kb *LCT* gene region, and carriers of *–13910*T* tend to have identical chromosomes extending for more than 200 kb, in many cases up to 1 Mb (Bersaglieri et al. 2004; Liebert et al. 2017; Poulter et al. 2003).

In vitro studies using promoter-reporter construct assays in a colon carcinoma cell line ($CaCo_2$) demonstrated that a 450 base-pair region (–14133 to –13684) surrounding *–13910C>T* has regulatory function and enhances the effect of the immediate promotor. The *–13910*T* allele increases transcription (Olds and Sibley 2003; Troelsen et al. 2003) and binds the transcription factor Oct-1 more strongly than the ancestral allele, providing a possible mechanism for escape from down-regulation of *LCT* (Lewinsky et al. 2005).

These observations were regarded by many as compelling evidence that the sole cause of LP had been identified, and some groups recommended using the absence of the *–13910*T* allele as a diagnostic test for LNP (Rasinpera 2004).

However, researchers from our group noted that *–13910*T* was extremely rare in sub-Saharan African populations, even in those populations where LP frequency had previously been reported to be high. Using a statistical procedure involving comparison of incidence in phenotyped and genotyped groups of similar origin, the study concluded that *–13910*T* could not be causal of LP throughout sub-Saharan Africa (Mulcare et al. 2004). This inference was confirmed when several other causal variations were found in Africa and the Near East.

In Sudan and Saudi Arabia, it was shown that both *–13910*T* and the A haplotype were too rare to account for LP ascertained by the breath hydrogen test, but a new variant (*–13915*G*) did associate with LP (Imtiaz et al. 2007; Ingram et al. 2007). In Tanzania and Kenya, a different novel allele (*–14010*C*) was found to associate with LP (Tishkoff et al. 2007). Both studies also identified other novel variants clustered within 100 bp of *–13910*T*, and some of these (*–13907*G*, *–14009*G*) also show a statistically significant association with LP in other non-European populations (Ingram et al. 2009b; Jones et al. 2013). These findings show that LP has evolved more than once in human history. Distributions of the LP-associated variants in the Old World are shown in Fig. 6.3.

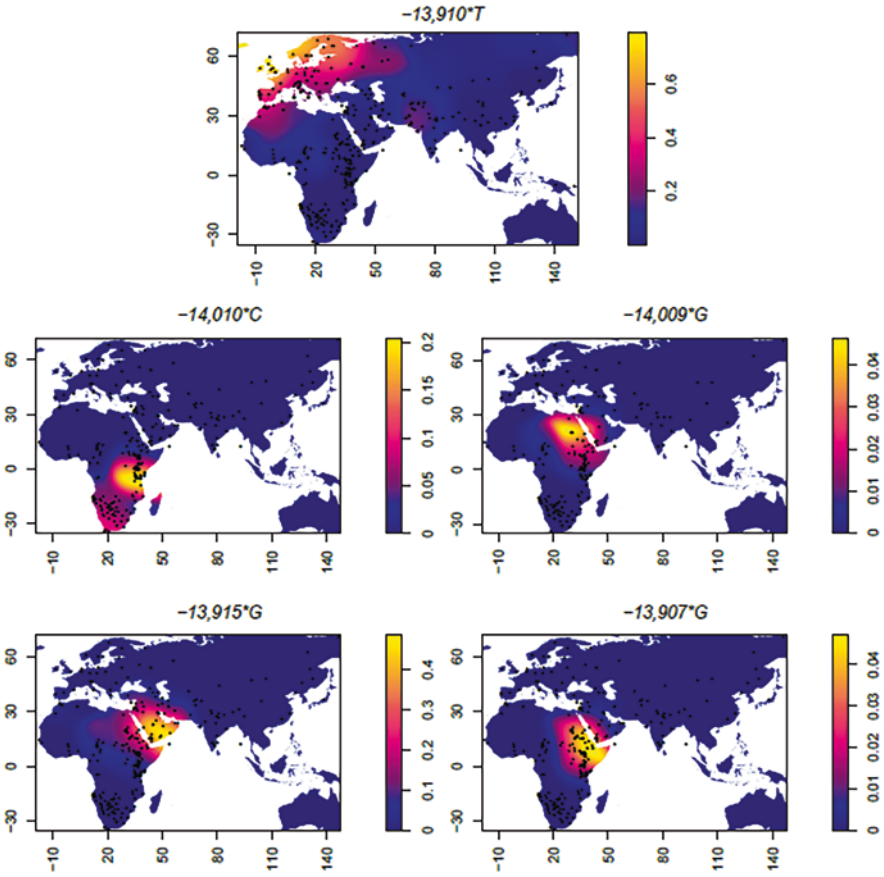

Fig. 6.3 Interpolated geographic distributions of LP causative variants *–13910*T*, *–14010*C*, *–14009*G*, *–13915*G*, *–13907*G*, in the old world. The scale shows allele frequencies and values on axes are degrees of latitude and longitude. (Data updated from https://www.ucl.ac.uk/biosciences/departments/genetics-evolution-and-environment/research/molecular-and-cultural-evolution-lab/glad)

6.6 Mechanism of Down-Regulation of *LCT*

Transcription factor recognition sequences for Cdx-2, GATA, HNF3α/Fox, HNF4α and Oct-1 have been identified in the *MCM6* intron 13 enhancer sequence (Fig. 6.4) (Jensen et al. 2011; Lewinsky et al. 2005), and several of the intron 13 variant alleles alter binding of one or more of these (and other) proteins, but notably not the same combinations of proteins (Ingram et al. 2007; Liebert et al. 2016; Olds et al. 2011).

Reporter gene assays (used for studying the regulation of gene expression) show that transcription from the *LCT* core promoter is enhanced twofold by addition of the ancestral *MCM6* intron 13 sequence, and this activity increases further when

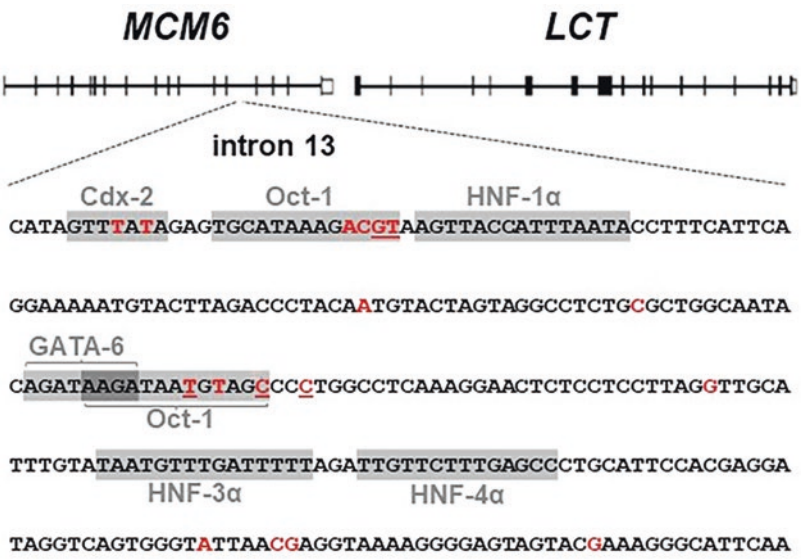

Fig. 6.4 Single-nucleotide variants (shown in red) located in *MCM6* intron 13, within the ≈−14 kb *LCT* enhancer sequence (sequence shown from –14035 to –13766 upstream of the *LCT* initiation site; chr2:135850932-1358512010 (reverse complement) human genome assembly GRCh38). SNPs for which the evidence of a functional role in LP is uncontroversial are underlined. Transcription factor binding sites identified by Lewinsky et al. (2005) and Jensen et al. (2011) are shaded. Darker shading indicates overlapping binding sites

particular variant alleles are present (Jensen et al. 2011; Liebert et al. 2016; Olds et al. 2011; Tishkoff et al. 2007). Table 6.1 details the effects of the ≈14 kb candidate LP SNPs on protein binding and gene expression. Some enhancer alleles have been reported to alter protein binding or increase transcription in vitro but are too rare to allow association studies, or such studies have not been done. Of these, *–14028T>C*, *–13779G>C*, could well be functional (Liebert et al. 2016), whilst others are more frequent in maldigesters (e.g. *–13913T>G*) so have not been pursued further.

Evidence suggesting epigenetic regulation of *LCT* was first reported in the 1990s, when immuno-histological staining showed that some individuals show patchy expression of *LCT* in the intestinal epithelia, which was interpreted to result from somatic cell changes such as methylation or histone acetylation (Maiuri et al. 1991). More recently, Labrie et al. (2016) demonstrated that there was significant variability in DNA methylation that correlated with *LCT* mRNA levels (Fig. 6.5). These differential methylation regions co-aligned with other epigenetic signals, namely DNAse hypersensitivity and histone modification sites, identified in the NIH Roadmap epigenomics project (http://www.roadmapepigenomics.org/). The DNA modifications were shown to be cell-type specific, with lower DNA methylation in *LCT*-expressing enterocytes than in enterocyte-deficient jejunum or white blood cells. Importantly, the authors also found a significant difference in DNA

Table 6.1 Functional analysis of nucleotide variants located in *MCM6* intron 13, within the ≈−14 kb *LCT* enhancer sequence. Those for which the evidence of a functional role is uncontroversial are shown in bold

MCM6 INTRON 13 SNP	rs number	Evidence of association with lactose digestion	Functional evidence	
			EMSA	Reporter gene
–14028T>C	rs759157971	Only candidate variant allele in –13910 CT heterozygote with homozygous high lactase mRNA expression (Poulter et al. 2003).	Decreased Cdx-2 binding. Increased HNF-4α binding (Liebert et al. 2016).	No difference in expression compared to ancestral allele (Liebert et al. 2016).
–14011C>T	rs4988233	Rare allele observed in Estonia (Lember et al. 2006), India (Gallego Romero et al. 2012), Ethiopia (Jones et al. 2013) and several other countries (Liebert et al. 2017).	Increased binding of Oct-1 and HNF-1α (compared to ancestral sequence). Possible changes in GATA binding (Liebert et al. 2016).	1.5× increased expression in undifferentiated cells, 1.8× increased expression in differentiated cells (Liebert et al. 2016).
–14010G>C	rs145946881	Statistically significant association with LP in Kenya, Tanzania (Tishkoff et al. 2007).	Increased binding of Oct-1 and HNF-1α compared to ancestral sequence (Jensen et al. 2011; Liebert et al. 2016).	Altered transcription in vitro (Jensen et al. 2011). 1.5× increased expression in undifferentiated cells, 2.5× increased expression in differentiated cells (Liebert et al. 2016).
–14009T>G	rs869051967	Statistically significant association with LP in Ethiopia (Ingram et al. 2009b; Jones et al. 2013).	Changes in binding to Ets transcription factor, possibly c-Ets-1 (Liebert et al. 2016).	1.4× increased expression in undifferentiated cells, 1.9× increased expression in differentiated cells (Liebert et al. 2016).

(continued)

Table 6.1 (continued)

MCM6 INTRON 13 SNP	rs number	Evidence of association with lactose digestion	Functional evidence	
			EMSA	Reporter gene
–13915T>G	rs41380347	Statistically significant association with LP in Sudan (Ingram et al. 2007), Kenya (Tishkoff et al. 2007), Ethiopia (Jones et al. 2013), and the Middle East (Imtiaz et al. 2007).	Alters Oct-1 binding (Olds et al. 2011), not found by (Enattah et al. 2008; Ingram et al. 2007).	Slightly increased expression (1.18–1.3×) in undifferentiated cells (Tishkoff et al. 2007). 2× increased expression of compound *–3712G/–13915*G* construct compared to *–3712T/–13915*T* in differentiated cells (Enattah et al. 2008).
–13913T>G	rs41456145	More frequent in maldigesters (Jones et al. 2013).	Weak binding to Oct-1, equivalent to *–13910*C* binding (Enattah et al. 2008) contradicted in (Ingram et al. 2007).	No data
–13910C>T	rs4988235	Statistically significant association with LP in Europe (Enattah et al. 2002; Poulter et al. 2003), N. Africa (Myles et al. 2005).	Increased binding to Oct-1 (Ingram et al. 2007; Lewinsky et al. 2005).	2× increase in expression (Lewinsky et al. 2005; Liebert et al. 2016; Olds and Sibley 2003).
–13907C>G	rs41525747	Statistically significant association with LP in Ethiopia (Ingram et al. 2009b; Jones et al. 2013) and Sudan (Tishkoff et al. 2007).	Increased Oct-1 binding (Enattah et al. 2008; Ingram et al. 2007).	Slightly increased expression (1.18–1.3×) in undifferentiated cells (Tishkoff et al. 2007).
–13779G>C	rs527991977	Somali maldigester (Ingram et al. 2009b). Occurrence in south Indian pastoralist groups (Gallego Romero et al. 2012).	Minimal changes compared to ancestral (Liebert et al. 2016).	Increased expression compared to ancestral (Liebert et al. 2016).

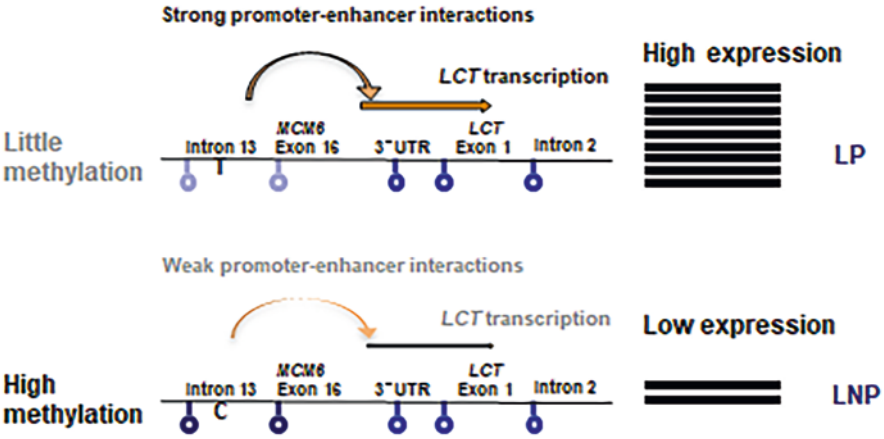

Fig. 6.5 Diagrammatic interpretation of the different methylation (indicated as circles) in the *LCT* enhancer correlated with the presence of T or C at –13910, and similar methylation in other regions. Labrie et al. (2016) showed that the SNP somehow drives epigenetic changes. Lower methylation leads to higher mRNA expression

modification (particularly at intron 13) between individuals homozygous for *–13910*C* or *–13910*T* (with heterozygotes being intermediate) and that these modifications increased with age in *–13910CC*, but not *–13910TT* individuals. Although they did not have access to samples from children, the study of Labrie et al. (2016) indicated that *LCT* down-regulation is directed by an age-dependent increase in epigenetic changes and that the European *–13910*T* allele in some way disrupts this process (Swallow and Troelsen 2016). It seems likely that the other LP variants have a similar effect.

Lactase activity may be additionally influenced by post-translational processes, such as glycosylation and/or transportation. Heterogeneity of the LNP phenotype has been reported by some research groups who have observed individuals with slower or abnormal processing (Sterchi et al. 1990; Witte et al. 1990), and other groups have reported a heterogeneous pattern of *LCT* mRNA level, LPH synthesis, and lactase activity in both LP and LNP subjects (Rossi et al. 1997).

6.7 Adaptations to Milk Consumption

6.7.1 Genetic Adaptation

The original observation of a positive correlation between LP frequencies and milk drinking led to the widely held notion that LP has been subject to positive selection. In the intervening years, molecular evidence in support of this has accumulated. The *–13910*T* allele occurs on an extremely extended haplotype background,

which is present in the northern European population at very high frequency (Bersaglieri et al. 2004; Hollox et al. 2001; Poulter et al. 2003) and gives one of the strongest signatures of selection in the human genome (Sabeti 2006). This is consistent with a model of recent positive selection in which alleles surrounding the selected locus 'hitch-hike' rapidly to high frequency and haplotype length is exaggerated, indicating a recent event where recombination has not decayed the allelic associations in the region. Diversity of so-called microsatellite polymorphisms (variable simple repetitive sequences that occur throughout the genome) can also be analysed and interpreted in a similar way—reduced microsatellite diversity (as seen on the *–13910*T* carrying chromosomes) indicates that this particular haplotype has risen in frequency quickly and recently (Coelho et al. 2005). These observations are consistent with selection for LP along with the recent practise of dairying, approximately 9000 years ago in the Near East (Evershed et al. 2008), and date estimates of *–13910*T* indicate it was selected for during the past 5000–10,000 years (Bersaglieri et al. 2004; Coelho et al. 2005; Itan et al. 2009).

Ancient DNA data confirm that *–13910*T* was rare in samples of >5000 years bp (Burger et al. 2007), and only reached appreciable frequencies in the last 4000 years (Mathieson et al. 2015), supporting the model that the cultural trait of dairying was adopted prior to LP becoming frequent.

The existence of other, non-European LP alleles shows that LP has been selected for independently in different geographic locations, which indicates that the ability to digest milk has been advantageous in certain circumstances. Some of these non-European LP alleles (*–14010*C*) also carry signatures of recent positive selection and occur on extended haplotypes (Liebert et al. 2017; Ranciaro et al. 2014; Tishkoff et al. 2007).

In Ethiopian populations where multiple persistence alleles occur, this shows as a different signature of selection, known as a soft selective sweep (Hermisson and Pennings 2005) where there is a greater nucleotide diversity in the enhancer of the milk drinkers than non-milk drinkers (Ingram et al. 2009b; Jones et al. 2013).

6.7.2 Cultural Adaptation

The correlation between pastoralism, milk drinking and LP is not observed in all populations, for example, the Dinka and Nuer in Sudan (Bayoumi et al. 1982) and the Somali in Ethiopia (Ingram et al. 2009b) have a low LP frequency despite cows or camels playing a very important role in their lifestyle. These populations are not completely dependent on milk despite its consumption being substantial, and the selective pressure for LP may have been less strong, but perhaps more importantly there has been cultural adaptation. Milk is processed to sour milk, yoghurts and cheese, which have reduced lactose content, and individuals also adapt their consumption habits by taking smaller quantities of milk at a time. These cultural adaptations enable non-persistent individuals to benefit from the calorific, mineral and vitamin constituents of milk without inducing the associated symptoms of lactose

malabsorption and are complemented by adaptations of the large intestinal bacterial flora, as described in the following section.

6.7.3 Adaptation of the Gut Microbiome

The gut microbiome consists of the entire collection of microorganisms present in the human gut. The microbiome is assembled at birth, develops with its host, and is influenced by environmental factors such as diet and other exposures (Goodrich et al. 2017). Association studies between human genetic variation and the gut microbiome have revealed an association between the *13910CC* genotype and the abundance of *Bifidobacterium* spp. (Blekhman et al. 2015; Goodrich et al. 2016; Wang et al. 2016), and this association has been shown to be dependent on milk consumption (Bonder et al. 2016). In LNP adults, the bacterial flora adapts to regular milk consumption by increasing the abundance of bacteria capable of metabolising lactose.

6.8 Selection for Lactase Persistence

Taking into account the various possible adaptive mechanisms, what were the selective forces that resulted in the high frequency of LP observed in certain populations? Milk is a good source of calories, protein and fat, but the selective advantage of LP may not simply be an increased food supply, as cultural practises (such as fermentation, or consuming milk in small quantities) enable LNP individuals to obtain many of the same benefits (Gerbault et al. 2011; Ségurel and Bon 2017). However, there may be some nutritional advantage, as LP is associated with increased height or BMI (Almon et al. 2012; Charati et al. 2020; Corella et al. 2011; Gugatschka et al. 2005; Hartwig et al. 2016; Kettunen et al. 2010; Lamri et al. 2013; Malek et al. 2013) and so may improve reproductive success and survival (Montalva et al. 2019).

Several theories were developed early on to explain the strong selection for LP.

6.8.1 Culture-Historical Hypothesis

Due to the observation that LP often occurs in historically milk-drinking populations, Simoons (1970) and McCracken (1971) independently suggested that dependence on fresh milk selected for LP.

However, LP frequency and fresh milk-drinking are not perfectly correlated. Some populations have either a high LP frequency without being milk dependent or rely heavily on milk products but have a low reported frequency of LP. As can be seen in Sect. 6.7, a degree of asymmetry may be explained by adaptations of the gut

flora (Goodrich et al. 2017) or by cultural practices such as milk processing. In addition, demographic processes such as population expansion (Itan et al. 2009) undoubtedly played a role.

Statistical analyses and computer simulations have been used to characterise the processes contributing to the current distribution of LP (reviewed in Gerbault et al. 2011). Aoki (1986) showed that an incomplete correlation between LP and dairying does not necessarily provide evidence against the culture-historical hypothesis and is to be expected if some LP populations have recently stopped milking, or LNP populations have only recently adopted the custom. Later, Holden and Mace (1997) performed an analysis that corrected for shared ancestry between pastoralist groups and showed that LP most likely evolved as an adaptation to dairying, and has never been observed at high frequency in peoples that have not previously adopted the practice. This is supported by archaeological evidence such as dairy residues in potsherds (Evershed et al. 2008), and the age and sex distribution of animals in archaeological skeletal assemblages (Helmer et al. 2007; Vigne 2008), along with ancient DNA studies (Burger et al. 2007; Mathieson et al. 2015, 2018) which show that dairying predates the first observation of the *–13910*T* variant. Detection of milk proteins in samples of dental calculus provides direct evidence of milk consumption and has recently been shown in ancient east African individuals who did not carry a genetic adaptation for LP (Bleasdale et al. 2021).

Environmental conditions were likely important in determining which populations adopted the custom of milk drinking, and therefore contributed to the geographical distribution of LP. Bloom and Sherman (2005) found that selection for LP occurs only in environments conducive to dairying or in a few nomadic African groups that probably maintained herds by escaping unfavourable environmental conditions.

Another source of evidence in support of the culture-historical hypothesis is the genetic analysis of milk genes in cattle, which shows a correlation between high intra-allelic diversity and the geographic incidence of LP in Europe, which is consistent with the maintenance of large herd sizes for dairying and selection for high milk yields (Beja-Pereira et al. 2003).

Currently, the term ‘culture-historical hypothesis’ has been reframed in the context of gene-culture coevolution, i.e., where culturally transmitted behaviours affect evolutionary outcomes (see Gerbault et al. 2011).

6.8.2 Arid Climate Hypothesis

First suggested by Cook and al-Torki (1975), the arid climate hypothesis proposes that milk provides an important food source, and in particular, a source of clean, uncontaminated water in desert climates (i.e., Middle and Near East). The advantage to persistent individuals may be even more pronounced during outbreaks of diarrhoeal disease, when non-persistent individuals are unable to utilise milk as a water source without exacerbating their condition. This hypothesis may be

particularly pertinent to nomadic camel herders as these animals continue to lactate for several days in the absence of water (Bekele et al. 2011).

6.8.3 Calcium Absorption Hypothesis

In northern Europe where the arid climate hypothesis does not apply, the calcium absorption hypothesis (Flatz and Rotthauwe 1973) may explain the distribution of LP. The low levels of sunlight experienced in northern Europe are associated with an increased risk of developing rickets and osteomalacia due to a lack of vitamin D (which is synthesised by the skin in the presence of sunlight; see review by Holick 2007). Calcium may help to prevent rickets by impairing the breakdown of vitamin D in the liver (Thacher et al. 1999) and is itself an important mineral required for bone health. Lactase non-persistent individuals could obtain calcium from yoghurt or cheese, dairy foods that contain reduced lactose. In addition, milk proteins and lactose are believed to facilitate the absorption of calcium (for review see Guéguen and Pointillart 2000), and hence, the ability to drink fresh milk which contains both calcium and components that stimulate its uptake (along with small amounts of vitamin D) may have provided an advantage to LP individuals. In support of the calcium-absorption hypothesis, a simulation study that modelled geographical structuring of the selection pressure according to latitude (Gerbault et al. 2009) found that positive selection was required for LP frequencies to reach their observed values in northern, but not southern Europe. However, an independent simulation study (Itan et al. 2009) found that a latitudinal effect was not required to explain the frequency distribution of LP across Europe and an ancient DNA study found that the calcium absorption hypothesis was insufficient to explain the spread of LP in Europe (Sverrisdóttir et al. 2014).

6.8.4 Other Hypotheses

One study has suggested that selection for LNP might explain the frequency distribution of LP. Because individuals with flavin deficiency may have a slightly reduced risk of malarial infection (Dutta 1991), Anderson and Vullo (1994) suggested that LNP would be beneficial in malarial regions as it would discourage the consumption of milk, which is rich in riboflavins. However, this hypothesis is not supported by other studies (Meloni et al. 1998, 1996) and the opposite hypothesis, that malarial mortality is reduced by a milk-rich diet, has also been proposed (Cordain et al. 2012).

Another intriguing hypothesis is that the benefit of fresh milk operates through increasing circulating insulin-like growth hormone (IGF-1), an effect which is lost from milk on processing (Wiley 2018). IGF-1 promotes growth and might explain the larger body size of LP individuals (Almon et al. 2012; Charati et al. 2020;

Corella et al. 2011; Hartwig et al. 2016; Kettunen et al. 2010; Lamri et al. 2013; Malek et al. 2013; Montalva et al. 2019).

Whatever the cause of selection for LP, it is hard to see how it could have been so strong, given that any of the selective agents would have varied geographically and temporarily, and required 'exposure' to fresh milk. The demographic effects of population expansion and migration contributed to the spread of LP (Itan et al. 2009), but it has also been speculated that a chromosomal rearrangement might possibly have affected the 'survival frequency' of some of the alleles (Liebert et al. 2017).

6.9 Health and Medical Considerations

Lactose intolerance (the presence of gastrointestinal symptoms due to lactose malabsorption) is difficult to distinguish from different causes of similar symptoms, in particular Irritable Bowel Syndrome (Misselwitz et al. 2019; Szilagyi and Ishayek 2018). Lactose intolerance may be confused with milk protein allergy by the lay public, though the causes and symptoms are different (as reviewed by Crittenden and Bennett 2005; Walsh et al. 2016). Many individuals avoid dairy foods to prevent symptoms they believe result from lactose maldigestion (Carroccio et al. 1998), and there seems to be a preoccupation with symptoms allegedly related to the consumption of lactose (Szilagyi and Ishayek 2018). However, a meta-analysis comparing low doses of lactose with placebo found that differences in the severity of gastrointestinal symptoms reported by lactose maldigesters were minimal to zero between the two groups (Savaiano et al. 2006), and self-reported lactose intolerance is an unreliable diagnostic (Jellema et al. 2010; Peuhkuri et al. 2000; Saltzman et al. 1999; Zheng et al. 2015). Lactase non-persistence has also been implicated in causing a variety of systemic conditions without clear evidence (Matthews 2005; Wilder-Smith et al. 2018). Despite these issues, the consumption of milk and milk products by those who cannot digest lactose is a relatively common cause of gastrointestinal complaint in Europe and the USA (Fassio et al. 2018; Vesa et al. 2000) and is underdiagnosed (Fassio et al. 2018). Therefore, testing for lactose maldigestion has a clinical role in either detecting or eliminating lactose malabsorption as the cause of gastrointestinal symptoms.

Several methods have been developed for molecular diagnosis of LP (examining the DNA for LP alleles) (Brasen et al. 2017; Janukonyté et al. 2010; Nilsson and Olsson 2008; Strand et al. 2014; Tag et al. 2008), although at present, DNA sequencing is the only method that can type all the LP-associated alleles simultaneously. As LP is nearly uniformly mediated by the *LCT –13910C>T* polymorphism in Europeans, genetic testing for this one variant can usually detect primary LNP in this population (Pohl et al. 2010) and is useful for epidemiological studies (Misselwitz et al. 2019). This single genetic test also permits a differential diagnosis between primary and secondary LNP to be made in lactose maldigesters (Fassio et al. 2018), without the need for invasive biopsies.

Genetic testing for LNP for clinical purposes is not on the other hand currently advocated for people with African or Asian ancestry (Misselwitz et al. 2019) due to the increased genetic complexity (Jones et al. 2015; Liebert et al. 2017) and the probable existence of unidentified causal variants (Ingram et al. 2009b; Jones et al. 2013).

Several studies have attempted to demonstrate the health benefits of milk consumption, and several others claim that high milk consumption has an adverse effect on health. These inconsistencies are common in nutritional epidemiology, reflecting the complexity of studying diet and its relationship with health in the presence of many other variables (Satija et al. 2015). Systematic reviews and meta analyses now indicate that dairy intake is associated with improved bone health and protection from osteoporosis (van den Heuvel and Steijns 2018; Matía-Martín et al. 2019) and may slightly reduce, or have no effect on the risk of type 2 diabetes (Bergholdt et al. 2015; Gijsbers et al. 2016) and cardiovascular disease (Drouin-Chartier et al. 2016; Fontecha et al. 2019). Likewise, meta-analyses suggest that dairy intake has a protective effect against some cancers (colorectal, bladder, gastric, breast) and does not seem to be associated with the risk of pancreatic, lung or ovarian cancer, whilst the evidence for prostate cancer risk is inconsistent (as reviewed by Szilagyi and Ishayek 2018 and Thorning et al. 2016). Whether or not milk intake confers specific health risks or benefits, the effects may be different in LP and LNP persons (Enattah et al. 2004, 2005; Meloni et al. 2001; Obermayer-Pietsch et al. 2003; Wu et al. 2017). Recent meta-analyses show that the LP genotype is not associated with osteoporosis (Bergholdt et al. 2018a), nor all-cause mortality (Bergholdt et al. 2018b) in people with high milk intake. Overall, current evidence indicates that whilst adjusting for individual variations in lactose tolerance, milk and dairy products can be appropriately included as part of a balanced diet for most people (Marangoni et al. 2019; FAO 2013).

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Chapter 7
Milk Oligosaccharides

Hannah K. Masterson, Tadasu Urashima, Rebecca A. Owens, and Rita M. Hickey

7.1 Abbreviations of Carbohydrate Structures

Oligosaccharide	Abbreviation	Structure
Lactose	Lac	Galβ1-4Glc
Galactose	Gal	Gal
Glucose	Glc	Glc
Fucose	Fuc	Fuc
N-Acetylgalactosamine	GalNAc	GalNAc
N-Acetylglucosamine	GlcNAc	GlcNAc
N-Acetylneuraminic acid	Neu5Ac	Neu5Ac
N-Glycolylneuraminic acid	Neu5Gc	Neu5Gc
Lacto-*N*-biose	LNB	Galβ1-3GlcNAc
Hexose	Hex	Hex
Hexosamine	HexNAc	HexNAc
Lacto-*N*-novopentaose 1	*novo*-LNP 1	Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
Isoglobotriose	α3′-GL	Galα1-3Galβ1-4Glc
3′-Sialyllactosamine	3′-SLN	Neu5Acα2-3Galβ1-4GlcNAc
Lacto-*N*-hexaose	LNH	Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6) Galβ1-4Glc

H. K. Masterson · R. M. Hickey (✉)
Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland
e-mail: HannahKate.Masterson@teagasc.ie; rita.hickey@teagasc.ie

T. Urashima
Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan
e-mail: urashima@obihiro.ac.jp

R. A. Owens
Department of Biology, Maynooth University, Maynooth, County Kildare, Ireland
e-mail: Rebecca.Owens@mu.ie

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Oligosaccharide	Abbreviation	Structure
β6′-Galactosyllactose	6′-GL	Galβ1-6Galβ1-4Glc
β3′-Galactosyllactose	β3′-GL	Galβ1-3Galβ1-4Glc
N-Acetyllactosamine	LacNAc	Galβ1-4GlcNAc
N-Acetylgalactosaminyllactose	GNL	GalNAcα1-3Galβ1-4Glc
6′-Sialyllactosamine	6′-SLN	Neu5Acα2-6Galβ1-4GlcNAc
Lacto-*N*-fucopentaose I	LNFPI	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-fucopentaose II	LNFPII	Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-fucopentaose III	LNFPIII	Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc
Disialyllacto-*N*-tetraose	DSLNT	Neu5Acα2-3Galβ1-3(Neu5Acα2-6) GlcNAcβ1-3Galβ1-4Glc
Disialyllacto-*N*-hexaose I	DSLNH I	Neu5Acα2-3Galβ1-3GlcNAcβ1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
Disialyllacto-*N*-hexaose II	DSLNH II	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
Fucodisialyllacto-*N*-hexaose I	FDSLNH I	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3(Fucα1-2Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
Fucodisialyllacto-*N*-hexaose II	FDSLNH II	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3[Galβ1-4(Fucα1-3)GlcNAcβ1-6]Galβ1-4Glc
Fucodisialyllacto-*N*-hexaose III	FDSLNH III	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3(Neu5Acα2-6Galβ1-4GlcNAcβ1-6) Galβ1-4Glc
Disialyllacto-*N*-tetraose	DSLNT	Neu5Acα2-3Galβ1-3(Neu5Acα2-6) GlcNAcβ1-3Galβ1-4Glc
Sialyl-3′-galactosyllactose	S3′-GL	Neu5Acα2-3Galβ1-3Galβ1-4Glc
Disialyllactose	DSL	Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc
Lacto-*N*-tetraose	LNT	Galβ1-3GlcNAcβ1-4Galβ1-4Glc
Lacto-*N*-neotetraose	LN*n*T	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-hexaose	LNH	Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6) Galβ1-4Glc
Lacto-*N*-neohexaose	LN*n*H	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6) Galβ1-4Glc
2′-Fucosyllactose	2′-FL	Fucα1-2Galβ1-4Glc
3-Fucosyllactose	3-FL	Galβ1-4(Fucα1-3)Glc
3′-Sialyllactose	3′-SL	Neu5Acα2-3Galβ1-4Glc
6′-Sialyllactose	6′-SL	Neu5Acα2-6Galβ1-4Glc
Sialyllacto-*N*-tetraose a	LSTa	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
Sialyllacto-*N*-tetraose b	LSTb	Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc
Sialyllacto-*N*-tetraose c	LSTc	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc
Lacto-*N*-difucohexaose I	LNDFH-I	Fucα1-2Galβ1-3(Fucα1-4) GlcNAcβ1-3Galβ1-4Glc
6′-*N*-Acetyl-glucosaminyl-lactose	NAL	GlcNAcβ1-6Galβ1-4Glc

7.2 Introduction

Milk contains from trace to ~13% carbohydrate, of which lactose (Galβ1-4Glc) usually constitutes more than 80%. The milk of most mammals also contains a variety of oligosaccharides, many of which have *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, glucose, fucose, and/or sialic acid (*N*-acetylneuraminic acid and *N*-glycolylneuraminic acid) residues attached to lactose, which is usually located at the reducing end (Urashima et al. 2013). Certain oligosaccharides in the milks of domestic animals including bovine milk contain Galβ1-4GlcNAc (*N*-acetyllactosamine) at the reducing ends. The ratio of milk oligosaccharides to free lactose in milk varies, depending on the mammalian species. For example, in mature human milk, milk oligosaccharides constitute 20% of the total carbohydrate content, whereas mature bovine and goat milk contain much lower amounts of oligosaccharides. The diversity of human milk oligosaccharides (HMOs) is large and major innovations in the field of glycomics have enabled the identification of over 200 HMO structures (Bode 2019; Urashima et al. 2018).

The biological functions of HMO have been extensively researched in recent years. According to many in vitro, in vivo, and clinical studies, the intake of HMO is associated with many benefits on the infant gastrointestinal and immune physiological systems. Other systems, such as the respiratory, central nervous, circulatory, locomotor, and urinary systems have also been found to be affected by HMO consumption. However, these protective effects ascribed to HMOs for the most part have been unavailable to formula-fed infants, with the exception of 2′-fucosyllactose (Fucα1-2Galβ1-4Glc, 2′-FL) and lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc, LN*n*T), which have been added to some formulas recently (Puccio et al. 2017; European Union 2017). Despite this advancement, the complexity of HMOs makes it almost impossible for their associated functions to be duplicated in formulas. Infant milk formulas are based on bovine and goat milk, which as mentioned contain lower concentrations of oligosaccharides (~0.03 g L^{-1} and ~0.3 g L^{-1}, respectively) (Kunz et al. 2000; Martinez-Ferez et al. 2006; Meyrand et al. 2013). However, a number of bovine (BMOs) and goat milk oligosaccharides (GMOs) share the same structure as certain HMOs, which could imply common functionalities (Barile et al. 2009; Mariño et al. 2011; Robinson 2019). Moreover, Meli et al. (2014) have shown that BMO-supplemented infant formulas were well tolerated and supported normal growth of healthy term infants. Therefore, value may lie in extracting and concentrating oligosaccharides from domestic animal milks with a view to adding them as an active ingredient to infant formulas.

In this chapter, we describe the biological significance of milk oligosaccharides, their gastrointestinal digestion and biosynthesis, their chemical structures, and methods for their structural analysis. In particular, we pay special attention to their prebiotic properties, their ability to prevent pathogen attachment to mucosal surfaces, thereby reducing infections, their involvement in improving gut barrier function, promoting immune development and tolerance, and modulating intestinal cell responses in addition, to providing the infant with a source of sialic acid, an essential nutrient in brain development and cognition. Current investigations into the production of milk oligosaccharides as functional ingredients will also be discussed.

7.3 The Chemical Structures of Milk Oligosaccharides

Oligosaccharides in milk are assembled in the mammary gland by combining the monosaccharides glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose (Fuc), and the sialic acids *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). The oligosaccharide structures contain either lactose (Galβ1-4Glc) or *N*-acetyllactosamine (Galβ1-4GlcNAc, LacNAc) at their reducing end, with additional monosaccharide residues branching off from the non-reducing galactose. BMOs and GMOs can possess lacto-*N*-biose (Galβ1-3GlcNAc, LNB) or LacNAc units linked to the lactose core, which are defining features of the type 1 and type 2 oligosaccharide structures contained within many HMOs (Robinson 2019; Urashima et al. 2001). However, the type 1 oligosaccharides, which contain LNB,

Table 7.1 Quantities of neutral oligosaccharides found in human milk and dairy milks

Oligosaccharide (abbreviation)	Human (g/L)	Bovine (g/L)	Goat (g/L)
2′-Fucosyllactose (2′-FL)	1.88–4.9	Trace	Trace
3-Fucosyllactose (3-FL)	0.25–0.86	Trace	Trace
Lacto-*N*-tetraose (LNT)	0.5–1.5	Trace	Trace
Lacto-*N*-neotetraose (LN*n*T)	0.04–0.2	Trace	–
Lacto-*N*-fucopentaose I (LNFPI)	1.2–1.7	–	–
Lacto-*N*-fucopentaose II (LNFPII)	0.3–1.0	–	–
Lacto-*N*-fucopentaose III (LNFPIII)	0.01–0.2	–	Trace
α-3′-Galactosyllactose (α3′-GL)	–	Trace	0.03–0.05
β-3′-Galactosyllactose (β3′-GL)	Trace	Trace	0.03
β-4′-Galactosyllactose (4′-GL)	Trace	–	–
β-6′-Galactosyllactose (6′-GL)	0.002	Trace	Trace
α-3′-*N*-Acetylgalactosaminyllactose (α-3′-GalNAcL)	–	0.003–0.065	Trace
Lacto-*N*-difucohexaose I (LNDFH-I)	0.58	–	–
Lacto-*N*-neohexaose (LN*n*H)	Trace	–	–
Lacto-*N*-hexaose (LNH)	0.13	–	0.001–0.005
6′-*N*-Acetyl-glucosaminyl-lactose (NAL)	–	Trace	0.02–0.04

2′-FL; Fucα1-2Galβ1-4Glc, 3-FL; Galβ1-4(Fucα1-3)Glc, LNT; Galβ1-3GlcNAcβ1-4Galβ1-4Glc, LN*n*T; Galβ1-4GlcNAcβ1-3Galβ1-4Glc, LNFP I; Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNFP II; Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc, LNFP III; Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc, α3′-GL (isoglobotriose); Galα1-3Galβ1-4Glc, β3′-GL; Galβ1-3Galβ1-4Glc, 4′-GL; Galβ1-4Galβ1-4Glc, 6′-GL; Galβ1-6Galβ1-4Glc, α3′-GalNAcL; GalNAcα1-3Galβ1-4Glc, LNDFH-I; Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc, LN*n*H; Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-3)Galβ1-4Glc, LNH; Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc, NAL (*iso*-lacto-*N*-triose, *iso*-LNTri); GlcNAcβ1-6Galβ1-4Glc. The ranges shown reflect differences due to variations in the analytical methods used in the different studies and reflect changes in abundance over lactation, i.e., from colostrum to mature milk. Compiled data from: Kunz et al. (2000), Gopal and Gill (2000), Wang et al. (2001), Nakamura et al. (2003), Nakamura and Urashima (2004), Sumiyoshi et al. (2004), Fong et al. (2011), Oliveira et al. (2012), Meyrand et al. (2013), Aldredge et al. (2013), Albrecht et al. (2014), Oliveira et al. (2015), Austin et al. (2016), Sprenger et al. (2017), Thurl et al. (2017), Ma et al. (2018), Tonon et al. (2019), Samuel et al. (2019), Sousa et al. (2019), van Leeuwen et al. (2020)

predominate over the type 2 oligosaccharides, which contain LacNAc, in human milk, while the type 1 oligosaccharides are rare in the milk of domesticated dairy animals (Urashima et al. 2012, 2017, 2013). Another significant difference between human and dairy-derived milk oligosaccharide pools is that human milk contains high levels of fucosylated oligosaccharides, accounting for approximately 70% of oligosaccharides in human milk, with high levels of 2′-fucosylactose (2′-FL) detected (e.g., 2.01–4.65 g L^{-1} in secretor donor milk) (Asakuma et al. 2008; Chaturvedi et al. 2001; Marriage et al. 2015). Dairy-derived milk also contains higher levels of sialylated oligosaccharides containing Neu5Ac or Neu5Gc unlike human milk which is dominated by neutral oligosaccharides (Urashima et al. 2001, 2013). The acidic oligosaccharides from the milk of cows contains mainly Neu5Ac (97%), while Neu5Gc contributes 64% and 94% of the total sialic acid content in those from the milk of goats and sheep, respectively (Albrecht et al. 2014). Neu5Gc-containing saccharides had not been observed in HMO, but recently, Quin et al. (2020) detected these types of HMO in low abundance. Since humans are not able to synthesize this sialic acid, the authors hypothesized that the Neu5Gc originated from the diet of the lactating women. Also, unlike HMO, BMOs and GMOs are known to contain α-linked Gal or GalNAc structures, sialyl derivatives of β3′-GL (Galβ1-3Galβ1-4Glc) or 6′-GL (Galβ1-6Galβ1-4Glc), disialyllactose (Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc), and, less commonly, ganglio-type oligosaccharides. Added to this, only about 40-50 BMO and GMO structures have been identified to date (Albrecht et al. 2014; Tao et al. 2008). However, despite these differences, structurally identical oligosaccharides are found in human and dairy-derived milk oligosaccharide pools. The quantities, where known, of neutral and acidic BMOs and GMOs identified to date are presented in Tables 7.1 and 7.2, respectively.

Table 7.2 Quantities of acidic oligosaccharides found in human, bovine, and goat milks

Oligosaccharide (abbreviation)	Human (g/L)	Bovine (g/L)	Goat (g/L)
3′-Sialyllactose (3′-SL)	0.1–0.3	0.035–0.119	0.03–0.05
6′-Sialyllactose (6′-SL)	0.3–0.5	0.014–0.088	0.05–0.07
Sialyllacto-*N*-tetraose (a) (LSTa)	0.03–0.2	Trace	–
Sialyllacto-*N*-tetraose (b) (LSTb)	0.01–0.16	–	–
Sialyllacto-*N*-tetraose (c) (LSTc)	0.1–0.6	Trace	–
6′-Sialyl-lactosamine (6′SLN)	–	0.009–0.176	Trace
Disialyl-lactose (DSL)	–	0.002–0.07	0.001–0.005
Disialyllactose-*N*-tetraose (DSLNT)	0.2–0.6	Trace	–

3′-SL; Neu5Acα2-3Galβ1-4Glc, 6′-SL; Neu5Acα2-6Galβ1-4Glc, LST a; Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LST b; Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc, LST c; Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc, 6′SLN; Neu5Acα2-6Galβ1-4GlcNAc, DSL; Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc, DSLNT; Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc. The ranges shown reflect differences due to variations in the analytical methods used in the different studies and reflect changes in abundance over lactation, i.e., from colostrum to mature milk. Compiled data from: Kunz et al. (2000), Gopal and Gill (2000), Wang et al. (2001), Nakamura et al. (2003), Nakamura and Urashima (2004), Fong et al. (2011), Oliveira et al. (2012), Meyrand et al. (2013), Aldredge et al. (2013), Albrecht et al. (2014), Oliveira et al. (2015), Austin et al. (2016), Sprenger et al. (2017), Thurl et al. (2017), Ma et al. (2018), Tonon et al. (2019), Samuel et al. (2019), Sousa et al. (2019), van Leeuwen et al. (2020)

7.4 Biosynthesis of Milk Oligosaccharides

Despite the intense interest in HMO in recent decades, many details of HMO biosynthesis remain unclear. While the many possible monosaccharide addition events are known, the order of the biosynthetic steps and many of the enzymes involved are less well characterized. For example, the lactose core is extended by alternating actions of β-1,3-*N*-acetylglucosaminyltransferases (b3GnT) and β-1,4-galactosaminyltransferases (b4GalT) or β-1,3-galactosyltransferase (b3GalT) while β-galactoside sialyltransferases (SGalT) and α-1,2-fucosyltransferases (including the FUT2 "secretor" locus) are responsible for some sialylation and fucosylation of a terminal galactose, respectively (Kellman et al. 2020a). However, each enzymatic activity in HMO extension and branching can potentially be catalyzed by multiple isozymes in the respective gene family. Direct evidence of the specific isozymes performing each reaction in vivo is extremely limited. Kellman et al. (2020b) recently used a systems biology framework that integrated glycan and RNA expression data to construct an HMO biosynthetic network and predict the glycosyltransferases involved. To accomplish this, models were constructed describing the most likely pathways for the synthesis of the oligosaccharides accounting for >95% of the HMO content in human milk. Through these models, the authors proposed candidate genes for elongation, branching, fucosylation, and sialylation of HMOs. The study further explored selected enzyme activities through kinetic assay and their co-regulation through transcription factor analysis. This type of knowledge can provide insights for advancements in large-scale synthesis of HMOs as ingredients.

A number of recent studies have also shed more light on BMO synthesis. Wickramasinghe et al. (2011) examined the genes coding for enzymes involved in BMO metabolism by comparing the oligosaccharide profiles in 32 milk samples across lactation with the expression of glycosylation-related genes. Ninety-two glycosylation-related genes were found to be expressed in milk somatic cells. Recently, Liu et al. (2019) measured the abundance of 12 major BMO in the milk of 360 cows, which had high density single nucleotide polymorphism (SNP) marker genotypes. Most of the BMO were found to be highly heritable. A genome-wide association study (GWAS) allowed the group to fine-map several quantitative trait loci (QTL) and identify candidate genes with major effects on five of the BMO [3′-SL (Neu5Acα2-3Galβ1-4Glc), *N*-acetylgalactosaminyllactose (GNL), sialyl-3′-galactosyllactose (Neu5Acα2-3Galβ1-3Galβ1-4Glc, S3′-GL), lacto-*N*-novopentaose 1 (Galβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc, *novo*-LNP-I), and lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc, LN*n*T)]. Among them, a putative causal mutation close to the *ABO* gene (encoding ABO blood group glycosyltransferases) on Chromosome 11 accounted for approximately 80% of genetic variance for two BMO, GNL and LN*n*T. This mutation lies very close to a variant associated with the expression levels of ABO. A third QTL mapped close to *ST3GAL6* (that codes for α-2-3-sialyltransferase) on Chromosome 1 explaining 33% of genetic variation of 3′-SL. The presence of major gene effects suggests that targeted marker-assisted selection could lead to a significant increase in the level of these BMO in milk.

In a similar study, also using GWAS, Poulsen et al. (2019) aimed to estimate genetic parameters in order to examine whether BMO in Danish Holstein and Danish Jersey milk are heritable. The group also aimed to identify underlying SNP markers affecting BMO variation in the dairy breeds. In total, 15 different BMO were monitored. The GWAS identified in total 1770 SNPs for five different BMO in Danish Holstein and 6913 SNPs for 11 BMO in Danish Jersey cows. In Danish Holstein cows, a major overlapping QTL was identified on BTA1 for lacto-*N*-hexaose (Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc, LNH) and lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNT) explaining 24% of the variation in these BMOs. The most significant SNPs were associated with *B3GNT5*, a gene encoding a glycosyltransferase involved in glycan synthesis. In Danish Jersey cows, a very strong QTL was detected for the BMO with composition 2 Hex 1 HexNAc on BTA11. The most significant SNP was assigned to *ABO*. This SNP has been reported to be a missense mutation and explains 56% of the BMO variation. Other candidate genes of interest identified for BMO synthesis were *ALG3*, *B3GALNT2*, *LOC520336*, *PIGV*, *MAN1C1*, *ST6GALNAC6*, *GLT6D1*, *GALNT14*, *GALNT17*, *COLGALT2*, *LFNG*, and *SIGLEC*.

A number of studies have also investigated the synthesis of oligosaccharides in goat milk. Crisà et al. (2016) sequenced and assembled the goat milk transcriptome at the colostrum stage and at 120 days of lactation. The analysis of 144 different oligosaccharide metabolism-related genes showed that most of these (64%) were more expressed in colostrum than in mature milk, with eight expressed at very high levels including the sugar transporters, *SLC2A3* and *SLC2A1*, a fucose synthesis gene, *GMDS*, a lactose synthesis gene, *NME2*, the galactosyltransferase, *B4GALT1*, the *N*-acetylglucosaminyl transferase, *B3GNT2*, a sialic acid synthesis gene, *NANS*, and the glycosidase, *HEXB*. More recently, this group (Crisà et al. 2019) identified the complete cDNAs of candidate genes implicated in sialylated oligosaccharide biosynthesis, namely β-1,4-galactosyltransferase 1 (*B4GALT1*), α-lactalbumin (*LALBA*) related to lactose synthesis (the precursor molecule for 3′-SL and 6′-SL biosynthesis), β-galactoside α-2,3-sialyltransferase 5 (*ST3GAL5*) related to 3′-SL biosynthesis, and β-galactoside α-2,6-sialyltransferase 1 (*ST6GAL1*) related to 6′-SL (Neu5Acα2-6Galβ1-4Glc) biosynthesis. The group then analyzed their expression during lactation in Garganica and Maltese goat breeds and measured the levels of 3′-SL, 6′-SL and disialyllactose (Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc, DSL) in their milk to make correlations between expressed genes and phenotype. Gene expression analysis demonstrated that *LALBA* and *ST6GAL1* had the highest and lowest expression in both the breeds, respectively. The interaction effects of the breeds and sampling times were associated with higher levels of *B4GALT1* and *ST3GAL5* gene expression in Garganica when compared to the Maltese goats at kidding. *B4GALT1*, *LALBA*, and *ST3GAL5* gene expression changed from kidding to 60 and 120 days in Maltese goats, while in Garganica goats, a difference was observed only for the *LALBA* gene. Breed and lactation effects were also found to influence the sialylated oligosaccharide profile. Positive correlations of *B4GALT1*, *LALBA*, *ST3GAL5*, and *ST6GAL1* with 3′-SL/6′-SL and DSL were found. These types of studies provide information of specific candidate

Table 7.3 Functional properties associated with bovine milk oligosaccharides (BMOs)

Activity	Potential benefits to health	Reference
Prebiotic	Produced a microbiota-dependent promotion of growth and metabolic changes indicative of improved nutrient use in germ-free mice and new-born piglets which had been colonized with a consortium of cultured bacterial strains isolated from the fecal microbiota of a severely stunted Malawian infant	Charbonneau et al. (2016)
	Supplementation of infant formulas with *Bifidobacterium lactis* (CNCM I-3446) and BMOs induced a shift toward bifidobacteria-dominated stools, resembling those of breastfed infants	Radke et al. (2017), Simeoni et al. (2016)
	Supported the growth of *Bifidobacterium longum* ssp. *longum* and *Parabacteroides distasonis*, while at the same time inhibiting the growth of *Clostridium perfringens* and *Escherichia coli*	Jakobsen et al. (2019)
	Modulated microbiota composition and volatile fatty acids profiles in piglet neonatal model	Wang et al. (2021)
Anti-infective	Inhibition of *E. coli* hemagglutination	Martín et al. (2002)
	Inhibition of *Salmonella fyris* adhesion to Caco-2 cells	Coppa et al. (2006)
	Reduced the cellular invasion and translocation of *Campylobacter jejuni* in HT-29 cells, in a concentration-dependent manner	Lane et al. (2012)
	Adherence inhibition of enteric pathogens, such as *Escherichia coli*, *Cronobacter sakazakii*, and *Salmonella enterica* serovar Typhymurium	Maldonado-Gomez et al. (2015)
	Inhibited the adhesion of *Salmonella enterica* IID604 to Caco-2 cells	Urakami et al. (2018)
	Reduced *Streptococcus pneumoniae* adhesion to pharynx and lung cells in vitro when tested at physiological concentrations	Ryan et al. (2018)
	Reduced adhesion of *Staphylococcus aureus* to Caco-2 cells	Yue et al. (2020)
Barrier function	Modulated host–bacterial crosstalk, leading to enhanced epithelial barrier function, as measured by paracellular ion flux through transepithelial electrical resistance following *Clostridium difficile* toxin A challenge	Duncan et al. (2020)
Immunomodulation/ inflammation	Oligosaccharides from bovine colostrum influenced the expression of various cytokines, chemokines, and cell surface receptors in HT-29 cells	Lane et al. (2013)
	Effects of a high-fat diet such as liver abnormalities, steatosis, and inflammation can be eliminated via regulating lipid and glucose metabolism through the consumption of BMOs and *Bifidobacterium longum* subsp. *infantis* in genetically predisposed animal models	Jena et al. (2018)

(continued)

Table 7.3 (continued)

Activity	Potential benefits to health	Reference
Obesity and intestinal permeability	Significantly reduced weight gain and intestinal permeability that is induced in mice consuming a high-fat diet	Hamilton et al. (2017)
	In combination with a weekly gavage of the probiotic *Bifidobacterium longum* subsp. *infantis*, increases in intestinal permeability associated with the high-fat diet were prevented	Boudry et al. (2017)
Brain function	A whey preparation enriched in BMOs improved spatial cognition, with effects on hippocampal genes related to sialic acid metabolism, myelination, and ganglioside biosynthesis in preterm pigs	Obelitz-Ryom et al. (2019)
	BMOs (derived from bovine whey and composed primarily of galacto-oligosaccharides and trace amounts of 3′-SL and 6′-SL) were found to have distinct effects on brain structure and cognitive performance in pigs	Fleming et al. (2020)

Table 7.4 Functional properties associated with goat milk oligosaccharides (GMOs)

Activity	Potential benefits to health	Reference
Prebiotic	Utilization of oligosaccharides by bifidobacteria and *Bacteriodes* spp. and the capacity for short-chain fatty acid (SCFA) production	Oliveira et al. (2012)
	Increased numbers of *Bifidobacterium* spp. have been demonstrated using in vitro fermentation models	Thum et al. (2015), Barnett et al. (2018)
	Consumption of GMOs by mice during gestation and lactation improved the development of their pups, and the relative abundance of bifidobacteria and butyric acid in the colon at weaning	Thum et al. (2016)
	Significantly enhanced the growth of bifidobacteria and lactobacilli in vitro	Leong et al. (2019)
Anti-infective	Inhibited the adhesion of *Salmonella enterica* IID604 to Caco-2 cells	Urakami et al. (2018)
	Inhibited the adhesion of *E. coli* NCTC 10418 and *Salmonella typhimurium* to Caco-2 cells	Leong et al. (2019)
	Reduced adhesion of *Staphylococcus aureus* to Caco-2 cells	Yue et al. (2020)
	In combination with *Bifidobacterium longum* subsp. *infantis* ATCC 15697, GMOs show synergism in vitro as anti-infectives against *Campylobacter jejuni*	Quinn et al. (2020b)
Effect on inflammation	Intestinal anti-inflammatory effect in a trinitrobenzenesulfonic acid-induced colitis in rats	Daddaoua et al. (2006)
	Reduction of intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis	Lara-Villoslada et al. (2006)
Barrier function	Enhanced transepithelial electrical resistance, mucin gene expression, and mucin protein abundance in epithelial co-cultures	Barnett et al. (2016, 2018)

genes related to milk oligosaccharide synthesis and have the potential to guide breeding strategies to achieve production of milk with higher diversity and concentrations of oligosaccharides.

7.5 Gastrointestinal Digestion and Absorption of Milk Oligosaccharides

Milk oligosaccharides are typically considered indigestible by human gastrointestinal enzymes. Ingested HMOs can be found to be intact and non-metabolized in infant feces. Nonetheless, there are several reports of HMO existing in urine of exclusively breastfed infants (De Leoz et al. 2013; Dotz et al. 2014; Rudloff et al. 2012, 1996) as well as in preterm infants. Underwood et al. (2015) suggested that portions of HMO are absorbed into plasma. Moreover, experiments have detected the presence of some milk oligosaccharides, specifically sialylated oligosaccharides, in plasma from formula-fed and partially breastfed infants (Ruhaak et al. 2014), and direct evidence of HMO in the circulation of breastfed infants has been established (Goehring et al. 2014). Vazquez et al. (2017) administered a single oral dose of the HMOs, 2′-FL, 6′-SL and LN*n*T at different concentrations to adult rats. The time course of absorption of HMO into the bloodstream and their appearance in urine was studied. The results showed that rats, similar to human infants, effectively absorb a portion of HMO from the intestine into plasma and excrete them in urine. A specific kinetic absorption study with 2′-FL, was then performed in 9- to 11-day-old rat pups and confirmed that a significant amount of 2′-FL was absorbed into the systemic circulation and subsequently excreted in urine during lactation in rats in a dose-dependent manner. Basal levels of these HMO were found in the plasma and urine of adult rats as well as rat pups as a natural result of nursing. Hirschmugl et al. (2019) provided direct evidence of HMOs in cord blood and suggested that the placenta transfers HMOs from the maternal to the fetal circuit. In the study, the researchers analyzed HMO concentration and composition in cord blood in comparison to maternal serum HMOs at delivery in a small pregnancy/birth cohort. Maternal-to-fetal 2′-FL transfer across the human placenta, using an ex vivo placental perfusion approach was also investigated and after 180 min perfusion, 22% of maternally offered 2′-FL was found in the fetal circuit without reaching equilibrium.

These studies collectively confirm that, although most HMO are excreted in feces, a portion of HMO is absorbed into plasma and may modulate or contribute to systemic functions. The degree of absorption appears to vary substantially by structure, and the biological implications of this absorption have yet to be fully elucidated. It has been hypothesized, however, that absorbed oligosaccharides can prevent urinary tract infections in infants and that consumption of 3′-SL and 6′-SL increases brain ganglioside-bound sialic acid content, suggesting that these carbohydrates make an important contribution to brain development and immune

function (discussed below). Milk oligosaccharides that are not absorbed are available for consumption by the gut microbiota. Human milk has long been known to influence the development of the infant gut microbiota in ways that confer health benefits to the infant, and more recent studies have determined that the milk oligosaccharides are key to providing this prebiotic functionality (discussed below). The studies providing evidence that BMOs and GMOs can mimic specific properties associated with HMOs are summarized in Tables 7.3 and 7.4, respectively, and discussed in detail in the following sections.

7.6 Brain-Stimulating Activity by Milk Oligosaccharides

HMOs have been associated with increased delivery of sialic acid for the developing brain (Wang 2009). The concentration of sialic acid in saliva and brain tissue is known to be higher in breastfed versus formula-fed infants (Tram et al. 1997; Wang et al. 2003, 2001). Animal studies have investigated the functional brain effects of sialic acid using rats of varying age (Morgan et al. 1981; Morgan and Winick 1980; Oliveros et al. 2018; Sakai et al. 2006), mice (Kikusui et al. 2012), and full-term neonatal piglets (Wang et al. 2007). Sialic acid supplementation has been shown to increase the sialic acid concentration in brain gangliosides and improve cognition in term newborn pigs. BMOs consist of a high proportion of sialylated structures and may therefore confer similar effects (Wang et al. 2007; Jacobi et al. 2016; Obelitz-Ryom et al. 2019). Hobbs et al. (2021) recently reviewed the effects of sialylated milk oligosaccharides on the brain and gut health of newborns. 3′-SL and 6′-SL have been found to support normal microbial communities and behavioral responses in mice during stress by modulating the gut–brain axis (Tarr et al. 2015). Similarly, these oligosaccharides have been shown to increase ganglioside sialic acid concentrations in the corpus callosum and cerebellum and modulate the colonic microbiota of formula-fed piglets (Jacobi et al. 2016). Recently, Wang et al. (2019) provided in vivo evidence that milk 3′-SL, 6′-SL and 6′-sialyllactosamine (Neu5Acα2-6Galβ1-4GlcNAc, 6′-SLN) can alter many important brain metabolites and neurotransmitters required for optimizing neurodevelopment in piglets using in vivo magnetic resonance spectroscopic (MRS) approaches. Dietary sialyllactose (3′-SL or 6′-SL) was also shown to influence sialic acid concentrations in the prefrontal cortex and magnetic resonance imaging measures in the corpus callosum of young pigs (Mudd et al. 2017). The structural and functional neurodevelopmental outcomes in preterm pigs with or without supplementation of an oligosaccharide-enriched whey with sialyllactose during the first 19 days after preterm birth was also investigated (Obelitz-Ryom et al. 2019). The whey preparation improved spatial cognition, with effects on the expression of hippocampal genes related to sialic acid metabolism, myelination, and ganglioside biosynthesis in the preterm pigs.

Hauser et al. (2021) recently investigated the long-term consequences of a selective lactational deprivation of 6′-SL in knock-out mice. To test whether lactational 6′-SL deprivation affects cognitive capabilities in adulthood, the researchers

assessed attention, perseveration and memory. To detail the associated endophenotypes, they investigated hippocampal electrophysiology, plasma metabolomics, and gut microbiota composition. To investigate the underlying molecular mechanisms, gene expression (at eye-opening and in adulthood) in two brain regions mediating executive functions and memory (hippocampus and prefrontal cortex) was assessed. Compared to control mice, offspring deprived of 6′-SL during lactation exhibited consistent alterations in all cognitive functions addressed, hippocampal electrophysiology, and in pathways regulating the serotonergic system (identified through gut microbiota and plasma metabolomics). These alterations were associated with reduced expression of genes involved in central nervous system development. Moreover, the reduced expression was site- (prefrontal cortex) and time-specific (eye-opening). The data suggest that 6′-SL in maternal milk adjusts cognitive development through a short-term upregulation of genes modulating neuronal patterning in the prefrontal cortex.

Apart from sialylated oligosaccharides, other oligosaccharides such as LN*n*T and 2′-FL have also been implicated in enhancing cognition during development. Docq et al. (2020) recently summarized the reported observations regarding the effects of HMOs on memory and cognition in rats, mice, and piglets. The impact of both BMOs and HMOs on cognition, brain development, and hippocampal gene expression in pigs was also recently assessed. HMOs (LN*n*T and 2′-FL) and BMOs (derived from bovine whey and composed primarily of galacto-oligosaccharides and trace amounts of 3′-SL and 6′-SL) were found to have distinct effects on brain structure and cognitive performance (Fleming et al. 2020). Pigs were tested on the novel object recognition task using delays of 1 or 48 h at postnatal Day 22. At postnatal Day 32–33, magnetic resonance imaging procedures were used to assess structural brain development, and hippocampal tissue was collected for analysis of mRNA expression. Pigs consuming only HMOs exhibited recognition memory after a delay of 1 h, and those consuming BMOs and HMOs exhibited recognition memory after a delay of 48 h. Both absolute and relative volumes of cortical and subcortical brain regions were altered by varying oligosaccharides in the diet. Hippocampal mRNA expression of GABRB2, SLC1A7, CHRM3, and GLRA4 were most strongly affected. The authors concluded that the HMOs and BMOs had distinct effects on brain structure and cognitive performance. A recent paper associated levels of 2′-FL in human milk at 1 month with cognitive function at 24 months in human infants (Berger et al. 2020a).

7.7 Effects of Milk Oligosaccharides on the Gut Microbiota

The human gut lacks glycoside hydrolases, other than lactase, and intestinal membrane transporters which can degrade milk oligosaccharides; therefore, HMOs are not digested in the upper part of the gastrointestinal tract of infants. As a result, the majority of HMOs reach the colon, where they act as a substrate for specific

bacteria, influencing the composition of the gastrointestinal microbiota. HMOs are specifically known to influence populations of beneficial bacteria such as *Bifidobacterium* (Akkerman et al. 2019), a dominant genus in the intestine of breast-fed infants, thereby acting as prebiotics. Bifidobacteria are involved in modulating the immune system, inducing anti-inflammatory and antioxidant responses, producing antimicrobial substances, as well as competitively excluding pathogens. These bacteria have the ability to use HMOs with dedicated enzymes (glycoside hydrolases), transporters, and other molecules that contribute to degradation. Genomic analysis of a prototypical infant-derived bifidobacterial strain, *Bifidobacterium longum* subsp. *infantis*, which grows well on HMOs, revealed a cluster of genes coding for enzymes dedicated to the degradation of HMOs, named HMO-1 cluster, suggesting co-evolution of this strain with human milk (Sela and Mills 2010). *Bifidobacterium bifidum* can extracellularly release monosaccharides from type 2 HMO and lacto-*N*-biose from type 1 HMOs, allowing them to be utilized by other bifidobacterial species (Sakanaka et al. 2019). In addition, several strains of *Bifidobacterium breve* and *B. longum* subsp. *infantis* have metabolic pathways, specific for fucosyllactose (Matsuki et al. 2016; Sakanaka et al. 2019).

In order to elucidate the prebiotic molecular mechanism for degradation of HMOs, several bacteria have been tested for their ability to grow on individual or total HMOs as the sole carbon source in vitro. A vast literature on the ability of bifidobacteria to metabolize HMO exists, and these studies have been summarized in recent review articles (Cheng et al. 2020; Hundshammer and Minge 2020; Walsh et al. 2020a). Also, other individual strains such as *Bacteroides* (Yu et al. 2013), *Enterococcus* (Yu et al. 2013), *Akkermansia* (Kostopoulos et al. 2020), *Lactobacillus* (Yu et al. 2013), *Streptococcus*, and *Clostridium* cluster IV/XIVa (Pichler et al. 2020) have been shown in vitro to have the ability to utilize oligosaccharides. However, few longitudinal studies exist which investigate the establishment of the infant gut microbiota in relation to changes in breastmilk HMO composition. Borewicz et al. (2020) followed 24 mother–infant pairs to investigate the associations between concentrations of selected HMOs in breastmilk, infant feces, and the fecal microbiota composition in healthy, breastfed infants at 2, 6, and 12 weeks of age. Lactation duration was found to have a significant effect on the HMO content of breastmilk, which decreased with time, except for 3-fucosylactose (Galβ1-4(Fucα1-3)Glc, 3-FL) and lacto-*N*-fucopentaose III (Galβ1-4(Fucα1-3) GlcNAcβ1-3Galβ1-4Glc LNFP III). The group confirmed that microbiota composition was strongly influenced by infant age and was associated with the mode of delivery and concentration of LNFP III in breastmilk at 2 weeks, infant sex, delivery mode, and concentrations of 3′-SL in milk at 6 weeks, and infant sex and levels of lacto-*N*-hexaose (LNH) in milk at 12 weeks of age. Correlations between levels of individual breastmilk HMOs and relative abundance of operational taxonomic units (OTUs) found in infant feces, including the most predominant *Bifidobacterium* OTUs, were weak and varied with age. The fecal concentration of HMOs decreased with age and was strongly and negatively correlated with relative abundance of OTUs within genera *Bifidobacterium*, *Parabacteroides*, *Escherichia-Shigella*,

Bacteroides, *Actinomyces*, *Veillonella*, *Lachnospiraceae Incertae Sedis*, and *Erysipelotrichaceae Incertae Sedis*, indicating the likely importance of these taxa for HMO metabolism in vivo.

While the role of HMOs as prebiotics is well characterized, the use of BMOs as prebiotics is less well investigated, and only a limited number of in vitro studies have been documented (Jakobsen et al. 2020, 2019; Perdijk et al. 2019). The ability of BMOs to modulate the gut microbiota in vivo has been the subject of some recent studies (reviewed by Quinn et al. 2020a). A study by Charbonneau et al. (2016) used animal models (gnotobiotic mice and piglets) of infant undernutrition to show that dietary supplementation with BMOs provides a microbially mediated increase in lean body mass and bone growth and generates metabolite profiles indicative of improved nutrient utilization. BMOs, derived from demineralized whey permeate and also containing galactooligosaccharides (GOS), were proven to be beneficial in two clinical trials. The supplementation of infant formulas with *B. lactis* (CNCM I-3446) and BMOs induced a shift toward bifidobacteria-dominated stools, resembling those of breastfed infants (Radke et al. 2017; Simeoni et al. 2016). In order to discriminate the prebiotic effects of BMOs from the probiotic effects of *B. lactis* and synbiotic effects of their combination, all the three conditions were tested separately by Marsaux et al. (2020). BMOs alone significantly induced acetate and lactate production (leading to pH decrease) and stimulated bifidobacterial growth in ten donors. A further in-depth study on two different donors proved the ability of *B. lactis* to colonize the infant microbiota, regardless of the competitiveness of the environment. BMOs further enhanced this engraftment, suggesting a strong synbiotic effect. In another study by Jena et al. (2018), BMO supplementation was also found to significantly increase the expression of butyrate-generating bacterial genes in mice fed a western diet, which is of importance as butyrate can have anti-inflammatory effects in the liver and colon. A recent study by Wang et al. (2021) demonstrated that supplementation of BMOs and HMO (as described for the study of Fleming et al. 2020, above) were found to modulate the microbiota composition and volatile fatty acid profiles in a neonatal piglet model. HMOs alone did not affect overall microbial composition, but increased the relative proportion of specific taxa, including *Blautia*, compared to other groups. Abundance of *Bacteroides* was increased in the ascending colon by BMOs and synergistically by BMOs and HMOs in the feces. Similar in vivo studies on oligosaccharides from goat milk are limited. Thum et al. (2015) found that consumption of GMOs by mice during gestation and lactation improved the development of their pups, and the relative abundance of bifidobacteria and butyric acid in the colon at weaning. Overall, these studies suggest that HMOs and dairy oligosaccharides exert distinct actions on the gut microbiota and formula-fed infants could benefit from formula containing a variety of oligosaccharides.

7.8 Effects of Milk Oligosaccharides on Obesity

Feeding newborn infants human milk is known to temper weight gain compared to formula (Gillman et al. 2001; Kramer and Kakuma 2012) and is most beneficial in the first 6 months. Animal studies provide evidence that exposure to oligosaccharides may diminish weight gain, adiposity, and caloric intake (Hamilton et al. 2017). Alderete et al. (2015) conducted a small pilot study in non-Hispanic white mother–infant pairs ($n = 25$), which revealed that individual fucosylated and sialylated oligosaccharides were related to infant adiposity at 6 months. This was in line with more recent findings (Larsson et al. 2019) in a separate but similar cohort and sample size ($n = 30$). A subsequent study by Berger et al. (2020b) aimed to determine whether HMOs at 1 month predicted infant weight gain at 6 months, and if associations varied by HMO secretor status. The participants were 157 Hispanic mother–infant pairs and human milk samples were collected at 1 month. Nineteen individual HMOs were analyzed using high-performance liquid chromatography, and secretor status was determined by the presence of 2′-FL or lacto-*N*-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNFPI). Infant weight was measured at 1 and 6 months. Path analysis was used to test the effects of HMO composition on infant weight gain, adjusting for maternal age, pre-pregnancy BMI, infant age, sex, and birthweight. The results suggested that higher lacto-*N*-fucopentaose II [Galβ1-3(Fucα1-4) GlcNAcβ1-3Galβ1-4Glc, LNFPII] in human milk may decrease obesity risk across all infants, whereas higher LN*n*T and disialyl lacto-*N*-tetraose (DSLNT) may increase obesity risk in infants of non-secretors only. To determine if HMOs are associated with eating behavior in the Hispanic infants, cross-sectional analysis of the cohort of Hispanic mother–infant dyads was performed. Several HMOs were both positively and negatively associated with infant food responsiveness, which is a measure of drive to eat (Plows et al. 2020).

Maternal obesity has also been associated with changes in HMO concentrations. Lagström et al. (2020) investigated the association between maternal HMO composition and child growth during the first 5 years of life. In addition, the association between maternal pre-pregnancy BMI and HMO composition was assessed. Human milk samples from 802 mothers were obtained from a prospective population-based birth cohort study in Finland. Maternal HMO composition 3 months after delivery was associated with height and weight during the first 5 years of life in children of secretor mothers. Specifically, HMO diversity and the concentration of LN*n*T were inversely associated and that of 2′-FL was directly associated with child height and weight *z* scores in a model adjusted for maternal pre-pregnancy BMI, mode of delivery, birthweight *z* score, sex, and time. Maternal pre-pregnancy BMI was associated with HMO composition. The authors concluded that the association between maternal HMO composition and childhood growth may imply a causal relation, and altered HMO composition may mediate the impact of maternal pre-pregnancy BMI on childhood obesity, both of which warrant further investigation. In a study by Saben et al. (2021), maternal obesity was associated with lower concentrations of several fucosylated and sialylated HMOs and infants born to obese women had

lower intakes of these HMOs. Maternal BMI was positively associated with LN*n*T, 3-FL, 3′-SL, and 6′-SL and negatively associated with DSLNT, disialyllacto-*N*-hexaose (DSLNH), fucodisialyllacto-*N*-hexaose (FDSLNH), and total acidic HMOs concentrations at 2 months. Infant intakes of 3-FL, 3′-SL and 6′-SL, DSLNT, DSLNH, and total acidic HMOs were positively associated with infant growth over the first 6 months of life.

Related to this, HMOs may be important modulators of gut–brain axis development and homeostasis. The brain reward system, specifically the mesolimbic dopamine (DA) projections from the ventral tegmental area (VTA) to nucleus accumbens (NAc) is involved in the motivation and preference for food. A recent study by Tuplin et al. (2021) aimed to determine if HMO fortified diets given during the critical period of reward system development (21 days after birth) could affect the structure of the reward system. At weaning (Day 21), Sprague-Dawley rats were randomized to one of four fortified diet groups: control, 3′-SL, 2′-FL, or a combination of 3′-SL and 2′-FL. Messenger RNA (mRNA) expression was quantified for DA and appetite associated markers in the VTA and NAc, and Western blots measured the immediate early gene FosB and its isoform ΔFosB. Females fed the 3′SL + 2′FL fortified diet displayed a decrease in DAT expression in the VTA and an increase in leptin expression in the NAc. Females displayed an overall lower expression of NAc D2, VTA ghrelinR, and VTA leptin. In males, VTA DAT and FosB were negatively correlated with body weight and systemic leptin. The authors concluded that sex differences in the expression of DA markers underscore the need to investigate this phenomenon and understand the functional significance in preventing or treating obesity.

In relation to BMOs, dietary supplementation was associated with reduced weight gain and adiposity in mice fed a high-fat diet. The study by Hamilton et al. (2017) showed that consumption of BMOs by mice could prevent the deleterious effect of a high-fat diet on the gut microbiota and intestinal permeability in addition to attenuating the development of an obese phenotype. Gut microbiota and intestinal permeability were assessed in the ileum, cecum, and colon. Addition of BMOs to the high-fat diet significantly attenuated weight gain, decreased adiposity, and decreased caloric intake. BMOs completely abolished the high-fat diet-induced increase in paracellular and transcellular permeability in the small and large intestines. BMOs also increased the abundance of beneficial microbes such as *Bifidobacterium* and *Lactobacillus* in the ileum.

7.9 Anti-Pathogenic Effect of Milk Oligosaccharides

Milk oligosaccharides are considered to be soluble receptor analogs of epithelial cell surface carbohydrates, because they are generated by the action of similar enzymes. These structures display structural homology to host cell receptors and thus function as receptor decoys that pathogens can bind to instead of the host. Oligosaccharides can also inhibit pathogens by competitive binding with the host

cell surface receptors. We refer the reader to the expansive literature that exists describing the action of HMOs against a variety of bacterial and viral pathogens as it is beyond the scope of this chapter to cover all anti-infective studies associated with HMOs (Hickey 2012; Hundshammer and Minge 2020; Laucirica et al. 2017; Li et al. 2014; Manthey et al. 2014; Morozov et al. 2018). In terms of in vitro studies, HMOs have been shown to interfere with the lectin–glycan association for pathogens such as enterohaemorrhagic, entereopathogenic, enterotoxic, uropathogenic *Escherichia coli*, *Entamoeba histolytica*, *Campylobacter jejuni*, *Clostridium difficile*, *Helicobacter pylori*, *Listeria monocytogenes*, *Neisseria meningitidis* C, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella enterica*, group B *Streptococcus*, *Vibrio cholerae*, human immunodeficiency virus, norovirus, influenza virus, and respiratory syncytial virus. Indeed, a recent study by Yue et al. (2020) demonstrated that HMOs, BMOs, and GMOs were found to reduce the adhesion of *Staphylococcus aureus* to Caco-2 cells in comparison to the lactose control. Oligosaccharides isolated and purified from the colostrum of Holstein-Friesians were found to dramatically reduce the cellular invasion and translocation of *Campylobacter jejuni* in HT-29 intestinal cells, in a concentration-dependent manner (Lane et al. 2012). Similarly, Maldonado-Gomez et al. (2015) demonstrated that oligosaccharides from bovine colostrum could prevent the adhesion of enteropathogenic *E. coli*, *Cronobacter sakazakii*, and *Salmonella enterica* serovar Typhimurium to HEp-2 cell monolayers cultured in vitro. Recently, neutral and acidic oligosaccharides also isolated from bovine colostrum were compared for their potency to inhibit the adhesion of *S. enterica* IID604 to Caco-2 cells using HMO as a positive control (Urakami et al. 2018). The oligosaccharides inhibited the adhesion of *S. enterica* to Caco-2 cells at concentrations ranging from 2.5 to 5.0 mg mL^{-1}. Another recent study by Ryan et al. (2018) examined BMOs extracted from demineralized whey, using a combination of membrane filtration and chromatography. The authors found that the BMOs were capable of reducing *Streptococcus pneumoniae* adhesion to pharynx and lung cells in vitro when tested at physiological concentrations. Two recent studies also investigated the direct anti-adhesive capacity of isolated GMOs. One study observed reduced adhesion of *Escherichia coli* and *Salmonella typhimurium* to Caco-2 cells when pre-incubated with GMOs (Leong et al. 2019). This was observed independent of beneficial microbiota. The second study showed the same results with *Salmonella* by green fluorescent antibodies against the *Salmonella* strain used (Urakami et al. 2018). Quinn et al. (2020b) examined the synergistic effect of GMO-treated *Bifidobacterium infantis* on preventing the attachment of a highly invasive strain of *Campylobacter jejuni* to intestinal HT-29 cells. The combination decreased the adherence of *C. jejuni* to the HT-29 cells by an average of 42% compared to the control (non-GMO treated *B. infantis*). Taken together, these studies highlight the significant antimicrobial activity associated with milk oligosaccharides and their potential as novel substitutes for antibiotics in preventing infection.

7.10 Immunomodulating Effect of Milk Oligosaccharides

HMOs are known to affect immune cell populations and cytokine secretion. HMOs are also absorbed into the blood, where they affect binding of monocytes, lymphocytes, and neutrophils to endothelial cells and the formation of platelet–neutrophil complexes. The immunological effects of HMO have been reviewed (Ayechu-Muruzabal et al. 2018; Plaza-Díaz et al. 2018; Triantis et al. 2018). Less is known about the role that BMOs and GMOs play in modulating the immunological system. Lane et al. (2013) compared the transcriptional response of colonic HT-29 epithelial cells to the entire pool of HMOs and bovine colostrum oligosaccharides (BCOs). RT-PCR analysis revealed that HMOs and BCOs influenced the expression of various cytokines, chemokines, and cell surface receptors, suggesting that BMOs may result in an intestinal immune response similar to that of HMOs. In a recent study, Cowardin et al. (2019) colonized germ-free mice with cultured bacterial strains from a 6-month-old stunted infant and fed the mice a diet supplemented with bovine sialylated milk oligosaccharides. Although this study was focused on bone biology, the diet was associated with BMO-dependent and microbiota-dependent increases in cecal levels of succinate, increased numbers of small intestinal tuft cells, and evidence for the activation of a succinate induced tuft cell signaling pathway linked to T helper (Th)2 immune responses. GMOs have shown to be anti-inflammatory in a rat model of hapten-induced colitis (Daddaoua et al. 2006). When compared with the control group, the GMO group showed decreased anorexia and body weight loss, reduced bowel wall thickening and longitudinal extension of necrotic lesions, and downregulated colonic expression of interleukin 1β, inducible nitric oxide synthase, cyclooxygenase 2, and mucin 3; and increased trefoil factor 3. Lara-Villoslada et al. (2006) also studied the effect of GMOs on colon inflammation in rats induced by dextran sodium sulfate (DSS). The GMO-treated rats showed less severe colonic lesions and a more favorable intestinal microbiota. After DSS treatment, histological analysis showed that the GMO-treated rats had no ulceration and recovered from inflammation, while the DSS control rats had significant ulceration and inflammation. Also, blood granulocyte levels were reduced in GMO-fed rats compared to control rats. In GMO-fed rats, the levels of myeloperoxidase activity, a proxy for neutrophil infiltration, did not increase upon DSS treatment, while in control rats, a five-fold activity increase is observed upon DSS treatment. These studies suggest that GMOs reduce intestinal inflammation and contributed to the recovery of damaged colonic mucosa, indicating they may have potential for the treatment of inflammatory bowel disease.

7.11 Influence of Milk Oligosaccharides on Intestinal Cell Properties

Beginning in the perinatal period and extending through the first year of life, the gastrointestinal tract undergoes numerous morphological changes that influence its physiological functions. Human milk provides trophic factors that influence maturation of the GI tract, an important developmental process in all infants but of particular importance in pre- and early-term infants. Necrotizing enterocolitis (NEC) is a leading cause of morbidity and death in preterm infants, occurring more often in formula-fed than in breastfed infants. In preclinical studies using a newborn rat model of NEC, pups fed with a formula containing DSLNT demonstrated a reduction in NEC severity and decreased mortality (Jantscher-Krenn et al. 2012). Wu et al. (2019) suggested that the mechanism for the prevention of NEC by HMOs is related to recovery of the colonic barrier function. The authors demonstrated that a HMO-gavage given to rat pups increases Muc2 levels and decreases intestinal permeability to macromolecular dextran. In vitro experiments showed that HMO-treated cells have increased Muc2 expression, decreased bacterial attachment and dextran permeability during challenge by enteric pathogens.

The effect of dairy-derived oligosaccharides on intestinal properties has also been explored. Kuntz et al. (2019) identified that BMOs from different cattle breeds were able to induce growth arrest and differentiation of non-transformed human intestinal cells by modulating the epidermal growth factor receptor (EGFR) signal pathways, and cell cycle associated gene expression in a similar way as was shown for HMOs (Kuntz et al. 2009, 2008). Studies investigating the ability of BMOs to modulate intestinal permeability have also shown promising results. The study of Hamilton et al. (2017) mentioned above showed that consumption of BMOs can significantly reduce the intestinal permeability that is induced in mice consuming a high-fat diet. In a similar study, introduction of BMOs to the diet of high-fat-fed mice, in combination with a weekly gavage of the probiotic *Bifidobacterium longum* subsp. *infantis*, prevented increases in intestinal permeability otherwise associated with the high-fat diet (Boudry et al. 2017). Duncan et al. (2020) recently explored the synbiotic action of BMOs and galactooligosaccharides (GOS) with *Lactobacillus rhamnosus* in imparting protection of epithelial barrier function against *Clostridium difficile* enterotoxicity, in a simplified in vitro host–epithelial barrier function model system. The authors found that the BMO-enriched preparation modulated host–bacterial crosstalk, leading to enhanced epithelial barrier function, as measured by paracellular ion flux through transepithelial electrical resistance following challenge with *C. difficile* toxin A. Interestingly, recent studies examining the impact of a GMOs on barrier function of epithelial cell co-cultures found that the GMOs at the maximum concentration tested (4.0 mg mL^{-1}) enhanced trans-epithelial electrical resistance, mucin gene expression, and mucin protein abundance in epithelial co-cultures, all of which are essential components of intestinal barrier function (Barnett et al. 2018, 2016).

7.12 Separation, Detection, and Quantification of Milk Oligosaccharides

Purifying milk oligosaccharides prior to analysis is sometimes challenging due to the similarity in physical properties between the oligosaccharides and lactose. The smallest oligosaccharides have a degree of polymerization of three and will often behave similarly to lactose when oligosaccharides are enriched by polarity or size-based methods. The general scheme for obtaining oligosaccharides from milk is first to precipitate fat and protein using different reagents. The defatting step is often performed simply by centrifugation or by solvent extraction. Protein precipitation is usually completed with organic solvents, such as ethanol, chloroform/methanol, acetone, or acetonitrile. In several studies, membrane separation has been applied to eliminate milk proteins. For more detail on the extraction and fractionation of milk carbohydrates, we refer the reader to a review by Nagaraj et al. (2018) where methods such as pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), and solid-phase extraction (SPE) are discussed. Oligosaccharides can be separated from lactose by gel filtration using various resins (e.g., Sephadex G25, Toyopearl HW50, and Biogel P2) or by stepwise elution from a column of activated charcoal using ethanol. High pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is often used to separate milk oligosaccharides. Reversed-phase high-performance liquid chromatography (RP-HPLC) is another widely used method for oligosaccharides analysis, although it requires sample derivatization since native milk oligosaccharides are typically polar and are not retained by the column. Derivatization refers to the addition of a chromophore or fluorophore to the carbohydrate analyte in order to provide a greater degree of sensitivity and often enhance the selectivity of the detection system. Milk oligosaccharides have also been separated by hydrophilic interaction chromatography HPLC (HILIC) which provides good isomer separation but requires oligosaccharide labeling. Capillary electrophoresis (CE) is suitable for both derivatized and underivatized carbohydrates and is also considered a powerful and adaptable separation method.

Recent advances in the analysis of human milk oligosaccharides by liquid phase separation methods have been reviewed by Porfirio et al. (2020) and Auer et al. (2020). To detect the oligosaccharides after separation, there is a choice between derivatized (labeled) and label-free detection, using e.g., refractive index (RI), evaporative light scattering detection (ELSD), pulsed-amperometric detection (PAD), and mass spectrometry (MS). RI has limited sensitivity and is limited to isocratic separation conditions and therefore this detection approach is not suitable for complex mixtures. Likewise, ELSD has limited compatibility with common oligosaccharide separation gradients. This means that label-free detection is essentially limited to PAD and MS. Identification of the individual oligosaccharide structures in milk has come with numerous analytical challenges, many of which have been resolved in recent years.

Over the past 10 years, a number of studies have documented the detection of oligosaccharides from dairy sources. Mariño et al. (2011) employed an analytical

scheme based on fluorescent labeling of BMOs from colostrum, pre-fractionation by weak anionic exchange chromatography and separation by HILIC-HPLC. Structural assignments for 37 free oligosaccharides including 20 sialylated species were obtained by combining HILIC-HPLC, exoglycosidase digestion and offline negative-ion mode MS/MS. Aldredge et al. (2013) also fractionated oligosaccharides from bovine colostrum pool by high-performance liquid chromatography, incubated each BMO with glycosidases of known specificity, and analyzed the changes produced with LC-MS. This labor-intensive approach determined a variety of glycosidic linkages and specific monosaccharide types for numerous BMOs. Albrecht et al. (2014) performed a study to obtain a comprehensive overview of oligosaccharides present in the milk of a number of domestic animals including cows and goats. A combination of weak anion-exchange chromatography, ultra-performance liquid chromatography–hydrophilic interaction liquid chromatography with fluorescence detection (UPLC-HILIC-FLD) and complementary quadrupole time-of-flight MS as well as exoglycosidase sequencing allowed for the determination of the oligosaccharide sequences and linkages as well as their relative quantification. A total of 35 BMO structures were identified of which five were novel, 12 neutral, three fucosylated, 21 acidic with two phosphorylated. In relation to the GMOs, 40 were identified of which 19 were novel, 16 neutral, three fucosylated, 23 acidic with one phosphorylated. Mehra et al. (2014) employed concentration techniques on dairy streams at pilot scale combined with advanced mass spectrometry, to discover numerous high-molecular weight fucose-containing oligosaccharides in a whey stream of bovine milk. Among the BMOs identified, 18 have high-molecular weight and corresponded in size to the most abundant oligosaccharides present in human milk. Lee et al. (2016) then went on to use a nanoLC separation coupled to a high-resolution and sensitive quadrupole time-o-flight (Q-ToF) MS system to detect over 30 BMOs that were previously elucidated. Martín-Ortiz et al. (2016) analyzed GMOs from colostrum using nanoflow liquid chromatography–quadrupole time-of-flight MS (Nano-LC-Chip–Q-TOF MS). Up to 78 oligosaccharides containing hexose, hexosamine, fucose, *N*-acetylneuraminic acid, or *N*-glycolylneuraminic acid monomeric units were identified in the samples, some of them detected for the first time in goat colostra. Also, in relation to goat milk, Lu et al. (2020) identified and quantified oligosaccharides by using UPLC-MS/MS. The elution conditions of the UPLC was optimized leading to successful identification of 64 oligosaccharides in Guanzhong and Saanen goat milk. Recently, Sunds et al. (2021) characterized oligosaccharides in the milk of native Nordic cattle breeds, to reveal whether such breeds hold unique oligosaccharide distribution and variation. The study involved 80 milk samples collected from eight native breeds originating from Norway (Norwegian Doela cattle and Norwegian Telemark cattle), Sweden (Swedish Mountain cattle), Denmark (Danish Red anno 1970), Iceland (Icelandic cattle), Lithuania (native Lithuanian Black and White), and Finland (Western Finncattle and Eastern Finncattle). The analysis was conducted using high-performance liquid chromatography chip/quadrupole time-of-flight mass spectrometry (HPLC-Chip/Q-TOF MS) and thereby created comprehensive libraries for each breed based on tandem MS, as well as a relative quantification of

all the BMOs identified. Eighteen unique monosaccharide compositions and a multitude of isomers were identified. No *N*-glycolylneuraminic acid was identified among the breeds. Western Finncattle milk was found to be the most abundant in neutral, acidic, and fucosylated oligosaccharides. Eastern Finncattle milk had significantly higher levels of acidic oligosaccharides, and Icelandic cattle milk had significantly higher levels of fucosylated oligosaccharides, compared to the mean (the average stage of lactation and the average parity per breed). Such studies are of interest for future exploitation of milk oligosaccharides via selective breeding strategies.

Considering the multitude of applications which may be assigned to milk oligosaccharides, and often to specific structures, it is very important to have robust methods to accurately determine the levels of these structures in milk, dairy streams, and infant formulas. Fong et al. (2011) developed a method for the quantitation of different BMOs using HILIC-HPLC with high-resolution selected reaction monitoring-MS. Concentrations of five BMOs (3′-sialyllactose (3′-SL), 6′-sialyllactose (6′-SL), 6′-sialyllactosamine (6′-SLN), disialyllactose (DSL), and *N*-acetylgalactosaminyllactose (GNL)) in bovine mature defatted milk, homogenized mature milk, non-pasteurized mature milk, bovine colostrum, and infant formula were determined. Liu et al. (2014) subsequently improved the quantitative analysis of 3′-SL, 6′-SL, and 6′-SLN in bovine mature milk using a method based on HILIC coupled to MS in negative ion mode. Milk from commercial dairy and beef cows in early lactation has also been compared for oligosaccharide content (Sischo et al. 2017). Early lactation multiparous cows (5–12 days into milking) from five commercial Holstein dairy herds and five Angus or Angus hybrid beef herds were sampled once. Oligosaccharide diversity and abundance within and between samples was assessed using LC-MS and principal component analysis. Overall, oligosaccharide relative abundance was consistently greater in the cluster dominated by beef cows.

Liu et al. (2017) performed a systematic survey on seasonal variation of 14 major oligosaccharides with 19 cows over the entire milking season using a LC-MS technique. This study revealed a number of significant correlations between structurally related and structurally nonrelated oligosaccharides and a substantial individual animal difference for all 14 oligosaccharides. In another study, relative quantities of oligosaccharides in a large collection of milk samples were recently measured using LC-MS and isobaric labeling (the use of mass tags which have an identical overall mass but vary in terms of the distribution of heavy isotopes around their structure), an analytical technique that improves instrumental throughput for large sample sets by allowing samples to be multiplexed prior to analysis by mass spectrometry (Robinson et al. 2018). In a subsequent study, Robinson et al. (2019) applied a high-throughput isobaric labeling technique to measure oligosaccharide abundances in 634 milk samples collected from Danish Holstein-Friesian and Jersey dairy cattle by LC-MS. Thirteen oligosaccharides that vary significantly by breed were identified, with most structures being more abundant in the milk of Jersey cattle. The abundances of several oligosaccharides were increased in second-parity cows, and correlations between the abundances of oligosaccharide pairs were identified,

potentially indicating similarities in their synthetic pathways. Fucosylated oligosaccharide structures were also widely identified among both breeds. Fischer-Tlustos et al. (2020) recently characterized the oligosaccharide profile of colostrum and transition milk from ten primiparous and ten multiparous Holstein cows. Samples were analyzed for oligosaccharide concentrations using LC-MS. The results demonstrated that colostrum and transition milk contain elevated concentrations of certain BMOs compared with mature milk. Parity differences were also detected for levels of 3′-SL, 6′-SL, and 6′-SLN, with multiparous cows having greater concentrations than primiparous cows over the first 7 days of milking.

The use of HPAEC-PAD has been reported in a number of studies to quantify BMOs and is advantageous because it requires minimal sample preparation and achieves good chromatographic separation of oligosaccharide isomers within 30 min. Lee et al. (2015) used the method to measure 3′-SL, 6′-SL, and 6′-SLN in colostrum whey permeate. Similarly, Quinn et al. (2020c) used HPAEC-PAD to monitor the impact of days post-parturition and parity on the oligosaccharide profile of cow's milk. Colostrum and milk samples were obtained from 18 cows 1–5 days after parturition. Three distinct phases were identified: colostrum (Day 0), transitional milk (Days 1–2) and mature milk (Days 3–5). LS-tetrasaccharide c (Neu5Ac α2-6Galβ1-4GlcBAcβ1-3Galβ1-4Glc, LST c), lacto-*N*-neotetraose (LN*n*T), disialyllacto-*N*-tetraose (DSLNT), 3′-sialyl-*N*-acetyllactosamine (Neu5Acα2-3Galβ1-4GlcNAc, 3′-SLN), 3′-SL, lacto-*N*-neohexaose [Galβ1-4GlcNAcβ1-3{Galβ1-4GlcNAcβ1-6}Galβ1-4Glc, LN*n*H], and DSL were found to be highly affiliated with colostrum. The cow's parity was also shown to have a significant effect on the oligosaccharide profile. CE has also been applied to quantify 3′-SL, 6′-SL, and DSLNT (Monti et al. 2015) and was used recently for the rapid characterization of the relative abundances of 33 BMOs in milk collected from exclusively grass-fed or grain/corn-fed cows at matched time points during the first week of lactation (Vicaretti et al. 2018). Sousa (2019) and van Leeuwen et al. (2020) as part of their reviews discuss the quantitative studies performed on GMOs. Claps et al. (2014) evaluated the influence of two goat breeds (Garganica and Maltese) on the oligosaccharide content in colostrum and observed a higher concentration of 3′-SL and 6′-SL in milk of the Garganica breed compared to Maltese in the period after parity. It was also observed that there was an increase in the 3′-SL and 6′-SL levels found in the breeds 24 h after parity, with the 3′-SL concentration always being higher when compared to 6′-SL in both the periods.

7.13 Industrial-Scale Strategies to Produce Milk Oligosaccharides

Four different approaches have been investigated regarding current commercial HMO production: chemical synthesis, whole-cell biotransformation (fermentation), enzymatic, and chemo-enzymatic routes (Craft and Townsend 2017; Fang et al.

2018; Prudden et al. 2017). Currently, biocatalytic methods are considered the most efficient in terms of HMO production yields reviewed by Pérez-Escalante et al. (2020). Only 2′-FL and LN*n*T are currently commercially available for addition to infant formula despite the existence of over 150 HMO structures. Microbial engineering has recently made it possible to produce these two compounds at industrial scale by fermentation using genetically modified *Escherichia coli* (Bode et al. 2016; Bych et al. 2019). Recent commercialization and regulatory approval of synthesized HMOs have now paved the way for expanding the HMO portfolio as future ingredients in foods other than infant formula. 2′-FL was an obvious starting point for HMO production given its simple structure and as the most abundant HMO in breastmilk (Thurl et al. 2017). In contrast, LN*n*T is less abundant in human milk when compared to lacto-*N*-tetraose (LNT) for example, but is easier to synthesize at large scale (Baumgärtner et al. 2015) and was therefore marketed first. Indeed, for more complex and larger structures, fermentation yields are often low (Sprenger et al. 2017; Faijes et al. 2019). Despite this, 42 HMO structures (including building blocks) have been produced using the cell factory approach to date (Faijes et al. 2019). The regulatory landscape surrounding HMO production was recently summarized by Bych et al. (2019) and Walsh et al. (2020b).

Considering the wide availability of dairy side streams from which oligosaccharides can be isolated, BMOs and GMOs show promise as future therapeutics that could be used to provide HMO-associated health benefits to infants (when breastfeeding is not possible) and adults on a large scale. Studies show that BMOs and GMOs can be isolated and purified from whey, permeate, and mother liquor following lactose crystallization, which provides abundant raw materials for their industrial production (Barile et al. 2009; Martinez-Ferez et al. 2006; Mehra et al. 2014; Wang and Yu 2021). Whey, the liquid part of milk that separates from the curd during cheese production, is a particularly attractive source of oligosaccharides. The average BMO concentration in whey is approximately 0.2 g/L, and similar levels of GMOs are found in goat cheese whey (Bode et al. 2016; Thum et al. 2015). Whey permeate is obtained from the process of whey ultrafiltration and is disposed of or used to produce food-grade lactose by crystallization. Milk oligosaccharides pass through the ultrafiltration membranes, ending up in the whey permeate (Barile et al. 2009; Mehra et al. 2014). In terms of oligosaccharide content in whey permeate, de Moura Bell et al. (2018) found that the concentration of BMOs in bovine whey permeate was approximately 0.21 g/L while Thum et al. (2015) found that the concentration of GMOs in goat whey permeate was approximately 0.2 g/L. As mentioned, the permeate can then be used to produce lactose by crystallization, thus improving the economic value and in turn reducing the lactose content. The liquid that is separated from lactose crystals is known as mother liquor and is usually disposed of in sewage plants or sold as animal feed. Mehra et al. (2014) found that bovine mother liquor contained approximately 170 mg/L of sialyllactose for a similar concentration of lactose (49 g/L) in bovine whey permeate. Moreover, the concentration ratio of sialyllactose/lactose in bovine mother liquor was approximately 2.5- to 3.5-fold more concentrated than that in bovine whey permeate. To the best of our knowledge, concentrations of oligosaccharides in goat mother liquor is

unknown. The enrichment of oligosaccharides from bovine and goat milk was reviewed recently by Quinn et al. (2020a) and Wang and Yu (2021).

Martinez-Ferez et al. (2006) were among the first to describe the use of membrane technology for the isolation of oligosaccharides from domestic animal milk. A two-stage tangential filtration process was used on pasteurized skimmed goat milk. At the end of the process, 80% of the oligosaccharides were obtained in the final retentate. Oliveira et al. (2012) also used ultrafiltration on goat whey to remove proteins and fat globules, and then the ultrafiltered permeate was further processed using a 1 kDa "tight" membrane. The final retentate was fractionated to yield 28 oligosaccharide-rich fractions using preparative-scale molecular size exclusion chromatography. Mehra et al. (2014) concentrated BMOs from mother liquor using membrane filtration. A combination of HPLC and accurate mass spectrometry allowed the identification of optimal processing conditions for the production of kilogram quantities of BMO-enriched powders. Among the BMOs identified in the powder, 18 had high-molecular weights and corresponded in size to the most abundant oligosaccharides present in human milk. Six oligosaccharides identified contained fucose, which until then had rarely been found in bovine milk (Mehra et al. 2014).

A combination of lactose hydrolysis and membrane filtration is more commonly used to isolate oligosaccharides from milk. de Moura Bell et al. (2018) developed a novel pilot-scale approach for the recovery of highly pure oligosaccharides from colostral bovine whey permeate. Given that the concentration of BMOs in colostrum is much higher than in mature milk (Fong et al. 2011; Nakamura et al. 2003), this represents a possible source from which to separate BMOs at large scale. The method is based on the integration of optimized processing conditions that favor maximum lactose hydrolysis and monosaccharide fermentation prior to oligosaccharide concentration using selective membrane filtration. After complete lactose hydrolysis and fermentation of the monosaccharides by yeast, nanofiltration of fermented whey permeate enabled the recovery of 95% of the oligosaccharides at high purity (de Moura Bell et al. 2018). This processing strategy has also been applied to the generation of GMOs at pilot scale with a 75% recovery of oligosaccharides (Aquino et al. 2017). Recently, the enzymatic hydrolysis used in the above study was further optimized by Thum et al. (2019), through maximizing the specificity of the β-galactosidase from *Aspergillus oryzae* which was used for lactose hydrolysis. Overall, processing conditions using temperatures ≤40 °C and an enzyme concentration of ≤0.25% resulted in a higher preservation/formation of GMOs from the whey. Martín-Ortiz et al. (2019) were also successful in the selective removal of lactose, and its constituent monosaccharides, from pooled goat colostrum using a procedure based on the combined use of β-galactosidase from *Kluyveromyces lactis* to hydrolyze lactose and *Saccharomyces cerevisiae* to remove the released galactose and glucose through fermentation.

Sousa et al. (2019) investigated the characterization and concentration of oligosaccharides naturally present in goat cheese whey obtained from two types of goat milk. The goat cheese whey was processed by a two-step cross-flow filtration process. A quadrupole time-of-flight (HILIC UPLC-HDMS-Q-TOF) method was used

to identify and measure oligosaccharides in the samples. A final product with recovery of 63–96% of oligosaccharides was obtained when compared with the original whey. Although membrane filtration is the most commonly investigated technique for producing dairy-derived oligosaccharides at large scale, there has been some recent success using scalable chromatography approaches to produce BMOs from whey streams (Marotta and Hickey, 2018). However, the advantages of membrane technology over chromatographic separation technology include the lower energy cost, easy modification of critical operational parameters, reduction of the processing time, scaling-up, and reduction of environmental pollution (Wang and Yu 2021).

7.14 Concluding Remarks

The valuable effects of HMOs in breastmilk are largely lost to formula-fed infants. Substitution of infant formula with BMOs and GMOs to impart HMO functions is a potential solution, in addition to the benefits already observed by supplementation of formulas with 2′-fucosyllactose. The safety and tolerability of dairy-derived oligosaccharides for human consumption were recently evaluated and showed promising results (Smilowitz et al. 2017). Such studies pave the way for dairy oligosaccharides to be evaluated further in human trials, including in infant formula production. The development and optimization of industrial scale methods to isolate and enrich oligosaccharides from bovine and goat milk will be important going forward if they are to be used as health-promoting ingredients. Knowledge of the genes related to oligosaccharides synthesis and the influence of genetics, environment, breed, parity, diet, and seasonality on their expression should have potential to guide breeding strategies in cows and goats. Such information should allow the production of milk with a higher diversity and concentration of oligosaccharides and ultimately facilitate their large-scale production.

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Chapter 8
Milk Salts: Technological Significance

John A. Lucey and David S. Horne

8.1 Introduction

Mammalian milk contains all the essential components to sustain the growth and development of the newborn suckling. Usually, this is taken to mean the protein, fat, and carbohydrate, but it also applies to the mineral components, the milk salts, including the citrate, phosphate, and chloride salts of H^+, K^+, Na^+, Mg^{2+}, and Ca^{2+}, whether as ions in solution or as colloidal species complexed with the caseins. These minerals are essential for bone growth and development, for efficient cellular function, and for maintaining osmolality with increasing carbohydrate (lactose) synthesis. Like the other components, all these mineral species are there for a purpose, and, until weaning, milk may often be the only source of these essential elements.

There have been a number of reviews on the topic of milk salts (Allen 1931; Pyne 1962; Jenness and Patton 1976; Walstra and Jenness 1984; Holt 1985, 1997; Gaucheron 2005; Fox et al. 2015). In this chapter, the term salts will be used to represent substances that are, or can be, present in milk as low molecular weight ions. This group includes both inorganic and organic (e.g., citrate) substances. We can distinguish between the major salt constituents and trace elements and the latter will not be considered in this chapter. The approximate concentration of milk salts is shown in Table 8.1, which approximates to an ionic strength of around 80 mM (Gaucheron 2010). The milk salts have a crucially important impact on many properties of milk, including the formation and stability of the casein micelles, acid–base buffering, and various colligative properties, as well as their key biological role (i.e., providing nutrition for the new-born). In addition, these salts have a powerful

J. A. Lucey (✉)
Department of Food Science, University of Wisconsin-Madison, Madison, WI, USA
e-mail: jalucey@wisc.edu

D. S. Horne
2 Boghall Farm Steadings, West Lothian, Scotland, UK

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Table 8.1 Approximate salt composition in milk (from various sources)

Cationic	Concentration		Anionic	Concentration	
	mg L^{-1}	mmol kg^{-1}		mg L^{-1}	mmol kg^{-1}
Calcium	1040–1280	26–32	Carbonate (including CO_2)	~200	~2
Magnesium	100–150	4–6	Chloride	780–1200	22–34
Potassium	1210–1680	31–43	Citrate	1320–2080	7–11
Sodium	350–600	17–28	Total phosphorus (PO_4) (all forms)	930–1000	30–32
			Inorganic phosphorus (as PO_4)	1800–2180	19–23
			Sulfate	~100	~1

Table 8.2 Approximate distribution of salts between the colloidal and serum phases in milk (from various sources)

	Colloidal (micellar) (%)	Serum (soluble) %
Calcium	69	31
Chloride	≤5	≥95
Citrate	14	86
Inorganic phosphate	53	47
Magnesium	47	53
Potassium	6	94
Sodium	≤5	≥95

influence on protein stability during processing (e.g., rennet coagulation, heat and alcohol stability), the texture of various types of milk protein gels, cheese texture and functionality, and emulsion stability.

Milk is supersaturated with respect to calcium and phosphate ions, and these ions exist in a *dynamic equilibrium* with undissolved or colloidal forms (there is no true equilibrium for the Ca phosphates but some type of *pseudoequilibrium* that is influenced by several factors, including the presence of caseins). It has been recognized since Hammarsten (1879) that this insoluble Ca phosphate fraction is associated with the casein micelles (at that time the micelles were called the Ca caseinogenate). The Ca and phosphate contents vary in proportion to the casein content of milk since much of the Ca and phosphate are associated with the casein micelles. The partition of salts between the colloidal (micellar) and serum (soluble) phases is shown in Table 8.2 (the distribution between these two phases depends on the environmental conditions, including pH, temperature, concentration, etc.). In the serum phase, milk salts may be present as ion pairs (e.g., anions with cations). The Ca and Mg in milk are present at low concentrations as free ions and some as complexes with citrate and phosphate, as well as around 70% associated with casein micelles (Table 8.3). Part of the insoluble calcium is associated with inorganic phosphate to form colloidal calcium phosphate (CCP), which is solubilized at pH values around 5.0. The remaining insoluble calcium (i.e., not in the serum phase) is associated

Table 8.3 Calculated values for the major forms of calcium and magnesium in milk (mainly adapted from Neville 2005)

Binding species	Concentration (mmol L^{-1})
Calcium	
$[Ca^{2+}]$	2.0
$[CaCit^{-}]$	6.9
$[CaPO_4^{-}]$	0.6
Casein	19.4
α-Lactalbumin	0.5
Magnesium	
$[Mg^{2+}]$	0.8
$[MgCit^{-}]$	2.0
$[MgPO_4^{-}]$	0.3
Casein	1.9

directly with caseins; this has sometimes been referred to as caseinate calcium and is only completely released from casein at pH values 3.5–4.0 (Le Great and Brulé 1993). Both Mg and citrate are present in the colloidal phase, which is remarkable since their concentrations (or activities) are not in excess of solubility (Walstra and Jenness 1984). The concentrations of the main salt components in the serum phase have been reported (Jenness and Koops 1962). Theoretical models have been used to calculate the salt equilibria in models of the milk serum phase (e.g., Holt et al. 1981; Mekmene et al. 2009).

This chapter updates and revises the previous version by Lucey and Horne (2009).

8.2 Methods of Analysis

Ashing (e.g., dry heating in a muffle furnace at >500 °C for several hours) of milk is an approximate method of quantifying the inorganic elements (0.7–0.8% in normal milk but values >1.3% can be found in colostrum). However, organic salts are lost during ashing. Some carbonates are lost during ashing (as CO_2) and some types of carbonates are formed from organic compounds. Phosphates from lipids (i.e., phospholipids) also appear in the ash. The sulfur of proteins is oxidized during incineration and appears as sulfate. Oxidation also results in the formation of metal oxides. Wet ashing involves the use of acids like nitric acid. Ashing is routinely used as a pretreatment (by oxidizing organic matter) step for elemental analysis, as the ash can be dissolved with acid and used for quantification of Ca, Fe, etc., by atomic absorption spectroscopy or inductively coupled plasma spectroscopy. The various techniques used for the analysis of milk salts were described by Fox et al. (2015) and Gaucheron (2010). Measurement of the partition of salts between the colloidal and dissolved forms can be achieved by dialysis, ultrafiltration, and the preparation of rennet whey (Davies and White 1960; de la Fuente et al. 1996) although some

adjustments (e.g., to account for excluded volume effects) must be made with these techniques to calculate the serum concentration.

8.3 Secretion of Milk Salts

The biosynthesis of components in milk and milk secretion have been reviewed many times (e.g., Blackwood and Stirling 1932; Petersen 1944; Linzell and Peaker 1971; Larson 1985; McManaman and Neville 2003; Osorio et al. 2016). The cytoplasm of lactating alveolar cells is filled with numerous mitochondria and an extensive rough endoplasmic reticulum network. In addition, there is a well-developed Golgi apparatus, and secretory vesicles containing casein micelles are present in the apical region of the cell. Epithelial cells are connected to each other through an apical junctional complex composed of adherens and tight-junctional elements that function to inhibit direct paracellular exchange of substances between vascular and milk compartments during lactation (McManaman and Neville 2003).

The secretion of milk salts has been reviewed by Holt (1981, 1985), Neville (2005), and Neville et al. (2020). Lactating mammals must supply large amounts of Ca to the mammary gland where it is transported across mammary epithelial cells and into milk. Calcium transfer into milk can be divided into four main steps (Neville et al. 2020): (a) transfer of calcium across the basolateral membrane from the extracellular fluid; (b) intracellular sequestration of calcium in the endoplasmic reticulum to help maintain free cytosolic calcium in the micromolar range; (c) transfer of calcium into the Golgi and secretory compartments where it binds to proteins, phosphate and citrate; and (d) export of calcium into milk across the apical membrane.

Calcium is pumped from the cytoplasm into the Golgi compartment and enters milk via exocytosis of secretory vesicles from the Golgi compartment with a membrane-associated Ca ATPase mediating the transport (Bingham et al. 1993). Circulating Ca concentration must remain relatively constant, i.e., Ca homeostasis; a number of diseases/conditions occur when this is not the case. Such stability relies on cooperation between several organs, principally the parathyroid glands, the kidneys, the skeleton, and the gut. Several important entities are involved in the feedback loop that regulates Ca fluxes to the mammary gland. These control features include an extracellular Ca-sensing receptor (CaR) and parathyroid hormone-related protein (PTHrP) (VanHouten 2005). Very high concentrations of Ca are transferred from the cytoplasm although the cytoplasmic Ca concentration remains relatively constant (in the μM range). This demand for Ca is associated with transient loss of bone mass (in humans), triggered, in part, by the secretion of PTHrP from the mammary gland into the circulation (Ardeshirpour et al. 2006). The CaR is a G-protein-coupled receptor that signals in response to extracellular Ca^{2+} (Ardeshirpour et al. 2006). It is responsible for coordinating Ca homeostasis by regulating both parathyroid hormone secretion and Ca handling in the renal tubules. Calcium activates basolateral CaRs to stimulate its own transport into milk (VanHouten et al. 2004).

The intracellular Na and K concentrations are established by a Na/K-activated ATPase on the basolateral surface of the secretory cell, and there is a dynamic electrochemical equilibrium of these ions across the apical membrane (Holt 1985).

It has been known for a long time that milk is in osmotic equilibrium with blood, i.e., milk is isotonic with blood (van der Laan 1915). Taylor and Husband (1922) were probably the first to suggest that the quantity of lactose produced by the mammary gland controls the daily volume of the milk. Koestler (1920) used the ratio of lactose and chloride as a method to indicate normal and mastitic (abnormal) milk; the Koestler number = (100 × chlorine %)/lactose %. Normal milk has a Koestler number less than 3, while that of mastitic milk is considerable higher (e.g., 15). One of the first studies of the possible mechanisms involved in the secretion of Ca and phosphate in milk was reported by Wright (1928).

The large amounts of phosphate required by the suckling for normal growth and development are also supplied through the milk in at least three chemical forms, namely free inorganic orthophosphate in solution, colloidal (inorganic) phosphate associated with Ca in micellar Ca phosphate, and the ester (organic) phosphate of the caseins. The major pathway for phosphate secretion into milk was believed to be the Golgi vesicle route by a Na^{+}-P_i co-transport mechanism (Shennan and Peaker 2000). However, Holt (1985) described another mechanism by which phosphate is generated in the Golgi lumen by hydrolysis of UDP during lactose synthesis (Kuhn and White 1977). This uridine-nucleotide cycle involves UDP-galactose and glucose. Within the vesicle, these precursors form UDP and lactose. The UDP cannot cross the vesicle membrane unless hydrolyzed to UMP and inorganic phosphate, both of which can re-enter the cytosol, avoiding product inhibition of lactose synthetase. Thus, it should be noted that there is a lot of phosphate released into the Golgi when lactose is synthesized from glucose and UDP-galactose in the Golgi compartment, and this phosphate could be used to phosphorylate casein early in the casein micelle biosynthesis process.

Citrate concentration in milk varies widely throughout lactation and within individual cows (Banks et al. 1984). In general, citrate levels are higher during the grazing season (Holt and Muir 1979) and during early lactation (Braunschweig and Puhan 1999; Garnsworthy et al. 2006). In studies on the goat, Linzell et al. (1976) found that the mammary epithelium is impermeable to citrate in both directions, suggesting that citrate is synthesized within the secretory cells and released into milk after exocytosis of Golgi vesicles. Citrate has an indirect role in fat synthesis by providing reducing equivalents in the form of NADPH, which are required for de novo synthesis of fatty acids (Faulkner and Peaker 1982). Citrate is in equilibrium with iso-citrate, which is converted to α-ketoglutarate in the production of NADPH. Thus, increased de novo synthesis of fatty acids is predicted to lead to a decrease in citrate concentration. Such a correlation was found in the studies of Banks et al. (1984), who used fat supplements to reduce de novo synthesis of fatty acids in the mammary gland and induce increases in milk citrate concentration and was confirmed in the more recent lactational studies of Garnsworthy et al. (2006). The latter authors found a significant correlation between milk citrate and the amounts of acetate required for chain elongation in de novo fatty acid synthesis.

Any change in the citrate concentration of milk would therefore directly influence the Ca^{2+} concentrations (as citrate readily binds Ca^{2+}), which could influence milk behavior, e.g., its rennet coagulation time. Diet-induced changes in the citrate levels in milk would thus alter Ca^{2+} concentrations, and this type of mechanism could account for at least some of the observed diet-related changes in milk functionality. In grass-based milk production systems, there are also seasonal variations (due to stage of lactation as well as feed effects) in the concentrations of minerals, such as calcium and citrate (O'Brien et al. 1999; Dunshea et al. 2019).

It has been proposed that casein-derived phosphopeptides disrupt tight junction integrity, and precipitously cause milk secretion to dry up, i.e., they may help trigger the involution process (Shamay et al. 2002). Serotonin is another likely candidate to trigger this process. It is known that plasmin activity increases near the end of lactation (Politis et al. 1989), and it is possible that some phosphopeptides are produced by this mechanism. Involution involves remodeling of the mammary gland tissue by various proteases (like plasmin) and other mechanisms.

8.4 Factors Influencing the Milk Salts Equilibria

There are numerous dynamic equilibria between the salts in milk, and changes in many environmental conditions influence these equilibria. Some of these changes occur relatively quickly, but those involving CCP can be slow. Mastitic infections of the udder result in a decrease in the concentrations of Ca^{2+} and K^+ in milk but an increase in the concentrations of Na^+ and Cl^- (due to leakage of these ions into milk from blood where their concentrations are much higher than in milk). It should be noted that, during milking and processing, most CO_2 is lost. The impact of various processing techniques on the milk salt equilibria has been regularly reviewed (Holt 1985; de la Fuente 1998; Gaucheron 2005, 2010).

8.4.1 Temperature

Milk as secreted by the cows probably contains about 20 mg of CO_2 per 100 mL (Jenness and Patton 1976). This gas is rapidly lost, and heating and agitation accelerate this loss. The pH of milk decreases as its temperature increases, although few measurements of pH have or can be made at very high temperatures.

The solubility of Ca phosphates decreases at high temperature and during heating heat-induced CCP is formed, which re-solubilizes when milk is subsequently cooled. Jenness and Patton (1976) gave that approximate reaction as:

$$3Ca^{2+} + 2HPO_4^{2-} \rightarrow Ca_3\left(PO_4\right)_2\left(\text{precipitate}\right) + 2H^+$$

The release of H^+ contributes to the decrease in milk pH observed on heating (with extreme heating, there is also the production of organic acids, principally formic from lactose) (Dalgleish 1989). This heat-induced CCP appears to associate with the existing CCP in casein micelles, possibly by increasing the size of the nanoclusters (Holt 1995). The original equilibrium is mostly restored (slowly) after cooling, but there is some hysteresis. Cooling and holding milk at low temperatures result in an increase in the solubility of Ca phosphate and thus a decrease in the concentration of CCP. The Ca^{2+} activity is also mostly restored if sufficient time is allowed for equilibration (Geerts et al. 1983; Augustin and Clarke 1991). At temperatures ≥40 °C, artificial milk serum buffers (or ultrafiltrate) are prone to precipitation. Caseins are effective stabilizers of CCP and usually prevent precipitation of these salts in milk. The absence of casein from these buffers alters the behavior of salts during heating and irreversible precipitation of Ca phosphate occurs (Holt 1995). The deposits found on the surfaces of ultra-high temperature heat exchangers (known as fouling) are rich in Ca phosphate. There are indications that very severe heat treatments (e.g., 120 °C for 15 min) cause a change in the nature of CCP as indicated by an altered acid–base buffering profile (Lucey et al. 1993a), e.g., to form hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; Visser et al. 1986). There can also be precipitation of Ca phosphate on the heating equipment during severe heating or sterilization, leading to increased fouling, which does not resolubilize upon cooling (Sadeghinezhad et al. 2013). Under these severe heating conditions, caseins are unable to prevent the precipitation of Ca phosphate onto metal surfaces (Holt 1995).

Holding milk at low temperatures causes dissociation of some caseins, especially β-casein (<20% of total β-casein) (Downey and Murphy 1970; Creamer et al. 1977) and some dissolution of CCP (Qvist 1979; Ali et al. 1980). Most of these changes are reversed readily by mild heating, e.g., pasteurization (Qvist 1979).

Freezing of milk is sometimes practiced where milk production is seasonal, e.g., goat and ewe milk for cheesemaking (Wendorff 2001). Freezing and thawing for a sufficient time results in the reversal of most of the changes in the salt equilibrium that may have been caused during freezing. Long-term storage (after several months) of milk at ≤−15 °C can result in protein precipitation (due to the low pH and elevated ionic calcium levels). Ovine and caprine milk stored frozen for a few months had similar levels after thawing of soluble Ca, Mg, and P as in the unfrozen milk (de la Fuente et al. 1997).

8.4.2 pH

Acidification solubilizes CCP, which is an integral part of casein micelles. The extent of solubilization increases markedly below pH 5.6 and is complete at approximately pH 5.0 (Pyne and McGann 1960; Brule et al. 1974; Pierre et al. 1983; van Hooydonk et al. 1986; Dalgleish and Law 1989; Mariette et al. 1993). The pH at which CCP is completely solubilized presumably varies with the conditions (e.g., rate and temperature) of acidification, due to the (slow) kinetics of CCP

solubilization. At pH values <5, milk is unsaturated with respect to most types of calcium phosphates (Lyster 1979). At high pH values (≥6), concentrated milk products (e.g., condensed milk) have an increased likelihood of precipitation of some type of Ca phosphate, especially during heating. Increasing the pH of milk results in the formation of additional CCP. McGann and Pyne (1960) described a method for increasing the CCP content of milk (by up to 200%). Milk pH is increased by the addition of NaOH at about 0 °C followed by exhaustive dialysis against a large excess of the original milk. Ozcan et al. (2011) proposed that, as the pH is increased, the serine phosphates become more negatively charged and less inclined to associate with the CCP. The impact of those changes could be some dissociation of the micelle as well as further precipitation of Ca phosphate causing growth of the nanoclusters, before the dialysis with normal milk restores the original pH value (thereby restoring the calcium-binding activity of the serine phosphate).

Lucey et al. (1996) studied the impact of (cold) acidification and neutralization of milk on the properties of casein micelles. Acidification of milk to pH 5.0 or 4.6, followed by neutralization to pH 6.6, resulted in a reduction in the buffering maximum of milk at pH ~5.1; this buffering peak is caused by the solubilization of CCP. The reduced buffering in reformed (acidified and then neutralized) milk suggests that little reformation of CCP occurs on neutralization; reformed milks also had an elevated Ca^{2+} activity. Acidification of milk to pH >5.5, followed by neutralization to pH 6.6, only slightly reduced buffering (at pH ~5.1), suggesting that either little CCP dissolved on acidification in that pH range or that reformation of CCP occurred on neutralization. Canabady-Rochelle et al. (2007) also reported that milk had a higher soluble Ca level after acidification and neutralization.

Gevaudan et al. (1996) used high-pressure CO_2 to acidify milk reversibly (pH was restored to the original value after depressurization). Acidification to pH ~5 with high-pressure CO_2 resulted in a reduction in the buffering peak at pH ~5.1, but this peak increased during chilled storage of this milk (Raouche et al. 2007).

Heat treatment has little impact on the pH-dependent release of Ca and phosphate from micelles during acidification (Law 1996; Singh et al. 1996).

8.4.3 Concentration of Milk

Concentrating milk by evaporation results in a decrease in milk pH, e.g., a decrease of ~0.3 and 0.5 pH units for 2:1 and 3:1 concentration, respectively (Walstra and Jenness 1984). The $[Ca^{2+}]$ increases with concentration but less than the concentration factor (Walstra and Jenness 1984). Presumably, the slower increase in Ca^{2+} is at least partly due to the formation of additional CCP (even though the pH decreases in evaporated milk). Membrane filtration of milk using either ultrafiltration or microfiltration results in retentates in which CCP is a greater proportion of the total Ca content, as some soluble Ca is lost in the permeate during processing (Lelievre and Lawrence 1988; Srilaorkul et al. 1989; Solanki and Rizvi 2001). In the production of highly concentrated (casein content ≥70%) milk protein powders (e.g., milk

protein concentrates, MPC), extensive diafiltration or washing is required to reduce the lactose content. This extensive washing reduces some of the CCP content and causes some casein dissociation. It is well known that extensive dialysis of casein micelles against water causes dissociation of caseins due to the loss of CCP.

8.4.4 *Effects of Ca Sequestrants (Chelating Agents) and Calcium Addition*

Sequestrants (e.g., citrates and phosphates) combine with polyvalent metal ions (e.g., Ca^{2+} or Mg^{2+}) to form soluble metal complexes. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), are complexes in which the metal ion is bound to two or more atoms of the chelating agent, usually in the form of a ring-type of structure. The addition of sequestrants or chelating agents to milk disrupts casein micelles by reducing the [Ca^{2+}] and CCP content (Munyua and Larsson-Raznikiewicz 1980; Visser et al. 1986; Udabage et al. 2000), which causes casein micelle dissociation (Morr 1967; Gaucheron 2005). Several studies have reported that some of the CCP crosslinks can be removed from micelles without causing a lot of protein dissociation; higher levels of Ca removal caused micellar disintegration (Lin et al. 1972; Griffin et al. 1988).

Removal of Ca from milk using an ion-exchange resin resulted in an increase in pH, a reduction in Ca^{2+}, an increase in ethanol stability, and an increase in the rennet coagulation time (Lin et al. 2006).

When comparing the various types of phosphates, the orthophosphates are relatively poor at complexing Ca. Comparing the ability to complex Ca, phosphates and citrates can be ranked in the following order: long-chain phosphates > tripolyphosphate > pyrophosphate > citrate > orthophosphate (Van Wazer and Callis 1958). Figure 8.1 shows a comparison of the [Ca^{2+}] remaining in solution in equilibrium with a 0.01 M solution of a number of sequestering agents (Van Wazer and Callis 1958). This figure demonstrates the relative complexing abilities of the orthophosphates (weak, more free Ca left in solution) with long-chain polyphosphates (strong, little free Ca left in solution).

In well-defined systems, the relative efficiency of sequestrants can be compared by considering the stability constants (formation constant, equilibrium constant) for a given metal (Furia 1972). In general terms, the stability constant of a metal (e.g., Ca^{2+}) complex can be calculated as follows (Furia 1972):

$$K = \frac{[\mathrm{ML}]}{[M][L]}$$

where M = metal ion, L = ligand (sequestrant, chelating agent), ML = metal complex.

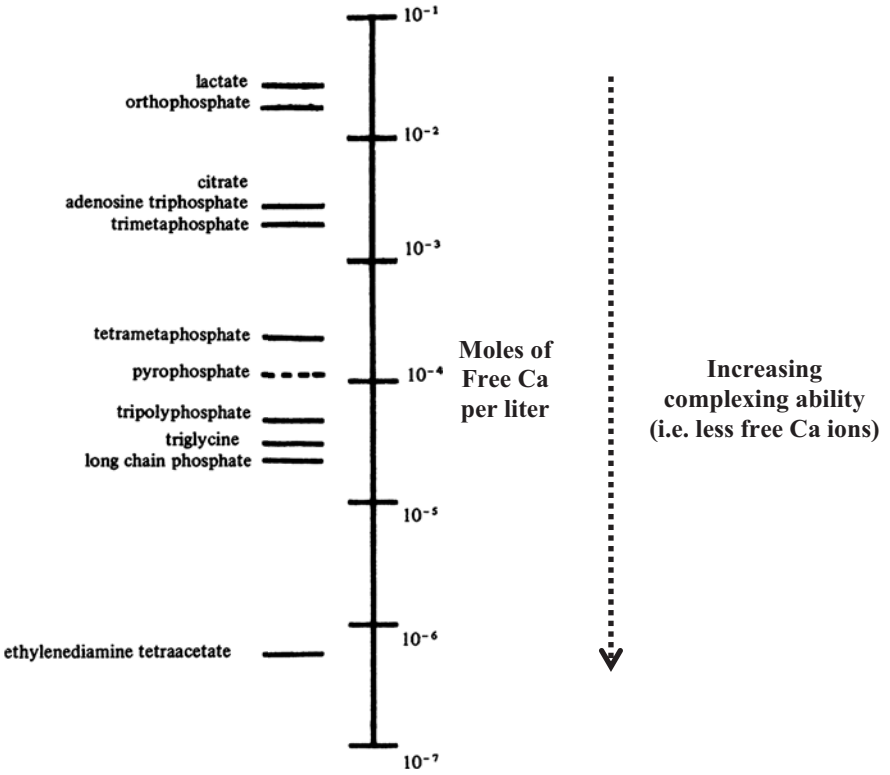

Fig. 8.1 The free calcium concentration (i.e., that not chelated or sequestered) for various types of complexing agents are estimated for the dissociation of a 0.01 M solution of the 1:1 Ca complex. Complexing agents that are lower on the scale (e.g., EDTA) are a stronger chelator for calcium. (Adapted from Van Wazer and Callis 1958)

The (log *K*) stability constants for Ca-chelates with citrate, pyrophosphate, and EDTA are 3.5, 5.0, and 10.7, respectively (Furia 1972). Higher values indicate a stronger tendency to form a complex.

The highly charged anionic nature of polyphosphates causes them to be attracted to, and to orient themselves along, the charged sites of other long-chain polyelectrolytes such as proteins (Van Wazer and Callis 1958). This should increase the charge repulsion between caseins at pH values above their isoelectric point (as in most dairy processing situations). At pH values below the isoelectric point, polyphosphates can induce protein precipitation by cation–anion interactions (Van Wazer and Callis 1958). Casein has been reported to precipitate or aggregate in the presence of phosphates (Fox et al. 1965). Some types of phosphates can crosslink caseins, e.g., pyrophosphates, and these can even induce casein gelation (Mizuno and Lucey 2005, 2007). Phosphate salts have also been used to cause heat-induced aggregation of caseins (Panouillé et al. 2004).

Calcium enrichment of milk is of interest for fortification purposes. Usually this involves adding different types of calcium salts, like those with chloride, lactate, gluconate, or citrate. The impact of calcium addition on milk properties was reviewed by Gaucheron (2010). Excessive levels of calcium addition can cause instability. Insoluble calcium salts like calcium phosphate have the unwanted issue of sedimentation. More common is the use of soluble salts like chloride or lactate. Addition of soluble calcium to milk causes an increase in the insoluble calcium fraction, even though much of the added salt remains in the serum phase; there is also a decrease in milk pH, reduced soluble casein, and an increase in turbidity (Philippe et al. 2003).

8.4.5 High Pressure

High hydrostatic pressure (HP) reduces the light-scattering of milk due to the disruption of casein micelles (Schmidt and Buchheim 1970). HP influences various properties of milk, including a reduction in the size of casein micelles, denaturation of β-lactoglobulin, and a reduction in CCP content (see reviews by Huppertz et al. 2002; López-Fandiño 2006; Munir et al. 2019). HP treatment influences the functional properties of proteins through the disruption of hydrogen bonds and hydrophobic interactions and the separation of ion pairs. The impact on the properties of casein depends not only on the pressure applied but also on factors such as the application time, pH, and temperature. It is well known that HP treatment at ≥300 MPa causes the disintegration of the casein micelles, as observed by a reduction in particle size (Needs et al. 2000; Garcıa-Risco et al. 2003). Micelle size is hardly unaffected, or is slightly increased, by pressures up to 250 MPa (Needs et al. 2000; Huppertz et al. 2004). Concomitant with these size changes, there is dissociation or aggregation (when there is an increase in size) of caseins. Huppertz and de Kruif (2006) proposed that the unfavorable exposure of hydrophobic surfaces at a pressure >200 MPa leads to the formation of larger casein particles from fragments of disrupted casein micelles during prolonged HP treatment. The interactions responsible for this re-association were likely to include van der Waals or hydrophobic interactions.

HP treatment solubilizes some of the CCP in raw (Schrader et al. 1997; López-Fandiño et al. 1998) and heat-treated milk (Gaucheron et al. 1997; Schrader et al. 1997). Some or nearly all of the CCP is restored during subsequent storage of HP-treated milk (Gaucheron et al. 1997: Schrader et al. 1997; Huppertz et al. 2006). Similar trends have been observed for milk of various species, although the magnitude of the changes in the state of the CCP varied (López-Fandiño et al. 1998; Huppertz et al. 2006). Some studies have found hardly any change in the concentration of soluble Ca after HP treatment (Law et al. 1998). It is presumed that, during HP, the solubilization of some of the CCP helps to cause casein micelle disintegration by disrupting one of the key crosslinking agents within micelles. Although pressure release helps to reverse the increase in soluble Ca during pressurization,

the original micelle structure is not reformed (Law et al. 1998). Micellar casein and MPC solutions that were HP-treated exhibited an increase in soluble calcium levels with treatments up to 350 MPa, and thereafter, a slight decrease in soluble calcium was observed (Cadesky et al. 2017).

8.5 Impact of Milk Salts on the Buffering Properties of Milk and Dairy Products

The buffering properties of dairy products have been reviewed by Singh et al. (1997) and Salaün et al. (2005). The affinity of acids and bases for H^+ may be expressed in terms of titration curves and dissociation constants. An acid–base titration curve is a plot of pH versus the amount of acid or base neutralized in the titration. The buffering value (index) at any pH may be determined graphically from the slope of the tangent to the titration curve at that pH. If the added alkali or acid is dB, and the resulting change in pH is dpH, then the average buffering value, i.e., the amount of acid or base required to cause a predetermined change in pH (dpH), is the differential ratio, dB/dpH (Van Slyke 1922), where:

$$\frac{\mathrm{dB}}{dpH} = \frac{\left(\text{ml of acid or base added}\right) \times \left(\text{normality of acid or base}\right)}{\left(\text{average volume of sample}\right) \times \left(pH \text{ change produced}\right)}$$

Apart from casein, the principal buffering components in milk are soluble phosphate, CCP, citrate, and bicarbonate. Srilaorkul et al. (1989) estimated that the contribution of casein, whey proteins and milk salts to the buffering of skim milk was 36.0%, 5.4%, and 58.6%, respectively. Lucey et al. (1993b) reported that, in the pH range 6.7–4.0, soluble salts and whey proteins (i.e., the substances in rennet whey), CCP, and casein contributed approximately 47%, 21%, and 32%, respectively, of the buffering in milk.

When milk is acidified (Fig. 8.2a), maximum buffering occurs at approximately pH 5.1 but, when acidified milk is back-titrated with base, there is low buffering at pH 5.1 and maximum buffering occurs at pH ~ 6.3. The maximum in the buffering curve at pH ~5.1 is due to the solubilization of CCP, which results in the formation of phosphate ions that combine with H^+ (to form HPO_4^{2-} and $H_2PO_4^-$), resulting in pH buffering (Lucey et al. 1993b). The removal of the CCP from milk, as in CCP-free milk made by the method of Pyne and McGann (1960) (cold acidification of milk to pH ~4.9 and extensive dialysis against normal milk to restore the original pH), results in the absence of the buffering peak at pH 5.1 during acid titration (Fig. 8.2b). When the acidified milk sample is back-titrated with base, buffering is low at pH 5.1, because CCP is already solubilized, but maximum buffering occurs

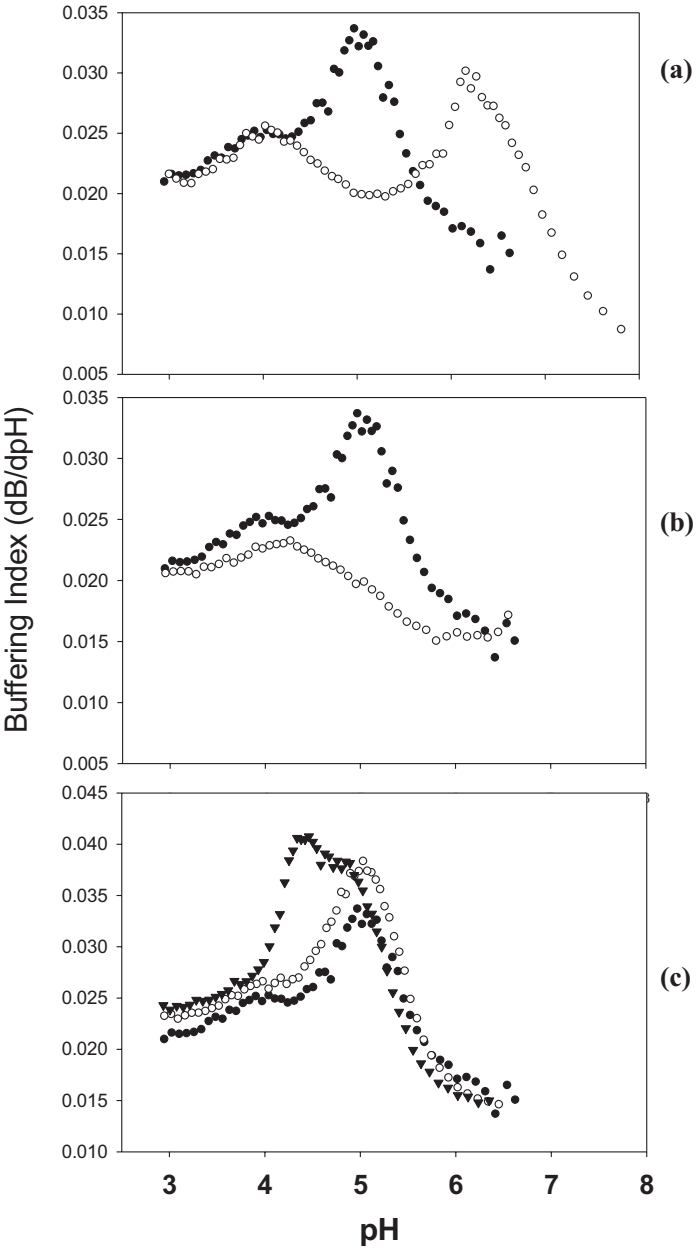

Fig. 8.2 Acid–base buffering curves of (**a**) milk titrated from its initial pH to pH 3.0 with 0.5N HCl (filled circle) and back titrated to pH 8.0 with 0.5N NaOH (open circle); (**b**) milk (filled circle) and colloidal calcium phosphate-free milk (open circle) titrated from the initial pH to pH 3.0 with 0.5N HCl; (**c**) titration of unheated milk (filled circle), milk heated at 100 °C for 10 min (open circle), milk heated at 120 °C for 15 min (filled inverted triangle). Milks were titrated from the initial pH to pH 3.0 with 0.5N HCl. (Adapted from Lucey et al. 1993a, b)

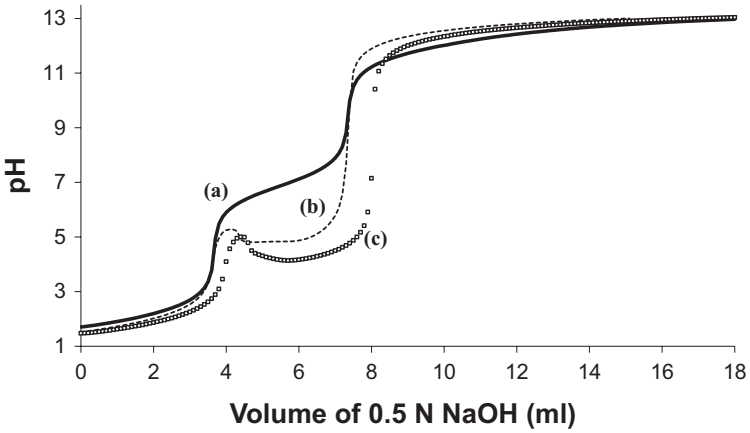

Fig. 8.3 Potentiometric titrations of 400 mg phosphoric acid with 0.5N NaOH in the presence of (a) no calcium, (b) 325 mg $CaCl_2$, (c) 650 mg $CaCl_2$; the method reported by Visser (1962) was used for these titrations (Salim and Lucey, unpublished data)

at pH 6.3, due to the formation of Ca phosphate (precipitation) with the release of H^+ (from HPO_4^{2-} and $H_2PO_4^-$) (Lucey et al. 1993b).

High heat treatments cause an increase in CCP due to the formation of heat-induced CCP (Fig. 8.2c). Some of the heat-induced CCP solubilizes on cooling (depending on the equilibration time allowed), but there is a substantial shift in the type of buffering curve observed during the acidification of very severely heated milk (e.g., 120 °C for 15 min) (Fig. 8.2c).

A strong buffering effect in the pH range 6–7 arises from the formation of Ca phosphate, as can be seen in the titration of phosphoric acid in the presence of Ca (Fig. 8.3). This buffering effect due to precipitation of Ca phosphate has been reported by many investigators, e.g., Visser (1962). Due to the precipitation of Ca phosphate around pH 6, the titration behavior of phosphoric acid in the presence of Ca is completely different compared to when this titration is performed in the absence of Ca (Fig. 8.3). In milk, both Ca and phosphate are present which suggests that this behavior would occur in dairy products. As is shown in Fig. 8.3, the onset of precipitation of Ca phosphate results in the release of H^+. We can speculate that this release of H^+ could also occur during the formation of CCP in the mammary gland and may contribute to the lower pH of milk compared to that of blood.

Acid–base buffering analysis is now widely used to indicate changes in the amount and type of CCP in milk as influenced by various treatments like the addition of chelating salts or heat treatment (e.g., Mizuno and Lucey 2005).

8.6 Interactions Between Milk Salts and Casein

8.6.1 Introduction

Caseins constitute approximately 80% of the protein in bovine milk, with four main types (α_{s1}-, α_{s2}-, β-, and κ-caseins; casein fragments can be produced as a result of proteolysis). Caseins are found in combination with appreciable quantities of micellar or CCP, sometimes called CCP nanoclusters, in the form of aggregates called casein micelles (Holt 1992). Casein plays a critical role in making milk super-saturated with Ca phosphate. As a packaging system, the micelles convert the milk into a free-flowing, low viscosity fluid and provide the means to (safely) transport the high levels of Ca and phosphate at concentrations which would normally precipitate in the mammary gland in the absence of the caseins. The CCP is completely soluble at pH values <5 (Pyne and McGann 1960; Lyster 1979) and the released Ca and phosphate are then available for absorption by the digestive system.

The caseins are a family of phosphoproteins found in the milks of all mammals. They are members of the group of Ca-phosphate-sequestering proteins which include dentine, bone matrix proteins and salivary proteins, among others (Kawasaki and Weiss 2003). Phosphorylation is a posttranslation modification of the caseins, and it occurs at serine residues, or rarely threonine, following a recognized template sequence Ser-X-Y, where X is any amino acid and Y=Glu, SerP or Asp. Due to the placing of the serine residues along the molecular sequences of α_{s1}-, α_{s2}-, and β-caseins, most of the phosphorylated residues are found in clusters. Thus, four of the five phosphorylated serine residues in bovine β-casein are found between positions 15 and 19, with the fifth at position 35. Four of the eight serines in bovine α_{s1}-casein are located between positions 64 and 68, with two more downstream at positions 46 and 48 and one upstream at position 75. Bovine α_{s2}-casein can have a variable level of phosphorylation from 10 to 13 mol P per mole of protein. The most abundant of these, α_{S2}-casein-11P, has three groupings of phosphorylated residues, one cluster of three from residues 8–10, four SerP spread as a group of three from 56 to 58 with the fourth member at 61, with the third cluster of two at positions 129 and 131. The remaining two single Ser-P residues of the total of 11 are located at positions 16 and 135 (Horne 2002). κ-Casein is unique among the caseins due to the absence of phosphoseryl clusters; most molecules of κ-casein contain only one phosphoserine residue, rarely two or three, and all are singlets located in the hydrophilic C-terminal region. The caseins (apart from κ-casein) are therefore sensitive to coagulation or precipitation by Ca. Horne and Dalgleish (1980) demonstrated that the logarithm of this critical coagulation time is a linear function of Q^2, where Q is the net negative charge on the protein, taking into account the binding of Ca to the casein. This linear correlation was also maintained when protein charge was changed following chemical modification of charged residues along the protein chain (Horne 1979, 1983; Horne and Moir 1984).

8.6.2 Casein Micelle Formation

The caseins are sensitive to precipitation due to the presence of approximately 30 mM Ca in milk. However, a key biological purpose of milk is to provide the high concentrations of the essential Ca and phosphate required for the growth of the newborn mammal. So how are these two conflicting factors resolved? The solution involves casein micelle formation, the formation of an insoluble CCP phase within the micelles, and the requirement for one of the caseins (usually κ-casein) to be insensitive to Ca and provide stability against Ca-induced precipitation to the other caseins. We also note that this insoluble CCP phase is never found outside the casein micelle but is an integral part of it. The details of how it forms within on-going micelle synthesis have been the subject of much debate and intensive study. For a discussion of the various casein micelle models, the reader is referred to various reviews (Farrell 1973; Slattery 1976; Rollema 1992; De Kruif and Holt 2003; Farrell et al. 2006; Qi 2007; Fox and Brodkorb 2008). We will focus our explanations on the dual-binding approach for micelle formation as described by Horne (1998, 2002, 2006, 2009), and our perspectives on the nature of casein interactions (Lucey and Horne 2018).

In the dual-binding model (Fig. 8.4), micellar assembly and growth take place by a polymerization process involving two distinct forms of bonding/interactions, namely association through clustering of hydrophobic regions/patches of the caseins

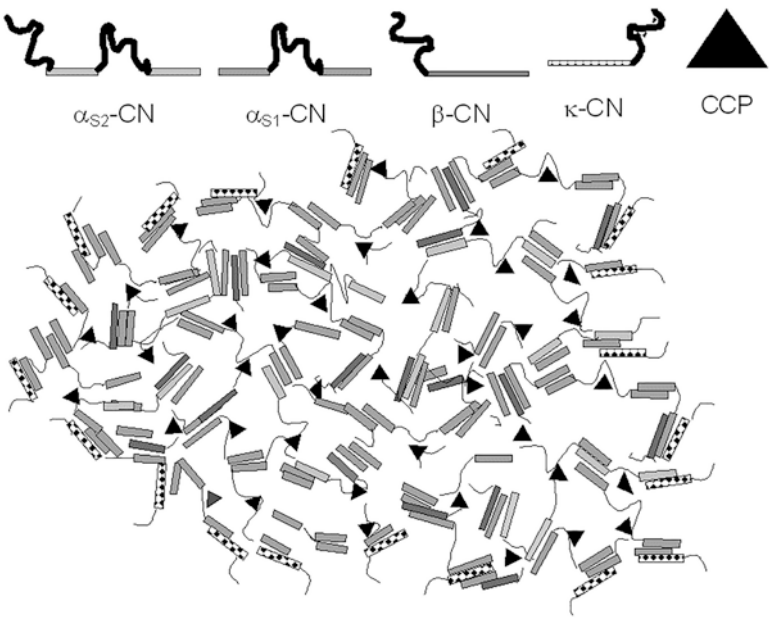

Fig. 8.4 Dual-binding model for the casein micelle. CN is casein and CCP is colloidal calcium phosphate (Horne 1998)

and, secondly, linking of several phosphopeptides into the Ca phosphate nanoclusters (insoluble CCP phase). Central to the model is the concept that bond formation is facilitated and, hence, micellar integrity and stability are maintained, by a local excess of hydrophobic attraction over electrostatic repulsion (otherwise, if the repulsive interactions were too large, little association of casein would occur and micelle formation would not be observed in milk). It should be noted that there are quite different ranges for these interaction components. Compared to hydrophobic interactions, electrostatic repulsion is a long-range force. Clustering of charged groups in specific regions of the protein molecule means that electric dipole moments may be large, so that their effects on interparticle interactions may be rather strong (Piazza 2004).

Each casein molecule effectively functions as a block copolymer, with the hydrophobic region(s) offering the opportunity for a multitude of individual, weak, hydrophobic interactions (driven by the thermodynamically favorable exclusion of water by this type of association). The hydrophilic regions of the casein molecules contain the phosphoserine cluster (or clusters), with the exception of κ-casein which has no such cluster, each offering multiple functionalities for cross-linking. α_{s1}-Casein can polymerize (self-associate) through the hydrophobic blocks, forming a worm-like chain. Further growth is limited by the strong electrostatic repulsion of the hydrophilic regions, but, in the casein micelle, the negative charges of the phosphoserine clusters are neutralized by inserting their phosphate groups into a facet of the Ca phosphate nanocluster.

This has two very important implications for the micelle. Firstly, by removal of a major component of electrostatic repulsion, it increases the propensity for hydrophobic bonding upstream and downstream of the nanocluster link, and thus effectively permits and strengthens those bonds. Secondly, it allows for multiple protein binding to each nanocluster (on different facets), allowing a network to be built up. β-Casein, with only two blocks, a hydrophilic region containing its phosphoserine cluster, and the hydrophobic C-terminal tail, can form polymer links into the network through both, allowing further chain extension through both. α_{s2}-Casein is envisaged in this model as having two of each block, two (possibly three, see below) phosphoserine clusters, and two hydrophobic regions. It is only a small fraction of the total complement of bovine casein but, by being able to sustain growth through all its blocks, it is likely to be bound tightly into the network. α_s-Caseins cannot be essential to micelle formation as human milk contains only trace amounts (~0.06% of total protein) of α_s-caseins (Lönnerdal 2004), and yet micelles are formed. κ-Casein is the most important of the caseins in the dual-binding model of micellar assembly and structure. It can link into the growing chains through its hydrophobic N-terminal block, but its C-terminal block is hydrophilic and cannot sustain growth by linking hydrophobically to another casein molecule. Nor does κ-casein possess a phosphoserine cluster, and therefore, it cannot extend the polymer cluster through a nanocluster link. Thus, chain and network growth are terminated wherever κ-casein joins the chain.

This polymerization process leaves the network with an outer layer dominated by κ-casein although other caseins are also present at, or close to, the surface (Dalgleish 1998). Srinivasan and Lucey (2002) studied the impact of plasmin on the rennet coagulation of skim milk. They found that even partial hydrolysis of β- and α_s-caseins accelerated the rennet coagulation time of milk. Plasmin has very little proteolytic activity against κ-casein. Srinivasan and Lucey (2002) hypothesized that plasmin could have degraded β-casein "hairs" present on the surface of micelles and that this could have reduced the repulsive barrier to aggregation of rennet-altered micelles such that aggregation could occur at a lower degree of κ-casein hydrolysis.

Other evidence that some β-casein may be on, or close to, the micelle surface is that a considerable proportion (up to about 20%) of β-casein can dissociate from the micelle at low temperatures; this also occurs in ovine micelles but to a much lesser extent in porcine milk due to its high CCP content (Umeda 2005; Umeda and Aoki 2005; Umeda et al. 2005). Some CCP also dissolves at low temperatures and that occurrence might also weaken the interactions between β-casein molecules and the rest of the micelle. Low temperature also reduces calcium binding by caseins, thereby enhancing electrostatic repulsion between caseins (Horne and Lucey 2014). How can a considerable proportion of β-casein dissociate at low temperatures while little κ-casein dissociates even though κ-casein is mainly on the surface? It is possible that κ-casein becomes polymerized by S-S bridging between κ-casein molecules (Farrell et al. 1996) after it has terminated the growth of the casein chains. If κ-casein polymers are formed (in vivo), it is likely that these polymers would have greater attachment/linkage to the rest of the micelle structure, making them more difficult to remove. Also, the attractive balance in κ-casein is not very sensitive to changes in phosphoserine involvement in CCP nanoclusters (so the loss of some CCP crosslinks at low temperatures does not have any major impact on its dissociation from the micelle) as they do not interact through CCP crosslinks. In contrast, for β-casein, if some of the CCP crosslinks are dissolved at low temperature, then the exposed negative charge on the phosphoserine residues would make the binding of β-casein to other casein molecules unfavorable. This type of process could allow some of the β-casein to dissociate as temperature is lowered. It is likely that the β-casein that dissociates is closer to the micelle surface, or if not, then the β-casein freed by this process would have some potential chances to re-attach/associate with other caseins as it diffuses through the inner micelle network out to the bulk solution.

κ-Casein-deficient mice, produced by genetic modification, were unable to lactate because of destabilization of the micelles in the lumina of the mammary gland (Shekar et al. 2006). The milk of most species appears to have a κ-casein or Ca-stabilizing casein (i.e., a casein that does not have a phosphate cluster), whereas a few milks contain little or no α_s-caseins (human milk) and various ratios of α_s- to β-caseins.

8.6.3 *Nature of Colloidal Calcium Phosphate and Size of Nanoclusters*

The nature of CCP or micellar Ca phosphate (as it is sometimes called) has been the subject of intense study and debate over the years. There have been several reviews of the nature of CCP (Pyne 1934; McGann and Pyne 1960; Schmidt 1980; van Dijk 1990; Holt 1992, 1995; De Kruif and Holt 2003). Schmidt (1980, 1982) considered CCP to be a ubiquitous coating or "cement" that bonded many casein molecules together. McGann et al. (1983a, b) reported that the CCP depositions in milk systems consist of spherical granules (other later names for this granule include nanoclusters) 2–3 nm in diameter. Such a large entity is incompatible with the small type of CCP structures proposed by van Dijk (1990) or Schmidt (1980) but is smaller than the nanocluster structures of 2.7 nm *radius* recently proposed by Holt et al. (See Bijl et al. 2019, for earlier references and latest developments).

For many years, CCP was believed to a basic Ca phosphate salt (e.g., Pyne and McGann 1960). Pyne and McGann (1960) and McGann et al. (1983a) reported that in CCP, the Ca/P_i ratio is >1.5, which would make it some type of basic salt, like apatite or tricalcium phosphate (e.g., $Ca_3(PO_4)_2$). Citrate and magnesium are also associated with the CCP phase. These studies only consider the inorganic phosphate content of CCP, and, if the ester phosphate content is taken into account, the Ca:P ratio moves closer to the stoichiometry of the mineral brushite (Holt 1997). Evidence that this is maintained across the milks of different species comes from the work of Jenness (1979), who observed linear correlations in plots of total milk calcium and total milk phosphorus versus the casein contents of the milks of 33 species. Assuming a casein monomer in these milks to have a mean molecular weight of 22,500 Da, the slope values of these plots corresponded to 20 Ca^{2+} ions/monomer and 18 mol of micellar phosphate (ester bound + inorganic P)/monomer, that is, close to the 1:1 stoichiometry of dicalcium phosphate. It should be noted that Ca^{2+} ions can also bind to carboxyl groups of the caseins and would then be included as micellar-bound ions in this calculation.

Evidence for the presence of crystalline brushite in the nanoclusters also comes from Holt et al. (1982), who found that the spectrum of milk calcium phosphate obtained by X-ray fine structure absorption was close to that of a brushite sample, but only the earliest peaks in the radial distribution functions could be obtained. Spectra recorded from the lyophilized micelles of pig, goat, rabbit, rat, and human milk samples showed the same short-range environment for Ca and closely resembled that of brushite (Irlam et al. 1985). The lack of longer-range structure in the EXAFS radial distribution functions was taken to imply that the nanoclusters were amorphous, lacking in crystal structure, following the approach of many earlier studies (Pyne and McGann 1960; Knoop et al. 1979; McGann et al. 1983b; Lyster et al. 1984). However, again, these studies only considered basic amorphous calcium phosphates with Ca:P ratios close to 1.5. Lu et al. (2019) carried out a study of the short-range structure of amorphous calcium hydrogen phosphate (ACHP), the acidic salt with Ca:P ratio 1.0. They found the X-ray powder diffraction (XRD)

spectra from these two forms to be typically amorphous and very similar, each with two peaks around the same 2 × theta values (angle between transmitted X-ray beam and the reflected beam), but both quite different from the XRD spectrum of milk CCP published by Wang et al. (2020) which has only one peak and a higher-angle shoulder, both at different 2 × theta values from the Lu et al. (2019) data. Horne et al. (2007) have argued previously that an apparent amorphous XRD spectrum could also be due to the small size of scattering entities, a view upheld by Lenton et al. (2016), who calculated the Ca phosphate XRD spectrum expected as the crystals grew in size, but the spectrum showed an amorphous nature below a 5 nm limit. Most measurements of CCP nanocluster size place the diameter around 2.5 nm (McGann et al. 1983b; McMahon and McManus 1998; Marchin et al. 2007; Kamigaki et al. 2018).

All of these observations are accommodated in the model for nanocluster structure and formation developed by Horne et al. (2007) and refined in Horne (2009, 2014, 2020) and Lucey and Horne (2018). In this model, the ester phosphates of the caseins are viewed as integrated into a brushite crystalline lattice structure. In this, they must have a surface location. We see the formation of nanoclusters as forming via a biomineralization mechanism, with the phosphate centers of the caseins as a facet of the nanocluster first initiating the reaction through the binding of calcium to serine (organic) phosphate. This is stronger than the calcium bond with an isolated (inorganic) phosphate anion and is therefore favored (Mekmene and Gaucheron 2011; Bijl et al. 2019). This structure then accumulates further phosphate and calcium ions in a brushite lattice framework, which is closed off to further growth and completed by other nearby phosphoserine clusters. These clusters can come from the same casein molecule or from other casein molecules in the vicinity. These, in turn, may be part of other phosphate nanoclusters in an extended interlinked network. A minimum of four phosphate centers or facets is envisaged for each nanocluster, giving a tetrahedral structure, though six such facets in a bi-pyramid is another possibility. Such structures preserve the stoichiometry between ester and inorganic phosphate required to allow the Ca:P_i ratio to be 1.5 and Ca:(P_i + P_{Ser}) to be 1.0. Their small size falls within the limits observed in electron microscopy studies and their molecular weights are in the range estimated for excised CCP nanoclusters by Choi et al. (2011).

Holt et al. (see Lenton et al. 2020 for references) provided an alternative mechanism for the creation of calcium phosphate nanoclusters, suggesting that the phosphate centers of the caseins inhibit the growth of a core of nucleating mineral calcium phosphate. The latest update of this concept has calcium ions binding to the phosphoserines before sequestering the mineral nuclei (Bijl et al. 2019) While this recognizes that the calcium binding to the serine phosphate is stronger than the bond to inorganic phosphate (Bijl et al. 2019; Mekmene and Gaucheron 2011), it raises the question of where the system finds the calcium ions necessary to grow the mineral nucleating calcium phosphate core. This is the question persistently raised by Horne (Horne 2006, 2009, 2014, 2020; Horne et al. 2007; Lucey and Horne 2018), all arising from the observation that in mixtures of α_{S1}-casein, phosphate, and calcium precipitate at far higher rates than would be predicted in the absence of protein

(Horne 1982). That the phosphate centers should simply coat a mineral core to form the nanoclusters is akin to suggesting that Shakespeare have the eponymous hero of his tragedy, Hamlet, appear for the first and only time in the last scene of the final act to close the curtains. This argues instead for the active role we have suggested above; growth of the nanoclusters is initiated, controlled and finally terminated by the phosphoserines.

The pK_a values for phosphate reported in chemistry textbooks are 2.1, 6.9, and 12.0, and Walstra and Jenness (1984) suggested that, on comparing the pH of milk (~6.7) with the pK_{a2} of phosphoric acid, one would expect $CaHPO_4$ to be the form of CCP. However, upon addition of NaOH, in the presence of Ca, all phosphates (pK_{a2} and pK_{a3}) are titrated around pH 6–7 due to the precipitation of Ca phosphate (Fig. 8.3). That observation, and the strong buffering at pH ~5.1 during acid titration of milk (Fig. 8.2a) caused by the protonation of the released phosphate ions (from CCP), suggests that the form of CCP in milk is less likely to be an acidic form (like $CaHPO \cdot 2H_2O$) and may be a more basic form (e.g., tricalcium phosphate). Other titration studies, including those with oxalate (Pyne and Ryan 1950; Jenness 1973), have indicated that most P_i in CCP is in the form of PO_4^{3-} (i.e., tricalcium phosphate). Holt (1985) suggested that there may be a difficulty in titration studies if it is assumed that the exposed phosphoserine groups, after the dissolution of CCP, do not contribute to some part of the titration. This suggestion by Holt (1985) does not explain the acid–base buffering behavior shown in Fig. 8.2a, as back-titration with base indicated that CCP does indeed contribute to the buffering at pH ~5. Removal of CCP resulted in the elimination of the buffering peak at pH ~5 (Fig. 8.2b). Any calculation of milk salt equilibria needs to take into account the unexpected pK_{a2} and pK_{a3} values of phosphate in milk/serum, although this does not appear to have always been the case.

8.7 Functional Properties of Milk Products

Milk salts greatly influence the functional properties of milk and various dairy products primarily by influencing the structural integrity of micelles or the sensitivity to aggregation of caseins. There have been a number of reviews on the effects of salts on the functionality of milk products (e.g., Augustin 2000).

8.7.1 Rennet-Induced Gels

Generally, it is thought that Ca does not directly affect the enzymatic phase of rennet gelation of milk, although addition of $CaCl_2$ does reduce milk pH, which accelerates the hydrolysis reaction of rennet on κ-casein (Lucey and Fox 1993). Rennet-altered micelles will aggregate only in the presence of free Ca^{2+}, and gelation occurs only if there is sufficient CCP present (i.e., it needs some type of casein

micellar structure, as sodium caseinate does not form a rennet-induced gel, even though there is release of the macropeptide). Addition of (<50 mM) Ca reduces the rennet gelation time, even at a constant milk pH, and flocculation occurs at a lower degree of κ-casein hydrolysis. Addition of Ca increases the rate of firming of renneted milk gels, mainly by neutralization of the negatively charged groups on the micelle surface and possibly by the formation of Ca bridges. Addition of high concentrations of Ca (e.g., >0.1 M) reduces the rate of gel firming, probably by increasing the effective (positive) surface charge on the micelles. Other alkali earth metals (like Sr and Mg) have similar impacts on rennet gelation to Ca addition (Cooke and McSweeney 2014).

Addition of up to 10 mM Ca increases the strength of rennet-induced gels (Lucey and Fox 1993). Low levels (≤0.02% $CaCl_2$) are often added by cheesemakers to help standardize the gelation process (e.g., cutting time); increased firmness and lower meltability are two other impacts likely in the cheese. Milk with high pH values (e.g., late-lactation, mastitic or non-coagulating individual milks) has poor renneting behavior that can be improved by addition of calcium chloride. Combinations of pH, NaCl, and $CaCl_2$ have been used to cause specific modification in the casein micelles in order to optimize rennet gelation properties (Lazzaro et al. 2020).

Reduction of the CCP content of casein micelles by ~30% prevents gelation unless [Ca^{2+}] is increased (Shalabi and Fox 1982). Udabage et al. (2001) investigated the effects of mineral salts and Ca sequestrants or chelating agents on the gelation of renneted skim milk. They found that, depending on the level of chelating agent, addition of citrate or ethylenediaminetetraacetic acid (EDTA) reduced the storage modulus (G') of rennet-induced gels, and, above a certain concentration, (rennet gelation was completely inhibited 10 mmol kg^{-1} milk).

Choi et al. (2007) demonstrated that the concentration of insoluble Ca phosphate (CCP) associated with the casein micelles had an important influence on the properties of rennet-induced gels. Removal of some CCP from milk prior to gelation using a Ca-chelator lowered the storage modulus of rennet-induced gels due to the reduction in the amount of CCP crosslinking in casein micelles. A reduction in the CCP content prior to rennet-induced gelation resulted in gels with higher loss tangent (LT) values, indicating greater bond mobility.

The swelling, hydration, and solubility of casein micelles in renneted milk are greatly increased in the presence of NaCl but markedly reduced if the brine solution contains Ca (Lucey and Fox 1993). The addition of high concentrations of NaCl causes a reduction in rennet coagulation time. In some cheese varieties, salt is added to the cheesemilk (e.g., Domiati), resulting in a slower set and weaker curd (Fahmi and Shahara 1950). These changes are largely reversible on removal of the excess NaCl by exhaustive dialysis against bulk milk (Huppertz 2007).

Casein concentrates (both liquid and dried forms) made using extensive diafiltration often require calcium addition to have adequate rennet coagulation properties. The diafiltration process removes soluble components, including Ca^{2+}, that are needed for renneting.

8.7.2 Acid-Induced Milk Gels

Acid-induced casein gels can be made from sodium caseinate, indicating that the presence of CCP is not a requirement for the formation of acid casein gels (Lucey et al. 1997). Since CCP is completely soluble at pH ≤5, CCP crosslinks do not contribute to the final (or at least at pH values <5) stiffness of acid milk gels. The rate and extent of CCP solubilization during the gelation process is an important variable influencing acid milk gel properties. In particular, the solubilization of CCP after gelation in acid gels made from heated milk results in an increase in the loss tangent (LT) value (which is the ratio of viscous to elastic moduli). Acid gels made from unheated milk do not exhibit this maximum in the LT behavior, due to their lower pH of gelation, because CCP had mostly dissolved prior to gelation.

The addition of Ca-chelating agents to milk has been reported to increase firmness of acid milk gels made with glucono-δ-lactone (GDL) (Johnston and Murphy 1992). Addition of EDTA also caused an increase in the LT value in acid-heat-induced skim milk gels (Goddard and Augustin 1995). Ozcan-Yilsay et al. (2007) studied the effect of trisodium citrate (TSC) on the rheological, physical properties, and microstructure of set yogurt. The storage modulus of gels increased significantly on addition of low levels of TSC, and highest values were observed in samples with 10–20 mM TSC; higher (>20 mM) TSC concentrations resulted in a large decrease in stiffness and longer gelation times. No maximum in LT was observed in yogurts made with ≥25 mM of TSC as CCP was dissolved completely prior to gelation. Partial removal of CCP resulted in an increase in the LT at pH 5.1. Ozcan-Yilsay et al. (2007) suggested that, at low TSC levels, the removal of CCP crosslinks may have facilitated greater rearrangement and molecular mobility of the micelle structure, which may have helped to increase the storage modulus and LT of gels by increasing the formation of crosslinks between strands. Ozcan-Yilsay et al. (2007) also concluded that the LT maximum observed in yogurts made from heated milk was due to the presence of CCP, as the modification of the CCP content altered this peak and removal of CCP eliminated this feature in the LT profiles. Ramasubramanian et al. (2008) reported that neither calcium addition nor chelation by citrate significantly altered the viscosity of stirred yogurt, although Kamal et al. (2017) indicated that addition of $CaCl_2$ did increase the firmness of acid set gels (made with GDL).

Ozcan et al. (2011) studied the effect of increasing the CCP content of heated milk on yogurt gelation properties; there was no major change in the storage modulus values when the CCP was increased to 116%, but the gelation pH did increase.

Roefs and van Vliet (1990) reported that increasing the concentration of NaCl added to cold-acidified skim milk samples resulted in a decrease in the dynamic moduli of the gels formed when these samples were warmed. This indicated that electrostatic interactions are important for particle interactions. At high ionic strengths, charged groups on casein particles would be screened, thereby weakening interactions between particles, which would result in a slower rate of increase of the storage moduli. In preparing Na caseinate gels by cold acidification, the addition of at least 0.1 M NaCl (to the acidified sample) was necessary to prevent precipitation

during the warming up procedure (Roefs and van Vliet 1990). Possibly, the primary effect of NaCl was to reduce rearrangement during the aggregation stage of gel formation. The addition of a high concentration of NaCl (>0.24 mol L^{-1}) to cold acidified milk prevented gel formation when it was subsequently heated to a higher temperature for gelation (Roefs and van Vliet 1990). Lucey et al. (1997) studied the impact of NaCl on the properties of acid casein gels. They found that the pH at gelation was lower, ≤5.0, in gels made with added NaCl than in gels made without added NaCl (pH ~5.1).

Low-methoxyl pectin is often used as a stabilizer in acid milk gel systems. Harte et al. (2007) proposed that, during the acidification of milk, the release of Ca^{2+} arising from the solubilization of CCP induces the formation of pectin-pectin complexes and, at lower pH values, these complexes interact with the casein particles. For acid casein gels made in the absence of Ca ions, a substantial reduction in the storage modulus was detected at pectin concentrations as low as 0.01–0.02% (w/v), and there was a significant increase in gelation time at pectin concentrations ≥0.05% (w/v) (Matia-Merino et al. 2004). Complete inhibition of acid-induced gelation of casein was noted at ≥0.8% (w/v) pectin. Addition of Ca at low pectin contents (<0.2%) reduced the modulus of acid milk gels but there was a large increase in the storage modulus at higher levels of pectin (≥0.2%, w/v).

8.7.3 Heat-Induced Whey Protein Gels

Salts have a major effect on the type, as well as the mechanical/sensory properties, of whey protein gels formed as a result of heat treatment. It is generally recognized that the addition of $CaCl_2$ to dialyzed samples of whey protein concentrate (WPC) or whey protein isolate (WPI) results in an increase in gel strength. Above a level of 10–20 mM $CaCl_2$, gel firmness starts to decrease (Schmidt et al. 1979; Kuhn and Foegeding 1991). It has been speculated that excessive Ca causes rapid protein aggregation (due to decreased protein stability), which limits protein unfolding and network formation (Mangino 1992). Caussin et al. (2003) reported that the addition of Ca to whey proteins resulted in the formation of very large protein aggregates during heating. Most commercially available WPC products probably have a Ca content that is higher than that required for optimal gel strength (Mangino 1992). There is considerable variability in the thermal aggregation behavior of commercial whey products and some of these differences could be removed by dialysis of these samples to a common ionic strength (McPhail and Holt 1999). The concentrations of divalent cations are higher in WPC made from cheese whey than in WPC made from acid whey, and these cations are not easily removed by dialysis, suggesting some binding by the whey proteins (Havea et al. 2001). Membrane filtration of acid whey WPC at low pH values resulted in a greater extent of demineralization. WPC made from acid whey is a superior heat-gelling product compared with cheese whey WPC (Veith and Reynolds 2004). These differences could be due to the absence of GMP and the low Ca concentration in acid whey WPC. Adjusting cheese whey to

low pH values prior to filtration/diafiltration can also cause flocculation of residual lipoprotein complexes, which has been used as a method to remove residual lipids from whey (Breslau et al. 1975).

8.7.4 Cold-Set Whey Protein Gels

Whey protein gels can also be produced using a two-step process that involves heat treatment at low ionic strength and/or far from the isoelectric point, followed by an increase in ionic strength and/or adjustment of pH (Barbut and Foegeding 1993; Britten and Giroux 2001). These gels are called cold-set gels, as the initial heat treatment produces a polymerized solution and gelation can occur at low temperatures (≤ ambient) if the repulsive forces are screened by the addition of mono- or poly-valent cations (e.g., Ca^{2+}) or a decrease in pH (e.g., through the addition of GDL or bacterial fermentation). To obtain gels via the cold-set gelation method, it is necessary to first prepare a solution of heat-denatured proteins, with a protein concentration below the critical gelation concentration. Heating (e.g., 80 °C for 30 min) results in the formation of soluble, denatured whey protein aggregates. Whey protein fibril-type gels are formed at very low pH values (e.g., 2) and cold-set fibril gels can also be made by the addition of Ca^{2+} (Bolder et al. 2006).

8.7.5 Emulsions

Caseins, especially caseinates, are widely used as emulsifiers (Dickinson 1997). The aggregation state of casein greatly influences surface activity, with sodium caseinate (non-micellar) having greater surface activity than micellar or Ca caseinate (Mulvihill and Murphy 1991). Dalgleish (1987) reported that emulsions prepared with α_s- or β-casein were sensitive to precipitation by Ca but that emulsions prepared with κ-casein did not aggregate on Ca addition. The phosphoserine residues in β-casein helped that molecules maintain a thick steric stabilizing monolayer on emulsion interfaces (Dickinson 1997). Increasing ionic strength by the addition of electrolytes screens out the double-layer repulsion and therefore reduces the electrostatic stabilization of proteins. Therefore, emulsions prepared with commercial milk protein ingredients of high salt content may be more susceptible to flocculation than model systems prepared with pure proteins dissolved in low ionic strength buffer solutions (Dickinson 1997). Calcium ions influence the stability of sodium caseinate-stabilized emulsions (Ye and Singh 2001). Addition of $CaCl_2$ before or after homogenization caused a decrease in the creaming stability of emulsions made with 0.5% sodium caseinate. In contrast, addition of $CaCl_2$ up to ~10 mM increased the creaming stability of emulsions made with 3% sodium caseinate, although the stability decreased again >20 mM $CaCl_2$. There was an increase in the surface

protein concentration with an increase in the level of $CaCl_2$, which was due to enhanced adsorption of the α_s-caseins (Ye and Singh 2001).

8.7.6 Foaming and Rehydration Properties After Spray Drying

Milk exhibits improved foam expansion when treated with EDTA (Ward et al. 1997), probably due to disruption of the micellar structure following the chelation of calcium from CCP. The Ca concentration influences the interactions of β-casein at the air–water interface; in the absence of Ca, a weak interfacial gel forms, whereas, with Ca addition, a strong interfacial gel forms quickly (Vessely et al. 2005). Added calcium chloride increases, and calcium-chelating agents decreases, the foam stability of skim milk (Kamath et al. 2011), presumably by altering casein–calcium interactions that contribute to the stabilization of the foam structure. The foamability of reconstituted skim milk powder increased as the NaCl concentration was increased from 0 to 0.8 M due to the gradually increasing dissociation of casein micelles (Zhang et al. 2004). The foamability of whey protein isolate increased when NaCl concentration was increased from 0 to 0.1 M, but decreased at higher NaCl concentrations (Zhang et al. 2004).

The addition of citrate or phosphate solutions to micellar casein suspensions before drying considerably increased rehydration rates, and this was related to the destruction of the micelle structure (Schuck et al. 2002). Water uptake in casein suspensions was improved by adding NaCl during rehydration. The addition of $CaCl_2$ considerably affected micelle organization and led to the formation of insoluble structures during spray drying. Partial demineralization, NaCl addition, or calcium chelating agents have been used to improve the solubility of high protein MPC.

8.7.7 Stability of Caseins

8.7.7.1 Ethanol

The stability of milk to various concentrations of added ethanol has been used as a milk quality index and is important in the production of drinks, such as cream liqueurs. Figure 8.5 is an attempt to illustrate the impact of pH on the ethanol and heat stability of caseins. Low pH values reduce stability, and stability increases sigmoidally with pH. The inflection point (pK) depends on the properties of individual milks. For a more complete description of this profile/behavior, see Horne (2003). Horne and Parker (1981) found that the addition of Ca or Mg to milk samples caused a shift of the ethanol stability (ES)/pH profile to more alkaline pH values. The addition of phosphate or citrate had little or no effect on the ES/pH profile, although addition of EDTA, a stronger sequestrant, caused a shift in the profile to more acidic pH.

The studies of Horne and Parker on ethanol stability, reviewed by Horne (2003), emphasized the role of the inorganic components of the milk serum, reinforcing the conclusions of Sommer and Binney (1923) that salt balance, the excess of Ca and Mg over citrate and phosphate in milk serum, was critical in alcohol-induced coagulation. Decreasing the salt balance ratio thus caused a shift in the ES/pH profile to acidic pH, whereas increasing the salt balance ratio shifted the profile to more alkaline values. The mechanism, proposed by Horne (1987) to explain these observations, suggests that ethanol has two competing effects on the micellar system, destabilization through loss of the hairy layer, and shifts in the Ca phosphate equilibria, first noted by Pierre (1985). If the ethanol promotes the precipitation of Ca phosphate external to the micelle, it would first reduce the concentration of free Ca, reduce the level of caseinate-bound Ca, and disrupt the binding through Ca phosphate nanoclusters. Moderate losses would increase the negative charge on the caseins and increase the thickness of the steric stabilizing layer. The higher the alcohol concentration, the faster and more extensive would be the precipitation of Ca phosphate. The ensuing adjustment in protein charge and conformation, although relatively rapid, still requires a finite response time. Countering these changes are the effects of ethanol as a non-solvent for the proteins, promoting cross-linking and collapse of the hairy layer. When the coagulation reaction occurs faster than the adjustment of charge and conformation resulting from shifts in Ca phosphate equilibria, or the extent of the latter is limited by insufficient ethanol, the aggregation reaction dominates and precipitation of micelles follows.

The origin of the sigmoidal ethanol stability/pH profile (Fig. 8.5) can also be explained through the effect of pH on Ca phosphate precipitation. Increasing pH brings about increased Ca phosphate precipitation, possibly further enhanced by the ethanol, which means that more ethanol is required to precipitate the protein, i.e., to overcome the increased energy barrier being erected following the transfer of Ca phosphate from the nanocluster state. Conversely, decreasing pH acts to diminish the influence of ethanol-induced precipitation of Ca phosphate by titrating away negative charge and reducing electrostatic repulsion between protein species. Other

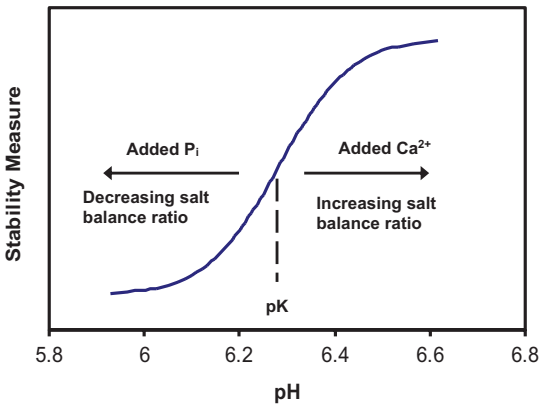

Fig. 8.5 Stability phase diagram of milk as a function of pH or other treatments; schematic, meant to indicate trends for both ethanol and heat stability behavior of milk. (Adapted from Horne 2003)

effects of milk serum composition, of forewarming the milk, and of modifying milk concentration and ionic strength can all be explained in similar fashion (Horne 2003).

Tsioulpas et al. (2007) reported that there is an inverse nonlinear relationship between free Ca ion concentration and ethanol stability ($r = 0.84$), confirming the earlier observations of Davies and White (1958). Citrate found naturally in milk acts as a stabilizing factor, as it slightly improved milk stability (Tsioulpas et al. 2007). Ethanol stability values for milks during lactation were reported to have a mean value of 83.2 ± 12.6% (range 62–100%) (Tsioulpas et al. 2007). Chavez et al. (2004) found that ethanol stability was positively correlated with the concentrations of chloride, potassium and ionic Ca in milk. Horne and Parker (1983) reported that the addition of NaCl reduced the ethanol stability of unconcentrated milk, primarily at pH > 6.5, which they suggested is a result of increased ionic strength. O'Kennedy et al. (2001) demonstrated that α_{s1}- and β-caseins were only minor components of the ethanol-induced precipitate, whereas α_{s2}- and κ-casein were the main proteins susceptible to aggregation.

8.7.7.2 Heat

The heat stability of milk has been regularly and extensively reviewed (Fox and Morrissey 1977; Singh and Creamer 1992; O'Connell and Fox 2003; Singh 2004; Dumpler et al. 2020). Older literature was reviewed by Pyne (1962). The effects of raising temperature on the status of the various Ca phosphate species have already been discussed above (Sect. 8.4.1). Heat stability is the ability of milk or concentrates to resist severe heat treatments without thickening, gelation, or coagulation (Augustin 2000).

Discussions on heat stability are complicated by the knowledge that heat-induced coagulation as a function of pH can follow two distinct profiles. In a Type A heat coagulation time (HCT) vs. pH profile, the time to induce coagulation at a fixed temperature first increases with pH, then enters a minimum before stability increases again at more alkaline pH values. In the Type B profile, heat coagulation time increases progressively with pH. Individual milks which follow type A behavior predominate in most countries, while all bulk milks show type A behavior (O'Connell and Fox 2003). When milk is heated, several competitive and often interdependent reactions occur, not all of them directly involving the milk salts. Fox (1981) listed a selection of these, but it is now generally agreed that the presence of the minimum in a type A profile is associated with the heat-induced formation of a complex between β-lactoglobulin and κ-casein. Such chemical reactions are outside the scope of this chapter and are covered in the reviews of Fox and Morrissey (1977), Singh and Creamer (1992), O'Connell and Fox (2003), and Singh (2004). However, milks showing type A characteristics can be converted into type B profiles and vice versa. For a list of methods and a discussion of these observations, see Horne and Muir (1990).

Interestingly, several of these methods involve manipulating the levels of milk salts, particularly Ca and phosphate. For many years, it was considered that

differences in the heat stability of milk were due to variations in the composition of milk salts, and this led Sommer and Hart (1919) to propose the salt balance theory referred to above in our discussion of ethanol stability. O'Connell and Fox (2003) have suggested that subsequent attempts to correlate heat stability with natural variations in the composition of milk salts are due to the original studies being based on deliberate additions of salts to milk at levels outside natural variability. This overlooks the fact that the experiments of Sommer and co-workers employed a different protocol for the heat stability assay, namely a measurement of the heat coagulation temperature, the temperature at which milk instantaneously coagulates (i.e., effectively coagulates within a short time, <2 min). Because this is a measure of instantaneous coagulation, it is unaffected by changes that occur on prolonged heating. Instead, the response to changing pH, as observed by Miller and Sommer (1940), is remarkably similar to the sigmoidal ethanol stability/pH profile. Moreover, the addition of Ca shifts this profile to more alkaline values while the addition of phosphate has the opposite effect of producing an acidic shift, just like the response of ethanol stability profiles. Horne and Muir (1990) suggested that such behavior indicated that heat-induced coagulation as measured by this assay might follow a similar, if not identical, pathway to alcohol-induced coagulation as described above, involving the precipitation of Ca phosphate and a decrease in Ca activity with increasing pH. Such a scenario also ties in with the observation that the amount of free Ca^{2+} has been associated by various authors with the heat stability of milk, powdered milk, and recombined milk (Augustin and Clarke 1990; Singh and Creamer 1992; Williams et al. 2005). Addition of Ca to milk results in a decrease in heat stability due to the increase in free [Ca^{2+}] (Philippe et al. 2004). Seasonal changes in milk salts (soluble Ca) have been correlated with changes in the heat stability of milk (Kelly et al. 1982). Salts, such as orthophosphates, are often added to milk concentrates (or ultra-high temperature sterilized milks) during processing to improve heat stability. Orthophosphates reduce the Ca^{2+} activity, which is mainly responsible for the improved heat stability (Augustin and Clarke 1990). O'Connell and Fox (2001) suggested that heat-induced precipitation of CCP is involved in the thermal coagulation of milk and that the specific effect of β-lactoglobulin at the pH of maximum stability may be related to its ability to chelate Ca.

Crowley et al. (2014) reported that Ca-ion activity of MPC suspensions increased with increasing protein content of MPC powers. During the manufacture of high-protein MPC powders, the extensive diafiltration involved removed many soluble salts including citrates. When the MPC is reconstituted into water, some CCP dissolves into the serum phase, but a higher proportion of serum Ca is in the form of ionic calcium due to the reduced concentration of anions available to form soluble complexes. Crowley et al. (2014) also reported that, at pH < 6.8, the heat stability of MPC suspensions decreased with increasing protein content of the MPC powders, due to the high Ca-ion activity (which could also help explain the protein aggregation and increasing insolubility observed during powder storage).

8.7.8 Cheese Texture and Functionality

The importance of calcium and phosphate interactions for cheese manufacturing properties, as well as textural properties, has been reviewed (Lucey and Fox 1993; McMahon and Oberg 1998; Lucey et al. 2003; Johnson and Lucey 2006). Processed cheese manufacture is based on the use of citrate or phosphate salts to sequester some of the Ca from the residual CCP, which solubilizes caseins that can then emulsify the released fat. The acidity of whey at drainage and rate of acid development are recognized as important parameters that determine the mineral content, acidity, and quality of cheese. Schulz (1952) developed a classification of cheese varieties based on their Ca contents. Monib (1962) was one of the first investigators to study the Ca phosphate–casein complex in cheese and concluded that very dilute cheese extracts did not represent cheese-like conditions and that their use would lead to incorrect conclusions about serum Ca concentrations (i.e., excessive dilution resulted in the dissolution of more insoluble Ca).

By the 1980s, it was recognized that acid development during manufacture determines the overall loss of Ca, which determines the basic structure of cheese (e.g., Lawrence et al. 1983). In the early 1990s, it became accepted that much of the residual Ca in cheese is associated with casein and that much of the CCP was not dissolved during cheesemaking (Lucey and Fox 1993). Previously, it was thought that almost all the CCP in cheese had dissolved, at least in most cheeses due to their low pH values (<5.3) since by this pH most of the CCP in milk is dissolved. It was also recognized that the insoluble Ca component is an important structural unit influencing cheese texture (Lucey and Fox 1993). Many subsequent studies have demonstrated the importance of pH and Ca content on the functional properties of cheese (e.g., Yun et al. 1993; Guinee et al. 2002; Joshi et al. 2002). It is now accepted that, during ripening, there are important changes in the amount of insoluble Ca (e.g., Guo and Kindstedt 1995; Hassan et al. 2004) and that these shifts in the Ca equilibrium contribute to textural changes during ripening (Lucey et al. 2005; O'Mahony et al. 2005). These initial (first few weeks) textural changes include increased fusion of milled curd, reduced rigidity ("curdiness"), and increased meltability. The proportion of insoluble Ca in cheese has been estimated by the expression of some of the aqueous phase ("juice") under high hydraulic pressure (Morris et al. 1988; Lucey and Fox 1993), centrifugation to extract some expressible serum in young high-moisture cheeses (Guo and Kindstedt 1995), measurement of acid–base buffering (Lucey and Fox 1993; Hassan et al. 2004) and water extraction methods (Metzger et al. 2001).

8.8 Other Uses/Applications of Milk Salts

Milk minerals (typical composition: <5% protein, <9% lactose, >70% ash, 25% Ca, 14% phosphorus) are produced by concentrating, alkalization to precipitate calcium and phosphates, and drying of de-proteinized whey. Acid whey contains higher

levels of milk salts like calcium and phosphate, making it an attractive starting material for the manufacture of milk minerals. Liquid–solid hydrocylones have been recently explored to remove the larger, more abrasive calcium phosphate precipitates often formed during the alkalization step (Crowley et al. 2019). This ingredient is often used for mineral fortification purposes in a range of food products. Milk minerals (or permeate powders) have a salty taste and have been used as a replacement for NaCl in foods.

A number of biologically active peptides are released during digestive breakdown of caseins, and they play a physiological role in newborn mammals (Kitts 2006). Casein phosphopeptides (CPPs) are resistant to further hydrolysis by mammalian digestive enzymes and accumulate in the small intestine. CPPs render Ca^{2+} in a relatively soluble form for potentially enhanced bioavailability by paracellular (passive) mechanisms. CPPs are produced commercially by a number of dairy companies and used as nutritional ingredients to enhance mineral absorption as well as provide anticariogenic benefits (Reynolds 1999; Tsuchita et al. 2001).

8.9 Concluding Remarks

Milk salts play a critical role in the formation and stability of casein micelles. They influence many of the important functional properties of dairy products, including gelation, protein stability, emulsification, foaming, and cheese texture. The concentration of milk salts can be varied by processing conditions including acidification or the addition of metal chelators/sequestrants. The nature and structure of CCP are still being investigated, but the main features are known. The manipulation of the amount of insoluble Ca in cheese is the major focus of ongoing studies related to controlling cheese performance. There is growing awareness of the nutritional benefits of Ca and P, which has resulted in the fortification of dairy products like dairy beverages, and cheese with Ca.

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Chapter 9
Partitioning Milk Constituents

M. J. Lewis

9.1 Introduction

Milk is one of nature's miracle creations. It is a colloidal system produced by mammals which has evolved to provide essential nutrients for their newborn. Milk comprises casein micelles and fat globules, dispersed in a soluble aqueous phase containing globular proteins, including soluble casein, whey proteins and enzymes, a whole host of smaller components which are the subject of this volume.

Relevant questions include where the components that are naturally present in milk reside and where will components that are added to milk, for whatever reason, will come to reside in this complex colloidal system.

In the main, there are three major possible locations. The first is within, or associated with, the casein micelle. The second is associated with fat globule, including the fat globule membrane material. The final location is in the soluble phase of milk. Often the boundaries between these locations are not so distinct, as is discussed below.

Milk is either drunk as liquid milk or converted into a wide range of products. When liquid milk is consumed its pH will fall quickly from about 6.7 to around 3.0 when it enters the stomach. Thus, it is quickly pushed through its isoelectric point of 4.6, into a pH region that is much less studied and where it is much less certain how specific components will partition.

A more gradual reduction in pH also occurs during the manufacture of a number of fermented milk products and this will also affect where some key components in milk become located. In cheese manufacture the whey protein is separated from the casein and liquid whey is removed.

M. J. Lewis (✉)
Department of Food and Nutritional Sciences, University of Reading, Reading, UK
e-mail: m.j.lewis@reading.ac.uk; http://www-dairy-solutions.com/

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This chapter will consider how the different constituents of milk associate with the casein micelles and, to a lesser extent, with the milk fat globules. Thus, the casein micelle is the starting point for further discussions.

9.1.1 The Casein Micelle

The reader is referred to chapters dealing with the casein micelle in these volumes (Huppertz 2013; McMahon and Oommen 2013), so this will be brief and pertinent to partitioning. The individual caseins in cows' milk are α_{s1}-, α_{s2}-, β- and κ-caseins. Their properties, relevant to this chapter, are summarised in Table 9.1. For modelling purposes, their concentrations are presented in molar terms. In some instances, where predictions from models are compared against experimental results, assumptions may be made about the breakdown of casein fractions, where data are only available for total casein in the sample (e.g., Davies and White 1958).

Also relevant is the number of ion binding sites of these casein fractions. Through these sites, the calcium and phosphate complex binds caseins together to form casein micelles, which have a range of particle sizes. Why this is so is not clear but has most recent being discussed by Holt (2021). κ-Casein exists predominantly on the surface and the micelle has a negative charge at the natural pH of milk.

Milk salts are partitioned between the casein micelles, where they are mostly in the form of nanoclusters of an amorphous calcium phosphate, sequestered by caseins through their phosphorylated residue, with the remainder in the continuous or soluble phase. According to the calcium phosphate nanocluster model, a typical micelle of 100 nm radius contains about 800 calcium nanoclusters, each of 60 kDa mass and radius 2.4 nm with an average spacing of about 18 nm between nearest neighbours. Each nanocluster is surrounded by a sequestering shell of the phosphorylated regions of the caseins (Bijl et al. 2019a). Most phosphorylated residues are close together in the casein sequences forming what are known as phosphate centres.

What is relevant for this discussion is how both the main minerals in milk and the caseins partition between the micellar and the soluble phases, bearing in mind that a small amount of casein exists in the soluble phase, along with other nitrogenous

Table 9.1 Information on main casein fractions of bovine milk, required for modelling studies

	Mol wt. (Da)	Strong binding sites	Mole fraction[a]
α_{s1}	23,623	2	0.378
α_{s2}	25,270	2	0.081
β-casein	23,983	1	0.355
κ-casein	19,280	–	0.147

[a] Mid-lactation: note there is also a very small amount of γ-casein which is produced by plasmin action from β-casein

components including the whey proteins and many of the over 60 indigenous enzymes in milk.

An important biological consideration and the primary function of the micelle is to provide a nutrient source that is rich in protein and minerals, but which also prevents the precipitation of calcium phosphate, especially within the mammary glands, where it would be disastrous for the health and welfare of the animal. This would apply to all milk from all lactating mammals.

The aim of many processing operations is to minimise precipitation of calcium phosphate, but also to keep the micelles apart or at least from causing excessive aggregation. Problems will arise if too much aggregation occurs, for example, in heating, and these problems become more noticeable in concentrated milk.

However, during the manufacture of cheese, casein products and fermented milk, aggregation of the micelles is encouraged. In practice, every batch of milk destined for processing will have a different susceptibility to coagulation. For cheesemaking it would be logical to use milk which is more susceptible to coagulation and to use milk which is not susceptible to coagulation to other products, such as UHT milk or milk powder (Leitner et al. 2011; Chen 2014). The best use for milk is an interesting and challenging concept (Chen et al. 2017a, b). In fact, we are moving toward a situation where it is possible to separate milk from individual cows into two batches, based on their coagulation properties (Leitner et al. 2011).

Looking at any of the models that have been proposed of the casein micelle, one might imagine that it is a static structure. However, this is far from being the case. Some examples will illustrate that a more dynamic situation persists.

When milk is heated, calcium phosphate becomes less soluble and it is said to "precipitate" (i.e., move from the soluble phase to the casein micelle) and most of this will associate with the casein micelle. In milk this "precipitation" will not be observed visually, but the pH will also fall as a result of generation of H^+ during this reaction. In contrast, if clear UF permeate from milk collected at 20 °C is heated, calcium phosphate will fall out of solution and the permeate will go cloudy. On cooling, it will go clear again. This cloudy appearance could be mistaken for whey protein being present in the permeate and denaturing on heating. However, when milk (or permeate) cools, the calcium phosphate will resolubilise and the pH will increase. Although calcium phosphate precipitation will occur when milk is heated and redissolve when milk cools, these changes go largely unnoticed in thermal processing of milk. If UF permeate from milk is collected at 80 °C it will also be clear. Also, its pH will hardly change when it cools. No such precipitation will occur until it is heated above 80 °C. This is discussed further in Sect. 9.4.1.

When milk temperature reaches above 65 °C some of the whey proteins will start to denature. They may become attached to the outer surface of the casein micelle or they may form aggregates with other denatured whey proteins or with some undissociated casein, although these aggregates will be much smaller than the casein micelle, depending upon the pH. It is also possible that a small amount of the micellar casein may dissociate from the micelle, most likely those casein molecules near the surface of the micelle. At higher temperatures, for example, in a UHT plant,

these casein micelles might themselves start to aggregate. Note that at 140 °C, both the pH and ionic calcium of the milk will be much lower than at 20 °C (On-Nom 2012).

If the product is homogenised, the surface area of the fat globules is massively increased (Walstra and Jenness 1984; Walstra et al. 2005; Tetrapak 2015; Fellows 2017). This freshly created surface needs to be stabilised, which is done by casein micelles migrating to the surface of the fat globule. This creates an interesting "particle", which is a fat globule in nature that has the surface properties of a casein micelle, and a density probably slightly higher than the soluble phase of milk. These particles may also aggregate on further heat treatment. In this way, some of the fat may end up in the sediment, when heated milk is centrifuged.

Milk is also acidified by a variety of means. Calcium phosphate becomes more soluble as pH is reduced and this then pushes Ca and P into the soluble phase. By the time the pH reaches 4.9–5.0, virtually all Ca and P are soluble, and a large proportion of this calcium is in its ionic form. Note that this is not the case in the soluble phase of cheese (Sect. 9.5.6). It is interesting that what happens at low pH values, for example, in acidified milk drinks, is rarely discussed.

The micelle will become "looser" as a result of losing Ca and P and its surface charge will increase and become less negative. Aggregation of micelles starts to occur and by the time this stage is reached it is largely reversible. However, if the milk is reduced in pH by a lesser amount, say to pH 6.0, for example, by acid or adding carbon dioxide, and then its pH is restored to its value before acidification, the process is more likely to be reversible. During pH restoration, Ca and P will re-enter the micellar structure, but almost certainly not back to their precise previous location. Thus, such pH adjusted and restored milk may well behave differently, and in some cases may be better suited for manufacture of some milk products, for example, cheese or other fermented products.

Virtually all milk is now cooled as soon as possible after it is expressed. The main events are that calcium phosphate solubility will increase and some Ca and P will also move from the micelle and pH will increase slightly. Another important change is that β-casein dissociation from the micelle increases, but the other casein fractions are not involved. These changes are further discussed in Sect. 9.5.1.

It is also possible to remove calcium from milk and there may be circumstances where it is beneficial to do this. One method is by ion exchange resin (Ranjith et al. 1999; Lin et al. 2015). Since ionic calcium in cow's milk is approximately 2 mM, compared to total calcium of 30 mM, one might expect that only about 6% of the total calcium could be removed. However, much more can be removed, because as ionic calcium is removed from the soluble phase, calcium moves from the micelle to restore the equilibrium. In fact, almost all the calcium in milk can be removed by this procedure. When approximately 60% of the calcium is removed the micelle starts to disintegrate and milk takes on a whey-like appearance. In these processes, calcium is replaced by the exchanging counterions (Na^+ or K^+ or H^+). Using H^+ would lead to a reduction in pH.

Addition of trisodium citrate (TSC) has a similar effect in terms of removing calcium from the micelle, although in this case the total calcium in the system remains the same. As citrate complexes ionic calcium in the soluble phase, calcium

and P move from the micelle and if sufficient is added the micelle will again be disrupted. Some of the micellar casein may also be transferred to the soluble phase. This may affect its heat stability (Sect. 9.5.3) and also its foaming capacity (Kamath et al. 2011).

Thus, there are a numerous examples of components moving to and from the micelle in milk processing operations, as well as some of the micelles coating newly created fat globule surfaces during homogenisation.

9.2 Partitioning of Milk: Practical Methods

9.2.1 Introduction

Milk can be partitioned in a number of ways; partitioning involves in most cases obtaining a soluble or diffusible phase of milk, free from the casein micelles, soluble protein and fat. However, if membranes with a larger pore size are used, some of the whey proteins and soluble casein may be present in this soluble phase.

Partitioning studies aim to establish how minerals (salts) in milk partition between the casein micelles and the soluble phase of milk. As well as milk at its normal pH, this chapter will examine how partitioning changes as milk pH is reduced, as its temperature is increased or as it is concentrated.

There is also an interest in the micelle itself and the relationship between soluble casein and micellar casein and how this relationship affects the structure of the micelle and its technological properties.

Fewer studies have looked at partitioning at high temperature, but those that have been undertaken have brought about some interesting results. Acidified milks are popular in the Far East, but there is also very little information about partitioning of components in the pH region of these products, say between pH 3 and 4.5.

Two widely used membrane techniques for partitioning milk include ultrafiltration and dialysis. If a membrane with a molecular weight cut-off value of about 10 kDa is selected, the resulting permeate will contain all the soluble components. Of special interest will be any of those components which will partially associate with either the protein fraction or the fat fraction. However, much tighter or looser membranes may be used. For membrane processing, this moves from looser UF membranes into the realm of microfiltration and for tighter membranes toward nanofiltration and reverse osmosis.

Another method is to “spin out” the casein micelles by ultracentrifugation. Casein micelles are denser than the soluble phase of milk but will take an infinitely long time to sediment out under normal gravitational forces. An ultracentrifuge will produce a casein micelle pellet and a soluble phase which is free from casein micelles (and fat) but which will contain soluble casein and the whey proteins.

9.2.2 Methods Used for Partitioning Studies

The three main methods are dialysis, ultrafiltration and ultracentrifugation. However, other techniques can be used including rennet coagulation and precipitation methods.

9.2.2.1 Dialysis

Dialysis is the simplest and oldest of the membrane techniques and one which has some interesting applications. It makes use of a semi-permeable membrane and relies on diffusion of components through this membrane, usually into water (diffusate). Time is allowed for the diffusate to come to equilibrium with the milk. It has been much used for partitioning processes (Davies and White 1960; de la Fuente et al. 1996; On-Nom et al. 2010, 2012; Tsikritzi 2011). For partitioning processes, a small amount of water is placed in a sealed dialysis bag and dialysed against a much larger volume of milk. Dialysis membranes with different molecular weight cut-off (MWCO) values are available. Typical values for milk partitioning experimental work are 10,000–20,000 Da, although De la Fuente et al. (1996) used one with a MWCO of 3500 Da.

Low molecular weight components will pass through the membrane until equilibrium is achieved. If the volume of water is low compared to that of milk, this removal of components from the milk will not bring out a fall in its concentration. Once equilibrium has been achieved, the dialysate can be analysed for specific components of interest, which would be any of the low molecular weight components which are not associated with the protein or fat fractions, which would be rejected by the membrane. The components transferring will have a range of sizes from H^+ upwards. Thus, dialysis is a simple technique to perform, although some patience is required as equilibrium may take 48 h at 20 °C. Where dialysis is performed at temperatures that will allow microbial growth, a microbial inhibitor must be added to prevent such microbial activity; depending on which organisms grow, the pH may well be reduced and hence change the equilibrium. Davies and White (1960) used chloroform (0.25 mL/100 mL milk). Sodium azide (0.1%) is widely used but care should be taken as it is highly toxic. Other options are thiomersal at 0.1% or 2-methylisothiazilinone at 0.01%.

It is important to establish how long it takes to achieve equilibrium and two important principles are in play (Davies and White 1960; On-Nom et al. 2010). The first relates to the size of the diffusing component of interest, as diffusion will be slower as molecular size increases. Thus H^+ will be the quickest, so if studies are related to pH measurement, then equilibrium will be achieved more quickly. The next class of components are the simple cations, for example, Na^+, K^+, Ca^{2+} and Mg^{2+}; other components such as lactose and some of the vitamins or undissociated salts will take longer to reach equilibrium. Freezing point depression (FPD) is one useful procedure for indicating how a dialysis process is progressing and whether it

has reached equilibrium. It is not specific for any one component, but it will indicate the totality of the components in the diffusate.

The second principle relates to the temperature of the process, and equilibrium will be achieved much more quickly as the temperature of the dialysis process increases. Typical milk dialysis experiments at 20 °C may take up to 24–48 h, but at 100 °C the time is reduced to a few minutes, especially for the lower molecular weight components.

There is potential to perform dialysis on a whole range of milk-based products. Milk can also be adjusted to different pH values prior to dialysis and dialysis can also be performed at different temperatures.

Dialysis can be done with simple laboratory equipment and many samples can be done at one time. It does not require any pumping or other sophisticated equipment and at the end of the experiment, the dialysates are removed and analysed for components of interest. It can also be used to investigate the formation of Maillard browning compounds (Deeth and Lewis 2017). Figure 9.1 shows milk that has been dialysed whilst being heated from 90 to 120 °C for 1 h; it can clearly be seen that browning in milk increases as the heating temperature increases. Of special note is that some of the brown reaction products appear in the dialysate, so this provides an opportunity of separating them from the milk protein fraction. This dialysis procedure not only generates Maillard reaction products in milk but also separates them from the milk fat and protein in one operation, which might facilitate analysis of the Maillard components. However, some of the higher molecular weight browning components may not be fully recovered in the dialysate.

Dialysis membranes are now available with molecular weight cut-off (MWCO) values of up to 1 MDa. Such a large pore size in principle to distinguish between soluble casein and micellar casein will be discussed further in Sect. 4.1 or to study β-casein dissociation at low temperature. It is more likely as well to detect casein in its monomeric form because of the nature of the dialysis process.

One of the main drawbacks of dialysis is that it cannot be easily scaled up. In addition, a factor that might slow the dialysis process is concentration polarisation,

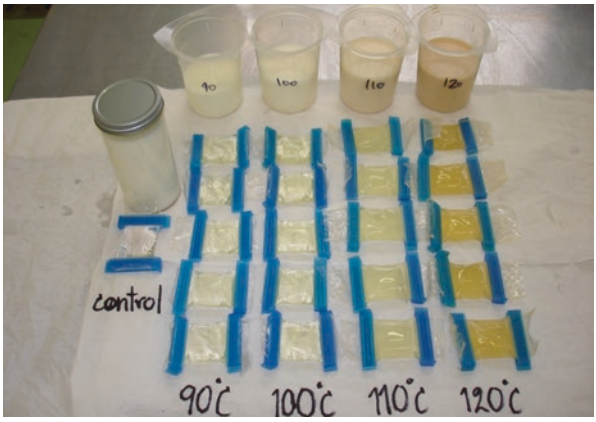

Fig. 9.1 Dialysis of milk at different temperatures, 90–120 °C for 1 h. (From Deeth and Lewis 2017)

which is the build-up of rejected material at the surface of the membrane. Agitation or circulation of the milk will reduce this effect.

Dialysis can also be used in a different mode for removing low molecular weight components from milk. For example, if milk is dialysed against large volume of water, the soluble salts, lactose and other components will be removed from the milk. If we assume that this mineral depletion takes place from the soluble phase of the milk, this in turn may induce some loss of minerals from the casein micelle, as the milk adjusts to the loss of soluble salts and if sufficient mineral (largely colloidal calcium phosphate) is removed, then the micelle may disintegrate. This allows some interesting experiments to be done.

If milk is dialysed against a 4.5% lactose solution, or against a simulated milk ultrafiltrate (SMUF) solution, losses of specifically lactose and mineral salts, respectively, would be minimised.

9.2.2.2 Ultrafiltration

Ultrafiltration (UF) has been widely used for partitioning studies (Rose and Tessier 1959; Pouliot et al. 1989b, c; On-Nom et al. 2010, 2012; Lin et al. 2015). All these investigators have also performed UF at a range of temperatures. Ultrafiltration uses membranes with similar MWCO values to those used for dialysis. In contrast, UF is a pressure-activated process and a UF module is slightly more complicated than a simple dialysis tube and a pump or a source of compressed air is required to apply the pressure. UF is a filtration process, with large components being rejected by the membrane. As for dialysis, membranes with a large range of MWCO values are available but most used in commercial processes are in the range of 10,000–25,000 Da. These will reject all the major whey proteins and caseins in milk. Also, UF membranes are now available with MWCO values up to 300 kDa but these have been less investigated. As part of the membrane spectrum, microfiltration membranes are also available with even larger pores sizes. These tend to be characterised in terms of microns and are used to remove bacteria but also to concentrate casein micelles during the production of micellar casein concentrate.

In contrast to dialysis, ultrafiltration and microfiltration are both widely used in the dairy industry for concentrating milk and whey proteins, for filtering bacteria from milk and for partitioning proteins on a large scale.

However, when ultrafiltration is being used for partitioning studies, it is normally carried out on small benchtop modules or pilot-scale equipment. It is a much quicker process than dialysis, but it may not be possible to do many samples at one time, as each sample will require its own ultrafiltration unit. Disposable centrifugal UF and MF filters are also available.

The material passing through the UF membrane is termed the ultrafiltrate or permeate. In partitioning studies, the volume of permeate collected should be small compared to the volume of milk that is being ultrafiltered, to avoid concentrating the milk. The pH of milk can be adjusted prior to UF and UF itself can be done at different temperatures. However, in contrast to dialysis, ultrafiltration can be scaled up for concentrating milk and whey proteins and it can be operated as a continuous

process. Industrial processes must produce a product which is microbiologically safe and for this reason UF processes usually operate below 5 °C or between 50 and 55 °C, to minimise microbial growth. Although there is a lot of information about UF how temperature affects flux rates, much less attention has been paid to the composition of the soluble phase, as the permeate is largely regarded as a by-product in industry.

A good account of the history of membranes processes has been provided by Price (2013) and an interesting overview on membrane dairy techniques by Pouliot (2008). Recently, UF has been investigated at higher temperatures, for two main reasons. These are to investigate fouling of UF membranes above 60 °C and for looking at milk partitioning at high temperatures. The maximum temperature that has been achieved for UF is 140 °C (On-Nom 2012).

9.2.2.3 Ultracentrifugation (UC)

UC is a laboratory technique which uses a very high centrifugal force to massively accelerate separations. One of its main applications in dairy research has been for spinning out casein micelles. In this sense, any soluble casein and the whey proteins will remain in the serum and the casein micelles will form a pellet.

What happens to the smallest of the micelles will depend upon a number of factors and is not often discussed.

The centrifugal force acting on a particle of mass (*m*) is

$$m\omega^2 r = m\,4\pi^2\,N^2\,r:$$

where ω = angular velocity (rad/s) and N = rev/s (N); note that $\omega = 2\pi N$.

The magnitude of the centrifugal force achieved is important and should always be mentioned in such separations processes. The number of G forces (g) is the ratio of the centrifugal force to the gravitational force.

$(g) = m\omega^2 r/mg = \omega^2 r/G$. For UC, conditions used are in excess of 50,000 g and these should always be recorded to allow experimental work to be replicated. Note that lower-case G = acceleration due to gravity which is 9.81 m/s^2.

Centrifugation as a general method has many uses in milk and dairy processing (Walstra et al. 2005; Tetrapak 2015). It is used for producing skim milk and cream from milk, which is then standardised to produce products such as semi-skim milk and creams with fat contents ranging from 12% to over 50% fat. It is used for removing small amounts of sediment in milk (desludging) and also for removing spores from milk (bactofugation). Centrifugation can also be used for assessing the stability of milk—in terms of emulsion stability and for measuring sediment in heat-treated milk or as an indicator of the long-term stability of products such as UHT milk and creams. In most of the applications above, the centrifugal force is quite low, usually below 10,000 g. Such G forces are not sufficient to separate casein micelles.

Ultracentrifuges are not cheap and feature more in research laboratories than in quality assurance. UC has been much used for partitioning studies and the following provide a selection of its applications: Kitchen (1974), Holt et al. (1986), De la

Fuente et al. (1996), On-Nom (2012), Bijl et al. (2014) and Poulsen et al. (2017). Most researchers interpret the ultracentrifuged supernatant giving an indication of the aqueous phase and any casein which is found in the ultrafiltrate is assumed to be soluble, or diffusible, casein. However, it is not quite so straightforward as this.

UC can be done at different pH values and after adding various components. However, it is more difficult to operate a UC at higher temperatures and the process itself will also generate heat, so it may not be possible to perform the operation at a constant temperature; temperature changes during UC are regrettably rarely reported.

In common with renneting, UC separates whey protein from casein micelles. However, that any whey protein (or mineral) that is associated with the micelle will be removed with the pellet. The separated micelles can be redispersed.

UC has been used as a tool to investigate whether certain factors, for example, heat stability, alcohol stability and renneting are influenced more by the casein fraction or by the soluble fraction. For example, experiments can be performed with milk of good or poor heat stability, by ultracentrifuging each sample and dispersing the casein micelle from one sample into the soluble phase of the other sample and comparing the heat stability, or other properties of the two new samples.

UC is not easy to scale up and thus has not been used as a large-scale process for recovering native casein micelles. UC has also been used to separate the milk fat globular membrane material in skim milk or cheese whey. In skim milk it will be observed as a "fluffy" type material, on top of any pelleted casein (Deeth 2018) (Sect. 9.6.2).

Differential ultracentrifugation has been used by Dalgleish et al. (1989) who produced eight fractions containing particles of different sizes. For each fraction, the contents of whole casein, calcium and inorganic phosphate were determined, and detailed analyses of the casein fraction were made. The results demonstrated the expected increase in the proportion of κ-casein as the micelle size decreased but that the proportion of α_{s1}- and α_{s2}-caseins was size-independent. The contents of calcium and inorganic phosphate were also largely independent of micellar size.

Therefore, pertinent questions to be asked of any study involving UC are (1) what proportion of the casein micelles will be removed and (2) is there a distinct cut-off point in terms of the size of the "particle" which will remain in the supernatant and how are these factors affected by properties such as product viscosity and temperature?

9.2.2.4 Milk Coagulation

Coagulating milk using milk clotting enzymes is another means of partitioning milk and in this case the bulk of the casein is removed as a coagulum, leaving behind whey proteins and other soluble protein, including soluble casein. Chymosin is the main coagulant enzyme used to produce rennet-coagulated cheeses, including hard and semi-hard varieties. Milk coagulation is a fundamental process for producing many dairy products, so it is a widely studied process, but not usually for

partitioning. Factors such as rennet coagulation time and the strength of the gel are important for cheesemaking. One could argue that the resulting whey will give an indication of what is not associated with the casein micelle, but specifically at the conditions used for that making that particular cheese. However, it is not quite so straightforward to interpret the results as cheesemaking is accompanied by a fall in pH which changes the partitioning of many components, especially P and Ca.

In cheesemaking, the temperature of the renneting process is usually fixed more to suit the starter activity, even though the optimum activity of rennet is around 40 °C; hence, the majority of studies on rennet coagulation have been performed at 30 °C (White and Davies 1958c; Tsioulpas et al. 2007), but other temperatures over range at 30–40 °C have been used. Therefore, there is scope for using chymosin to investigate temperature effects of partitioning, over the range 25–45 °C. Above 45 °C the enzyme may start to denature before coagulation is completed. Thus, as a partitioning technique, renneting coagulation has a limited temperature range but it may be the most appropriate method for studies related to the thermodynamic stability of milk, at around body temperature (Sect. 6). It is also possible to look at a range of pH conditions, within the activity range of the enzyme.

Milk coagulation thus offers another means of partitioning milk, although the resulting products have not been extensively analysed with this in mind. It is noteworthy that raw milk and pasteurised milk can be coagulated by rennet, but very rarely will UHT milk be coagulated. UHT processing causes extensive denaturation of β-lactoglobulin which attaches to κ-casein; this and other changes alter conditions on the surface of the micelle which has negative effects on both the first and second stages of rennet coagulation. Although this is a negative consideration as far as cheesemaking is concerned, it is positive for UHT milk in that it removes one of the possible mechanisms for gelation of UHT milk during storage. There are other sources of proteolytic enzymes which will cause gelation of milk, for example, ginger and some fruit enzymes. Also, it may be possible to use other proteases, such as from ginger, which is claimed to have its optimum temperature at 60 °C (Su et al. 2009). Coagulation of milk in general, by enzymes, acid and heat has been discussed (IDF 2007), although the effect of temperature on coagulation is hardly discussed.

Factors affecting rennet coagulation time are of great importance for cheesemaking and have been studied in considerable depth. One of the first studies is that of White and Davies (1958c); others include Tsioulpas et al. (2007) and Poulsen et al. (2017). Chen et al. (2014) measured variations in rennet coagulation time from a bulk herd over a complete year; values ranged from 12 to 24 min.

There are considerable losses of Ca and P from the caseins to the whey fraction during cheesemaking, depending very much on the pH at which the coagulation takes place. Whey protein is also lost. One drawback of the cheesemaking process is that the mineral fraction of milk is not as effectively utilised compared to milk consumption, as not all the calcium (and phosphorus) in milk is recovered in the curd (see Sect. 9.5.6). Nevertheless because of this concentration, cheese as a product is uniquely rich in Ca and P and contains much more of these nutrients compared to milk, per 100 g of product. However, it takes approximately 10 kg of milk

to make 1 kg of Cheddar cheese, leaving 9 kg of whey as a by-product. Therefore, it is important to recover most of these minerals and proteins from whey to ensure that full potential is made of these nutrients in milk. One advantage of renneting compared to membrane techniques is that the Donnan effect does not need to be considered (see Sect. 9.2.3).

9.2.2.5 Precipitation Methods: Isoelectric Precipitation and Salting In/Salting Out

As well as renneting, isoelectric precipitation at pH 4.6 is also an effective means for separating caseins from whey protein and can be used to fractionate the different forms of nitrogen compounds in milk. For example, casein N corresponds to the N fraction of milk that is insoluble in an acetic acid/sodium acetate buffer at pH 4.6 and 20 °C. Non-casein N is the soluble fraction under the same conditions. Non-protein nitrogen (NPN) is the fraction soluble in 12% trichloroacetic acid (TCA), and whey protein N refers to the difference between non-casein N and NPN. Thus, partitioning has been used for a long time for protein and non-protein nitrogen measurements. NMR is now a powerful method for more detailed analysis of the non-protein nitrogen fraction of milk (Foroutan et al. 2019). Although it has been less used as a tool for looking at partitioning of milk salts and it is limited to one pH value, isoelectric precipitation could be a useful technique to investigate temperature effects.

A classical salting out procedure is employed in the turbidity test, sometimes known as the Aschaffenburg test (Aschaffenburg 1950). At one time, this was the required test for ensuring milk was adequately sterilised, by measuring whey protein denaturation. It involves adding 4 g ammonium sulphate to 20 mL milk. This will cause precipitation of casein micelles in milk together with any denatured whey protein, leaving undenatured whey protein in solution. This is then filtered and in the classical test, the filtrate is boiled. Thus, any undenatured whey protein that was in the original milk sample would be present in the filtrate and would go cloudy when it was denatured on boiling. This is a very effective and easy test to do in the laboratory to determine the extent of whey protein denaturation in a milk sample. Sterilised milk had to produce a negative turbidity result. It was later found out some UHT milk also gave a negative turbidity, especially produced by indirect processing with longer heating and cooling values (e.g., high C^* values). Most boiled milk samples would also produce negative turbidity, although boiling at 100 °C would not inactivate bacterial spores.

In effect, the salting out procedure provides another means of removing casein micelles. The resulting filtrate is clear. During my time at Reading University many different milk samples were evaluated by students using the turbidity test and it was found to be very effective for quickly and cheaply determining how much whey protein had been denatured. Occasionally, samples of milk which would not filter would be encountered. The fact that a clear filtrate was produced provides options for making the test more quantitative, for example, by A_{280} measurements or by

HPLC or capillary zone electrophoresis, although one should remember that the filtrate may contain substantial ammonium sulphate, which itself could be removed by dialysis. The turbidity test is discussed in more detail by Burton (1988). Kitchen (1974) made use of a 50% saturated ammonium sulphate solution and dialysis in the preparation of fat globule membrane material, from both cream and skim milk.

Another reagent used for precipitating protein is trichloroacetic acid (TCA). A mixture of 4 parts of 10% TCA to 1 part of milk gave the most complete precipitation and was almost as effective as tungstic acid (Sanders 1933). Two percent TCA is often used as a general protein precipitating agent. Eight percent TCA is useful for preparing GMP as it is soluble at this concentration, unlike other polypeptides and 12% TCA-soluble nitrogen is generally related to small peptides and free amino acids (McSweeney, personal communication). DeVries et al. (2017) recently compared three selected protein precipitation methods, using TCA, tannic acid and ultrafiltration for measuring NPN compounds, as a means of detecting adulterants in milk powders. The most serious NPN adulterant in milk in recent times has been melamine, added to boost the "apparent" protein content of milk. MacMahon et al. (2012) investigated analytical procedures for six other potential NPN contaminants that might be added to protein foods (cyromazine, dicyandiamide, urea, biuret, triuret and amidinourea).

9.2.3 Some Early (Pioneering) Papers on Partitioning

Studies with regard to mineral partitioning most probably started in the 1950s; no reference to dialysis or UF being used for such studies is made in Cronshaw (1947) and Davis (1955). Using TCA and fractionation of N compounds started well before then.

One of the first papers to subject milk to UF and look at the composition of the soluble phase was Rose and Tessier (1959). This investigation was well ahead of its time, as they performed UF experiments up to 93.3 °C but they analysed the resulting permeates when they had cooled down to room temperature. The effects of cooling are discussed in more detail in Sect. 8.1. The authors argued that this procedure would be appropriate for measuring total calcium, phosphate and citrate as they would not be affected by cooling but the interpretation for pH and ionic calcium would be more difficult as both these parameters may change during cooling.

Figure 9.2 shows their reported results for partitioning of milk and concentrated milk at different temperatures and was the first paper to look at mineral partitioning at high temperature. They found that total calcium, ionic calcium and pH all decreased in the permeate as UF temperature increased. This was the case both for skim milk and concentrated milk (×2). Citrate was not included in these figures but was measured and was found hardly to change with UF temperature. They also reported that there was no significant change in the levels of magnesium, sodium or potassium in ultrafiltrate at any temperature studied.

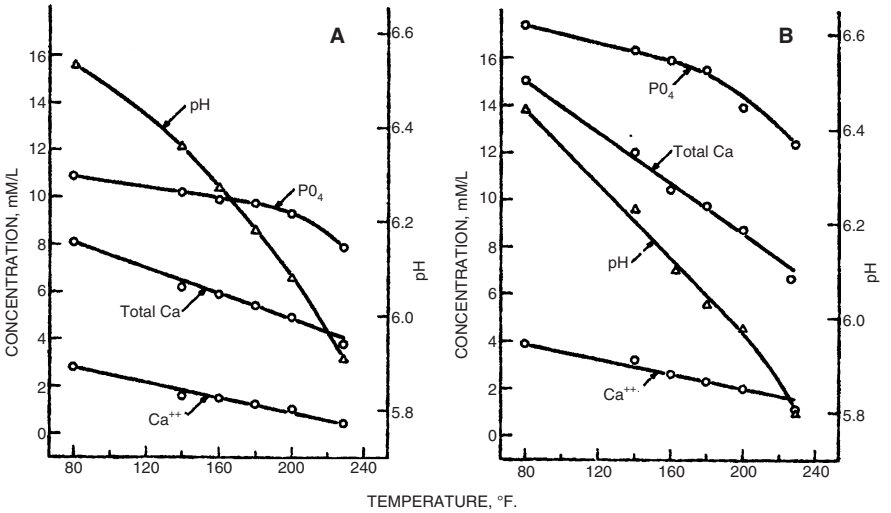

Fig. 9.2 Partitioning of (**a**) skim milk and (**b**) concentrated milk by UF (16.3% TS) at different temperatures. (From Rose and Tessier 1959, with permission)

If it is assumed that pH and ionic calcium do not change during cooling (see Sect. 8.1), then it would suggest that milk pH and ionic calcium in milk both decrease with increasing temperature. Rose and Tessier (1959) also attempted to measure pH and ionic calcium directly at high temperatures, but with less success. These authors further suggested that reactions which gave rise to changes in pH and ionic calcium were fast and that these were mainly attributable to calcium phosphate precipitation and dissolution, rather than to the production of organic acids or other components. There was still a debate about whether ionic calcium would decrease as temperature increases and these results would support that argument. Increasing the temperature of milk induces two changes which have competing effects. The reduction in pH might be expected to increase calcium phosphate solubility, but calcium phosphate becomes less soluble at high temperature, and this appears to predominate, as both soluble calcium and Ca^{2+} were later found to decrease as temperature increases. Nevertheless, Rose (1962) proposed that concentrations of Ca^{2+} would increase with increasing temperature, due mainly to the fall in pH, despite some contradictory evidence reported by Tessier and Rose (1958). The importance of these findings remained largely unnoticed for 30 years, until the studies of Pouliot et al. (1989a, b, c), which are discussed in Sect. 3.2.

Another interesting observation was that partitioning milk at UF at high temperatures seemed to be more informative than subjecting milk to heat treatment and then partitioning the heat-treated milk. In fact, the two experimental approaches gave very different results. Rose and Tessier (1959) also proposed that many of the

processes that took place when milk was heated were reversible on cooling. Misleading results on the effect of high temperature on milk partitioning could well be obtained by partitioning cooled milk.

In fact, one conclusion from studies on heated milk would be that heating and subsequent cooling does not noticeably change pH and levels of ionic calcium, which are two important determinants of heat stability. However, this conceals the reality that both pH and ionic Ca are both much lower at high temperature but revert toward their initial values during cooling, which will be further addressed in Sect. 9.3. This misunderstanding could also make interpretation of factors affecting heat stability more difficult, as Rose and Tessier (1959) pointed out.

Another clever approach was to see whether the properties of milk measured by partitioning at high temperature would correlate with heat stability as measured by the heat coagulation time (HCT) test. This was a very perceptive approach and Rose and Tessier (1959) were the first researchers to investigate this. They collected many different bulk milk samples and milk from some individual cows and subjected them to UF at 26.7 and 93.3 °C. They also determined the heat coagulation time at 140 °C. Unfortunately, none of the attributes determined by UF at 26.7 or 93.3 °C correlated well with heat stability, neither did differences in any of these attributes at the two temperatures. "Grouping the samples in terms of their heat stability failed to indicate any relation between these groups and the composition of the ultrafiltrates"; this must have been a disappointment to the authors.

There are several reasons why no correlations were found:

- HCT itself is not a fool-proof method for measuring heat stability (Singh 2004; Deeth and Lewis 2017).
- HCT is very pH dependent and goes through a maximum and minimum. Correlations may have been found if the maximum and minimum heat coagulations times had been determined.
- HCT was determined at 140 °C, whereas ultrafiltrate was removed at 26.7 and 93.3 °C, so they are not directly comparable.

Table 9.2 shows some results for different samples of bulk milk throughout lactation showing that milk varies in its composition. In each case the range of values found is given for the different measured parameters. The paper also presents similar data for milk from individual cows collected through lactation and would be of interest to those studying individual cows.

Table 9.2 Milk pH, mineral composition and heat stability for bulk milk. Also recorded are pH, total calcium and total phosphates in permeates collected at 26.7 °C (80 °F) and 93.3 °C (200 °F) (from Tessier and Rose 1958)

	Milk				Permeate (°F)					
					80	200	80	200	80	200
	pH	Total Ca	Total PO_4	Heat stability (min)	pH		Total Ca		Total PO_4	
Bulk	6.47–6.63	24.2–31.6	27.2–39.1	8.5–33.3	6.57–6.80	6.03–6.26	7.6–9.0	3.5–5.0	9.9–11.7	7.7–8.8

Rose and Tessier (1959) used the murexide method to measure ionic calcium. This method cannot be used directly on milk and is now rarely used and its limitations are further discussed by Lewis (2011). Values in permeates values ranged from 2.5 to 3.8 mM at 26.7 °C and 1.0 and 1.4 mM at 93.3 °C and are considered to represent the values in milk. It was also interesting that pH values in permeates at 26.7 °C were always higher than those in milk at the same temperature by 0.1–0.2 pH units.

The first paper to compare ultrafiltration and dialysis for partitioning milk was that of Davies and White (1960). Although not the first persons to investigate UF and dialysis for partitioning milk, they were the first to present a detailed comparison of the two methods for obtaining a sample of the aqueous phase of milk. The dialysate was considered to be identical to that of the soluble phase of milk and the results obtained by ultrafiltration were in good agreement with this. They reported that dialysis took 48 h to equilibrate. However, a closer inspection of their data for lactose and ionic calcium showed that these components equilibrated in 24 h. It is likely that pH values would equilibrate in an even shorter time period. Their results also showed some temperature dependence—both pH and ionic calcium were lower at 20 °C than at 4 °C. However, they did not use temperatures higher than 20 °C.

A summary of some of the conclusions of Davies and White (1960) is as follows:

- UF at different pressures had no effect on ionised calcium, pH and lactose.
- UF using different membranes had no effect on pH.
- Storing milk chilled for up to 3 days showed no differences in permeate composition.

Further observations from their dialysis experiments suggested that:

- There was little effect changing milk to water ratio from 25:1 up to 200:1.
- Equilibrium was achieved for many of the components that were analysed after 24 h, when values were compared to those after 48 h.

Table 9.3 shows a comparison of result obtained by UF and dialysis. These authors briefly discussed the Donnan effect which refers to the behaviour of charged particles near a semi-permeable membrane that sometimes fail to distribute evenly across the two sides of the membrane. It is relevant as it might either hinder or promote the transfer of charged particles such as Na^+ K^+, H^+ and Cl^-. Davies and White (1960) proposed that the Donnan effect might have an influence on membrane processes but would not have an influence on ultracentrifugation or renneting. On comparing these different methods, they concluded that for UF treatment of milk there was hardly any observable Donnan effect (see Table 9.4).

The casein micelle has a net negative charge, and membranes being for the partitioning process may also be charged which may also need to be considered, especially as casein micelles will be at a higher concentration adjacent to the membrane surface, resulting from concentration polarisation. Occasionally negative rejections have been reported for charged particles, i.e., their concentration is higher in the permeate than in the feed. The Donnan effect is more likely to be contributory in nanofiltration (NF) than in UF processes.

Table 9.3 A comparison of results for partitioning of minerals in milk by UF and dialysis, using different membrane for UF at 20 °C and different temperatures for dialysis; from Davies and White (1960), with permission

	Ultrafiltrate		Diffusate		
	Cellophane 20 °C	Visking 20 °C	Visking 20 °C	Visking 3 °C	Total in milk
	mg/100 g milk				
Total calcium	38·7	39·4	39·8	43·2	115·9
Ionized calcium	11·7	11·8	11·8	13·5	—
Magnesium	8·3	8·3	8·0	8·2	11·4
Sodium	46	46	47	47	48
Potassium	139	140	142	142	149
Total phosphorus	39·4	39·6	40·2	41·4	87·5
Inorganic phosphorus	32·7	33·1	33·9	34·6	—
Citric acid	162	166	168	166	175
Chloride	104·3	104·6	104·9	104·8	102·6
Total nitrogen	22·5	21·3	22·2	21·6	21·9[a]
Lactose (anhydrous)	4790	4837	4923	4947	4810
pH	6·82	6·80	6·82	6·89	6·77

[a] Non-protein nitrogen, not total nitrogen

Table 9.4 Composition of soluble phase (mg/100 g) obtained by dialysis, rennet coagulation and ultracentrifugation, adapted from data in Davies and White (1960)

	Diffusate[a]	Whey[a]	Ultracentrifugation[a]	Original milk
Total Ca	38.1	39.9	40.9	114.2
Magnesium	7.4	7.8	8.1	11.0
Sodium	46	47	47	50
Potassium	137	143	141	148
Total P	37.7	37.4	37.9	84.8
Citric acid	156	152	154	166
Lactose	4800	4800	4800	4800

[a] Results corrected for bound water

Davies and White (1960) also looked at partitioning at different pH values and these results are summarised in Table 9.5. On acidification, there was a great deal of movement of Ca and P from the casein micelle to the soluble phase.

Other observations were that lactose and citric acid permeation were affected by applied pressure in UF and that not all sodium and potassium was freely permeating. They concluded that ultrafiltration of milk and dialysis of milk will both give a serum or dialysate whose composition is reasonably close to that of the aqueous phase of milk. They also concluded that the Donnan effect was very small in UF of milk, as differences in pH and chloride values in permeates and milk were very small.

The effects of pH on mineral partitioning are clearly illustrated in Fig. 9.3 (Walstra and Jenness 1984), over the pH range 7.0–4.6. Again, it can be seen that soluble Ca, and P increase substantially as pH is reduced over this range. Soluble

Table 9.5 Composition of diffusate from separated milk (pH 6.77) and from portions of the milk acidified with hydrochloric acid (average results from two milk with same initial pH, corrected for bound water), taken from Davies and White (1960), with permission

	pH of milk						
	6·77		5·60		4·57		Total in milk
	mg/100 g milk	% of total	mg/100 g milk	% of total	mg/100 g milk	% of total	mg/100 g milk
Total calcium	36·0	30·5	80·5	68·1	115·0	97·3	118·2
Magnesium	7·6	65·5	9·1	78·5	11·1	95·7	11·6
Sodium	49	98·0	52	104·0	51	102·0	50
Potassium	140	93·9	146	98·0	151	101·4	149
Total phosphorus	43·4	45·6	64·0	67·2	69·5	73·0	95·2
Inorganic phosphorus	34·7	—	53·0	—	60·5	—	—
Citric acid	158	93·5	165	97·6	166	98·2	169
Total nitrogen	28·6	—	28·6	—	28·7	—	30·1[a]
Lactose (anhydrous)	4790	—	4790	—	4790	—	4790
pH	6·81	—	5·78	—	4·62	—	6·77

[a] Non-protein nitrogen, not total nitrogen

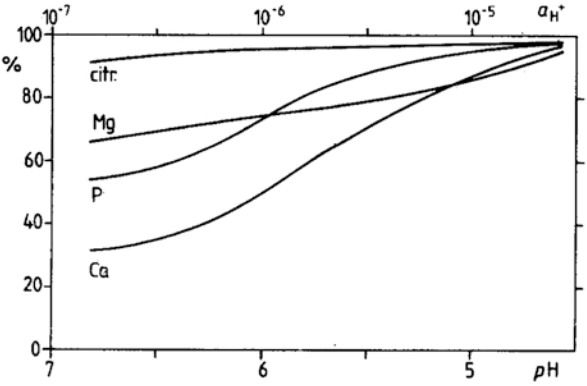

Fig. 9.3 Amount of minerals associated with the soluble phase of milk at different pH values. (From Walstra and Jenness 1984, with permission)

Mg also increases but less so and soluble citrate hardly changes. These changes will be discussed later in the context of milk fermentation processes (Sect. 9.5.6). Such changes also imply movement from the casein micelle to the soluble phase during acidification. What happens when the pH increased following acidification and whether these changes are reversible are discussed in Sect. 9.3.

9.2.4 Other Studies on Mineral Partitioning

Pouliot et al. (1989a, b, c) used ultrafiltration to look at heat-induced changes in the salt balance in cows' milk. Their experimental set-up involved a hollow-fibre UF module which was incorporated into a heating system which allowed holding milk at high temperatures, from seconds to hours, up to temperature of 90 °C. The milk samples initially at 4 °C were heated to 20, 40, 60, 80 or 90 °C. Samples of permeate were collected and analysed for their pH, Ca, Mg, Na, K, P and citrate levels over a period of 40 min. Measurements were taken when the permeate had cooled down to room temperature; ionic calcium was not measured in this study. For measurements of total amounts of minerals, this would not be a problem, but it is likely to be the case for pH and ionic Ca, as these may change as the permeate cools down, as was also pointed out by Rose and Tessier (1959). The pH was found to decrease as the UF temperature increased. It also decreased as the heating time increased, especially at the higher temperatures, perhaps due to Maillard reactions. There were clear reductions in pH, calcium, P and citrate as UF temperature increased. Results for magnesium were less clear, although results between 80 and 90 °C were lower than at 20, 40 and 60 °C (Pouliot et al. 1989b). These authors also found that decreases in Ca and P were proportional to the increase in temperature. Smaller reductions in Mg and citrate were observed and Na and K were not affected by temperature. An initial sharp decrease in concentration occurred within the first seconds of holding time and was followed by a slower and smaller decrease. The possible occurrence of a two-stage mechanism for the heat-induced salt precipitation is discussed. The precipitation of dicalcium phosphate is believed to occur together with some tricalcium citrate precipitation.

Pouliot et al. (1989c) then looked at reversibility on cooling, following milk heated to 85 °C for 40 min and then cooled down to temperatures from 4 to 60 °C. Ultrafiltration was performed at those temperatures for times up 120 min. The time concentration curves showed a two-step reaction for Ca P and pH recovery, with a rapid initial steep recovery, followed by a slower longer term recovery when cooled down to 4 °C. The recoveries for Ca and P were calculated as 90–95% and 93–99%, respectively, depending upon the cooling temperature. Results for Mg and citrate were much more variable, and recoveries were not calculated.

Ionic calcium was not measured in these studies, and they compared and contrasted their results with those of Rose and Tessier (1959). Pouliot et al. (1989b, c) also performed some calculations on how much of the different salt components were transferred to the micelle at different heating temperatures. Overall, they concluded that the majority of the reactions involving transfer of minerals in milk during heating and cooling took place quickly, but there were then some on-going reactions taking place at a much slower pace. However, since we are now storing products such as UHT milk for up to 1 year, these changes may contribute to how product quality might change over time.

Glover (1985) reported that the ratio of soluble to total divalent cations (TDVC) decreased from 29% in raw milk down to 7% in milk concentrated fivefold by

UF. Changes in pH have been found to influence the amount of minerals in the final retentate during UF of whey and buttermilk (Hiddink et al. 1978). It was observed that maximum mineral removal was obtained by UF at pH 6.6, followed by diafiltration at pH 3–3.5. It has been reported that the rejection of calcium, sodium and phosphorus was higher during diafiltration than UF and that diafiltration of acidified milk gave rise to lower rejections of calcium, phosphorus and sodium (Bastian et al. 1991). Calcium recovery and P in UF concentrates (CF = 5) were 84% for calcium and 66% for P. These were reduced by diafiltration and UF of acidified milk. Premaratne and Cousin (1991) reported the following concentration factors for some different divalent and trivalent cations resulting from a fivefold concentration of milk by UF: Zn (4.9), Fe (4.9), Cu (4.7), Ca (4.3), Mg (4.0) and Mn (3.0); this suggested that there were differences in their binding capacities to casein and whey proteins.

Retentates produced by UF of skim milk were able to withstand sterilisation at 120 °C for 7 min and their heat stability was improved by procedures which reduced the levels of salts in the retentates (Sweetsur et al. 1985). In milk concentrated twofold by UF, it was noted that Ca^{2+} in the retentate immediately after production was slightly lower than in the original milk, but increased slightly during storage, by up to 15% Ca^{2+} in permeate was reported to be only one-third of that in the milk (May and Smith 1998).

Partitioning of some other cations in milk and some important micronutrients from data presented by Hunt and Nielsen (2009) are shown in Table 9.6. Abdulghani

Table 9.6 Some observations for partitioning of some micronutrients in milk. Compiled from information in Hunt and Nielsen (2009)

Micronutrient	Concentration in bovine milk	Is bovine milk a good source	Partitioning in bovine milk
Calcium (mg)	1043–1283	Good	67% associated with colloidal phase, 33% with soluble phase
Magnesium (mg)	98–146	Medium	35% is colloidal, 65% soluble
Iron (μg)	200–700	Poor	14% of total iron in fat phase; 26% in whey proteins; 32% bound to casein 26%; casein 24%, 32% soluble
Zinc (mg)	3.5–3.9	Medium	1–3% in fat phase: 32% casein bound; most of remaining is bound to colloidal calcium phosphate; only 5% in soluble phase
Copper (μg)	90–100	poor	2% in fat fraction; 8% to whey proteins; 44% to casein fraction; 47% in soluble phase
Manganese (μg)	20–40	Poor	67% casein bound; 14% whey protein, 18% soluble
Phosphorus (mg)	830–992	Good	46% casein bound; 54% soluble
Iodide (μg)	48–661	Good	Largely as inorganic iodide >90%
Selenium (μg)	17–23		Mostly as selenomethionine in α-lactalbumin and β-lactoglobulin; 25–33% soluble

et al. (2015) fortified milk with iron, zinc and magnesium, prior to UHT treatment with levels up to 100% RDA per litre of milk. They also measured partitioning of these minerals between casein and the soluble and fat phases. Zinc is partitioned mainly to the casein; 87.7–92.7% for zinc alone to between 77.3% and 88.0% when fortified with all three minerals. Magnesium was between 27.2% and 31.6% alone and between 18.2% and 34.2% when it was fortified with all three minerals and iron was 76.4–89.0% alone and between 82.7% and 87.0% when fortified with all three minerals. In all cases only small amounts are partitioned to the fat phase, with amounts being higher for iron and zinc, compared to magnesium. It was concluded that fortification with magnesium and zinc showed considerable potential, but fortification with iron was more difficult because of potential oxidation reactions, leading to production of off-flavours.

9.2.5 Protein Dissociation from the Casein Micelle

Another facet of partitioning is casein dissociation from the micelle. This is important as it could be involved in the stability of casein micelles to heat, rennet coagulation and acidity. Most investigations measuring casein dissociation have been done using ultracentrifugation. One point of discussion is that without further investigation, it would not be clear what the aggregation status of the dissociated protein would be in the serum phase. All that can be said without further investigation is that does not reside in the pellet.

De la Fuente et al. (1996) reported the following concentrations (mg/L) of caseins in the micellar and soluble phases of unheated milk, respectively: α_{s1}- (10,900 and 700); α_{s2}- (3000 and 100); β- (9000 and 1300) and κ- (2900 and 500).

There is considerable evidence that casein dissociation from the micelle takes place both during cooling and heating of milk. Most investigations have focused on the dissociation of κ-casein from the micelle and its interactions with whey proteins, especially β-lactoglobulin. One line of thought is that dissociation of κ-casein from the surface of the micelle will make it more susceptible to calcium-ion-mediated heat-induced aggregation, so this has a marked effect on the heat stability of the milk. β-Casein dissociation during cooling is also important.

Studies have also focussed on the pH dependence of these interactions. This topic has been reviewed by Anema (2009); there have been a number of studies on the effects of pH on dissociation of caseins and their subsequent interaction with denatured whey proteins. At pH values >6.7 the amount of non-sedimented protein increases in heat-treated milk. Kudo (1980) showed that at pH 6.5, the amount of non-sedimented protein heated at 140 °C for 5–20 min was less than in non-heated milk. As pH was increased, the amount was also found to increase in heated milk, until at pH 6.7 it had exceeded that in non-heated milk.

Anema and Li (2000) reported that when milk at pH > 6.7 was heated, the quantity of α_S- and β-caseins dissociated increased with increasing temperature to a maximum at about 60 °C, decreased at temperatures between 60 and 100 °C and

then increased again at temperatures above 100 °C. This produced a local minimum in the dissociated casein–temperature curve at about 100 °C. The dissociation of κ-casein increased essentially linearly with increasing temperature up to 100 °C (Anema and Li 2000). Singh and Latham (1993) studied the aggregation and dissociation of protein in milk heated at 140 °C and found that initial heating gave rise to the formation of high molecular weight complexes of whey proteins and κ-casein.

With continued heating, the quantities of these complexes remained more or less constant, but the amounts of intermediate-sized protein material cross-linked through covalent bonds (other than disulphide bonds) increased gradually. Increasing the pH at heating resulted in increased quantities of whey protein–κ-casein complexes and monomeric protein in the ultra-centrifugal supernatant. Anema (2009) concluded that further detailed investigations are required to clarify whether dissociation of κ-casein occurs before or after interaction with the denatured whey protein.

It has been reported that urea addition increases levels of soluble casein (Dalgleish et al. 1987a, b). Addition of 10 mM urea was found to increase protein solubilisation in milk heated at 130 °C. Although this might result in a reduction in heat stability, it has been postulated that urea more likely acts to prevent formation of aggregate and to also diminish pH drop as it degrades to ammonia on heating.

Holt et al. (1986) used dialysis to alter milk composition in different ways and to observe the effects on casein partitioning. Some interesting dialysis experiments were performed:

- Milk was dialysed against phosphate-free buffers and found that both colloidal Ca and P decreased and that about 30% of colloidal P could be removed without significant casein dissociation. It would have been very interesting to look at the heat stability of these milk samples.
- Milk was also dialysed against calcium phosphate buffers containing different levels of free calcium ions. This resulted in no loss of colloidal P, but colloidal calcium increased with the free Ca^{2+} of the buffer. Little change in casein partitioning was observed at or above 1 mM ionic calcium. Serum casein increased markedly at levels below 1 mM. The strength of binding of caseins in the pelleted casein was in the order $\alpha_{s2} > \alpha_{s1} > \beta > \kappa$. Note that a similar order comes up in casein dissociation at very high pressures (Sect. 9.5.8). A summary of their findings in relation to casein dissociation is shown in Fig. 9.4.

There are many ways that concentrations of free Ca^{2+} can be reduced, for example, by addition of TSC, sodium hexametaphosphate (SHMP) and disodium hydrogen phosphate (DSHP) and by removal of calcium by ion exchange. Note that most of these will adversely affect heat stability when added in excess, even though they may increase the pH of milk.

There are relatively few studies on the extent of protein dissociation at high temperature. In an ideal world this could be achieved by directly ultracentrifuging or otherwise partitioning milk at high temperature. However, as most ultracentrifuges are not able to maintain milk at high temperature, it is unclear if this has been attempted.

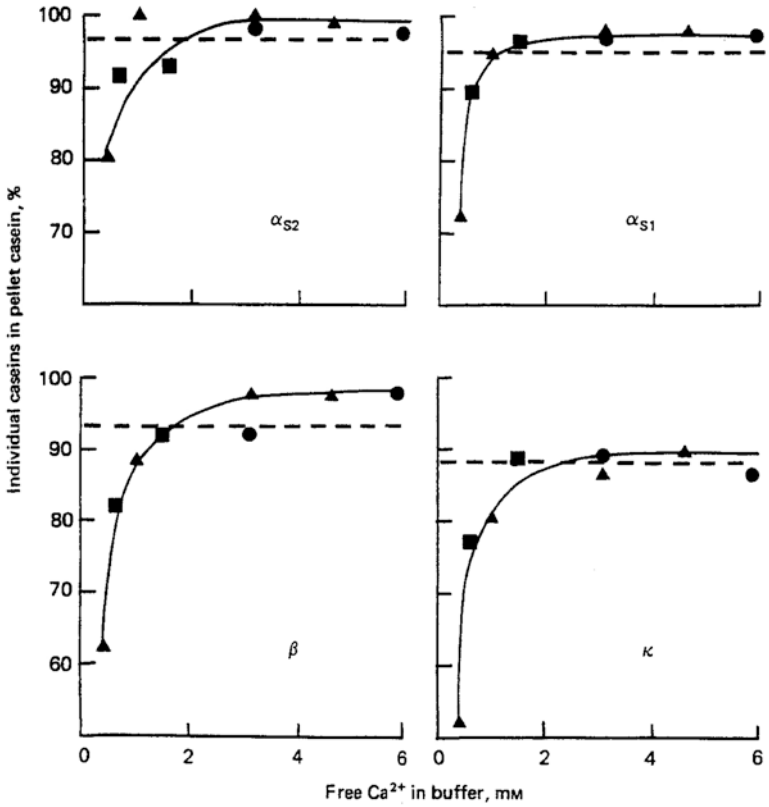

Fig. 9.4 Partition of individual caseins after dialysis of skim milk against calcium phosphate buffers having different buffers with different free Ca^{2+} concentrations: dashed line mean partition of individual caseins in the undialysed starting milk samples. Note that three different milks were used in these studies, represented by different symbols. (From Holt et al. 1986, with permission)

On-Nom (2012) compared ultrafiltration, dialysis and microfiltration for partitioning milk at different temperatures. The UF membrane used had a MWCO of 200,000 Da and dialysis membranes had MWCO values of 300,000 Da and 1 million Da. $CaCl_2$, DSHP and TSC were added to milk and pH, Ca^{2+}, total calcium, magnesium (Mg), phosphorus (P) and protein concentration were investigated using these different partitioning methods in the temperature range 20–120 °C. It was found that pH, mineral content and soluble protein decreased as temperature increased for the control milk. The addition of TSC and DSHP to milk increased pH, phosphorus and soluble protein but decreased Ca^{2+}, whereas $CaCl_2$ had the opposite effect. TSC addition resulted in a higher amount of soluble casein than DSHP, as illustrated in Fig. 9.5.

The protein composition of dialysates collected at 20 and 100 °C is shown in Fig. 9.5 for milk with added DSHP and TSC. This clearly shows that at 20 °C these membranes allow whey proteins and soluble casein to permeate. Note that for the

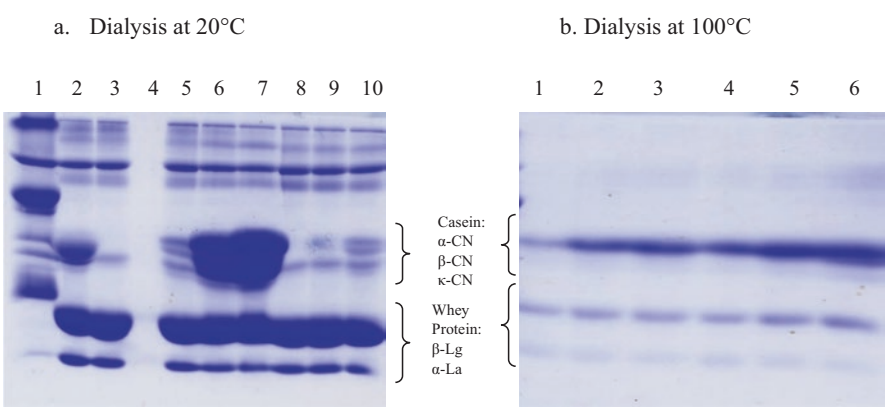

Fig. 9.5 Dialysis of milk with different levels of added trisodium citrate (TSC) and disodium hydrogen phosphate (DSHP) (from On-Nom 2012). Dialysis was performed at (**a**) predominantly 20 °C and (**b**) 100 °C. (**a**) Lane 1: Marker 3913; Lane 2: Dialysate of milk at 4 °C; Lane 3: Dialysate of milk at 20 °C; Lane 4: Dialysate of milk at 100 °C; Lane 5: Dialysate of milk + 10 mM TSC at 20 °C; Lane 6: Dialysate of milk + 20 mM TSC at 20 °C; Lane 7: Dialysate of milk + 30 mM TSC at 20 °C; Lane 8: Dialysate of milk + 10 mM DSHP at 20 °C; Lane 9: Dialysate of milk + 20 mM DSHP at 20 °C; Lane 10: Dialysate of milk + 30 mM DSHP at 20 °C. (**b**) Lane 1: Dialysate of milk + 10 mM TSC at 100 °C; Lane 2: Dialysate of milk + 20 mM TSC at 100 °C; Lane 3: Dialysate of milk + 30 mM TSC at 100 °C; Lane 4: Dialysate of milk + 10 mM DSHP at 100 °C; Lane 5: Dialysate of milk + 20 mM DSHP at 100 °C; Lane 6: Dialysate of milk + 30 mM DSHP at 100 °C

control milk dialysed at 100 °C (lane 4) there is no whey protein or soluble casein, but when samples with added TSC and DSHP are dialysed at 100 °C, it is mainly α_s-casein that is found. Also, at 100 °C differences between TSC and DSHP are not as noticeable as they are at 20 °C, where TSC results in much more soluble casein. Microfiltration was also investigated (Sect. 9.5). When comparing these three separation techniques, dialysis was considered to be the best method for investigating these properties at high temperature. It is likely that dialysis with these membranes may be more specific to monomeric casein. Further work needs to be done in this area.

Appropriate procedures were used by On-Nom (2012) to evaluate the effects of pH adjustment and ethanol addition on the soluble protein concentration. At pH 5.5, soluble protein concentration was less than it was in the range of pH 6.0–7.5. Increasing the concentration of ethanol slightly decreased the amount of soluble protein, although differences between 25% and 100% addition were relatively small. Overall, the amounts of soluble caseins found were also small.

Some of these observations on casein dissociation from the micelle might provide an explanation for the observed decrease in heat stability found for in-container sterilised milk when pH is increased by addition of stabilisers, when their addition exceeds the optimum level (Chen et al. 2012, 2014). This is discussed in more detail in Sect. 9.5.3.

9.2.6 *Effects of Adding Components to Milk*

A wide range of salts can be and have been added to milk. Some are added for practical purposes, for example, to improve heat stability or encourage coagulation in cheesemaking. Many of these will affect both pH and ionic calcium and will result in movement of both Ca and P between the micellar and soluble phase and some dissociation of casein from the micelle. Often, for scientific curiosity, amounts added are well in excess of what would be added in milk processing operations.

Perhaps the two most important additives used are what are called calcium chelating/sequestering agents or stabilising salts and a range of calcium salts for calcium fortification of milk. Chelation involves binding a cation in a ring type structure (e.g., EDTA) and thus compounds such as TSC and DSHP are strictly speaking are not chelating agents, but rather binding or sequestering agents.

Table 9.7 summarises the effects of additions of calcium chloride, di-Na EDTA and TSC on pH and levels of ionic calcium and the partitioning of minerals and casein. Udabage et al. (2000) reported that adding mixtures of DSHP and DHSP and $CaCl_2$ increased sedimented casein and calcium phosphate, whereas adding TSC or EDTA had the opposite effect. Note that the disodium salt of EDTA was used and samples were adjusted to a constant pH of 6.65. The effects of adding phosphate, citrate and/or EDTA at pH 6.65 followed by the addition of calcium chloride demonstrated the limits of reversibility of the dissolution and formation of the micellar calcium phosphate. Adding calcium chloride to milk containing more than 20 mM added EDTA or 30 mM added citrate did not result in complete reformation of the casein micelles, as determined by particle size and light scattering experiments. Dissolution of small and moderate amounts of colloidal calcium phosphate was reversible, whilst dissolution of larger amounts resulted in large reductions in micellar size and was irreversible.

Phosphates and EDTA additions have been widely studied. In such studies care must be taken to ascertain which form was used and whether pH was adjusted. For phosphates, DSHP and/or SDHP additions are common and for EDTA, either the disodium salt or tetrasodium salts have been used. For both phosphates and EDTA addition, it is possible to use a mixture of these two salts which will not change the pH of the milk, but this is only done occasionally. As mentioned, most of these compounds will change both pH and concentrations of ionic calcium. If experiments are done by restoring pH, only ionic calcium will change but if no pH restoration is performed, both pH and ionic calcium will change, making it more difficult

Table 9.7 Effect of addition of calcium chloride, trisodium citrate (TSC) and the disodium salt of EDTA (EDTA-di) on some partitioning properties of milk, adjusted back to pH 6.65

	pH[a]	Ca^{2+}	Alcohol[a] stability	Soluble Ca	Soluble P	Soluble casein
$CaCl_2$	Decrease	Increase	Decrease	Increase	Decrease	Decrease
TSC	Increase	Decrease	Increase	Increase	Increase	Increase
EDTA-di	Decrease	Decrease	Decrease	Increase	Increase	Increase

[a] pH and alcohol stability change, before pH readjustment

to determine which (if any) has the predominant effect. Polyphosphates have also been used; SHMP is a very strong calcium binding agent, but it does not change pH by much.

In situations where phosphates and citrates are used to improve heat stability, there is often an optimum addition and higher concentrations will cause heat stability to deteriorate. TSC and DSHP additions are accompanied by an increase in pH, a decrease in ionic calcium and an increase in casein dissociation, especially for TSC (On-Nom 2012). It is possible that this optimum level is one which will change pH and ionic Ca sufficiently but not cause too much dissociation of caseins. Once excessive dissociation of caseins occurs, as visually observed by its loss of milkiness, it would be very difficult to restore micelle integrity and restore milkiness, so at this stage the process would become irreversible.

There are some different options for calcium fortification. If calcium chloride is used, there will be a reduction in pH and an increase in ionic calcium. It takes only 2–3 mM addition to cause a detrimental reduction in heat stability, especially to UHT processing and the milk will produce a lot of sediment. Note that 3 mM addition would be equivalent to less than 10% fortification. Some illuminating papers involving calcium chloride addition include Le Ray et al. (1998), Sievanen et al. (2008), McKinnon et al. (2009) and Wang et al. (2020).

Calcium fortification, even of milk, is now commonplace. As well as calcium chloride, sparingly soluble salts such as calcium gluconate and calcium lactate can be used (Omoarukhe et al. 2010; Wang et al. 2020) and also insoluble salts such as calcium carbonate, calcium phosphate and calcium citrate (Omoarukhe et al. 2010). One advantage of adding insoluble salts is that they do not change pH or ionic calcium, but the challenge is to keep them suspended in solution and to ensure that they do not contribute to a gritty or powdery mouthfeel. Some partitioning studies on adding different calcium salts are described in Sect. 9.3.

De la Fuente et al. (2004) looked at partitioning of P in a selection of commercial UHT milk with added calcium and phosphates and concluded that polyphosphates were added to a number of these products. It is interesting that there were considerable ranges for many of the parameters measured; for example, pH from 6.49 to 6.94, total calcium from 1122 to 1793 mg/L, ionic calcium from 1.10 to 2.28 mM and soluble calcium from 23.6% to 37.2%. For those interested in phosphate chemistry, this paper should be investigated.

Thus, care needs to be paid to interpreting results from any studies involving addition of stabilising and calcium salts to milk. Figure 9.6 shows data for pH and ionic calcium for milk from individual cows (Lin et al. 2006). There are wide variations for milk from individual cows. The process of bulking milk will reduce these variations but not eliminate them. Also shown in Fig. 9.6 (by directional arrows) are the effects of different additives and some processing operations on pH and ionic calcium. For example, adding HCl or calcium chloride to milk will decrease its pH and increase ionic calcium. Note that most of these "processing events" will change both variables. The least certain is what happens to ionic calcium after in-container sterilisation. It always results in a considerable reduction in pH, but sometimes levels of ionic calcium have been found to increase and sometimes to decrease.

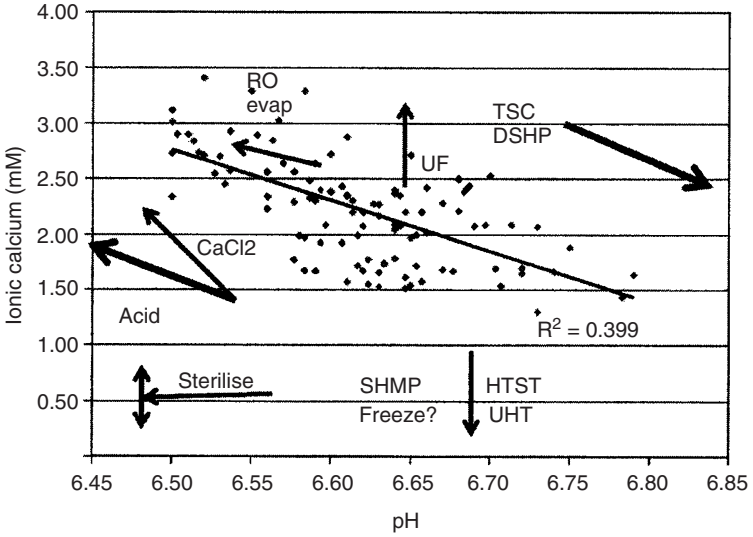

Fig. 9.6 Ionic calcium and pH values for milk from individual cows. Also shown are changes in pH and ion calcium brought about by adding various components and following various processing operations (for guidance only). (From Deeth and Lewis 2017 with permission)

9.2.7 Modelling Studies

Modelling studies offer a different perspective to the experimental approaches discussed above and also provide the opportunity to compare results obtained from the models with experimental data. Milk salts, in particular Ca, P and citrate, are partitioned between the casein micelles, where they are mostly in the form of nanoclusters of an amorphous calcium phosphate, sequestered by caseins through their phosphorylated residue, with the remainder in the soluble phase. In parallel with experimental work on partitioning described earlier, Holt et al. (1981) undertook some challenging work on modelling the soluble phase of milk; challenging because the physical chemistry involved is complex.

Known association constants for the various ionic species are used in the models and a table is provided in Holt et al. (1981). The composition of a typical dialysate (soluble phase) predicted by the model is shown in Table 9.8. The main compositional information required as input data for the model is summarised in the footnote to this table. This paper dealt specifically with the salts in the soluble phase of milk and established the principle of using modelling studies. It was found that most of the calcium and magnesium in the soluble phase was complexed with citrate as $Ca\ Cit^{-}$ and $Mg\ Cit^{-}$. Values predicted from the model for ionic calcium were plotted against datasets from 18 Ayrshire cows in Scotland and 28 individual cows in a Minnesota herd. There was a reasonable agreement with the experimental data. The model predicted ionic calcium values somewhere between those found by the murexide method and a selective ion electrode.

Table 9.8 Calculated concentrations (mM) of ions and complexes in a typical milk diffusate, from Holt et al. (1981)

Anion	Free ion	Complex Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}
H_2Cit^{-}	+[a]	+	+	+	+
$HCit^{2-}$	0.04	0.01	+	+	+
Cit^{3-}	0.26	6.96	2.02	0.03	0.04
$H_2PO_4^{-}$	7.50	0.07	0.04	0.10	0.18
HPO_4^{2-}	2.65	0.59	0.34	0.39	0.52
PO_4^{3-}	+	0.01	+	+	+
Glc 1-PH^{-}	0.50	+	+	0.01	0.01
Glc 1-P^{2-}	1.59	0.17	0.07	0.10	0.14
H_2CO_3	0.11	–	–	–	–
HCO_3^{-}	0.32	0.01	+	+	+
CO_3^{2-}	+	+	+	+	+
Cl^{-}	30.9	0.26	0.07	0.39	0.68
HSO_4^{-}	+	+	+	+	+
SO_4^{2-}	0.96	0.07	0.03	0.04	0.10
RCOOH	0.02	–	–	–	–
$RCOO^{-}$	2.98	0.03	0.02	0.02	0.04
Free ion		2.00	0.81	20.92	36.29

The following is the milk composition data which is used in the model; minerals (all mM): Ca, 10.2; Mg, 3.4; Na, 22; K, 38; Cl, 32.3; Cit, 9.4 Pi, 12.4; GLc-1-P, 2.6, H_2SO_4, 1.2; assumed CO_2, 0.44; RCOOH, 3.1: ionic strength, 73

Other components (all g/L) fat 37; lactose 46; whey protein 6; water 880

[a] Concentrations shown as (+), <0.005 mM; (–), not determined

Table 9.9 Calculated ultrafiltrate concentrations (mM) of the reference bulk skim milk calculated by Model 1 and Model 2 and comparisons with measured values. From Bijl et al. 2019a

		Calculated		Free ion		
UF	Measured	Model 2	Model 1		Model 2	Model 1
Ca	10.2	11.9	9.6	Ca^{2+}	2.02	2.00
Mg	3.4	3.6	3.2	Mg^{2+}	0.75	0.80
Citrate	9.4	8.6	8.8			
P_i	12.4	12.2	11.4			

Holt (2004) expanded this work not only to include the partitioning of salts but also binding of the different casein fractions to the calcium phosphate nanoclusters. He introduced the concept of thermodynamic stability of milk, in terms of preventing calcium phosphate precipitating out in the mammary gland. Stability will be maintained if there is an excess of phosphate binding sites provided by the casein fractions to ensure that all the calcium phosphate in the nanoclusters was tightly bound and hence not able to precipitate. This model was later described as Model 1 and some results described for partitioning of salts predicted by this model are shown in Table 9.9.

Model 1 showed that some of the caseins are bound more tightly than others, as discussed. Also, when milk is cooled, β-casein tends to dissociate from the micelle, whereas the α_s-caseins and κ-casein tend not to. Some results for the partitioning of the different caseins are presented in Fig. 9.7, where proportion of casein bound is plotted against log α, where α is defined as the fraction of the strong binding sites that have reacted with calcium phosphate and α ranges between 0 and 1 for milk which are thermodynamically stable. This diagram shows how much of the individual caseins are bound at different values of α, showing again as stronger binding to α_{s2}-casein and the lowest to β-casein.

Figure 9.8 shows the mole fraction of the different caseins bound to calcium phosphate nanoclusters at different pH values. It is interesting that α_s-caseins and β-caseins do not bind at pH less than 5.8, becoming more like κ-casein in this respect. This does not imply that the micelle breaks down at this pH, as some weaker binding forces remain in play, but it might explain some of the findings when milk which is acidified to this pH and restored is not changed much, compared to milk which have been reduced to lower pH values and restored (Sect. 9.3). Below pH 5.8, casein will not have a major influence on salt partitioning.

Also discussed were difficulties in comparing results from models for casein binding with what was found experimentally, as the soluble phase, which is usually recovered by ultracentrifugation will contain more than casein monomers. It is interesting that dialysis experiments on milk at different pH values (On-Nom 2012) found no major differences in soluble casein over the pH range 6.7–5.5.

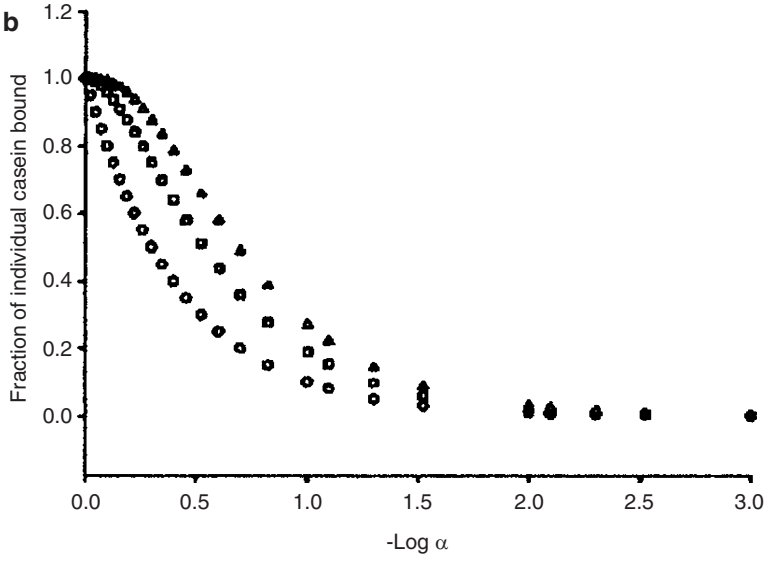

Fig. 9.7 Mole fraction of β- (circles), α_{s1}- (squares) and α_{s2}-casein (triangles) bound to the calcium phosphate particles, plotted against −log α (from Holt 2004, with permission). α is defined as the fraction of the strong binding sites that have reacted with calcium phosphate and α ranges between 0 and 1 for milk which are thermodynamically stable

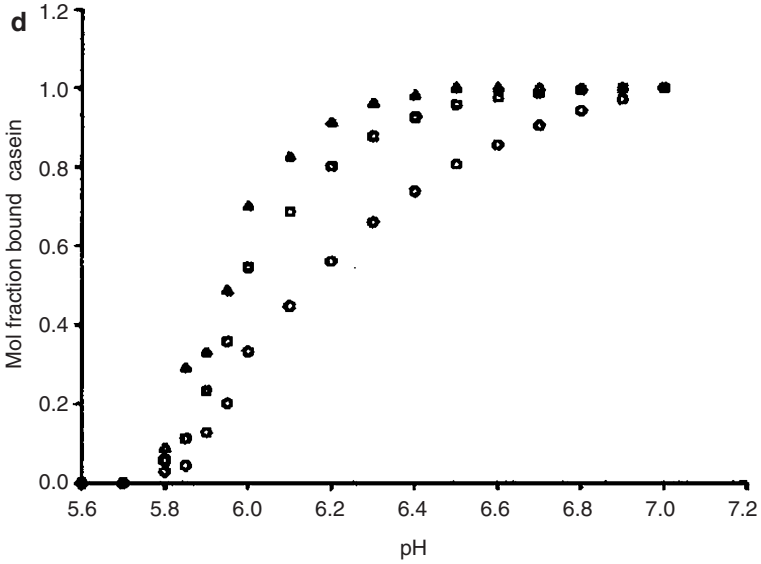

Fig. 9.8 Mole fraction of β- (circles), α_{s1}- (squares) and α_{s2}-casein (triangles) bound to the calcium phosphate particles, at different pH values. (From Holt 2004, with permission)

Model 1 has been further developed, as described by Bijl et al. (2019a), and this refined model is known as Model 2. Some of the main points involved in using and comparing Model 1 and Model 2 are discussed below:

The structure of the nanocluster is discussed in the introduction to this chapter. Most phosphorylated residues are close together in the casein sequences forming what is known as phosphate centres. The term sequestered calcium phosphate is preferred to the older term of colloidal calcium phosphate because the nanocluster is a thermodynamically stable complex of calcium phosphate with the sequestering casein phosphopeptides.

Casein mole fractions are calculated and used in two ways, namely the partition mole fraction and the composition mole fraction. The partition mole fraction is the proportion of an individual casein that is either bound or free of any linkage to the nanoclusters of calcium phosphate. The composition mole fraction is the mole fraction of an individual casein in whole casein. Bijl et al. (2019b) provide an online resource for determining mole fractions of the caseins.

Bijl et al. (2019a, b) compared and contrasted results from the original model of Holt (2004) (Model 1) and their revised model (Model 2). Both models predict the composition of the soluble phase of milk and also how individual caseins partition to calcium phosphate nanoclusters. A brief summary of the main differences between the two models is given below:

Model 1 α_s- and β-caseins have a proportion of their phosphorylated casein residues in either one or two phosphate centres and these were proposed to react with

the nanoclusters equally and independently. In this case a casein molecule with two phosphate centres can be free of any linkage or bound through either one or both of the phosphate centres.

Model 2 In this model all phosphorylated residues in what Bijl et al. (2019a) refer to as competent caseins act together to bind and sequester the nanoclusters. So, in Model 2, individual casein molecules are either not bound or fully bound through all binding sites. As there are no intermediate binding sites a higher proportion of casein is free, compared to Model 1. This model has been described to provide a better agreement with experiment of the partition of caseins between the free and bound states and equally good results for partition of milk salts. Model 2 predicts more spare capacity for sequestration than Model 1. A further assumption of Model 2 is that competent sequestering caseins must contain at least one strong divalent cation binding site occupied by a divalent cation which is essential to the initial binding event. Strong cation binding sites are formed by phosphate centres.

Table 9.10 shows the mole fractions of caseins which are bound to calcium phosphate and the charge on the casein micelle in the reference bulk milk sample. This is based on the bulk milk composition data found by White and Davies (1958a, b, c). Since only total casein was measured in this study, assumptions are made about the distribution of the different casein factions. The bound mole fraction according to Model 2 has 0.44 β-casein, 0.44 α_{s1}-casein and 0.12% α_{s2}-casein but no κ-casein. Model 1 predicts a similar distribution. In Model 2, the free fraction is in enriched in κ-casein (but not as much as predicted by Model 1), but all the other caseins are also represented until $\alpha = 1$, when only the κ-casein remains free. Model 2 is also able to predict the charge on the different caseins in their free and bound forms. The predicted total charge for the bound and free caseins is +0.84 and −8.64 mV.

Figure 9.9 shows how the charge on the casein micelle is affected by divalent cation concentration and pH. What is unusual about this figure is that it goes down to a pH of 3, and so should be of great interest to those producing acidic milk products, which are briefly discussed in Sect. 9.6. Few data are available on partitioning of acidified milk products, with pH values in the range 3.6–4.2.

Table 9.10 Composition of the casein mixture found bound to calcium phosphate and free in the example bulk milk at its natural pH, according to Models 1 and 2, and the charge in each state according to Model 2, taken from Bijl et al. (2019a)

	CaP-bound			Free		
	Mole fraction			Mole fraction		
Casein	Model 1	Model 2	Z	Model 1	Model 2	Z
κ-	0.00	0.00	−4.06	0.76	0.40	−4.06
β-	0.45	0.44	−1.76	0.24	0.27	−9.46
α_{S1}-	0.45	0.44	−1.44	0.00	0.27	−15.86
α_{S2}-	0.10	0.12	+18.45	0.00	0.07	−4.31
Total	1.00	1.00	+0.84	1.00	1.00	−8.64

Models 1 and 2 were used to look at the stability status of cows in the studies of White and Davies (1958a, b, c). The α values are calculated for all cows by these two models. Model 2 calculates that 98% of the milk samples are in the thermodynamically stable region, whereas Model 1 predicts that 23% of the samples were in the meta- or unstable regions.

Model 2 also allows the partition of the individual caseins between bound and free states and the net charge on each of the casein fractions to be determined. These calculations were performed using the composition data obtained for 18 cows, measured by Bijl et al. (2014) and the results are presented in detailed tables for each cow. Also shown in these tables for each milk are values for the average number of moles of divalent cations bound to the casein but not part of the complexes with calcium phosphate, expressed per mole casein (MCas) and the average number of moles of divalent cations bound to the calcium binding sites in whole casein ($MSerP_s$).

Model 2 is also claimed to calculate more accurately the thermodynamic stability curve as a function of pH, to identify among other things, whether there is a pH above which there is insufficient casein to ensure thermodynamic stability, that is to prevent the calcium phosphate precipitating as milk pH is increased. Calcium phosphate precipitation would be very undesirable for the problems and distress it would cause the animal, for example if calcium phosphate precipitation occurred within the mammary gland.

Figure 9.10 shows the stability curve for the "standard milk", whose composition is discussed earlier. The zone of stability lies to the left or above the line and that of meta-stability or instability lies to the right or below the line. Once the stability curve has been calculated from the model for each milk, the location of the milk itself can be pinpointed from its casein composition and pH and its thermodynamic stability status established by determining whether it lies in the stabile or unstable zones. This curve for every milk can be described by a mathematical equation. Key

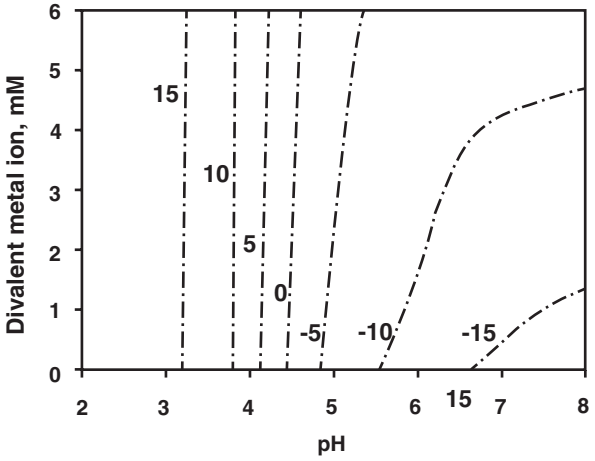

Fig. 9.9 Contours of net charge on whole casein as a function of free divalent cation concentration and pH. (From Bijl et al. 2019a, with permission)

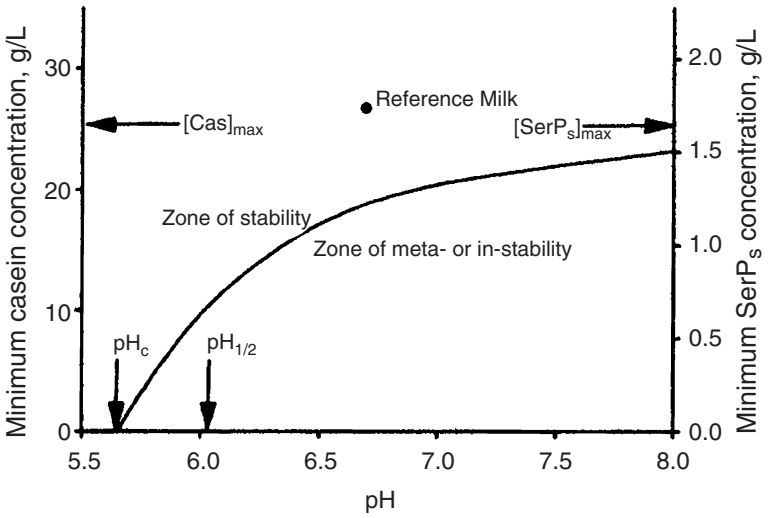

Fig. 9.10 Stability diagram of the standard reference milk showing the zones of stability and meta- or instability with respect to calcium phosphate. The right axis gives the minimum concentration of strong divalent cations required to achieve thermodynamic stability ($SerP_{min}$) with respect to precipitation of calcium phosphate. The left axis gives the corresponding minimum concentration of casein (Cas_{min}). Also shown is the position of the reference milk, which is stable as it is above the curve (from Bijl et al. 2019a, with permission). This curve can be described by a mathematical expression, containing four key parameters: pH_c is critical pH above which the solution requires a finite concentration of a competent sequestering agent for it to be stable. $pH_{1/2}$ is the pH at which $SerP_{min} = SerP_{max}/2$. $SerP_{min}$ and Cas_{min} are the minimum concentrations of sequestering agents and caseins, respectively, required for stability. $SerP_{max}$ and Cas_{max} are the maximum values found from the curve, respectively. Values for these four parameters are tabulated for 18 different milk samples by Bijl et al. (2019a)

parameters in this equation are also shown in the diagram, namely pHc, $pH_{½}$, (Cas_{max}, $SerP_{max}$), which are explained in the legend to the figure.

The stability curves were determined for 18 milk samples, which were analysed by Bijl et al. (2014). From the actual plateau values of the concentration of strong binding sites a stability ratio was calculated for each milk (R_{stab}). If $R_{stab} > 1$, the milk was predicted to be stable to alkaline pH adjustment. Of the 18 milk samples, only about half were considered to be stable. A reasonable generalisation from these findings is that a milk is likely to be stable to alkaline pH adjustment if its casein concentration is at least 30 g/L.

It is fascinating to speculate that casein micelle structure has evolved, since lactating animals first roamed the earth, to provide the newborn with adequate nutrition in terms of minerals and nutrients. It is also noteworthy that lactating animals may contain as little as 8 mM total Ca, to over 100 mM total calcium, but an important principle in all species is that calcium phosphate precipitation must not occur with the mammary gland, throughout a long lactation period and through successive lactations.

Those who are interested in the modelling approach should consult the three main papers discussed in this section. Although the outcomes are clear regarding theoretical predictions about casein binding to the nanoclusters, whether this relates to how susceptible that milk is during food processing operations is less clear, as will be briefly discussed in the next section. Other modelling studies have been conducted by Mekmene et al. (2009) on salt equilibria in mineral enriched milk; Wang et al. (2020) on partitioning with added calcium salts and Gao et al. (2010a, b) in simulated milk ultrafiltrate (SMUF).

9.2.8 Summary of This Work and Some Limitations

The modelling approach has introduced the concept of thermodynamic stability of milk and whether there are enough casein binding sites to prevent calcium phosphate precipitation. The models can be used if the appropriate compositional information is known for the milk. They will predict a great deal of information relating to salt concentrations in the soluble phase and a number of parameters related to how the different casein fractions bind to calcium phosphate. Models can also predict whether the calcium phosphate is likely to precipitate out when pH is increased. This concept of stability is certainly important in terms of the health of the cow and preventing calcium phosphate precipitation in the mammary gland. There are also similar mechanisms in place preventing calcium phosphate precipitating out in other biological fluids, such as blood and urine (Lenton et al. 2020). However, milk is exceptional in that it contains far more total calcium than blood and urine and, to accommodate this high concentration, far more calcium phosphate sequestering sites.

The physical chemistry underpinning modelling studies is complex, and many assumptions are made to permit the calculations to be made. It is also not straightforward to verify the model, especially for predictions made about how casein binds to the calcium phosphate nanoclusters, or how likely are milk samples which are predicted to be thermodynamically unstable are in fact likely to calcify in situ. Fortunately, there are very few situations where milk pH is increased in commercial processes, the two most obvious being additions of TSC and DSHP. TSC addition in particular leads to considerable production of all the main soluble casein fractions. There is also a limited number of datasets that can be used to evaluate the models and the two that have been extensively used are those of Davies and White (1958) and Bijl et al. (2014). In principle, modelling could also be used to predict the soluble phase of concentrated milk products and of protein-enriched products produced by ultrafiltration.

One of the main limitations is that the association constants for the different species that are required are not known with confidence at temperatures other than 20 °C, so it is not possible to predict how casein partitions at other temperatures, and when milk is heated. Some of the more recent approaches taken to partitioning milk at high temperature might provide data to allow estimation of association constants

and other parameters required for the models at high temperature. In terms of cow health, the most important temperature would be that of the cow's body temperature of 38.6 °C, or for human milk it would be at 36.8 °C. Some experimental work to determining how milk partitions at high temperature is described in Sect. 9.4.

Since the models can predict the amounts of the different casein fractions that are strongly bound to the calcium phosphate nanoclusters and those which are free, a logical extension is to ask whether those caseins which are not tightly bound remain in the micelle itself or migrate into the soluble phase. One might speculate that there may be some relationship between how much casein is bound to the nanoclusters and how much casein might be found in the serum following milk ultracentrifugation. However, there is doubt about the size range of the casein found in the supernatant from ultracentrifugation. Further clarification may come from analysis of dialysates obtained under conditions used by On-Nom (2012) and described in more detail in Sect. 4.1. Overall, the predictions provided by the models for casein binding are much more difficult to verify experimentally than those predictions for salt binding.

One might further speculate that parameters describing "thermodynamic stability" might also correlate with some other important technological properties, such as heat stability, ethanol stability, rennet coagulation time or foaming capacity. Detailed composition data were provided by White and Davies (1958a). Their studies also include measurement of heat stability, rennet coagulation time and ethanol stability for all these milk samples. There may be a possibility to look at the thermodynamic model in the context of these results.

Overall, Bijl et al. (2019a, b) have compared and contrasted the results from the two models very clearly and extended the earlier models of Holt et al. (1981) and Holt (2004). Milk is still being produced which provides a challenge to the dairy technologist. Some examples are: milk produced at the milk flush, which is the transition from indoor feeding to pasture feeding (Grimley et al. 2009); milk produced during heatwaves, or caused by heat stress and milk which is produced in various parts of the world with a low ethanol stability, which is caused by a salt imbalance and not due to poor microbiological quality (personal communications). All these factors could be influenced by how much of calcium phosphate is sequestered by caseins. Modelling may also offer an explanation why most milk samples will be stable after freezing and defrosting, whereas a small proportion are not, and those which are not stable produce a massive amount of sediment (Sect. 9.2).

9.3 Reversibility

It has been clearly shown that as milk pH is reduced, then calcium and phosphate will leave the micelle. Partitioning studies also appear to stop at the isoelectric point of casein, which is about 4.6, which was also confirmed in modelling studies by Bijl et al. (2019a, b). Information below pH 4.6 would be relevant, for example, for acidified milk drinks. These are popular in the Far East and are produced by

acidifying milk with fruit juices or various acidulating agents. They also need stabilising with either pectin or hydrocolloids. The pH range is normally 4.0–4.2 with sodium carboxymethylcellulose (CMC) as stabiliser or 3.8–4.0 with pectin as the stabiliser. No studies appear to have been done on mineral partitioning or the status of the casein in these products. Figure 9.9 derived from modelling studies (Bijl et al. 2019a, b) shows the surface charge on the casein micelle at different pH and divalent cation concentrations and covers the pH range of these acidic drinks.

Some of the data from Holt (2004) also suggest that some of the casein is not so tightly bound to the micelle or may not be bound at all, at a pH of about 5.8, although the structural integrity of the micelle is still maintained. Therefore, what will happen to these casein molecules which potentially have more freedom to migrate from and return to the micelle?

Another interesting question is what happens when the pH of acidified milk is restored. It is certain that the minerals will go back into the micelle, but it is unlikely that they will ever go back to exactly its same state, in terms of location and binding status and so the milk will be physically different. Such restored milk may be better suited to some milk processing operations because of transfers of components back into the micelle.

Lin et al. (2015) subjected milk to UF at discrete pH values over the range 6.7 down to about 5.4. Figure 9.11 shows how total calcium and ionic calcium changed over that pH range. The pH of the same milk was then increased back to 6.7 and concentrations of both total and ionic calcium were higher at all pH values during the pH recovery process, showing that the process was not reversible. The ethanol stability of the restored milk was also lower.

Sweet whey and permeate were also similarly treated. For both whey and permeate, ionic calcium increased slightly as pH was reduced. As pH was restored, ionic calcium remained constant and by the time pH reached 6.7, ionic calcium had reverted back to its value before acidification. There may have been some movement for a whey protein system but any changes seemed to be reversible.

Table 9.11 shows the effects of reducing the pH of milk to different values and then restoring the pH to its original value (Lin et al. 2015). Ethanol stability and ionic calcium were measured in the milk at the reduced pH and the restored pH. The results showed that the ionic calcium was higher in the pH-restored milk and its value increased as the pH prior to restoration was reduced. Also, the ethanol stability in the pH-restored milk was always lower and by the time milk had been reduced to pH 5.98 and then restored, its ethanol stability had fallen from 83% to 71%. According to guidelines for UHT processing, this milk would not be suitable for UHT processing.

Lucey et al. (1996) investigated the effect on milk of acidification to pH 5.0 and 4.6 followed by reversal of pH. Electron micrographs of milk acidified to pH values $\leqslant$5.5 prior to neutralisation showed increased clustering of casein particles. The original micellar appearance was not restored on neutralisation or dialysis of reformed milk. The authors concluded that if milk was reduced below 5.0 the micellar system is not readily reversible; once disintegrated by acidification, micelles do not reform on neutralisation. Rennet coagulation was reduced in the restored milk

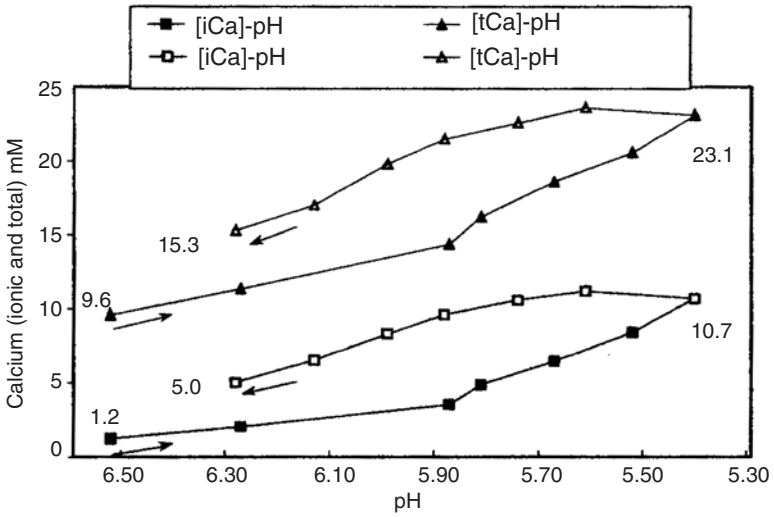

Fig. 9.11 Total (tCa) and ionic (iCa) calcium plotted for ultrafiltration of skim milk whilst decreasing and increasing pH. (From Lin et al. 2015, with permission)

Table 9.11 Ethanol stability and ionic calcium in milk reduced to different pH values and restored (from Lin et al. 2015, with permission)

pH	Ethanol stability	Ca^{2+}	Restored ethanol stability	Restored Ca^{2+}
6.75	83	1.62	83	1.62
6.58	65	2.33	82	1.85
6.38	53	2.72	78	1.94
6.16	37	3.34	75	2.02
5.98	29	3.99	71	2.14
5.80	25	4.85	63	2.31
5.59	21	6.34	55	2.42
5.40	16	7.78	50	2.33
5.19		9.82	0	2.51
4.97		11.53	0	2.55

samples and the ionic calcium was higher. In contrast, milk acidified to pH > 5.5 showed less drastic changes in their structure when their pH was restored.

Ezeh and Lewis (2011) reduced the pH of milk to 5.6 and then kept it at that pH for different time periods before restoring the pH to its original value. For the pH-restored milk, there was an increase in ionic calcium, a reduction in rennet coagulation time, no change in milk viscosity and a slight reduction in heat stability, but changes were small. Overall, there were some minor effects of reducing pH and these increased as the pH reduction increased. As pH was not reduced below 5.6, no substantial changes were noticed.

9.4 More Recent Investigations on Temperature and Other Factors Affecting Partitioning

As discussed, there has been no modelling of the effects of temperature on salt partitioning, or casein binding to the calcium phosphate nanoclusters, mainly because the effects of temperature on the dissociation constants and other properties required to complete these calculations are not known. Earlier practical investigations have been discussed above (Rose and Tessier 1959; Pouliot et al. 1989a, b, c).

On-Nom et al. (2010, 2012) investigated dialysis for partitioning milk at temperatures up to 120 °C. It was important to remove the dialysis bag from the milk as quickly as possible after the heating process. Visking membranes with a MWCO of 12,000 Da were used, as they were found to have good heat resistance, as there was no difference in permeation of hydrogen ions and calcium ions after the membranes had been sterilised at 120 °C for 1 h. Note that heat stability shown by membranes with a MWCO value of 1 MDa was very good. As milk temperature increased, pH and total and ionic calcium decreased and there was a linear relationship between both pH and ionic calcium and the slope of this relationship was the same of different milk samples. It was also the same as that found for variations in pH and ionic calcium with temperature measured during ultrafiltration of milk. It is discussed later that a dialysate collected at any high temperature would show hardly any change in pH and Ca^{2+} when it was cooled below that temperature.

Milk dialysed at 20 for 24 h and 80, 90, 100 and 110 °C for 1 h was also evaluated by On-Nom et al. (2010) and values for ionic calcium are shown in Fig. 9.12. Again, the relationship between Ca^{2+} and temperature appeared to be linear. This procedure allowed pH and ionic calcium to be determined in milk at high temperatures. In experiments with up to 7.2 mM added calcium chloride, pH and ionic calcium were measured at both 20 °C and after dialysis at 110 °C (Fig. 9.13). It was observed that 5.4 mM addition caused coagulation of the milk and the pH and ionic calcium at the point of calculation were 6.0 mM and 0.43 mM, respectively. This was the first time that pH and ionic calcium have been reported at the temperature of coagulation.

On-Nom et al. (2012) measured pH and Ca^{2+} in milk with small incremental additions of calcium chloride, over the temperature range 60–120 °C and observed how much calcium chloride was required to induce coagulation at each temperature; some results are shown in Table 9.12. Using dialysis, it was possible to establish pH and ionic calcium values at the point of coagulation and measured at the temperature of coagulation. Experiments were performed on dialysing milk with different levels of calcium chloride at 115 °C for 30 min. Dialysis bags were removed immediately and from cooled milk after 24 h. For comparison, the same milk was dialysed at 20 °C for 24 h; results are presented in Table 9.13 and show the reversibility for the milk when it cools and also for the dialysate which was left in the milk for 24 h, which resembled closely that of the dialysed unheated milk. One conclusion from these results is that when milk is sterilised its pH and ionic calcium will both be substantially reduced, e.g., from about 1.5 mM and pH 6.7 to 0.3 mM and pH 5.9

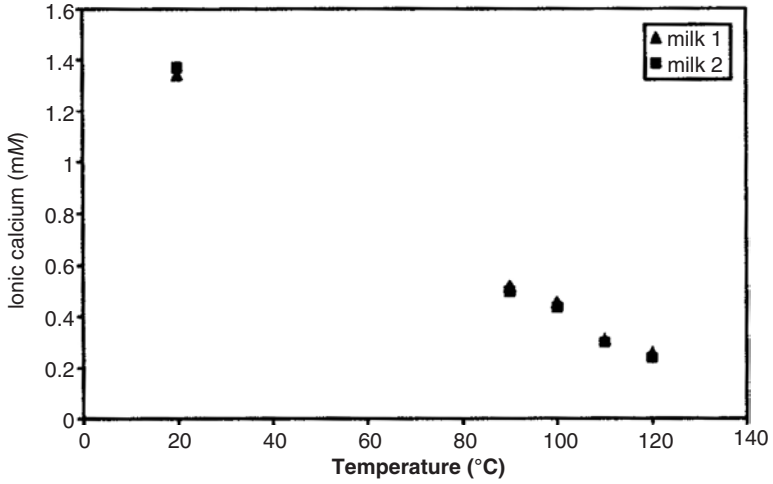

Fig. 9.12 Ionic calcium in milk dialysate heated at 90–120 °C for 1 h and also measured at room temperature. (From On-Nom et al. 2010, with permission)

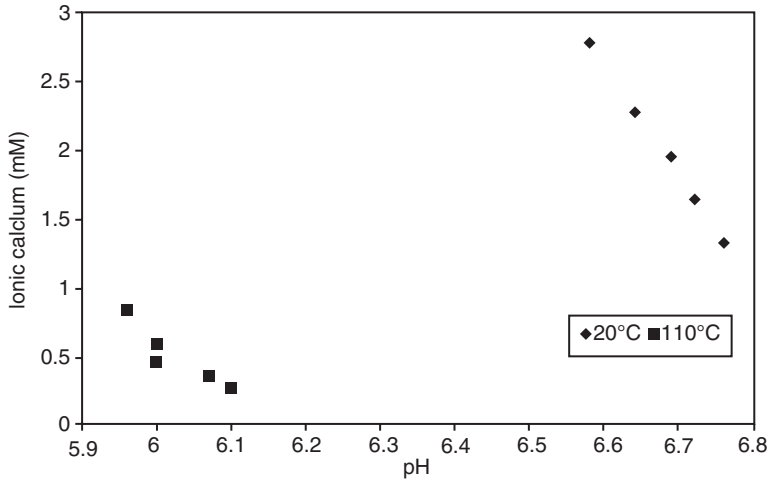

Fig. 9.13 pH and ionic calcium measured in milk at 20 °C with increasing levels of calcium chloride, and in milk dialysates collected at 110 °C. (From On-Nom et al. 2010, with permission)

at 115 °C. However, when the product cools, both pH and ionic calcium recover. pH can often make a full recovery, but ionic calcium recovery takes place in two stages: a rapid initial recovery followed by a much slower stage as has been described by Geerts et al. (1983) for UHT milk. The main component responsible for these changes is calcium phosphate which precipitates when milk is heated and dissolves when milk is cooled.

Table 9.12 pH and concentrations of ionic calcium for the highest concentration of added calcium chloride that did not cause coagulation (from On-Nom et al. 2012, with permission)

Temperature (°C)	Added $CaCl_2$ (mM)	Skim milk properties just before coagulation		Dialysates	
		pH	Ca^{2+} (mM)	pH	Ca^{2+} (mM)
60	16.2 (19.8)	6.29	6.43	6.13 (6.04)	5.36 (6.88)
70	10.8 (14.4)	6.45	3.65	6.15 (6.04)	2.64 (3.86)
80	7.2 (10.8)	6.55	2.49	6.17 (6.07)	1.56 (2.37)
90	7.2 (10.8)	6.52	2.60	6.24 (6.19)	1.47 (2.06)
100	3.6 (7.2)	6.64	1.67	6.32 (6.19)	0.74 (1.23)
110	3.6 (7.2)	6.52	1.68	6.15 (6.08)	0.53 (0.94)
120[a]					

Values in parentheses are the first set of conditions that caused coagulation
[a] At 120 °C, all samples coagulated

Table 9.13 Analysis of dialysates of 9% reconstituted skim milk powder with added calcium chloride; (1) after heating at 115 °C for 30 min; (2) followed by storage at 20 °C for 20 h; and in unheated milk dialysed for 24 h (from On-Nom et al. 2012, with permission)

Added $CaCl_2$ (mM)	115 °C, 30 min		115 °C, 30 min (20 °C, 24 h)		20 °C, 24 h	
	pH	Ca^{2+} (mM)	pH	Ca^{2+} (mM)	pH	Ca^{2+} (mM)
0	6.39 ± 0.04^{a}	0.25 ± 0.04^{a}	6.84 ± 0.12^{a}	1.18 ± 0.02^{a}	6.88 ± 0.16^{a}	1.25 ± 0.23^{a}
3.6	6.30 ± 0.07^{b}	$0.48 \pm 0.06^{a, b}$	$6.71 \pm 0.12^{a, b}$	1.70 ± 0.12^{b}	$6.75 \pm 0.16^{a, b}$	1.83 ± 0.32^{a}
7.2	6.24 ± 0.02^{b}	0.92 ± 0.15^{b}	$6.59 \pm 0.12^{b, c}$	2.39 ± 0.20^{c}	$6.66 \pm 0.14^{a, b, c}$	$2.59 \pm 0.52^{a, b}$
10.8	6.13 ± 0.03^{c}	1.71 ± 0.27^{c}	$6.53 \pm 0.11^{b, c}$	3.57 ± 0.27^{d}	$6.56 \pm 0.13^{b, c}$	$3.75 \pm 0.76^{b, c}$
14.4	$6.08 \pm 0.02^{c, d}$	2.74 ± 0.38^{d}	6.44 ± 0.12^{c}	4.75 ± 0.43^{e}	6.48 ± 0.14^{c}	5.23 ± 1.12^{c}
18.0	6.02 ± 0.02^{d}	3.95 ± 0.62^{e}	6.37 ± 0.12^{c}	6.38 ± 0.42^{f}	6.41 ± 0.12^{c}	6.77 ± 1.38^{d}

[a–f] Values (mean ± SD) in the same column followed by the same letter are not significantly different ($p > 0.05$)

Milk was subjected to ultrafiltration (UF) at high temperature, up to 140 °C, by placing the UF module in the holding tube of a UHT plant by On-Nom (2012). The pH and Ca^{2+} concentration of dialysates and UF permeates decreased as temperature increased and the pH and Ca^{2+} had fallen to 5.6 mM and 0.2 mM, respectively, at 140 °C. Thus, the amount of soluble Ca and P further decreased as temperature increased. Milk in the holding tube of a UHT plant is therefore very different to what it was before heat treatment and also when it cools, although measurements

taken on the milk before and after heat treatment would not indicate that this was the case.

One interesting conclusion is that the composition of UF permeate from milk varies according to the temperature at which UF is performed. This will include its pH and mineral content. This may be relevant to those milk processors who use UF permeate to standardise the protein content of milk. The UF module was also placed in the holding tube of a direct UHT unit. Milk which had been heated from about 20 °C to up to 90 °C by direct heating was subject to UF. These experiments confirmed that reductions in pH and ionic calcium took place extremely quickly, in a timescale of probably less than 1 s.

On-Nom (2012) also compared the compositions of UF permeates and dialysates obtained at temperatures of 80, 100 and 120 °C (see Table 9.14). The results show similar trends that pH and ionic calcium fall as temperature increases and freezing point depression (*m* °C) increases. Some of the properties obtained from dialysis are slightly different to those obtained from UF. This might be because in UF, milk was heated quickly to the experimental temperature (20–30 s) whereas dialysis is measuring the properties of milk which is held for a much longer time at the high temperature. The UF results would suggest that pH and Ca^{2+} fall quite quickly when milk is heated. At all temperatures, pH and FPD are lower than in UF permeates. This could reflect the longer time, which at high temperature would further reduce the pH of milk. FPD values are also lower for dialysates. This might have been due to the prolonged heating which leads to low molecular weight components associating with the micelle or some of those components which contribute to FPD take longer to equilibrate. These changes in pH and ionic calcium allow us to speculate how these factors change during UHT processing and in-container sterilisation of milk (Fig. 9.14).

Table 9.14 Comparison of UF permeate and dialysate samples obtained from ultrafiltration and dialysis at 80, 100 and 120 °C of whole pasteurised milk (average results from three milk), taken from On-Nom (2012), with permission

	80 °C		100 °C		120 °C	
	UF permeate	Dialysate	UF permeate	Dialysate	UF permeate	Dialysate
pH	6.50 ± 0.01[a]	6.47 ± 0.03[a]	6.38 ± 0.00[a]	6.29 ± 0.01[b]	6.16 ± 0.01[a]	5.92 ± 0.06[b]
Fpd* (*m* °C)	430 ± 0.58[a]	407 ± 12.01[b]	513 ± 0.58[a]	431 ± 5.03[b]	535 ± 1.53[a]	479 ± 12.01[b]
Ionic calcium (mM)	0.47 ± 0.06[a]	0.52 ± 0.05[a]	0.37 ± 0.12[a]	0.33 ± 0.05[a]	0.26 ± 0.15[a]	0.27 ± 0.06[a]
TDC** (mM)	6.53 ± 0.23[a]	7.16 ± 0.40[a]	5.73 ± 0.23[a]	6.53 ± 0.23[b]	5.47 ± 0.23[a]	5.87 ± 0.23[a]

[a] Mean ± standard deviation in the same row of each temperature followed by the same letter is not significantly different ($p > 0.05$)

* freezing point depression

** total divalent cations

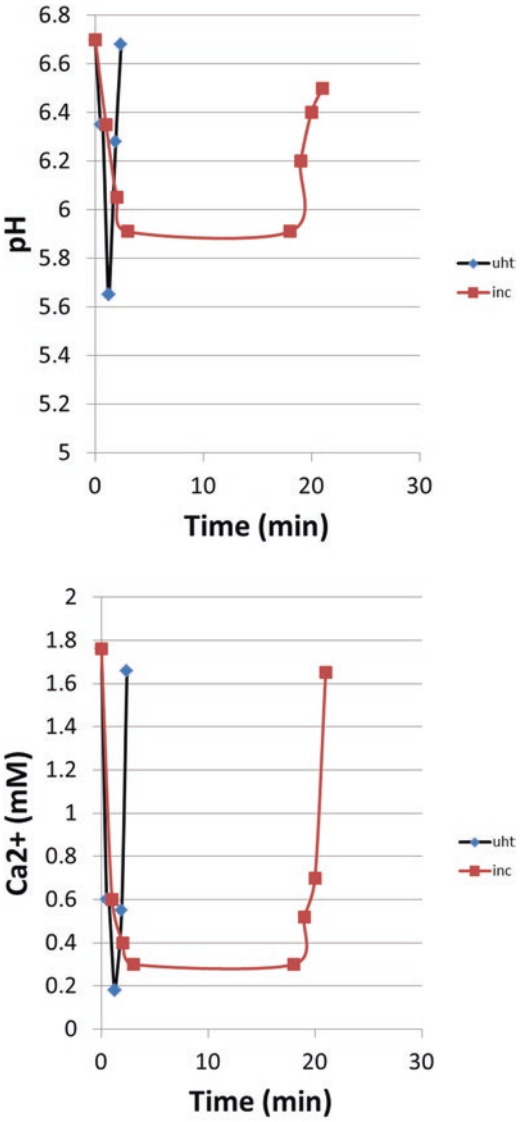

Fig. 9.14 Changes in pH and ionic calcium in milk during UHT processing and in-container sterilisation. (From Deeth and Lewis 2017, with permission)

9.4.1 Cooling of UF Permeates and Dialysates Obtained at High Temperatures

When performing UF or dialysis at high temperature, a key question is what happens to pH and ionic calcium when permeates/dialysates are cooled down; this was first noticed by Rose and Tessier (1959). When milk cools down, both its pH and

levels of soluble ionic calcium will increase. However, this was found not to be the case for permeates and dialysates, as the following experiments will show.

Table 9.15 shows milk UF permeate collected at 20 °C and dialysed at 20, 50 and 80 °C (On-Nom 2012). It can be clearly seen that all the components show considerable changes as the dialysis temperature is increased. Table 9.16 shows UF permeate collected at 80 °C and dialysed at 20, 50 and 80 °C. In this case, the values show hardly any changes and are independent of temperature. The main conclusion to be drawn is that permeate or dialysate removed at high temperature will show little change in these properties when it is cooled and hence doing these experiments at high temperature is a means of capturing the soluble phase at that temperature. However, if UF permeate collected at 80 °C was dialysed at 100 or 120 °C, it would show a further decrease in all those measured parameters.

In conclusion, as milk temperature increases, both pH and concentrations of ionic calcium will decrease (Rose and Tessier 1959; On-Nom et al. 2010). Also, as previously reported, levels of soluble Ca, P and Mg also decrease. Levels of soluble citrate do not seem to change as much. When performing dialysis at high temperatures, it has been shown that if dialysis bags are removed quickly, the dialysates provide a reasonable estimation of the components of the soluble phase in milk at that high temperature. Most of these changes result from calcium phosphate precipitation during heating and its subsequent dissolution during cooling, and the bulk

Table 9.15 Properties (pH, Ca^{2+}, total calcium, Mg and P) of UF permeate collected at 20 °C when it was dialysed (D of P20) at temperatures of 20, 50 and 80 °C, taken from On-Nom (2012), with permission

	pH	Ca^{2+} (mM)	Total Ca (mg/100 mL)	Mg (mg/100 mL)	P (mg/100 mL)
D of P20 at 20°	6.86 ± 0.21[a]	1.57 ± 0.61[a]	13.79 ± 1.22[a]	4.78 ± 0.59[a]	28.83 ± 2.50[a]
D of P20 at 50°	6.63 ± 0.08[a]	0.87 ± 0.12[a]	10.60 ± 2.63[a]	4.28 ± 0.85[a]	24.36 ± 2.00[a]
D of P20 at 80°	6.46 ± 0.11[a]	0.53 ± 0.09[a]	6.96 ± 0.25[a]	2.77 ± 0.83[a]	19.75 ± 2.03[a]

[a] Mean ± standard deviation in the same column followed by the same letter is not significantly different ($p > 0.05$)

Table 9.16 Properties of UF permeate collected at 80 °C when it was dialysed (D of P80) at temperatures of 20, 50 and 80 °C, taken from On-Nom (2012), with permission

	pH	Ca^{2+} (mM)	Total Ca (mg/100 mL)	Mg (mg/100 mL)	P (mg/100 mL)
D of P80 at 20°	6.41 ± 0.08[a]	0.63 ± 0.06[a]	9.30 ± 1.11[a]	4.47 ± 0.74[a]	26.40 ± 2.93[a]
D of P80 at 50°	6.40 ± 0.07[a]	0.62 ± 0.07[a]	8.72 ± 1.50[a]	3.93 ± 0.92[a]	24.53 ± 2.37[a]
D of P80 at 80°	6.38 ± 0.07[a]	0.61 ± 0.06[a]	8.53 ± 0.95[a]	3.18 ± 0.62[a]	22.41 ± 1.38[a]

[a] Mean ± standard deviation in the same column followed by the same letter is not significantly different ($p > 0.05$)

of these reactions take place quickly. Thus, dialysis and ultrafiltration are useful analytical tools for determining what happens to various components of milk when it is heated, acidified or subjected to addition of stabilisers, mineral supplements or other additives (e.g., phosphates, citrates, EDTA).

9.4.2 Other Observations and Uses for Partitioning Methods

Ezeh et al. (2011) performed dialysis on samples heated in cans. They also included control cans, without any dialysis bags and established that the dialysis procedure was not changing the composition of the milk. Experiments have been performed to see how quickly dialysis bags needed to be removed from cans at high temperature. The cans were allowed to cool down after heating with dialysate bags kept in situ. These were then removed after different time periods to see how long it took before pH and ionic calcium started to increase. The conclusion was that removing them as quickly as possible after the heating process did not provide sufficient time for the readings to increase and was therefore a valid procedure. Dialysis can also be used to alter the composition of milk for experimental purposes; in this case, milk is dialysed against a much larger volume of water. In place of water, other dialysing fluids can be used for selectively removing specific components, e.g., a 4.7% lactose solution to retain lactose, or simulated milk ultrafiltration permeate (SMUF) to retain all the mineral components and to maintain the integrity of the casein micelle. Ultrafiltration could be used in a similar fashion, with water or other solutions which have been used in dialysis studies, being used to replace the permeate which is removed. If water is added to the retentate during the UF process it is known as diafiltration. With some imagination, other fluids could be added instead of water, e.g., 4.7% lactose or SMUF (as discussed for dialysis, where Holt et al. (1986) used a variety of dialysis conditions to look at casein dissociation from the micelle; Sect. 4.1).

UC provides an opportunity to interchange the micellar phase with the soluble phase and has been used in studies where it may be important to determine which phase has the greatest influence on properties such as foaming, heat stability, alcohol stability or rennet coagulation time. The separated micelles from one milk sample could be reintroduced into the soluble phase from another sample. There may also be an opportunity for some differential centrifugation, with the aim of recovering casein pellets with different size distributions, as performed by Dalgleish et al. (1989). A real advance would be able to centrifuge directly at high temperature, thus providing experimental data to allow some of the association constants and partitioning data to determine some important parameters required for modelling.

Dialysis is one pre-treatment process for analysing some of the components in the soluble phase, especially where protein or fat will interfere with the analysis. On-Nom (2012) reported NMR results obtained by dialysis and ultrafiltration. NMR-analysis is fast becoming an effective means of analysing soluble phase components of biological fluids, including some organic acids, free amino acids and

other non-protein nitrogen compounds. The range of compounds that have been analysed in milk by NMR is described by Foroutan et al. (2019). Tenori et al. (2018) compared three other pretreatments: chloroform and deuterated chloroform (instead of dichloromethane) used in 1:1 ratio with the sample, ultrafiltration using a 10 kDa cut-off membrane and ultracentrifugation for 75 min followed by centrifugation, but *G* forces used (Sect. 9.2.2.3) were not mentioned in this paper.

9.5 Overview of Some Milk Processing Operations Where Changes in Partitioning of Salts and Casein May Occur

This section looks at partitioning reactions that may be occurring in milk processing operations and how they might influence those particular processes. There are many types of partitioning events taking place and attention is drawn to how these will influence pH and ionic calcium, as well as conditions on the surface or within the casein micelle.

9.5.1 Chilling of Milk

Most milk is chilled immediately after expression to reduce microbial activity, ideally to below 5 °C. The major partitioning involvement during milk chilling is some solubilisation of β-casein (see Sect. 6.1) and some mineral solubilisation. This will always occur, and β-casein dissociation may be as high as 20–25% and occasionally going above 30% after 20 h at 4 °C (Aoki et al. 1990; Creamer et al. 1977). This is attributed to weakening of the hydrophobic bonds as temperature is reduced. It is less clear exactly how quickly β-casein dissociation takes place and it is interesting that the other casein fractions are not involved, and it is attributed to the effect of temperature on hydrophobic interactions. This dissociation is also claimed to be reversible, but it is unlikely that its conformation and location will be exactly the same, for example, its binding to the calcium phosphate nanocluster. Also, a wide variety of things may happen to this chilled milk, depending upon its intended use. Thus, a supplementary question is does this dissociation of β-casein affect milk quality and how it behaves during subsequent processing? For example, if milk is cooled and pasteurised how will that milk compare with milk that is not cooled prior to pasteurisation?

As mentioned, another important effect of cooling is that calcium phosphate will become more soluble. Thus, levels of soluble ionic calcium will increase, pH will increase, and small amounts of Ca and P will move from the micelle to the soluble phase. Milk fat will also start to crystallise during the cooling process and crystallisation rate is at its maximum at around 20 °C. Overall, chilling of milk may appear to be a simple operation, but many important physicochemical changes are taking

place alongside a reduction in microbial activity. There may be an increase in dissolved oxygen concentration, as oxygen solubility increases as temperature decreases.

de la Fuente (1998) reported that long storage times at refrigeration temperatures resulted in slower clotting, higher losses of fat, weaker curds and lower cheese yield but heating at 60–65 °C reduced the effects of cooling. He suggested that pasteurisation or warming, which reinforces hydrophobic interaction among the caseins, was demonstrated to be effective in bringing about reabsorption of β-casein and restoring the molecular structure. Note that β-casein dissociation was attributed to weakening of hydrophobic interactions between casein molecules within the micellar structure.

Chilling of goat's milk is also practised and is worthy of further investigation especially as β-casein is the predominant casein in caprine milk. Chilling might be more detrimental to goat's milk than it is to cow's milk and goat milk may be ideal to study for gaining a better understanding whether β-casein dissociation has any detrimental effect on milk processing operations.

9.5.2 Freezing of Milk

Freezing of milk is a complex process. How water in milk freezes is discussed in Chap. 11. An enthalpy chart is presented showing the amount of water that is frozen at different values of temperatures and total milk solids. According to this chart the percentage of frozen water in milk at 12% TS at different temperatures is as follows: −1 °C, 40%; −2 °C, 68%; −5 °C, 86%; −10 °C, 92%; and −20 °C, 95%. Thus, most of transition from water to ice takes place over the temperature range −1 to −5 °C in milk. If stored at a constant temperature, e.g., −20 °C, the amount of frozen water remains constant, but if storage temperature fluctuates, some water melt and freeze again.

It can be argued that freezing milk is a partitioning process in itself, with water being converted to ice rather than being removed as water vapour in evaporation. Freezing of milk is simple to do but it is a complex process in terms of its physicochemistry. As ice is formed, it will lead to an increase in concentration of soluble components in the unfrozen liquid. However, the temperature would be much lower than that used in chilling and it would be interesting to speculate on the solubility of calcium phosphate and the environment at −20 °C, where about 8% of the water is unfrozen, especially as calcium phosphate is known to become more soluble as temperature decreases. Thus, the pH and calcium ion concentration in the concentrated phase are unknown, as is the amount of casein that is bound to the calcium phosphate nanoclusters. This could be important as it will affect how these interact with the casein and ultimately whether it will cause destabilisation of the micelle. Of course, all this could be influenced by the rate of freezing, which will depend upon the method of freezing and also the dimensions of the frozen milk consignments. In fact, there are three processing factors in play, which are not always

clearly distinguished. These are the rate of freezing, the storage temperatures and how long the product is frozen before it is defrosted.

Freezing of milk has been studied for a long time. A starting point is a paper of Webb and Hall (1935), who drew attention to freezing of milk and its potential for longer term storage and the problem of casein precipitation. Briefly, some of their conclusions were:

- Slow freezing of milk or cream caused a gradual precipitation of the caseinate system and immediate destruction of the fat emulsion.
- Freezing did not alter the stability of the skim milk until it has been frozen for several months at −18 °C. The destruction in fat emulsion in cream during slow freezing was lessened by adding sugar of increasing milk solids not fat before freezing.
- Fresh whole milk was pasteurised, evaporated to a third of its weight, canned and frozen without any detrimental effects to its body or flavour. If it was held frozen for less than 4 weeks and reconstituted it often could not be distinguished from fresh milk when restored to its original solids content.

Thus, the main defects caused by freezing are destabilising the casein micelles and disrupting the fat globule. Casein micelle destabilisation is very noticeable as it will lead to excessive sediment after thawing which is not readily dispersed by stirring. Some partitioning of salts into the casein was considered to be responsible by Muir (1984). This destabilisation could be thought of as a salting out effect of the casein and this has been observed in a number of studies, Webb and Hall (1935), Desai et al. (1961), El-Negoumy and Boyd (1965), Chen (2014) and Li et al. (2021).

In practical terms, frozen milk is likely to be stored in the region of −20 °C to −30 °C. Some studies have taken place at higher temperatures than this, for example, about −10 °C (Desai et al. 1961; El-Negoumy and Boyd 1965), and some at much lower temperatures, down to −80 °C (Gaber et al. 2020a, b). This lower temperature (−80 °C) is thought to contribute to longer term storage stability, but faster freezing rates do not necessarily improve storage stability, as discussed by Li et al. (2021). There appear to be no studies done on freezing milk in liquid nitrogen, or using ultra high pressures, which would result in very rapid freezing. Where freezing of milk is practised, it is most likely to be frozen slowly, especially if this is done in large containers in a home freezer or by air-blast freezing.

Longer term storage of frozen milk will lead to excessive sediment formation when the milk is thawed. Why this happens is not clear but there will be movement of calcium and P between the micellar and the soluble phase in the unfrozen portion. Desai et al. (1961) showed that the caseinate system suddenly destabilised when 85–90% of the lactose was in the alpha form. The time required to reach this critical stage was longer when samples contained sucrose. The inhibiting effect of sucrose addition was attributed to retardation of the rate of lactose crystallisation. Bound phosphorus was also found to dissociate slowly from the casein micelles into the serum, but they pointed out that it was not clear whether this is a cause or an effect of the destabilisation reaction.

Fluctuating storage temperatures may also contribute to instability, as some of the ice will melt and then freeze again. One implication is that these destabilising factors are time-dependent and slow reactions. These longer terms effects were observed when storing both raw milk and UHT milk at −18 °C. Twenty-four batches of raw milk were stored at −18 °C and after about 150 days storage, all of them produced large amounts of sediment when defrosted (Chen 2014). This was also observed in storage studies of UHT milk, described by Deeth and Lewis (2017). Those stored at −18 °C showed a massive amount of sediment and a greenish supernatant when defrosted. Gaber et al. (2020a, b) compared frozen storage at −20, −40 and −80 °C in casein concentrates; serum Ca and P decreased at −20 and −40 but increased at −80 °C but increased at −80 °C. Some casein aggregation was observed during storage, especially at −20 and −40 °C.

Goats' milk was recommended not to be stored for longer than 30 days, for similar reasons (Nurliyani et al. 2015). Pazzola et al. (2013) looked at stability of frozen sheep milk, including its rennet coagulation time. Many non-coagulating samples were observed for long-term storage. Milk clotting times and curd firmness were diminished after 5 months storage at −20 °C. There was evidence of casein aggregation and changes to rennet coagulation time.

If milk is to be frozen, there may be some useful pretreatments that might improve stability to freezing. El-Negoumy and Boyd (1965) worked on frozen milk and milk concentrate, which was stored at −9.4 °C. Pre-treatments investigated included about 50% lactose removal and some soluble calcium removal, both by dialysis. Calcium removal gave a stable product for 30 weeks, even in the presence of 20% lactose. They also suggested that precipitation of calcium phosphate in frozen milk and its interaction with the caseinate micelle is not the main factor responsible for instability.

Concentrated milk is one product, where milk solids are increased and ice cream is another frozen system which shows an excellent resistance to freezing. There is no evidence that concentrated milk systems have poorer stability to freezing; in fact, they may be improved. Concentrated milk is also now transported frozen, and one perception is that it will be indistinguishable for fresh milk, as an alternative to transporting milk powder (Fonterra 2021a). Again, there is evidence that higher total solids, in the form of sugar or increased milk solids would reduce or improve casein destabilisation (Desai et al. 1961).

Casein will also be present in ice cream formulations, which are kept frozen. There appear to be no reports of textural defects caused by casein precipitation. The most usual defects being ice-crystallisation or lactose crystallisation. Jonkman (2000) studied the properties of casein micelles in ice cream plasma obtained at −10 °C. The structure and behaviour of the casein micelles were found not to differ greatly from those in milk. A slightly increased amount of salts was associated with the micelles in ice cream plasma and fewer submicelles and small micelles were present. The increase in size was primarily due to fusion of micelles.

Webb and Johnson (1935) proposed an interesting procedure for producing a mixture of fat and casein by allowing homogenised frozen cream to thaw at a temperature below the melting point of the fat. The serum could be drained from the

frozen mass leaving behind a mixture of fat and absorbed casein. This fat–casein mixture could be washed with ice water to remove traces of serum without loss. A clear separation was only achieved when the fat content of the cream was above 25%. This fat–casein mixture was found to have some practical value, both as an ingredient for ice cream mixes and if the fat was separated, as a casein dispersion with good heat stability and as a source of casein in its native state.

Thus, there are many unknown factors in play during freezing: what will happen to the pH of the concentrated unfrozen phase, or what the freezing process will do to the soluble phase of milk or to milk stability in general; how will it affect casein micelle size and when the milk has defrosted how will it influence factors such as alcohol stability, heat stability, rennet coagulation time and susceptibility to foaming? Of course, milk from some of the many different species of mammals will vary in their stability to freezing.

An informative summary is provided by Gaber (personal communication) who cautions that those engaged in the process of freezing milk and later using that thawed milk for making products should not assume that the freezing process will not have had considerable effect on the quality of the final product. The thawing process is also crucial, especially as higher temperatures may be used to thaw the milk and some of the milk may become very hot before all the frozen mass has melted. Li et al. (2021) recently investigated different thawing methods for milk stored at −80 °C.

9.5.3 Heating of Milk

Firstly, it is important to distinguish between the effects of prior heat treatment on partitioning of components and the effects of partitioning milk at different temperatures. Most studies on heat-treated milk look at the changes caused by the heat treatment, by comparing the milk before and after heat treatment. Fewer aim to look at specific properties, for example, pH, ionic Ca and soluble casein, at the high temperature. Thus, these are very different topics (Rose and Tessier 1959).

Almost all milk undergoes some form of heat treatment; exceptions are some raw milk which is consumed and raw milk used to make certain cheeses. Thermal processes for milk vary widely in their intensity, ranging from thermisation (57–68 °C for 15 s) pasteurisation, extended shelf life (ESL), ultra high temperature (UHT) and in-container sterilisation, in increasing order of severity. The severity of heat treatment can be expressed in many different ways; in chemical terms two most useful are the extent of whey protein denaturation and the amount of vitamin B_1 (thiamin) loss, which is measured by the C^* value (a C^* value of 1 indicates that vitamin B_1 loss was 3%; Deeth and Lewis 2017). Heat treatments also vary significantly in terms of how they alter the partitioning of the salts and protein fractions.

The casein micelle will survive the most severe of these processes, usually remaining unchanged or sometimes modified. For example, pasteurised milk can be coagulated by rennet, whereas UHT milk can rarely be coagulated. Milk may also

be concentrated and dried, which involves further exposure to heat. One of the most severe thermal processes to which milk is subjected is in-container sterilisation for normal milk and especially for canned evaporated milk production, because of its higher concentration of protein and other milk solids. During the heating and cooling periods of all these processes, components move backwards and forwards between the soluble and micellar phases. These movements usually go unrecognised because heated milk after cooling appears to be largely unchanged, as discussed below.

A very important property is the heat stability of the milk. Two important determinants of heat stability are pH and ionic calcium; pH and ionic calcium values for milk measured before and after UHT processing are similar. One might thus conclude that nothing much is happening during the UHT treatment. However, this is not the situation as milk pH is typically reduced from 6.7 to about 5.6 in the holding tube but reverts back to almost 6.7 after it cools (On-Nom 2012). The same is true for levels of ionic calcium in the soluble phase, which will fall from about 1.8 mM initially, to a value of about 0.2 mM in the holding tube and revert to about 90% of its original value after cooling. Geerts et al. (1983) showed that ionic calcium of heated milk will slowly recover further during extended storage. It is also interesting that UHT milk is whiter than raw milk, due to alteration of its light scattering properties (Rhim et al. 1988). Some residual calcium phosphate precipitation, aggregation of whey proteins to the surface of the micelle and some aggregation of the micelles may contribute to this. Similar changes in pH and ionic calcium will also occur when milk is subject to in-container sterilisation, in which case the pH will not fully recover, and the milk will be noticeably browner due to the Maillard reaction. In this case the pH probably reached about 5.9 and the ionic calcium about 0.4 mM. Figure 9.14 shows how these factors change during typical in-container and UHT sterilisation processes.

There have been some important review articles on factors affecting the heat stability of milk (Rose 1963; Fox and Morrissey 1977; Singh 2004). Most of these focus on results from the classic heat coagulation time (HCT) test. This involves heating milk at 140 °C and measuring the time taken to coagulate; stable milk may remain stable for 20–30 min. The method is subjective, and it will take some time for a skilled operator to obtain reproducible results. Nevertheless, researchers and even food factories still use this approach to assess heat stability. It is worth mentioning that HCT studies subject the milk to more thermal stress than they would encounter in commercial processes. Even though temperatures around 140 °C are commonly used in UHT processing, the conclusions from HCT testing may not be applicable to UHT processes because of the very different time scales. In fact, some of the earlier reviews on heat stability were conducted before UHT processes were used commercially.

Singh (2004) stated that the heat coagulation time (heat stability) often correlates very poorly with the stability of milk on commercial sterilisation. He also pointed out that from an industry point of view, the use of a pilot-scale or laboratory scale steriliser which simulates sterilisation conditions used in practice provides more reliable results and prediction of behaviour of milk in commercial plants.

Table 9.17 pH and Ionic Ca values for milk with 30 mM added calcium salts; measurements in milk at 20 °C and in milk dialysates at 100 °C, from Omoarukhe et al. (2010), with permission

Sample	pH_{20}	pH_{100}	$Ca^{2+}{}_{20}$ (mM)	$Ca^{2+}{}_{100}$ (mM)
Control	6.73	6.41	1.19	0.26
Calcium chloride	6.13	5.80	14.8	11.2
Calcium lactate	6.26	5.88	9.79	7.43
Calcium gluconate	6.48	5.91	8.10	5.43
Calcium carbonate	6.76	6.41	1.08	0.27
Calcium citrate	6.73	6.38	1.07	0.41
Calcium phosphate	6.77	6.42	1.11	0.28

All measurement were taken at 20 °C

Omoarukhe et al. (2010) measured pH and ionic calcium in milk fortified with 30 mM of different calcium salts; results are summarised in Table 9.17. These added salts included a very soluble salt, sparingly soluble salts and insoluble salts. Dialysis was performed at 20 and 100 °C to look at how pH and ionic calcium changes with temperature. As discussed earlier, one advantage offered by the insoluble calcium salts was how similar those values were to the control milk without added calcium.

For unconcentrated milk, poor heat stability will be manifested by heat exchanger fouling and sediment formation and not by product gelation. Fouling will lead to premature termination of a process and a heavy cleaning process. Longer UHT run times (up to 30 h) have been found possible by incorporating a protein stabilisation tube or an intermediate holding section, where the milk is held between 76 and 80 °C for 40–70 s or 65 and 95 °C for 5 and 25 s (Mottar and Moermans 1988; Prakash et al. 2015).

In concentrated milk products, poor heat stability results in an increase in viscosity and perhaps coagulation and gel formation. Sediment formation is rarely an issue in these products. Concentrated milk products have a lower pH than normal milk and are heated at lower UHT temperatures, typically at 120 °C.

In conclusion, pH and ionic calcium are important determinants of the heat stability of milk and their individual contributions toward influencing heat stability will be better understood by measuring their values at high temperatures rather than at room temperature.

It is interesting to speculate whether the "thermodynamic stability" of milk, as discussed in Sect. 9.2.7 is anyway related to its heat stability. It would appear that any reactions that promote any of the following will result in poor heat stability, calcium phosphate precipitation, casein micelle aggregation or an increase in the overall size of the micelles will result in poorer heat stability. Chen et al. (2012, 2015) reported that the same milk samples subjected to UHT and in-container sterilisation will show different heat stabilities. This has been shown both for goats' milk and for cows' milk; heat stability was assessed by measuring the amount of sediment formed. UHT milk was more unstable than in-container sterilised milk when its pH was reduced slightly using calcium chloride, which would also increase its level of ionic calcium. In contrast, when milk pH was elevated, by addition of

TSC or DSHP, it was more unstable to in-container sterilisation than to UHT processing. Note that pH changes will not be the only property that is changed. For goat milk it was found that small additions of DSHP and TSC improved heat stability to UHT processing, but higher additions then lead to a reduction in heat stability. It has also been shown that fairly low additions of these two stabilisers result in some dissociation of all the casein fractions from the micelle, TSC causing more than DSHP (On-Nom 2012) and it was concluded that this must then induce high temperature casein induced aggregation reactions. Higher additions of TSC will cause the casein micelle to fall apart at room temperature.

Thus, the heat stability of milk is not only dependent upon its composition, but also on how the heating is performed. When milk is heated by in-container sterilisation, both heating and cooling rates are much slower, and the milk is held for a longer time period at the sterilisation temperature of around 120 °C. What is obvious from the experimental observations is that these conditions confer poorer heat stability on milk with increasing additions of TSC and DSHP (Chen et al. 2012, 2015). It is possible that the longer heating periods allow casein dissociation and some dephosphorylation reactions to take place, which renders the micelle more susceptible to heat-induced aggregation. These reactions are not given sufficient time to proceed during a UHT process. Conversely, milk with a slightly reduced pH is more unstable to UHT processing. This discussion involves moderate additions of these different salts. Higher additions will confer poor heat stability in both UHT and in-container sterilisation procedures (Chen et al. 2012, 2015).

9.5.4 Milk Concentration

Investigations on concentrated milk have been conducted for the last 80 years. For detailed accounts of some of the early work, please see Cronshaw (1947) and Davis (1955). In milk, the soluble phase is supersaturated with calcium phosphate, so as more water is removed, some precipitation occurs, and the pH will fall. Also in concentrated milk, the casein micelles will be packed closer together, which will make them inherently more unstable to heat. Classic tests on heat stability of concentrated milk tend to use 120 °C, whereas for unconcentrated milk it is usually 140 °C.

For production of evaporated milk, the main issues are stabilising the milk proteins, controlling viscosity and preventing excessive thickening of the product and even more seriously, product coagulation. This is achieved by an intense forewarming process and by adding stabilising salts prior to the final sterilisation process. Forewarming processes are severe in chemical terms and range from temperatures of 90–95 °C for 10–20 min and using continuous processing at temperatures in the range 120–140 °C for 25 s (Webb and Bell 1942). It is also important to avoid salt crystallisation during storage in milk containing TSC; calcium citrate has been implicated, arising most probably from too much added TSC (Deysher and Webb 1952). This problem seems to be specific to TSC addition and may be the reason

why most evaporated milk products use DSHP as stabiliser. The standard manufacturing procedure is to determine the correct level of stabiliser to be added to each batch of evaporated milk by performing small scale trials with different levels followed by retorting (Singh 2004).

A further consideration arises when evaporated milk is manufactured by reconstituting milk powder. For these products, an important starting consideration is selecting the best powder to use; high heat powders generally provide the best heat stability. One advantage of using reconstitution is that the appropriate level of stabiliser addition needs only to be established for every consignment of powder, rather than for every batch of milk. The quality of the water used for reconstitution is also important, especially its hardness (calcium and magnesium contents), as this might influence heat stability.

Nevertheless, partitioning in concentrated milk is also relevant, but studies are few. The first was that of Tessier and Rose (1958) on skim milk concentrated to 16.3% TS. Trends for all components were similar to unconcentrated skim milk in terms of temperature effects (see Fig. 9.2). pH values for the concentrate were between 0.1 and 0.2 units lower at any given temperature. Soluble P was between 1.5 and 1.6 times higher and soluble calcium between 1.8 and 1.9 times higher for the concentrate across the temperature range. However, extensive concentration was not performed in this study.

The most detailed study was undertaken by Nieuwenhuijse et al. (1988) who subjected milk and concentrated milk samples to UF and analysed the resulting permeates. Milk was concentrated to 31.3% total solids after two different forewarming conditions. Partitioning studies were used to look at changes caused by forewarming and concentration on partitioning of Ca and P. Sterilisation took place with and without phosphate addition at 18 mM concentrated milk. A mixed phosphate (40:60 on a molar basis) of SDHP and DSHP was used.

Ca and P became colloidal during the preheating and the evaporation procedure but scarcely during the sterilisation procedure, unless some sodium phosphate had been added. However, pH changes were both significant following the concentration and also during the sterilisation process.

Some typical data are shown in Fig. 9.15 for ultrafilterable calcium and phosphorus for the milk, preheated milk and concentrated milk. Preheating sightly increased pH and slightly reduced soluble P and Ca. The evaporation process significantly increased both soluble P and Ca, apparently by approximately the concentration factor (Nieuwenhuijse et al. 1988). Further data were presented for values of soluble P and Ca following an intense in-container sterilisation process. In this case one set of experiments was done with and without added stabiliser (Figs. 9.16 and 9.17).

In concentrate with no added stabiliser, changes were very small for ultrafilterable calcium, sometimes a slight increase and sometimes a slight decrease and for ultrafilterable P, a small decrease was normally found. However, where phosphate was added, sterilisation caused a significant amount of Ca and P to become colloidal. For those with an interest in milk concentrates, Nieuwenhuijse et al. (1988) contain detailed discussion on amounts of Ca and P transferred from the soluble to colloidal states at different conditions and also some heat stability studies on the products.

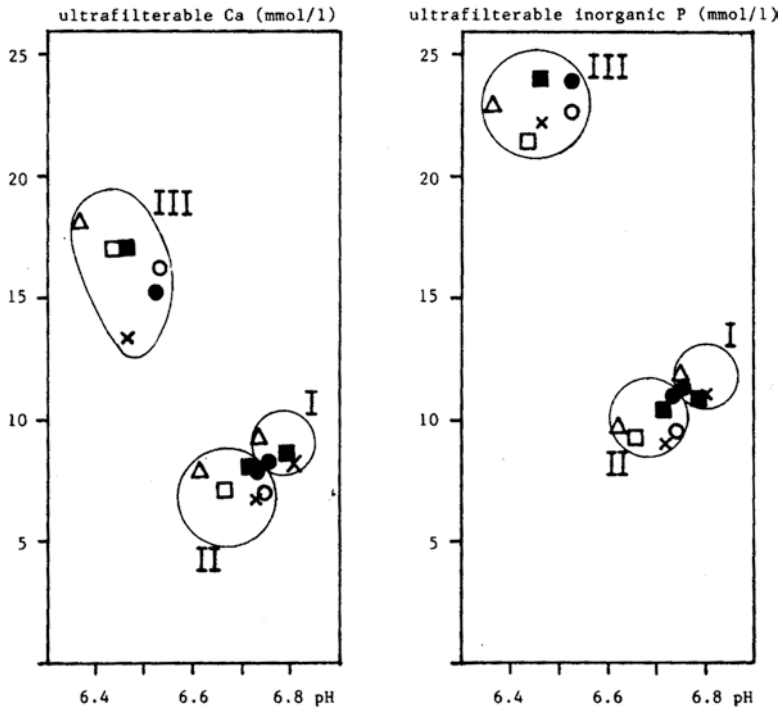

Fig. 9.15 Ultrafilterable Ca and inorganic P in raw milk and after preheating and concentration; I, milk; II, preheated milk, III, concentrated milk. (From Nieuwenhuijse et al. 1988, with permission)

More recently, a study by Tsikritzi (2011) applied dialysis at room temperature and at high temperatures to milk powder reconstituted to different total solids contents (Tables 9.18 and 9.19). Dialysis results at 20 °C showed that soluble P increased as total solids increased, ionic calcium increased slightly but total calcium remained almost constant. Dialysis at 115 °C showed the temperature dependence of these reactions, with pH, ionic calcium, soluble calcium and soluble phosphate being much lower than at 20 °C. All these factors showed some slight concentration dependence at high temperature, although soluble calcium was the property that changed least as total solids concentration increased. This was found to be the case in dialysates at 80, 90 and 100 °C. A dialysis membrane with a MWCO value of 250,000 Da showed that α_s-, β- and κ-caseins were found in dialysates at 80 °C using SDS electrophoresis. As dialysis temperature increased up to 115 °C, α_s- and β-caseins were still present, but there was no κ-casein found above 90 °C.

This study looked at the heat stability of reconstituted milk powders at 20% and 25% TS with different stabilising salts, including phosphates, EDTA salts, TSC and SHMP. Samples were sterilised at 115 °C for 15 min and assessed for their heat stability. Data for pH and ionic calcium prior to heat treatment are shown in Fig. 9.18, which also shows which samples coagulated and which did not. Two clusters can be seen, according to heat stability. The heat-stable samples appear on

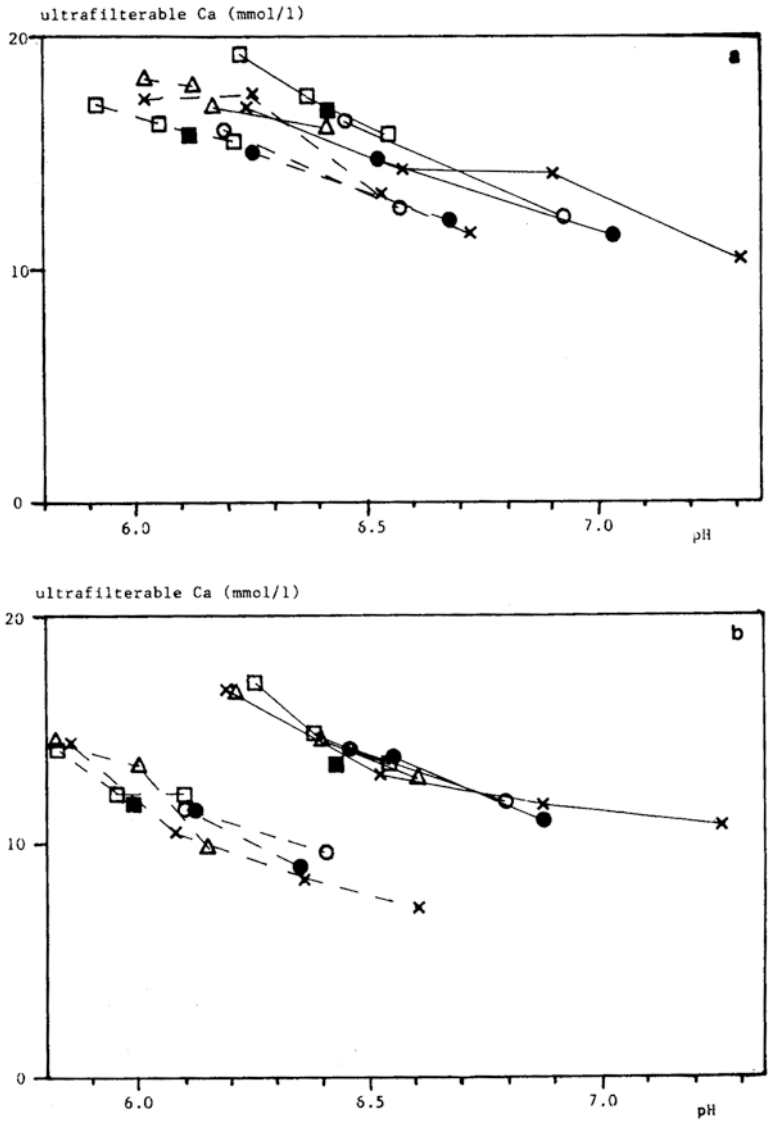

Fig. 9.16 Ultrafilterable Ca in concentrated milk before (solid line) and after sterilisation (dashed line): (**a**) without added phosphate; (**b**) with 18 mmol PO_4 per L concentrated milk. Closed symbols represent preheating at 74 °C for 2 s; all other symbols represent preheating at 120 °C for 3 min. (From Nieuwenhuijse et al. 1988, with permission)

the right side of the graph, showing a wide range in pH (6.47–6.70), as well as Ca^{2+} concentration (0.47–1.20 mM). The samples which had poor heat stability, and which coagulated appear on the left and all of them have pH lower than 6.47 and higher ionic calcium levels. SHMP addition at 0.4% and 0.5% (encircled

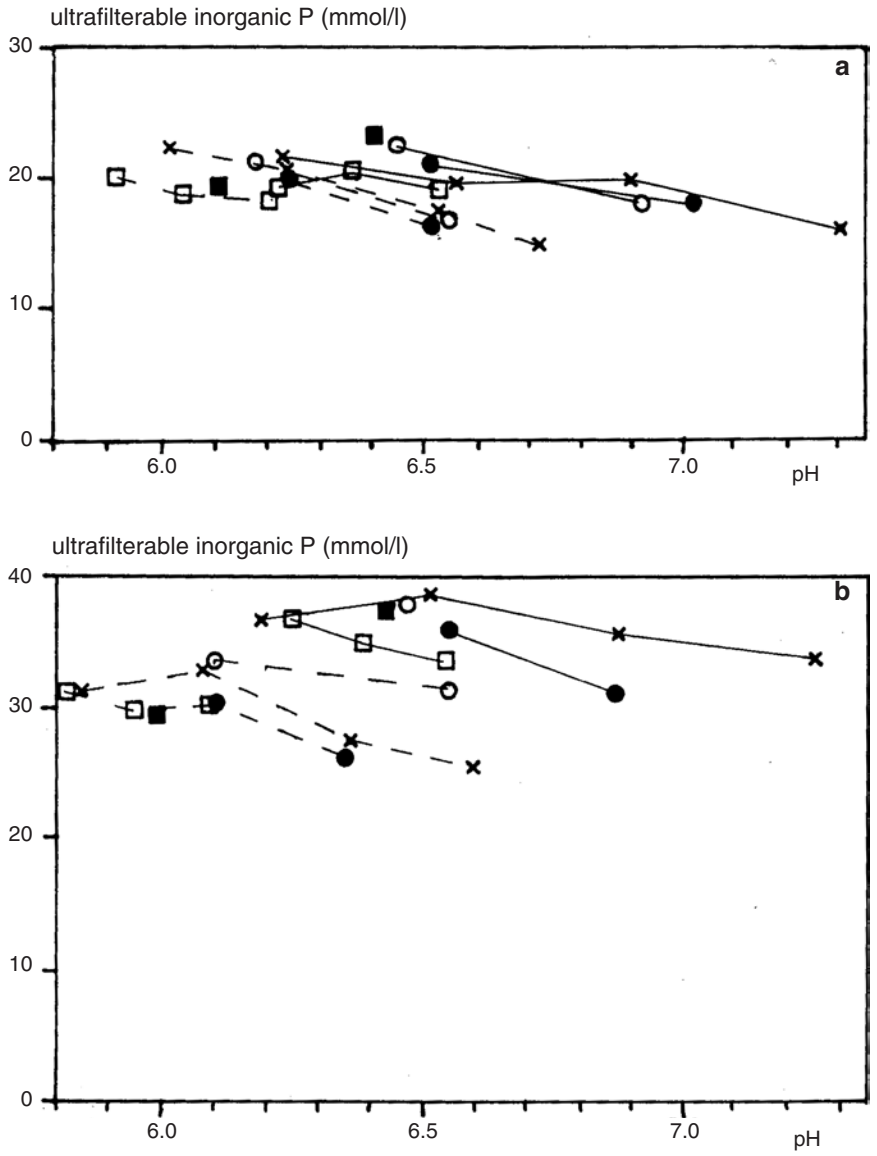

Fig. 9.17 Ultrafilterable inorganic P in concentrated milk before (solid line) and after sterilisation (dashed line): (**a**) without added phosphate; (**b**) with 18 mmol PO_4 per L concentrated milk. Closed symbols represent preheating at 74 °C for 2 s; other symbols represent preheating at 120 °C for 3 min. (From Nieuwenhuijse et al. 1988, with permission)

treatments) reduced Ca^{2+} concentration to lower than 0.70 mM, whilst the pH of those samples was below 6.45. Faka et al. (2009) showed that heat stability of a low heat skim milk powder could be improved using two strategies that reduced ionic calcium: to remove some calcium by ion exchange or to add TSC prior to drying.

Table 9.18 Dialysis at 20 °C for 24 h for skim milk powder reconstituted to different total solids, taken from Tsikritzi (2011), with permission

Solids (%)	pH	Ca^{2+}	Total P	Total Ca
9	6.68 ± 0.07	1.16 ± 0.04	15.3 ± 0.04	8.22 ± 0.12
15	6.62 ± 0.03	1.34 ± 0.03	18.3 ± 0.03	10.0 ± 0.28
20	6.53 ± 0.01	1.45 ± 0.01	23.1 ± 0.01	8.33 ± 0.16
25	6.48 ± 0.02	1.49 ± 0.06	23.7 ± 0.06	9.06 ± 0.19

All concentrations are mM

Table 9.19 Dialysates of skim milk powder at different total solids taken at 115 °C for 15 min, from Tsikritzi (2011), with permission

Total solids (%)	pH	Ca^{2+}	Total P	Total Ca
9	6.11 ± 0.05	0.29 ± 0.01	5.04 ± 0.84	3.52 ± 0.36
15	6.01 ± 0.03	0.32 ± 0.01	8.48 ± 0.76	4.34 ± 0.26
20	5.94 ± 0.07*	0.37 ± 0.02*	11.6 ± 1.00	5.66 ± 0.01
25	5.94 ± 0.10*	0.39 ± 0.02*	10.6 ± 0.65	5.50 ± 0.18

All concentrations are mM

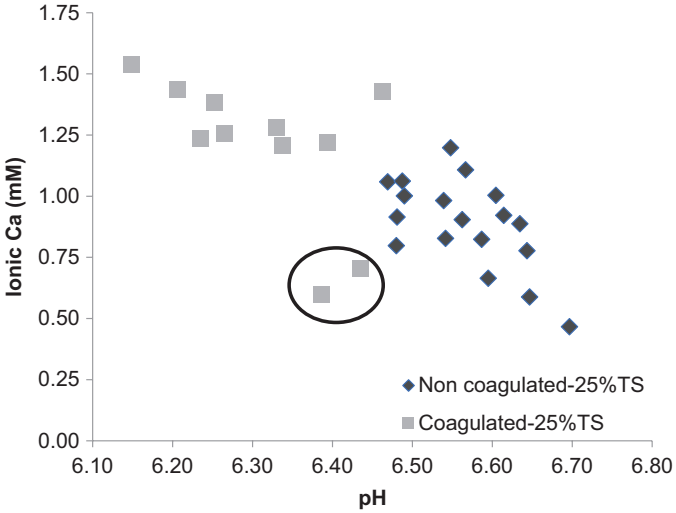

Fig. 9.18 Ca^{2+} concentration as a function of pH for reconstituted skim milk powder at 25% total solids before sterilisation at 115 °C for 15 min, with different added salts. Also shown are those samples which coagulated during heating and those that did not. (From Tsikritzi 2011)

Overall, there is a limited amount of information on partitioning of milk concentrates. Reliable data may not necessarily be obtained using UC and ultrafiltration, as there may be viscosity limitations. Dialysis may provide the best experimental approach, especially using membranes with high MWCO values, which will also show whether any monomeric casein is present. Some examples of its use have been illustrated here.

9.5.5 Membrane Processing

Concentration of milk can be achieved by reverse osmosis (RO) and by UF. Concentration by RO is not dissimilar to evaporation as the permeate is mainly water. Thus, as milk is concentrated, its pH will fall, levels of ionic calcium in the soluble phase will increase slightly and alcohol stability will fall. When powders produced from RO-treated milk were reconstituted to 25% TS, they were not stable when sterilised in the can (Syrios et al. 2011). Reverse osmosis is an excellent option for increasing the capacity of evaporation plant in terms of capital and running costs for milk and whey products, by up to 25–30% TS. In addition, the water recovered will also be potable, thus saving on water costs.

Concentration by UF is a much more interesting procedure. UF treatment as a partitioning method has been discussed in Sect. 9.2.2.2. It is also widely used as a method for modifying the composition of dairy streams, for example, for increasing protein in skim milk and whey and for increasing both protein and fat in full cream milk.

Concentration by UF is interesting because a permeate equivalent to the soluble phase of milk is removed continuously. For the permeate, both its total concentration of divalent cations and its levels of ionic calcium increase only slightly as milk is concentrated by UF. Lin et al. (2015) found that at a concentration factor of 4, the concentration of divalent cations in the permeate was about 12.8 mM, compared to 9 mM at the start of the process.

In contrast to RO, levels of ionic calcium and pH in the retentate hardly change as concentration factor increases. Proportionally more of the total Ca and P is associated with the casein micelle fraction, than with the soluble phase, mainly because the casein fraction is considerably concentrated, and it may be this which is responsible for the differences in rheological properties of products made from UF concentrates.

Throughout a UF process a permeate containing Ca and P is being continuously removed. It is less clear whether casein micelles differ considerably in their mineral composition compared to the original milk and also whether any significant casein dissociation takes place as a result of UF treatment.

Two options for manipulating the mineral content of UF concentrates are to use diafiltration and to do some ultrafiltration at a lower pH, or a combination of these processes (Hiddink et al. 1978; Bastian et al. 1991; Gaber et al. 2020a, b). Diafiltration (DF) offers an opportunity to remove more of the low molecular weight fraction. Water is added to the UF concentrate, which is then subjected to further ultrafiltration, usually to remove an amount of permeate similar to the water added. DF is essentially non-selective and will remove all components not bound to the membrane in equal proportions. The action of adding the water and removing more of the salts may cause both salts and some casein to dissociate from the micelle, but this mechanism is not clear.

Performing UF at different pH conditions allows the mineral fraction to be manipulated. UF at reduced pH will remove more Ca and P but will still retain all

the protein. Quarg produced from UF milk has improved flavour (see Sect. 9.6). When UF milk is used for making "cheese-type" products, there may be no further whey drainage. This will improve the protein recovery in the product, but it could adversely affect both its rheological properties and the flavour (Winwood 1983; Tamime and Robinson 1999). Also, in theory, UF could be done at a wide range of temperatures, which also gives scope for manipulating the mineral composition. The two options available to reduce microbial growth are below 10 or above 55 °C. Thus, there is scope for altering the amount of Ca and P that is lost in the permeate and retained in the product by a variety of methods.

Microfiltration (MF) is a more recent membrane process. Commercially, it is used to filter out bacteria from skim milk; filtered milk is increasing in popularity worldwide. In this process all the milk components pass through the membrane. However, some MF membranes will also retain casein micelles but allow whey proteins to permeate, allowing production of a casein-enriched fraction (micellar casein concentrate) that is not made by casein precipitation.

MF is of increasing interest for the cheese industry, mostly for its protein separation selectivity, where high casein retentates and native whey protein permeates can be obtained, which may offer some cost reduction possibilities (Lagrange et al. 2015). Such MF retentates can have some textural and flavour defects due to their higher mineral contents, but there are opportunities using diafiltration with various acidifying agents to remove some of these minerals (Gaber et al. 2020a, b). This would suggest that these membranes would also allow soluble casein to permeate and would thus be useful for casein partitioning studies.

On-Nom (2012) attempted to use small microfiltration PVDF units, to obtain permeates whilst milk was held at high temperature. Results using these microfiltration cartridges clearly showed that this method has potential, as MF performed on milk at 20 and 50 °C clearly showed the presence of the major whey proteins and some soluble caseins in the permeate, as determined by SDS electrophoresis. Of the membrane techniques, this would be the most appropriate to explore for further studies of casein partitioning at high temperature. Ceramic microfiltration modules are available and would provide an excellent opportunity to partition casein at high temperatures and obtain data on casein partitioning that could be used to compare with those found from modelling studies.

9.5.6 Fermentation Processes

Fermentation is involved in the production of cheese, yoghurt and other fermented milk products and involves conversion of lactose to lactic acid. This is usually monitored by measuring pH and/or titratable acidity, but freezing point depression could also be used. This fall in pH will be accompanied by an increase in minerals in the soluble phase or an increasing loss from the curd itself. Since all cheesemaking processes are different, the amount of minerals retained in the curd will be different and will depend very much on the ultimate pH of the cheese.

There are two partitioning issues related to cheese fermentation. The first concerns how the various components in milk partition between the cheese and the whey. The second is perhaps more subtle and concerns mineral partitioning within the cheese matrix itself, i.e., casein bound or dissolved in the cheese aqueous phase (Cooke and McSweeney 2017). In terms of partitioning between the cheese and whey, mineral loss in the drained whey is a major difference between conventional cheese manufacture and yoghurt production. Thus, during yoghurt manufacture all the mineral components would be predominantly in the soluble phase but they are all retained within the product, but in cheese manufacture those that are in the soluble phase will be lost in the whey on drainage.

Calcium is an important nutrient in milk and in most cheesemaking processes not all the calcium in the milk is recovered in the cheese curd. In most cheesemaking processes involving whey drainage, the majority of the calcium ends up in the cheese whey, along with the whey proteins. All cheeses will contain calcium but for reasons discussed its concentration is very variable; values for calcium (mg Ca per g protein) in a range of varieties include Cheddar cheese, 28.2; Edam cheese, 29.6; Feta cheese, 23.0; and cottage cheese 5. The values for the starting milk are 36 mg Ca per g protein, based on total protein and about 46 based on casein protein. So, not only does the cheesemaking process partition casein and whey proteins, but it also has a drastic effect on the amount of total calcium that is retained in the cheese. In most other fermented products, e.g., yoghurt, there is no separation involved and there may be some further fortification with milk solid to boost the texture of the product. This will not be the case for drained or strained fermented products.

The pH at whey drainage during cheesemaking not only has a huge effect on Ca retained in the curd but it also has a major effect on texture. High pH at drainage (e.g., Emmental) gives an elastic texture, whilst low pH at drainage (e.g., Cheshire) gives a crumbly, short texture (Cooke and McSweeney 2017).

There is also a soluble or aqueous phase present in cheese and there will be a partitioning of calcium within the cheese matrix, between that which is casein bound and that which is in the aqueous phase of cheese. During ripening Ca migration will take place from the curd into the aqueous phase and this can influence the texture of the cheese (Cooke and McSweeney 2017).

Cooke and McSweeney (2017) describe how soluble and insoluble calcium contents can be determined for cheese, by comparing the total calcium content of "juice" to that of the cheese. The soluble phase or cheese juice can be obtained by applying hydraulic pressure to the grated cheese and measuring its composition and comparing it to that in the cheese. Alternatively, its insoluble calcium content can be obtained from buffering capacity measurements. It is claimed that these two methods of measurement give results of similar accuracy for insoluble calcium.

The level of insoluble calcium reduces significantly during the ripening period, typically from about 72% to about 58% during the first 3 months of ripening. This transition will cause a softening of the curd and other textural changes. However, the level of insoluble calcium was found not to decrease below a level of 41% of total calcium during ripening of cheese, even when the pH of the curd was as low as

4.9 (Lee et al. 2005). Note that this is very different to the situation in milk where almost all the calcium would be soluble at pH.4.9.

In contrast, mould ripened cheeses such as Camembert show an increase in pH during the ripening period, due to liberation of ammonia. Changes in pH in such cheeses have been studied by Liu and Peri (2005). This generation of ammonia gives rise to a pH gradient within the cheese as the ripening time increases, with pH in the surface regions being higher than at the centre. The pH at the surface was found to be about 4.5 on Day 1 and approximately 7.5 after 35 days ripening. This is one of the rare situations where pH increases significantly during milk processing operations and, as discussed, pH increases make calcium phosphate precipitation more likely.

Thus, partitioning of Ca and other components plays an important role in both cheese texture and flavour and hence in the overall quality of the cheese.

Manufacturing soft cheeses or quarg by UF is interesting (Puhan and Gallmann 1980). The process involves concentrating skim milk by a factor of about four times and fermentation with a lactic acid culture. The order is very important. Some minerals and lactose will be lost during the UF process in the permeate, but whey protein will be incorporated not the curd. Experience has dictated that too much mineral retention results in an unpleasant off-flavour, described as bitter or metallic (Winwood 1983) as well as becoming smeary, gluey and shiny in appearance. Puhan and Gallmann (1980) reported that a higher calcium content is responsible for these unwanted defects which can be avoided by fermenting first, followed by the UF process. This will involve larger fermentation tanks and UF treatment of a more viscous product and also higher losses of Ca and P from the quarg but results in a product with a much cleaner flavour. Lawless et al. (2003) examined the taste characteristics of various calcium and magnesium salts which were described as salty, metallic, stringent, sour and sweet, generally in decreasing order of intensity.

Fermenting protein concentrates produces flavour problems which are most likely to be caused by the higher mineral content. The mineral content will most likely also affect the rheological properties of the final product and hence its texture, so using UF technology to produce fermented milk products is a challenging task (Tamime and Robinson 1999). Thus, if texture or flavour problems are encountered when using membrane technology for making fermented problems, it would be worthwhile looking at the mineral content of the problematic products. One example of this is discussed for quarg by Winwood (1983).

9.5.7 Carbon Dioxide Treatment

Carbonation of milk is a relatively simple process, especially if it is performed at atmospheric pressure. Carbonated soft drinks can be produced in high volume throughputs. Carbonation has been investigated for inhibiting microbial activity in milk. We also consume copious quantities of carbonated soft drinks, so it is curious that carbonated milk products have never become popular. Saturating milk with

CO_2 at atmospheric pressure will reduce its pH to between 5.9 and 6.2, but the final pH depends upon the CO_2 concentration, as described by Ma and Barbano (2003). When the milk is degassed, the pH can be restored to its original pH. Since pH changes are relatively small, the changes brought about to the casein micelle in milk with added CO_2 are small. Guillaume et al. (2002) concluded that the effects of acidification of reconstituted milk to pH 5.8 were completely reversible after CO_2 was removed by vacuum treatment.

If CO_2 treated milk is to be used for further processing, it would probably be best to remove the CO_2, although this may not always be necessary. CO_2 has also been investigated at higher pressures, in which case the pH change will be greater. A comprehensive review on the microbial effects of CO_2 and the use of CO_2 treated milk for production of yoghurt, cheeses and frozen desserts has been provided by Loss and Hotchkiss (2003).

De La Fuente (1998) speculated whether changes brought about by acidification are simply due to pH reduction, or whether other effects were evident. He reported that effects of CO_2 were more drastic than acidification processes at the same pH. However, milk which was saturated with carbon dioxide (pH 6.0) was more heat stable when heated to 80 °C, compared to milk which was reduced to the same pH by hydrochloric acid addition, which completely blocked the equipment. One explanation is that heating itself degases the CO_2, thereby increasing its pH and improving its heat stability.

Thus, a small amount of disruption will occur to the micelle as a result of CO_2 addition, but whether any casein dissociation takes place is not clear. It would be interesting to dialyse milk saturated with CO_2, to measure how much minerals and casein partition. It may even be possible to ultracentrifuge such saturated milk. Freezing point depression readings may also give some indication of changes brought about by the movement of salts to or from the micelle. Sometime in the future we may see a much wider range of carbonated dairy products selling alongside carbonated soft drinks.

9.5.8 High Pressure Processing (HPP)

Another process which is of interest is high pressure processing (HPP) of milk. HPP is a complex operation that requires expensive equipment and is limited to batch sizes of about 500 L. The one company in the UK offering contract high pressure processing currently has two machines of 135 and 420 L capacities. As far as dairy applications are concerned, the following products have been investigated; cheese and ham snacks, yoghurt-oat-fruit based breakfast drinks, a variety of cheeses, and smoothies containing milk, although milk products are only a small percentage of the total business. HPP may also be used to alter the properties of milk for technological processes, although it would need to be a drastic improvement to justify the high costs involved. High pressures have been investigated in considerable detail for inactivating the diverse microbial flora in milk and also the different enzymes in milk. However, I am only aware of one commercial process, which is that of

cold-filtered milk produced in Australia (Made by Cow 2021). Deeth and Lewis (2017) recently reviewed HPP of milk.

HPP enhances reactions that lead to a decrease in volume, in accordance with Le Chatelier's principle, and exerts a disruptive effect on the ionic and hydrophobic interactions. Pressure may have a greater disruptive effect at low temperatures, in terms of quicker disintegration of casein and shifts in pH values. However, in most cases changes caused by high pressure are usually measured after the treatment and are as a result of the treatment. What is happening and the properties of the milk whilst it is at high pressure is not known with any certainty, so it cannot be stated whether the changes induced by high pressure are reversible. Samaranayake and Sastry (2010) describe a pH sensor which can be operated in extreme environments and was tested at 25 °C for in situ pH measurement of several buffer solutions under high pressure up to 784.6 MPa. An increase in acidity was generally found for the buffer solutions with increasing pressure. Sensor response and pH changes were found to be completely reversible upon depressurisation at the same temperature. However, for milk, the resulting product from high pressure processing is subtly different to what it was before processing.

Applications of pressures between 100 and 200 MPa at 20 °C for 30 min generally caused little or no changes to casein micelles. Pressures of 250 MPa applied for more than 15 min lead to a significant increase (approx. 25%) in the average size of casein micelles caused by the aggregation of caseins. Applying pressures above 400 MPa reduced the average size of casein micelles by up to 50% (Bravo et al. 2015; Lopez-Fandino 2006). As a result of this, light scattering properties are changed and it loses its milky appearance. The milky appearance can be restored by heating the HPP milk. Also prolonged HPP (e.g., 250–300 MPa for 1–3 h) results in reassociation of dissociated casein aggregates. Most of the results have been performed on normal milk, say with up to 4% protein. HPP has been found to cause milk with upward of 10% protein to form a gel (Deeth and Lewis 2017).

Some dissociation of casein takes place during HPP. Their dissolution at pressures >400 MPa is in the following order: β-casein > κ-casein > α_{s1}-casein > α_{s2}-casein. This order is related to the binding sites of the caseins and their hydrophobic behaviour (Lopez-Fandino 2006).

The dissolution of colloidal calcium phosphate has a big impact on milk minerals (Lopez-Fandino 2006). According to the literature, the solubility of calcium and phosphorus increased by 42% and 63%, respectively, under pressures of 150–350 MPa (Kielczewska et al. 2009). HPP milk was found to have a higher content of soluble Ca and P after processing, but concentration of ionic calcium is about the same.

For goats' milk, moderate pressures of 300–350 MPa at 45 °C caused the formation of large micelles and increased the level of serum κ-casein substantially. Higher pressure resulted in a breakdown of these micelles (Law et al. 1998). At 20 °C, no large micelles were found and soluble casein did not change too much with pressure and appeared to peak between 300 and 400 MPa.

A recent review article examined the benefits of using HPP milk for manufacture of cheese, yoghurt and other dairy products (Ravash et al. 2020). It is interesting that whilst heat treatment produces subtle changes to the micelle once the milk has

cooled, high pressure processing has a much more drastic effect after removal of the pressure. Nevertheless, UHT milk can very rarely be coagulated by rennet, whereas HPP milk can be coagulated by rennet and it may even improve the process (de Castro et al. 2016).

9.5.9 Soya and Other Plant Protein Beverages

There is now more interest in plant protein beverages and soymilk is the most popular of these. The use of the term "milk" for these products is now prohibited within the EU and most of the raw materials used for their production have a much lower mineral and vitamin content than milk. Most also have a much lower protein content than milk and need fortification with calcium and with vitamins.

Pathomrungsiyounggul et al. (2012) used dialysis to measure pH and calcium in soymilk at high temperature, with and without added calcium (as chloride, lactate and gluconate salts). As for cows' milk, these calcium salts decreased pH and increased ionic calcium. At 100 °C coagulation was found when adding 3 mM calcium chloride, 3 mM calcium gluconate and 4 mM calcium lactate. Dialysis was performed at 80 °C and 100 °C for 1 h and 121 °C for 15 min. pH and levels of soluble ionic calcium decreased as temperature increased, which is similar to that found for cows' milk. Changes between 20 and 100 °C in soymilk with 3 mM added calcium chloride were pH reduced from 6.25 to 5.8; Ca^{2+} concentrations reduced from 0.21 to 0.03 mM.

Levels of soluble calcium were much lower than in cows' milk. In dialysate at 80 °C its values ranged from 12.0% to 13.4% of total calcium and at 100 °C it was 15.4–21.8% calcium, which is opposite to that found for milk. Ionic calcium was also very low but showed no variation with temperature. It is not clear whether the proportion of soluble calcium will increase in soymilk as pH is reduced, as it does in cow's milk. Most researchers report that heat treatment of soymilk results in a reduction in pH. This contradicts some of our pilot plant trials where UHT treatment of soymilk by direct heating at 145 °C resulted in an increase in pH of 0.3–0.4 units (unpublished results). There are many other plant protein beverages now on the market, such as almond, rice, oat and hemp. Most of these would be low in both minerals and vitamins without fortification and there is currently no information available about how minerals partition with the protein fractions in these beverages.

9.6 Further Partitioning Processes

Two other processes, where partitioning is involved are foaming of milk and the relocation and recovery of material from the milk fat globule membrane and skim milk membrane material.

9.6.1 Foaming of Milk

Milk has a lower surface tension than water, with values of about 50 mN/m, compared to 72.6 mN/m for water. Factors affecting the surface tension of milk are discussed in Chap. 12. Milk has a lower surface tension because it contains a number of surface-active components. These are numerous and they vary in their molecular size and complexity. One consequence of this is that milk is susceptible to foaming.

Foaming of milk can be described as a partitioning process, as it leads to an accumulation of these surface-active components at the gas–liquid interface. In principle, this foam could then be removed and allowed to drain, thereby selectively removing any compounds which have accumulated at the gas–liquid interface. Foam fractionation, as a separation process, has potential where the surface-active components may be of high value or be causing other problems, for example, having a bitter flavour, such as polyphenols in apple juice. However, foam fractionation is not widely practised in dairy processing. One possible reason is that there appears to be no significant enrichment of any specific proteins in the foam when compared to milk (Kamath et al. 2011). Nevertheless, milk foaming is of great interest, as it can be a problem in milk processing operations. Examples are when equipment is operating under vacuum, for example, in flash cooling, in vacuum evaporation or on some filling lines where it is very difficult to fill containers because of excessive foam formation. In contrast, milk which forms a stable foam is highly prized, for example, in some speciality coffees and other beverages.

There are various methods available to produce a milk foam: by aeration and agitation; by heating and agitation and by agitation and steam injection heating (Huppertz 2010; Ho et al. 2019). The design and engineering aspects of foaming devices are important to get the best foam from any milk sample, which would entail producing a large volume of foam that has good stability in a short time period. Links between injector design, steam pressure and milk foam quality were reported by Jimenez-Junca et al. (2015) for steam injection systems, whereas Ho et al. (2019) compared different types of devices for milk foaming.

Huppertz (2010) reviewed the foaming properties of milk. Good foam stability is a valued functional property of milk. Some milk foaming principles are that proteins encourage foam formation, whereas lipolysis tends to suppress foaming. Bubble coalescence, foam drainage, film viscosity and disproportionation are all factors that will influence foam stability. There is evidence that protein contributes to this in a positive manner, although the total amount of protein is not the key factor. Skim milk usually foams well. It shows its best stability at 45 °C (Kamath 2007); stability was hardly affected over the pH range 6.2–7.0, but was best at pH 7.0, which was attributed to slow drainage due to the higher viscosity of the milk. These results may also have been influenced by how the pH was adjusted. Heat treatment is not considered to have any major effect on foam stability and the main influence of mineral balance arises from how it influences casein micelle dissociation. Huppertz (2010) reported that dissociation of casein micelles has been shown

to improve the foamability. Sodium hexametaphosphate (SHMP) has been reported to improve foam volume and retarded drainage of foams. In contrast Kamath et al. (2011) found the opposite with reconstituted skim milk. In fact, EDTA, TSC and SHMP reduced the stability of foams, although they each had little effect on foam volume (Kamath 2007). It is interesting that skim milk can be diluted up to fourfold without affecting the volume or stability of foam. In contrast, whole milk which is not homogenised does not foam well. Foaming is improved by homogenisation but homogenised milk foams poorly up to 45 °C, but above that temperature it is improved and its foaming is almost the same as that of skim milk.

Xiong et al. (2020) measured foam stability of milk samples with different ratios of casein to whey protein. Foam stability was found to be higher as this ratio increased, which was possibly due to adsorption and spreading of the micellar caseins at the air–liquid interface compared with the whey proteins.

Kamath et al. (2011) provided further evidence that casein micelles perform better than some of the smaller protein entities and reducing casein micelles had a detrimental effect on foaming. Factors that affect casein dissociation were examined. TSC, SHMP and EDTA all decreased foam stability, which was attributed to an increase in soluble casein. Calcium chloride improved foam stability, which is stated to decrease levels of soluble casein, but generally levels required were too high to be used commercially, as they would decrease pH significantly, increase ionic calcium and could even impart a bitter note to the milk. Milk with added calcium chloride would also have a reduced heat stability. However, other proteins cannot be ignored, especially as foaming is a prime functional property of whey protein concentrates. Chen et al. (2014) performed a standard foaming test on 25 bulk milk samples collected from the same herd over a 12-month period and the time required to produce a stable foam ranged from 24 to 205 s. There were variations in all other measured properties; the range of values recorded was pH, 6.73–6.87; protein (%), 2.89–3.56; fat (%), 3.62–4.77; ethanol stability, 84–100% and rennet coagulation time, 12–24 min. Foaming showed the widest variations from all the properties that were measured. No correlation was found between foaming capacity and any of the milk compositional factors that were measured (including casein and total protein). However, free fatty acids and phospholipids were not measured in this study.

Kamath et al. (2011) also examined the protein fractions in the drained foam. Although this approach has real potential, no major differences were found in the overall balance of proteins found in the drained foams compared to that in the milk. This approach could be used to look at differences in the low molecular weight components in foams from milk that foam well and those which perform poorly. Foaming of milk from other species has not been so well studied.

The role of some of the compounds that inhibit foaming is more complex (Huppertz 2010). In milk foams, if polar lipids occupy the interface, coalescence occurs. However, phospholipids (PL) alone may stabilise foams, but combinations of PL and protein can have a destabilising effect, which is described as a mutually incompatible means of foam stabilisation. It is interesting that up to 60% of PL in milk is in the skim milk fraction (Sect. 10.2). Lipolysis and excessive lipase activity contribute to poor foaming; some of the free fatty acids may have stronger foam

suppression properties than others. Thus, the presence of phospholipids, free fatty acids and partial glycerides strongly impairs foaming of milk. It is claimed that they compete with the proteins for being located at the interface; it might be that they are more mobile than the proteins. Buttermilk does not foam well, which is attributed to its high phospholipid content.

Wilde et al. (2014) provide an explanation that the stabilisation mechanisms by proteins and phospholipids are mutually incompatible to explain why systems containing both polar lipids and protein show poorer foam stability than systems stabilised by those components alone. The same applies to free fatty acids, which along with phospholipids are known to stabilise foams.

As mentioned, the foaming properties of milk have become more apparent recently, as increasing amounts of milk are used by coffee producers for their speciality beverages and the popularity of kitchen-scale milk frothing devices. The main requirement here is that the foam can be produced quickly and remains stable whilst the coffee is being consumed, e.g., 10–15 min. What is apparent to both supplier of milk to coffee outlets and end-users is that there are considerable variations in the foaming capacity of individual milk samples. Those compositional factors that contribute to these qualities are recognised but it is still not easy to predict how well a milk will foam from its composition, although some general principles have been discussed earlier. Foamability is also not related to fat content.

For suppliers of milk to the coffee industry, it is important to know whether poor foaming is caused by a deficit of those proteins that contribute most to foaming, especially casein micelles, or a surplus of low molecular weight surfactants, such as free fatty acids and phospholipids. It is possible that both situations may exist, but the latter is most likely to provide the explanation, especially as it has been observed that milk can be diluted without altering its foaming potential. Of course, the situation is further complicated because coffee is both hot and acidic and its pH can easily range from 4.8 to 5.8. This too could be another important factor, especially in situations where poor stability is observed after the foam is added to the coffee. There may also be inhibitory surface-active components in the coffee itself and calcium and other minerals in the water used to make the coffee.

Another approach would be to take milk that foams poorly and look at how this could be improved, for example, by selectively removing some of its low molecular weight components, e.g., by dialysing against SMUF with lactose. These observations suggest that the foaming of milk cannot be predicted from simple analytical tests that can be done in a quality assurance laboratory. Therefore, those supplying milk to be used in foaming applications (e.g., coffee) should establish that it foams well by using one of the many available milk frothing devices that are now available.

9.6.2 Milk Membrane Material and Its Isolation

The milk fat globule is covered and stabilised by a layer of material known as the milk fat globule membrane. This is a complex layer discussed by Oliveira and O'Mahony (2020) and Lopez (2020). This layer keeps the fat and aqueous phases

apart in full cream milk. This is one of its most important biological functions, but it is now recognised that some of the components that comprise this membrane have useful functional and health related benefits (Hernell et al. 2016). Thus, recovering these components and producing products that are enriched with these components is currently of great interest. One such simple product is buttermilk.

The MFGM serves two main functions. The first is to disperse the fat into small globules and to stabilise these globules. The second is to protect the fat from the action of the indigenous lipoprotein lipase in the milk. Lipase action causes release of free fatty acids, which would impart an objectionable taste in milk and inhibit foaming, as discussed above. These rancid off-flavours resulting from damaging the membrane in raw milk at 30–40 °C, for example, by agitation or homogenisation, are very objectionable.

The structure of the membrane is also complex. It comprises three layers (Oliveira and O'Mahony 2020): one might expect that material in the layer close to the fat phase will be more difficult to remove and thus remain with the fat, whereas material in the outer layer may be more easily removed, but this might be too simplistic an approach. We will see later that many of the structural compounds found in MFGM are also found in skim milk.

Briefly the main components of MFGM are summarised in Table 9.20 and are lipids, proteins and carbohydrates, but each of these fractions may be more complex. They are present in approximately the following ratios: protein: lipids: carbohydrate: 4:3:1. The most abundant protein in that fraction is butyrophilin, the two most common enzymes are xanthine oxidase and alkaline phosphatase and there are several other minor proteins. The predominant polar lipids are the phospholipids, which are probably the most valued fraction from a health perspective. The main phospholipids and their approximate compositions are phosphatidylcholine (25–30%); phosphatidyl ethanolamine (25–30%) and sphingomyelin (25–30%). Smaller amounts of ceramides 5–8% and glycolipids such as gangliosides (2–4%) are also present.

Table 9.20 Main components of milk fat globule membrane material (MFGM) and skim milk membrane (SMM), compiled mainly from Deeth (2018) and other sources

Component	MFGM	SMM
Proteins	Butyrophilin (40%), others	Similar enzymes to MFGM, but their activities are different
	Enzymes: xanthine oxidase, alkaline phosphatase	
Lipids	Neutral lipids (triglycerides) and cholesterol, phospholipids and glycolipids	SMM have the same lipid components as MFGM, although amounts of each lipid are different
Carbohydrates	Associated with proteins and lipids as glycoproteins and glycolipids	Believed to be fractions of plasma membrane of mammary secretory cells
Other facts	Ratio proteins:lipids:C/H—4:3:1	SMM: no identifiable function
	MFGM	Skim milk membrane (SMM)
		Obtained by UC of skim milk

In practice, MFGM is removed by churning cream, which is a phase inversion process, resulting in butter and buttermilk. Thus, if considering recovering MFGM material, buttermilk is one starting material. An excellent source of buttermilk is that produced by churning cream at about 75% fat, which is produced by a double centrifugation process and is known as the Ammix Process (Ammix 2021).

MFGM fragments are produced in the churning process and can be further enriched by microfiltration. However, one problem in using MF for enrichment is the size similarity between MFGM fragments and the casein micelles. The size distribution of the main species involved in these separations is:

whey proteins: 3–6 nm
casein micelles 60–300 nm
MFGM fragments 100 nm to 4 μm
intact milk fat globules 100 nm to >1 μm
Holzmüller and Kulozik (2016)

Thus, two approaches for enriching MFGM fragments by removing casein are to induce dissociation of the casein micelle or to remove casein micelles by coagulation. Whey cream would be another useful starting material for churning, as casein micelles would have been previously removed. Recovery has been described in more detail by Deeth (2018).

In terms of measuring the efficiency of a partitioning process for MFGM fragments, the situation is complicated because many of the compounds found in the MFGM are also found in skim milk; for example, only 40% of the phospholipids found in milk are present in MFGM. The remaining 60% is found in the skim milk fraction in the form of what have been termed "extracellular vesicles", which have been described as being without a triglyceride core (Christie et al. 1987; Arranz and Corredig 2017). Thus, the totality of these compounds which are found in skim milk and which are similar to compounds found in the MFGM is now known as *skim milk membrane material* (SMM), which is believed to be the same as what others call extracellular vesicles (Deeth, personal communication).

In the laboratory, SMM material can be visualised by ultracentrifuging skim milk, with produces a pellet of casein and a lighter "fluff" layer which is SMM material (Deeth 2018). Kitchen (1974) prepared SMM and MFGM material from the same batches of milk and presented detailed tables on their differences in composition, enzyme levels and activities, molecular weights of their major protein fractions and amino acid compositions of their proteins. A brief overview of these results is provided in Table 9.21 which shows that some of these enzymes are present at higher activity in SMM, whereas others are more active in MFGM. Electrophoretic profiles of the protein fractions are presented by Kitchen (1974) and the amino acid data showed hardly any differences in their amino acid profiles. It may not be clear whether these components ever had any involvement in the MFGM.

Thus, SMM material can be enriched in products, starting from renneted skim milk or cheese whey. In both cases enrichment is by microfiltration, and with these materials, there will be no casein to interfere with the process. In fact, cheese whey after separation still contains lipid material, as very small fat globules which are not

Table 9.21 Composition of material from skim milk membrane (SMM) and milk fat globular membrane (MFGM) (from cream), compiled from information in Kitchen (1974)

Component	SMM	MFGM (cream)
Total lipid	1.01 ± 0.07	1.06 ± 0.11
Phospholipid	0.43	0.33
Cholesterol	0.110	0.035
Natural hexose	0.154	0.111
Sialic acid	0.052	0.019
Xanthine oxidase	0.075	0.24
Alkaline phosphatase	0.56	0.32
Acid phosphatase	0.0058	0.0125
Nucleotide pyrophosphate	1.85	0.39
Sulphydryl oxidase	0.70	0.27
Most abundant protein M wt. and (abundance)	85,000 (32.5%)	70,000 (34%)

Units are mg/mg protein for chemical components or units of activity/mg for enzyme activity

removed by centrifugation in addition to SMM material; the lipid material can be further concentrated by microfiltration. A lipid fraction is also removed by microfiltration during production of whey protein isolate. This fraction is maintained in the retentate and its production and properties are discussed by Levin et al. (2016).

This can be dried and sold as an enriched product. Whey protein phospholipid concentrate is now commercial product obtained during manufacture of WPI by microfiltration of whey. Examples of phospholipid-rich products include Lacprodan PL-20 (containing 49–55% protein) produced by Arla (2021) and milk phospholipid concentrates at 70% and 90% protein, produced by Fonterra (2021b).

Therefore, whilst the MFGM membrane provides an effective barrier between the milk fat and the aqueous phase, its components also play an important role as functional ingredients and in infant nutrition. Recovering these components from milk and producing products enriched in these components is an excellent example of using partitioning technologies to provide added value from milk. Hansen (2019) evaluates more gentle processes to isolate material from the MFGM, starting with microfiltration and diafiltration of raw milk, to enrich the MFGM protein and phospholipids in the final product.

9.7 Concluding Remarks

The main partitioning events in milk involve the movement of components to and from the casein micelle; this movement may affect how the milk behaves in various manufacturing processes.

Methods have been described to measure how various components migrate under different conditions and models have been discussed which predict partitioning of salts. Modelling has progressed further to predict how the different casein factions bind to the calcium phosphate nanoclusters. This latter approach can be used to assess the thermodynamic stability of milk and whether it is likely that calcium phosphate precipitation will take place. Such precipitation could be very distressing and even life-threatening to the cow.

It is well known that the casein micelle provides adequate nutrition in terms of protein and minerals. Compared to most other proteins in our biological world, it also shows great heat stability. Milk processing operations involve doing one of the two things: trying to keep the casein micelles apart or trying to bring them together. Thus, an interesting question to ask is whether the modelling approach will be able to predict the "best use for milk". Another major challenge for modelling is to extend it to study partitioning at high temperatures. Perhaps more experimental work on partitioning at high temperature might provide ammunition to help determine some of the association constants required for the model.

The casein micelle might appear to be a static entity. However, this grossly simplifies what is happening. The casein micelle is a very dynamic environment where components move backward and forward between the micelle and the soluble phase as milk is subjected to different processing procedures.

Ultracentrifugation provides one means of removing the micelles from the soluble phase. Dialysis, ultrafiltration and microfiltration are also capable of separating micellar casein from soluble casein and whey protein, if appropriate membranes are selected, but development of an ultracentrifuge that could work at high temperatures would also be of great benefit.

Using dialysis and UF procedures provides another means of retaining casein micelles and removing a soluble phase and these techniques can be used to evaluate partitioning at high temperatures. Some of these membranes will remove only the salts and retain all the proteins, but membranes with larger MWCO values are now available which will remove whey proteins and dissociated casein but retain micellar casein, which offer further opportunities to look at partitioning processes at high temperature. Being able to measure pH, ionic calcium, salt partitioning and casein dissociation at high temperature will help to provide a better understanding of factors affecting the heat stability of milk.

It has been challenging to think about the different concepts of stability that the casein micelle imparts to milk. That the micelle can provide more than adequate mineral and protein nutrition, but also remain stable and not precipitate out is remarkable. As is the interaction of the casein micelle with the soluble phase of milk to impart unexpected high heat stability and also be transformed and incorporated into a wide and varied range of products for our continued enjoyment.

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Chapter 10
Vitamins and Minerals in Milk: Levels and Effects of Dairy Processing

T. R. Hill

10.1 Introduction

Milk is a rich dietary source of many vitamins and minerals, which are central to the functioning of many physiological and biochemical systems in organisms. The vitamin fraction of milk is composed of lipophilic (vitamins A, D, E, and K) and hydrophilic (B vitamins and vitamin C) vitamins. Lipophilic vitamins are often present in the milk fat fraction due to their hydrophobic properties (cream, butter), while the hydrophilic vitamins are found in milk's aqueous phase.

Vitamins are a heterogeneous group of organic substances that are present in our natural foods that are of very high biological potency, and are required in extremely small concentrations for growth and maintenance of normal cells and body function. They need to be supplied in the diet, either because the body cannot make them, or cannot do so in amounts that are essential for growth, maintenance and normal and body function. Vitamin families are chemically heterogeneous and are generally classified according to their physical properties, i.e., as being either fat-soluble, or water soluble. The fat-soluble vitamins tend to have predominately aromatic or aliphatic character, whereas the water-soluble vitamins tend to have one or more polar or ionizable group (carboxyl, keto, hydroxyl, amino, or phosphate).

The first half of the twentieth century saw the identification, extraction, and purification of many vitamins. The synthesis and production of these essential nutrients

T. R. Hill (✉)
Population Health Sciences Institute, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, UK
e-mail: tom.hill@ncl.ac.uk

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soon followed and cures for many classical nutritional diseases, including scurvy, beriberi, pellagra, and rickets, were demonstrated. More recently, several epidemiological studies have examined the association between vitamin intake/status and various chronic diseases, such as cancer, cardiovascular diseases, and osteoporosis. Recognition of the prominent role of some micronutrients as antioxidants in preventing free radical-mediated tissue damage has awakened a re-evaluation of vitamin status and has shed new light on the importance of some vitamins in the prevention of some chronic diseases and the influence of these nutrients at the cellular and molecular levels. Milk makes a significant contribution to the intake of vitamins A and D in American children and adults, with a minor contribution to the intake of vitamins C, E, and K. For example, milk contributes between 10% and 30% to vitamin A intake in children and adults, while fortified milk contributes 40% and 60% of vitamin D intake by toddlers and adults and is thus the most important source of this vitamin in the American diet (O'Neil et al. 2012). Milk is also a major contributor to vitamin B_{12} intakes in the American and British diet, typically providing between 25% and 40% of total vitamin B_{12} intake (O'Neil et al. 2012; Bates et al. 2014).

Minerals, including trace elements, are inorganic substances that have a physiological function in the body. Elements must be provided in the diet, as they cannot be interconverted. The requirements for minerals and trace elements vary from grams (g) per day (e.g., potassium) to milligrams (mg) per day (e.g., iron) and micrograms per day (μg) (e.g., copper). Fourteen of the minerals present in bovine and human milk (calcium, chloride, cobalt, copper, iodine, iron, magnesium, manganese, molybdenum, sodium, phosphorus, potassium, selenium, and zinc) have well-established essential physiological functions that range from structural components of body tissues to essential components of many enzymes and other biologically important molecules. Another seven minerals (arsenic, boron, chromium, fluorine [as fluoride], nickel, silicon, and vanadium) are not considered essential but may be beneficial, based on the evidence that they have a role in some physiological processes in one or more mammalian species. In Western diets, milk is a major contributor to the dietary intake of a range of minerals, including calcium, phosphorus, iodine, selenium, and zinc.

This chapter will summarize the vitamins and minerals in milk. Each nutrient will be described under the following headings; nutritional significance, biological roles, methodological aspects in foods (mainly for fat-soluble vitamins), milk composition and the effects of processing on milk nutrient composition.

10.2 Fat-Soluble Vitamins

10.2.1 Vitamin A

Vitamin A is a generic term used to designate any compound possessing the biological activity of retinol. The term "retinoids" was designated to include compounds consisting of four isoprenoid units joined in a head-to-tail manner. All retinoids may

be formally derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional terminal group at the terminus of the acyclic portion. All three basic forms (retinol, retinal, and retinoic acid) are found in two variants: with the β-ionone nucleus (vitamin A_1) or the dehydrogenated β-ionone nucleus (vitamin A_2), with about half the vitamin A activity. The parent retinoid compound all-*trans*-retinol, is a primary alcohol with molecular mass of 286 Da. In most animal tissues, the predominant retinoid is retinyl palmitate but other fatty acid esters, such as retinyl oleate and retinyl stearate, are also found. Most of these metabolites occur in the all-*trans* configuration. The 11-*cis*-aldehyde form, 11-*cis*-retinal, is present in the retina of the eye, and several acid forms, such as all-*trans* and 13-*cis*-retinoic acid, are metabolites of retinol found in many tissues. Carotenoids may contribute significant vitamin A activity to foods of both animal and plant origin. Of the estimated 500 known carotenoids, ~50 exhibit some provitamin activity (i.e., are partially converted to vitamin A in vivo).

For a compound to have vitamin A or provitamin A activity, it must exhibit certain structural similarities to retinol, including, (a) at least one intact mono-oxygenated β-ionone ring, and (b) an isoprenoid side chain terminating in an alcohol, aldehyde, or carboxyl functional group. The vitamin A-active carotenoids such as β-carotene are considered to have provitamin A activity until they undergo oxidative enzymatic cleavage of the central C15:C15′ bond in the intestinal mucosa to yield two molecules of retinal, which can either be reduced to retinol or oxidized to retinoic acid. Carotenoids with ring hydroxylation or the presence of a carbonyl group exhibit less provitamin A activity than β-carotene if only one ring is affected and have no activity if both rings are oxygenated.

Until recently, the term ‘retinol equivalents’ (REs) was used to convert all sources of preformed retinol and provitamin A carotenoids in the diet into a single unit (NRC 1989). Nutritionally, 1 μg RE = 1 μg of all-*trans*-retinol = 2 μg of supplemental (in oil) all-*trans*-β-carotene = 6 μg of dietary all-*trans*-β-carotene = 12 μg of other dietary provitamin A carotenoids. When defining RE, it was assumed that the efficiency of absorption of provitamin A carotenoids was relatively good. Recent studies have shown, however, that absorption of carotenoids is much lower and appears to be quite variable. In addition, a number of factors such as protein-energy malnutrition, zinc deficiency, dietary fat, alcohol, infections and degree of food processing and the food matrix affect the bioavailability and bioconversion of retinol and carotenoids (Parker et al. 1999; van het Hof et al. 1999). Based on these and other studies, it is estimated that 1 retinol activity equivalent (μg RAE) is equal to 1 μg of all-*trans*-retinol, 2 μg of supplemental all-*trans*-β-carotene, 12 μg of dietary all-*trans*-β-carotene, or 24 μg of other dietary provitamin A carotenoids (e.g., α-carotene, β-cryptoxanthin) (Parker et al. 1999; Food and Nutrition Board 2000; Trumbo et al. 2001).

Most retinoid and carotenoid analyses have been performed by partition RP-HPLC on octadecylsilane (C18) columns, but C30 columns have proven useful for certain demanding separations of carotenoids (Sander et al. 1994). The most commonly used detectors are UV and visible light absorbance detectors, although fluorescence detectors (retinol and retinyl esters are fluorescent but other retinoids

and most carotenoids are not), electrochemical detectors and mass spectrometers are also used (Furr 2004). Detailed reviews of HPLC analysis of retinoids and carotenoids, including sample preparation techniques have been published (Barua et al. 2000; Song et al. 2000). A number of analytical methods have been described for the determination of vitamin A in milk and milk-based infant formulae either alone (Strobel et al. 2000; Miyagi et al. 2001) or with other fat-soluble vitamins (see vitamin E section for details).

The retinol, carotene, and total vitamin A content (as retinol equivalents) for bovine and human milk from the British National Food Composition Dataset is shown in Table 10.1. The mean content of vitamin A and β-carotene in cow's milk is 40 μg/100 g (range 10–100) and 20 μg (range 3–50), respectively (Walstra and Jenness 1984; Renner et al. 1989). The retinol content averages 16.3, 32.6, and 52.2 and that of β-carotene 9.6 μg/100 g, 16.7 μg/100 g, and 3.0 μg/100 g in milk (1.9% fat), milk (3.9% fat), human milk, respectively (Ollilainen et al. 1989). The contents of the fat-soluble vitamins and β-carotene in milk are highly dependent on the amount consumed in the feed (Jensen et al. 1999). Vitamin A and β-carotene concentrations of milk follow a seasonal trend, with higher values being obtained during the outdoor grazing period (O'Brien et al. 1999). Higher concentrations of

Table 10.1 Average concentrations of fat soluble vitamins (A, D, E, and K) and vitamin C in human and bovine milks (per 100 ml or 100 g as appropriate) from the British National Food Composition Database (McCance and Widdowson 2018[a])

Food name	Retinol (μg)	Carotene (μg)	Retinol equivalents (μg)	Vitamin D (μg)	Vitamin E (mg)	Vitamin K_1 (μg)	Vitamin C (mg)
Milk, condensed, whole, sweetened	110	70	122	Trace	0.19	0.36	4
Milk, evaporated, whole	105	100	122	2.7	0.19	0.50	1
Milk, human, colostrum	155	135	178	N	1.30	ND	7
Milk, human, mature	58	24	62	N	0.34	ND	4
Milk, semi-skimmed, pasteurized	19	9	20	Trace	0.04	ND	2
Milk, skimmed, pasteurized	1	Trace	1	Trace	Trace	0.02	1
Milk, whole, pasteurized	36	14	38	Trace	0.06	0.60	2
Milk, whole, UHT	54	31	59	0.0	0.08	0	Trace

N significant amounts present but no reliable indication of the amount
ND no data
[a] Data from Composition of foods integrated dataset (CoFID)—GOV.UK (www.gov.uk) accessed 12/03/2021

vitamin A and β-carotene are present in retail milk during both the outdoor grazing (June to October) and indoor feeding (December to March) periods compared to manufacturing milk, which reflects the higher feed concentrate input in retail milk production (O'Brien et al. 1999). Seasonal variation has also been observed by Hulshof et al. (2006), who observed that winter milk contains 20% less retinol and β-carotene than summer milk. Higher amounts of retinyl esters are found in colostrum (233–369 μg/100 mL) than in mature milk (33–57 μg/100 mL) (Debier et al. 2005). The age of the cow also appears to exert an effect on the concentration of vitamin A in colostrum and milk. In cows, primiparous females exhibit significantly higher vitamin A concentrations in plasma, colostrum and milk than multiparous females (Kumagai et al. 2001).

Kim et al. (1990) reported that the retinol concentration (mean ± SD) in human milk is 57 ± 25 μg/100 g, and carotenoid concentrations (μg/100 g) are 4.6 ± 1.6 for β-carotene, 3.2 ± 0.9 for α-carotene, 3.8 ± 2.1 for lycopene, and 11.5 ± 3.4 for lutein. Meneses and Trugo (2005) determined the concentrations of retinol, β-carotene, and non-provitamin A (lutein + zeaxanthin) carotenoids in mature human milk. Nutrient concentrations (μm/L, mean ± SE) in milk were retinol, 1.4 ± 0.1; β-carotene, 0.018 ± 0.002; lutein + zeaxanthin, 0.006 ± 0.001. Similar retinol levels have been described for the milk of well-nourished lactating women (Roy et al. 1997; Canfield et al. 1998; Rice et al. 1999). The milk of multiparous women contained higher levels of retinol than milk of primiparous women. Colostrum samples from human donors showed considerable variation in total carotenoid concentrations (34–757 μg/100 mL) (Patton et al. 1990). Multiparous mothers had higher mean colostrum carotene concentrations than did primiparae, 218 ± 194 vs. 114 ± 132 μg/100 mL, respectively.

Loss of vitamin A activity of retinoids and carotenoids in foods occurs mainly through reactions involving the unsaturated isoprenoid side chain, by either autoxidation or geometric isomerization. The 9-*cis* and 13-*cis* isomers, resulting from all-*trans*-retinol isomerization reactions, have been found in many types of foods, including cheese, UHT milk and butter, at different concentrations, depending on the processing and/or storage conditions (Woollard and Indyk 1986; Fellman et al. 1991). Pasteurized milk heated at temperatures ranging from 72 to 76 °C for 15 s had an average 13-*cis*: all-*trans* ratio of 6.4% (Panfili et al. 1998). Milk subjected to a more severe heat treatment had a higher degree of isomerization (UHT milk, 15.7%; sterilized milk, 33.5%), consistent with increased thermal conversion of the retinol isomers. Photochemical isomerization of vitamin A compounds occurs both directly and indirectly via a photosensitizer (Pesek and Warthesen 1990). The ratios and quantities of *cis* isomers produced differ depending on the photoisomerization. The type of packaging material has a significant effect on net retention of vitamin A activity in food exposed to light during storage. Vassila et al. (2002) reported vitamin A losses ranging from 15.1% to 73.6% in whole milk stored in various flexible monolayer and multilayer co-extruded pouches held under fluorescent light at 4 °C for up to 7 days. Zygoura et al. (2004) reported losses of 8.8–50.9%, depending on the packaging material, when pasteurized milk was stored under fluorescent light at 4 °C for 7 days.

10.2.2 Vitamin D

The term ‘vitamin D’ was given during the early 1920s to a group of closely-related secosteroids with antirachitic properties. The two major dietary forms of vitamin D in foods are cholecalciferol (vitamin D_3, derived from animals) and ergocalciferol (vitamin D_2, derived from plants). Both chole- and ergo-calciferol are also formed by photoirradiation from their precursors 7-dehydrocholesterol and ergosterol in vertebrates and some fungi, respectively. The chemical structures of vitamin D_2 and vitamin D_3 differ only in their side chain at C-17, which in vitamin D_2 has a double bond and an additional methyl group.

The various vitamin D compounds found in foods include chole- and ergo-calciferol, their pro- and pre-vitamins and their hydroxylated metabolites. The determination of these in foods provide a good estimate of the total vitamin D content of foods (Mattila et al. 1996). Previous analytical methods based on biological assays and were unable to distinguish between different vitamin D compounds, were laborious and very costly. High-performance liquid chromatography (HPLC) with UV detection is now the most widely used analytical method for measuring vitamin D compounds in foods because of its reliability, ease, and ability to separate the various vitamin D compounds (Takeuchi et al. 1993; Mattila 1995).

Older HPLC methods only characterized ergo- and cholecalciferol, but recent advances in analytical methodology can separate and characterize the 25-hydroxyvitamin D compounds in addition to ergo- and cholecalciferol. For example, the ability to detect 25 (OH) D_3, especially in meat products, from the mid-1990s produced large amounts of data on the vitamin D content of meat which was previously unavailable (Mawer and Gomes 1994; Mattila 1995; Clausen et al. 2002; Ovesen et al. 2003). This is of importance because 25 (OH) D_3 is absorbed better and more rapidly than native vitamin D_3 and it may be up to 5 times more active (Ovesen et al. 2003). Unfortunately, to date, no reliable HPLC method has been developed which is capable of quantifying 1,25-dihydroxyvitamin D compounds, due to the fact that these compounds are present in very low amounts. When estimating the total vitamin D content of foods, it is important to note the varying antirachitic activities of the various vitamin D compounds. For example, it is assumed that vitamin D_2 and D_3 are equal whereas 25(OH)D is assumed to have 5 times the antirachitic activity (i.e., the therapeutic potential to cure rickets) of either vitamin D_2 or D_3 (Ovesen et al. 2003). The vitamin D content of foods is usually described in μg or international units (IU), where 1 μg = 40 IU.

Vitamin D is naturally present (mainly as vitamin D_3) in only a few foods: oily fish, meat, egg yolk and milk and the level present in such foods can be highly variable. Since animals can synthesize vitamin D and obtain it from their feed, both ergo- and cholecalciferol-based compounds can be found in milk. In general, bovine and human milk are not good sources of vitamin D (Table 10.1), containing between 0.1 and 1.5 μg/L (Søndergaard and Leerbeck 1982; Reeve et al. 1982). Both vitamin D_3 and 25(OH)D_3 have been identified in milk (Mattila 1995). Milk also contains significant amounts of its water-soluble analogue, vitamin D sulfate, although the

biological activity of this compound is low (Hollis et al. 1981). The most important determinant of the vitamin D content of milk is sunlight exposure and seasonal variation in the vitamin D content of cow's milk has been reported, with higher values in summer (~0.35 μg/L) than in winter (~0.25 μg/L) (Scott et al. 1984a, b). The antirachitic activity of human milk is variable and is predominately affected by season and maternal vitamin D intake. In addition, the circulating 25(OH)D concentrations in breast-fed infants are related directly to the vitamin D content of the mothers' milk (Cancela et al. 1986).

In the US, nearly all milk is fortified with vitamin D_3 to a level of approximately 10 μg/L (400 IU). In such countries, fortified milk makes a substantial contribution to the mean daily intake of vitamin D. Furthermore, the effect of vitamin D-fortified milk on the serum 25(OH)D level shows marked increases. For example, in a study of healthy adults, aged between 17 and 54 years, the consumption of fortified milk (12 μg vitamin D_3/L) reduced the seasonal decline in serum 25(OH)D by >50% (McKenna et al. 1995). However, in countries where fortification of milk is not mandatory, such as the UK and Ireland, milk contributes <10% to the mean daily intake of vitamin D (Hill et al. 2004). Stability studies show that, on exposure to light, there is a slight loss of vitamin D_3 from fortified milk (Renken and Warthesen 1993), although this is unlikely to be caused during normal handling and storage of milk. Air exposure does not affect stability in milk (Renken and Warthesen 1993).

10.2.3 Vitamin E

Tocochromanols are a group of four tocopherols (α-, β-, γ-, and δ-) and four tocotrienols (α-, β-, γ- and δ-) produced at various levels and in different combinations by all plant tissues and some cyanobacteria. The polar head group is derived from aromatic amino acid metabolism and the hydrophobic tail from phytyl-diphosphate (phytyl-DP) or geranygeranyl diphosphate (GGDP) for tocopherols and tocotrienols, respectively (DellaPenna and Pogson 2006). The term 'vitamin E' is used to describe all tocopherol and tocotrienols that qualitatively exhibit the biological activity of α-tocopherol. Tocopherol is methylated at C-5, C-7, and C-8 on the chromonal ring, whereas the other homologues (β-, γ-, and δ-) differ in the number and positions of the methyl groups on the ring. Tocopherols have a fully saturated 20-carbon phytyl side chain attached at C-2 and have three chiral centers that are in the R configuration at positions C-2, C-4^1 and C-8^1 in the naturally occurring form, which are given the prefix 2R, 4^{1}R, and 8^{1}R (designated RRR). They are more biologically active than their synthetic counterparts, which are mixtures of all eight possible stereoisomers and are given the prefix all-*rac*.

Tocotrienols differ from the corresponding tocopherols in that the 20-carbon isoprenoid side chain is unsaturated at C-3^1, C-7^1 and C-11^1 and possesses one chiral center at C-2, in addition to two sites of geometric isomerism at C-3^1 and C-7^1. Natural tocotrienols have the 2R, 3^1-*trans*, 7^1-*trans* configuration. The phenolic hydroxyl group is critical for the antioxidant activity of vitamin E, as donation of

hydrogen from this group stabilizes free radicals. The presence of at least one methyl group on the aromatic ring is also critical. Vitamin E biological activity is defined in terms of α-tocopherol equivalents (α-TE) whenever possible. RRR-α-tocopherol has an activity of 1 mg α-tocopherol equivalents (α-TE/mg compound). The activities of RRR-β, RRR-γ, and RRR-δ-tocopherol are 0.5, 0.1, and 0.03, respectively. Synthetic all-*rac*-α-tocopheryl acetate has an activity of 0.74 mg α-TE/mg. Of the tocotrienols, only α-tocotrienol has significant biological activity (0.3 mg α-TE/mg). Lengthening or shortening the side chain results in a progressive loss of vitamin E activity (Ingold et al. 1990).

In the determination of vitamin E, solid-phase extraction (Luque-Garcia and Luque de Castro 2001) and supercritical fluid extraction (Turner and Mathiasson 2000; Turner et al. 2001) are now used extensively for sample extraction and clean-up. Methods used for the determination and quantitation of vitamin E include normal-phase high-performance liquid chromatography (NP-HPLC), reversed-phase high-performance liquid chromatography (RP-HPLC), gas chromatography (GC) and supercritical fluid chromatography (SFC) (Pyka and Sliwiok 2001; Ruperez et al. 2001; Turner et al. 2001). Detection of fat-soluble vitamins after HPLC resolution can be accomplished by UV (using diode array detection), fluorescence (FLD), electrochemical (ED), or evaporative light scattering (ELSD) detection methods (Ruperez et al. 2001). The most commonly used detector for vitamin E analysis is FLD, which is considerably more sensitive and selective than UV, but less sensitive than ED. A number of analytical methods have been described for the determination of vitamin E in milk and milk-based infant formulae, either alone (Rodrigo et al. 2002; Romeu-Nadal et al. 2006a) or simultaneously with other fat-soluble vitamins (Turner and Mathiasson 2000; Rodas Mendoza et al. 2003; Heudi et al. 2004; Chavez-Servin et al. 2006).

Average total vitamin E content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.1. The concentration of vitamin E in animal products is usually low but, there may be significant sources of the vitamin because of their high level of consumption. Different authors have reported concentrations of α-tocopherol between 0.2 and 0.7 mg/L in bovine milk (Renner et al. 1989; Jensen 1995a, b). γ-Tocopherol has also been found and trace amounts of some other vitamers. Barrefors et al. (1995) reported α-tocopherol levels of 7.4–10.0 mg/g lipid for different herds and also observed the presence of low levels of γ-tocopherol and α-tocotrienol. Colostrum contains about 1.9 mg/L of α-tocopherol and the level was shown to decrease in approximately 4 days to the level in fresh milk (0.3 mg/L) (Hidiroglou 1989). γ-Tocopherol is also present in small amounts in colostrum. The transfer of vitamin E into colostrum does not appear to occur through a passive mechanism following the transfer of lipid (Debier et al. 2005). A mechanism involving low-density lipoproteins (LDL) may be responsible for the high vitamin E concentration in colostrum compared to mature milk (Schweigert 1990).

Also, tissue delivery of α-tocopherol into milk may be promoted by the action of lipoprotein lipase on triglyceride-rich lipoproteins (Martinez et al. 2002). The quantitative secretion of α-tocopherol (also β-carotene) from plasma into bovine milk appears to follow Michaelis-Menten kinetics for active transport across membranes

(Jensen et al. 1999). According to these authors, the daily secretion of α-tocopherol and β-carotene is limited in quantity and is independent of the yield of milk and fat content. The concentration of vitamin E in milk appears to be dependent principally on the amount consumed by the cow. Indyk et al. (1993) observed that the vitamin E content of cow's milk exhibits a significant seasonal pattern that is largely independent of fat content, with pasture maturity and quality the dominant factors. However, this seasonal effect was not observed in an Irish study, probably because farm management practices include supplementation of the diets of spring-calved cows with silage or concentrates from mid-September onward (O'Brien et al. 1999).

The vitamin E concentration in human milk is also much higher in colostrum than in mature milk. Boersma et al. (1991) observed that human colostrum contained 22 ± 14 mg/L as α-tocopherol equivalents compared with transitional milk (14 ± 8) and mature milk (8 ± 5). Barbas and Herrera (1998) also observed significantly higher levels of vitamin E in human colostrum (14.4 ± 2.3 mg/L) compared with 3.1 ± 0.5 mg/L in mature milk. The vitamin E/linoleic acid ratio in human colostrum is higher (89.8 ± 14.7 μg/g) compared with 25.9 ± 3.49 μg/g in mature milk. Vitamin E does not consistently cross the placental barrier (Quigley and Drewry 1998) and, as a consequence, plasma vitamin E concentration in pre-suckled newborn infants (Sinha and Chiowich 1993) and calves (Nonnecke et al. 1999) is very low. Colostrum ingestion is therefore important to provide mammalian neonates with an adequate source of vitamin E to protect against oxidative stress and enhance the immune response. Following birth, colostrum intake induces a sharp increase in the circulating and tissue levels of vitamin E in the young (Hidiroglou et al. 1993).

The stability of vitamin E in foods is affected by environmental factors and food-related factors such as water activity, the degree of unsaturation of biomembranes and the presence of trace elements such as copper and iron (Frankel 1998). Vidal-Valverde et al. (1993) observed that α-tocopherol in UHT milk stored at 30 °C decreased by 3–14% at 1 month and by 9–30% at 2 months. After storage of UHT milk at −20 °C α-tocopherol levels were stable for 2 months, but decreased by 10–20% after 4–8 months. Supplementation of animal feed with vitamin E increases the oxidative stability of milk (Barrefors et al. 1995; Focant et al. 1998). Elevated α-tocopherol levels contribute to lower lipid and cholesterol oxidation in whole milk powders during storage at elevated temperatures (McCluskey et al. 1997; Morrissey and Kiely 2006).

10.2.4 Vitamin K

Vitamin K (the coagulation vitamin) was discovered in the 1940s as a result of investigations into the cause of an excessive bleeding disorder in chickens fed on a fat-free diet. The term 'Vitamin K' is a group name for a number of related compounds, which have in common a 2-methyl-1,4-napthoquinone ring system, but differ in the length and degree of saturation of their isoprenoid side chain at the 3-position. Three vitamin K compounds have biological activity.

Phylloquinone, vitamin K_1 (2-methyl-3-phytyl-1,4-napthoquinone), is found in green leafy vegetables and represents the main dietary source of vitamin K in the Western diet (Bolton-Smith et al. 2000). Menaquinones (MKs), vitamin K_2 (2-methyl-3-1,4-napthoquinone), are synthesized by the gut microflora, with fully or partially unsaturated isoprenoid side chains of various length at the 3-position. The predominant forms of the MK compounds contain between 6 and 10 isoprenoid units, but MKs containing up to 13 units have been isolated (Suttie 1985). The parent structure of the vitamin K group of compounds is 2-methyl-1,4-naphthoquinone, commonly called menadione (vitamin K_3), is not found in nature but is a synthetic form which can be metabolized to phylloquinone or menaquinone and thus may be regarded as a provitamin. Menadione is also used as an animal feed supplement and in this way may indirectly enter the human food chain as preformed MK-4 (Shearer et al. 1996).

Previous analytical techniques to measure vitamin K compounds, such as the chick bioassay, were cumbersome and tended to overestimate the vitamin K content of foods. However, at present, the method of choice for vitamin K analysis in foodstuffs is HPLC separation after lipid extraction (Booth et al. 1993, 1995). Electrochemical or fluorescence detection (after reduction to the hydroquinone form) offers the sensitivity and selectivity needed for quantification of the small amounts of the vitamin K compounds. Food composition data for vitamin K derived from HPLC are generally lower than earlier data derived from the chick bioassay (Booth et al. 1993). The use of these HPLC-derived data on the vitamin K content of foods allows for a more accurate determination of the phylloquinone content of a typical western diet.

The average vitamin K_1 content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.1. Milk is not a good dietary source of vitamin K, containing between 3.5 and 18 μg/L as phylloquinone (Haroon et al. 1982) and contributes minimally to vitamin K intake in adults (Booth et al. 1996). Mature human milk contains less phylloquinone than cow's milk (~0.25 μg/L) (Haroon et al. 1982). However, vitamin K levels are higher in colostrum than in mature milk (von Kries et al. 1987). The menaquinone concentration in human milk has not been accurately determined but appears to be much lower than that of phylloquinone. Phylloquinone concentration in infant formula milk ranges from 3 to 16 μg/L in unsupplemented formulae and up to 100 μg/L in fortified formulae. The average intake of phylloquinone by infants fed human milk during the first 6 months of life has been reported to be less than 1 μg/day, which is approximately 100-fold lower than the intake in infants fed a typical supplemented formula (Greer et al. 1991). A study in Germany concluded that a minimum daily intake of about 100 mL of colostrum milk (which supplies about 0.2–0.3 μg of phylloquinone) is sufficient for normal vitamin K homeostasis in a baby of about 3 kg during the first week of life (von Kries et al. 1987). Similar conclusions were reached in a Japanese study that showed a linear correlation between the prevalence of undercarboxylated coagulation protein and the volume of breast milk ingested over 3 days (Motohara et al. 1989).

Newborn infants are at serious risk of hemorrhaging because of poor placental transfer of vitamin K, lack of intestinal bacteria, and the low vitamin K content in breast milk. For this reason, they receive intramuscular vitamin K at birth. In children and adults, "clinical" vitamin K deficiency in terms of blood coagulation is rare. However, "subclinical" vitamin K deficiency in extrahepatic tissues, particularly in bone, is not uncommon in the adult population. The multitude of proteins which require carboxylation of Glu to Gla residues for proper functioning suggests that poor vitamin K status may contribute to certain chronic vascular and skeletal diseases (for a comprehensive review on this topic please see Harshman and Shea 2016).

10.3 Water-Soluble Vitamins

10.3.1 Thiamine (Vitamin B_1)

Thiamine is a water-soluble vitamin, which is unstable in light and loses its biological activity in alkaline solutions (pH > 7). The chemical name of thiamine is 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium; its coenzyme form is thiaminepyrophosphate (TPP) which is involved in four enzyme systems involved in carbohydrate and energy-yielding metabolism. Historically, thiamine deficiency affecting the peripheral nervous system (beriberi) was a major public health problem in south-east Asia following the introduction of the steam-powered mill that made highly polished (thiamine-depleted) rice widely available.

Thiamine is used in pharmaceutical and other preparations in the form of water-soluble thiazolium salts (thiamine chloride hydrochloride, thiamine mononitrate); synthetic lipophilic derivatives are also available (the so-called allithiamins). The latter can move through biological membranes more easily and in a dose-related manner, allowing for the production of thiamine stores through supplementation, which are usually low and only adequate for 4–10 days (Biesalski and Back 2002a). Thiamine is converted to thiochrome, a fluorescent material used to assess the thiamine content of feeds, foods, and pharmaceutical preparations in the presence of oxidizing agents and in highly alkaline solutions (Alonso et al. 2006).

The thiamine content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.2. Heat treatment and storage conditions can cause thiamine losses in foods: low pasteurization causes a loss of 3–4%; boiling causes a loss of 4–8%; spray-drying causes a loss of 10%; roller drying causes a loss of 15%; pasteurization causes a loss of 9–20%; manufacture of condensed milk causes a loss of 3–75%; sterilization causes a loss of 20–45%; and evaporation causes a loss of 20–60%. Fresh milk stored in dark bottles loses 24% of its initial thiamine content after 24 h of storage at 4 °C, 14% at 12 °C, and 16% at 20 °C. Evaporated milk loses 15–50% over a 12-month span, while spray-dried

Table 10.2 Average concentrations of B vitamins in human and bovine milks (per 100 ml or 100 g as appropriate) from the British National Food Composition Database (McCance and Widdowson 2018[a])

Food name	Thiamine (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (μg)	Folate (μg)	Pantothenate (mg)	Biotin (μg)
Milk, condensed, whole, sweetened	0.09	0.46	2.3	0.07	0.7	15	0.85	3.9
Milk, evaporated, whole	0.07	0.42	2.2	0.07	0.1	11	0.75	4.0
Milk, human, colostrum	Trace	0.03	0.8	Trace	0.1	2	0.12	Trace
Milk, human, mature	0.02	0.03	0.7	0.01	Trace	5	0.25	0.7
Milk, semi-skimmed, pasteurized	0.03	0.24	0.7	0.06	0.9	9	0.68	3.0
Milk, skimmed, pasteurized	0.03	0.22	0.8	0.06	0.8	9	0.50	2.5
Milk, whole, pasteurized	0.03	0.23	0.8	0.06	0.9	8	0.58	2.5
Milk, whole, UHT	0.04	0.18	0.9	0.04	0.2	1	0.32	1.8

[a] Data from Composition of foods integrated dataset (CoFID)—GOV.UK (www.gov.uk) accessed 12/03/2021

whole milk shows no change in thiamine content. Thiamine is lost during cheese production, mostly during the first whey draw, but no major changes are observed during maturation (Biesalski and Back 2002a). Under some conditions (cheese, fresh milk), UV-light-induced thiamine inactivation can be counterbalanced by thiamine-synthesizing microorganisms (Biesalski and Back 2002a).

10.3.2 Riboflavin (Vitamin B_2)

The chemical name for riboflavin is 7,8-dimethyl-10-(10-D-ribityl)isoalloxazine; riboflavin forms the coenzymes flavin mononucleotide (FMN) (riboflavin phosphate) and flavin adenine dinucleotide (FAD), which act in a wide variety of enzymes involved in oxidation and reduction reactions and energy-yielding metabolism. Some enzymes also contain covalently-bound riboflavin.

Riboflavin is found in the diet in both free and protein-bound forms, with milk being the richest source. In bovine milk, the free form, which has a higher

bioavailability (61% riboflavin, 26% FAD, 11% hydroxyethyl form, and others), predominates, while, in other foods, the protein-bound, and therefore less bioavailable, form predominates. It is estimated that between one to two thirds of riboflavin occurs as FAD in human breast milk. While riboflavin is very heat-stable, it is extremely photosensitive. Under alkaline or acidic conditions, riboflavin is photodegraded to lumiflavin or lumichrome, which are biologically inactive metabolites (Ahmad et al. 2006). UV light induces a high degree of natural fluorescence in riboflavin, which is used to detect and evaluate it in yoghurt or non-fat dried milk (Liu and Metzger 2007).

Table 10.2 shows the average riboflavin content of bovine and human milk from the British National Food Composition Dataset. Heat treatment has a minimal effect on riboflavin concentrations, whereas milk exposed to sunlight loses 20–80% of its riboflavin content. As a result, dark bottles, light-tight wax cartons, or special PET bottles are recommended for storage of milk (Mestdagh et al. 2005). Photochemical oxidation and the depletion of ascorbic acid are catalyzed by riboflavin degradation. In liquid milk, 10 Gy of gamma radiation destroys about 75% of the riboflavin; however, milk powder shows no losses even at higher doses. When stored at 8–12 °C for 2 years or 10–158 °C for 4 years, condensed milk loses 28% and 33% of its initial riboflavin content, respectively; ice cream loses 5% when stored at −23 °C for 7 months. Fresh milk stored at 4–8 °C for 24 h or dried milk powder stored for 16 months showed no losses of riboflavin (Nohr and Biesalski 2009). The majority of the original riboflavin losses (66–88%) in cheese tend to occur during whey drainage, while ripening has almost no effect. However, due to microbial synthesis, the concentration of riboflavin is higher in the outer layers of certain cheese types (Nohr and Biesalski 2009).

10.3.3 Niacin (Vitamin B_3)

Two compounds have the biological activity of niacin: nicotinic acid and nicotinamide. In the USA, niacin is commonly used specifically to mean nicotinic acid, with niacinamide for nicotinamide. Both vitamers can be interconverted and two coenzymes exist, nicotinamide-adenine dinucleotide phosphate (NADP+) and nicotinamide-adenine dinucleotide (NAD+). The nicotinamide nucleotide coenzymes, NAD and NADP, participate in a number of oxidation and reduction reactions in the body. In addition, NAD is the donor of ADP-ribose for the modification of tissue proteins, with a role in the repair mechanism after accidental damage to DNA. NAD derivatives play a role in cell signaling in response to hormone action as well (FSAI 2018). These coenzymes are found at highest concentrations and with high bioavailability in animal food sources, in contrast to nicotinic acid which is found, at lower bioavailability and lower concentrations, mainly in plants.

Free nicotinic acid is found in very little quantities, if at all in foods; the majority of niacin is found in the form of nicotinamide nucleotide coenzymes. Nicotinic acid is found in cereals, but it is almost exclusively in the form of niacytin, esters of

complex carbohydrates. Except in the case of alkali-treated maize meal, which is used in the traditional preparation of tortillas, less than 10% of niacytin is biologically accessible. The nicotinamide ring of the coenzymes can also be synthesized in the body from the amino acid tryptophan; it is conventional to express total niacin intake as the sum of preformed nicotinic acid and nicotinamide plus 1/60 × tryptophan (FSAI 2018). The average niacin content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.2. Almost all of the niacin in milk is in the free form. Sunlight, different storage conditions, and heat treatments used in dairy production have little or no impact on niacin content. When liquid milk is exposed to gamma radiation (10 Gy), it loses about 30% of its niacin, while niacin in milk powder survives higher doses of radiation. The majority of niacin passes to the whey during cheese processing, which can be partially offset by an increase in niacin content in the rind ~~outer layers~~ due to microbial activity during maturation (ten- to 25-fold) (Nohr and Biesalski 2009).

10.3.4 Pantothenic Acid (Vitamin B_5)

D(+)-pantothenic acid is the natural form of pantothenic acid and the only stereoisomer with biological activity. However, the alcohol (D)-panthenol can be converted to pantothenic acid, giving it biological activity, though indirectly. Pantothenic acid is a light-yellowish viscous oil that is highly hygroscopic and soluble in both water and ethanol (Biesalski and Back 2002b). Pantothenic acid is used to from coenzyme A (CoA), which is involved in fatty acid metabolism, the entry of products of carbohydrate and amino acid metabolism into the citric acid cycle as acetyl CoA, and a number of metabolic processes involving the conversion of acetyl or other acyl groups, such as cholesterol and steroid hormone synthesis, as well as the production of the neurotransmitter, acetylcholine (Bender 2003). It also forms the prosthetic group of the fatty acid synthase multi-enzyme complex. About 50–95% of pantothenic acid occurs as CoA or pantetheine (fatty acid synthetase complex) in the overall diet.

The average pantothenic acid content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.2. Milk is considered a relatively good dietary source of pantothenic acid containing between 0.5 and 0.6 mg/100 mL, which is equivalent to 10% of the US Recommended Dietary Allowance of 5 mg/day (Bender 2003). In milk, about 25% of pantothenic acid is protein-bound, but this value rises to 40–60% in cheese, depending on the type of cheese (Nohr and Biesalski 2009). The amount of pantothenic acid in cheese is determined by the degree of proteolysis. Although some types of cheese (e.g., Limburger, Camembert, and Brie) lose a significant amount of their content (mostly in the free form) during manufacturing, the concentration in others (e.g., Limburger, Camembert, and Brie) increases due to microbiological synthesis (Nohr and Biesalski 2009).

10.3.5 Pyridoxine (Vitamin B_6)

Pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), as well as their phosphates, have the biological activity of vitamin B_6. In the intestinal mucosa, the phosphorylated compounds are dephosphorylated, and tissues pick up the unphosphorylated vitamers and phosphorylate them. The vitamers are readily interconvertible in the body; the metabolically active compound is pyridoxal phosphate (PLP). Pyridoxal phosphate acts as a coenzyme for a number of enzymes involved in amino acid metabolism; as a coenzyme for glycogen phosphorylase (and hence the release of glucose from glycogen stores in the liver and muscle); and in mitigating the effects of steroid hormones by displacing hormone receptors from DNA binding (FSAI 2018).

PN and PM and their phosphorylated forms are the predominant forms in plant-derived foods, while in animal-derived foods PL and PLP predominate (Bitsch 1993). Vitamin B_6 is sensitive to heat and light, although PM and PN are more stable than PL, particularly to light. Table 10.2 shows the average concentrations of vitamin B_6 in human and selected bovine milks. In bovine milk, the vast majority (86%) of vitamin B_6 exists in the free form, with the remainder in the bound form (Jensen 1995b). UV light destroys vitamin B_6 in neutral and alkaline solutions. In acidic environments, PL, PM, and PN are normally heat stable, but in an alkaline medium, they are heat-sensitive. Different studies report different losses of vitamin B_6, possibly due to differences in experimental procedures. Approximate losses (in %) in vitamin B_6 after heat treatment has been estimated by Biesalski and Back (2002c) as follows: insignificant effects in dried milk products; pasteurized milk: 0–8%; UHT milk: <10%; boiled milk: 10%; sterilized milk: 20–50%; evaporated milk: 35–50%. During cheese production, the majority of vitamin B_6 passes into the whey, and its concentration drops even more during early maturation, whereas it rises in later stages, especially on the surface of some varieties (due to synthesis by yeasts and moulds). Camembert and Brie have the highest vitamin B_6 content, along with very hard, hard, and semi-hard cheeses.

10.3.6 Biotin (Vitamin B_7)

Only one of the eight isomeric forms of biotin, D(+)-biotin, is found in nature. It acts as a coenzyme in reactions that catalyze the addition of bicarbonate to a substrate by binding to four carboxylase enzymes. It is required for the metabolism of energy, carbohydrates, lipids, and amino acids. Biotin is required by many microorganisms, animals, and humans, but it can also be produced by the colonic microflora. Although recent findings show that colonocytes can absorb water-soluble vitamins (including biotin), it's unclear whether the amount produced is enough to cover all physiological functions or whether it's just a fine-tuning of body homeostasis (Said and Mohammed 2006).

Table 10.2 shows the average biotin concentrations in human and selected bovine milks. Biotin occurs in the free form in milk, most foods from animal sources or cereals contain it in an enzyme-bound form named biocytin (ε-*N*-[d-biotinyl]-L-lysine). Biotin loss during food processing or storage is usually minimal. There is no loss of biotin in frozen milk after a few weeks, in dried milk kept at room temperature for a year, or in milk exposed to sunlight or 10 Gy gamma radiation for 2 h (Biesalski and Back 2002d). UHT sterilization causes no losses of biotin, while pasteurization and in-container sterilization cause less than 10% loss of biotin. Less than 15% loss of biotin was observed in evaporated, condensed dried whole milk (Biesalski and Back 2002d). Biotin concentrations in cheese differ depending on processing or maturation (microbiological synthesis; e.g., Limburger or Brie); the highest concentrations are mostly found in the outer parts but can spread across the cheese.

10.3.7 Folates (Vitamin B_9)

"Folates" are a group of compounds with a chemical structure and nutritional activity similar to that of folic acid (pteroyl-L-glutamic acid). Folic acid and folate are often used interchangeably but there are important differences. Folic acid is a synthetic source of folate. Natural folates (found in human tissue and plant and animal foods) are a mixture of reduced folates (mostly 5-methyltetrahydrofolate) in the polyglutamylated form which contain a variable number of glutamate residues. Unlike normal folates, folic acid is completely oxidized and is a monoglutamate with just one glutamate moiety. Because of these chemical variations, folic acid is more stable and bioavailable than naturally occurring food folates at similar dietary intake levels. The synthetic vitamin form of folic acid is only present in fortified foods and supplements in the human diet, but it is easily converted to the normal cofactor forms after ingestion. It is a crystalline solid that's yellow-orange in color, tasteless and odorless, and mildly soluble in pure water (FSAI 2018). Folate is required for one-carbon metabolism which involves the transfer and utilization of one-carbon units in biochemical pathways involving amino acid metabolism, methylation processes and in DNA and RNA biosynthesis.

Folates are the vitamins most susceptible to oxidation, leakage, and enzyme activity during processing and storage. Approximate losses (in %) in folates after various storage and heat treatment have been estimated by Witthoft and Jägerstad (2002) as follows: pasteurized milk: 0–10%; UHT milk: 0–20%; blanched broccoli or spinach: 40–90%; oven baked trout and chicken breast: 30–45%: cooked, chilled/reheated various vegetables: 25%; soaked and cooked pulses: 30–70%; ionized radiation of vegetables: 10–60%; stored spinach, liver, or strawberries in the freezer over 6–8 months: 17–40%; chilled storage of yoghurt or fermented milk for 2 weeks: 0%. It is important to note that the bioavailability of folates from various sources differs greatly, for example, 5% from vegetables, 55% from liver, 70–75% from egg yolk, and up to 100% from pharmaceutical formulations taken as supplements.

Folic acid fortification of wheat flour has been introduced in 78 countries, including the USA, Canada, and Australia as a means to reduce the burden of neural tube defects (Morris et al. 2016). In 2009, the UK Scientific Advisory Committee on Nutrition made a recommendation to introduce mandatory wheat flour fortification with folic acid but no regulatory action was taken. Table 10.2 shows the average content of folates in human and various bovine milks from the British Food Composition Database. While the folate content of milk is low, its bioavailability has been shown to be good (Pfeuffer and Schrezenmeir 2007). Another significant factor in bioavailability appears to be milk's folate-binding protein (FBP), which has been shown to facilitate folate uptake by the intestinal mucosa in animals (Achanta et al. 2007).

10.3.8 Cobalamin (Vitamin B_{12})

The chemical structure of vitamin B_{12} is the most complex of all the vitamins. Cobalamins are a group of cobalt-containing compounds that make up vitamin B_{12}. The vitamin exists in a variety of forms, including methylcobalamin and deoxyadenosylcobalamin, which are both metabolically active; other dietary cobalamins are easily converted to these forms. Vitamin B_{12} is needed for a variety of methylation reactions, including the methylation of DNA, RNA, myelin basic protein, neurotransmitters, and amines, and thus has similar biochemical functions to folate. It is also essential for the metabolism of branched chain amino acids and odd chain fatty acids. Vitamin B_{12} can only be synthesized by intestinal microorganisms, which supply certain animal species with ample vitamin B_{12}. The synthesizing species in humans, on the other hand, are found in the colonic portion of the intestine, which is too far away from the small intestine (ileum), where vitamin B_{12} must be absorbed. As a result, humans only get vitamin B_{12} from their diet, and only animal-derived foods contain adequate quantities of the vitamin.

The intrinsic factor, which is secreted by gastric parietal cells and promotes ileal cobalamin uptake, is required for vitamin B_{12} uptake (Said and Kumar 1999). The average concentration of vitamin B_{12} in human and various bovine milks from the British Food Composition Database is shown in Table 10.2. Milk is an excellent source of vitamin B_{12} and the cobalamin content of cow's milk is very stable regardless of feed, breed, season, or stage of lactation. Human milk concentrations, on the other hand, are slightly lower than those found in bovine milk and differ depending on the aforementioned factors. Milk is a major contributor (up to 30%) to dietary vitamin B_{12} intakes in many countries. Though storage and radiation have minor effects on cobalamin concentration in milk (30–40% loss in sterilized milk after 90 days at room temperature), destruction by heat has a significant impact on vitamin B_{12} content. Vitamin B_{12} losses in cow's milk after various heat treatments include: sterilization: 20–100%; evaporated milk: 50%; boiling: 20%; pasteurization: <10%; UHT: 5–10% (Nohr and Biesalski 2009). In Gruyere cheese, the vitamin B_{12} content is increased due to vitamin B_{12}-synthesizing microorganisms (Biesalski and Back 2002e). However, an overall loss of vitamin B_{12} between 10% and 50% in most other cheeses is typical (Nohr and Biesalski 2009).

10.3.9 Vitamin C

Ascorbate, also known as ascorbic acid (AA) or vitamin C, is synthesized from guanosine diphosphate (GDP)-mannose and the pathway shares GDP-sugar intermediates with the synthesis of cell wall polysaccharides and those glycoproteins that contain D-mannose, L-fucose, and L-galactose (Smirnoff 2000). Humans and non-human primates, guinea pigs, the Indian fruit bat, several birds, and some fish have lost the ability to synthesize ascorbate de novo as a result of a gene mutation that has rendered inactive a key ascorbate biosynthetic enzyme, L-gulonolactone oxidase, which is required to catalyze the last step in the biosynthesis of ascorbate (Woodall and Ames 1997). Ascorbate is quantitatively the predominant antioxidant in plant cells, it is found in all subcellular compartments, including the apoplast, and has an average cellular concentration of 2–25 mM or more in the chloroplast stroma (Smirnoff 2000).

Several methods have been developed for determining the amount of vitamin C in biological specimens, foods, and pharmaceutical products in which spectrophotometric, fluorometric, chromatographic, and electrochemical techniques are used. High-performance liquid chromatography (HPLC), using a UV detector, is currently the most commonly used technique for the analysis of ascorbic acid in foods (Esteve et al. 1995; Perez-Vicente et al. 2002; Sanchez-Moreno et al. 2003; Romeu-Nadal et al. 2006b). Some HPLC methods require electrochemical (Margolis and Schapira 1997) or fluorometric detection (Nielsen et al. 2001), because of the low absorptivity of DHA in the ultraviolet range of the spectrum. A HPLC/UV method for determining total vitamin C (AA and DHA) and ascorbic acid in human milk has recently been described (Romeu-Nadal et al. 2006b). The National Institute of Standards and Technology (NIST) has developed food matrix reference materials and methods for the measurement of naturally occurring levels of vitamins C, A, and E (Sharpless et al. 2000).

The mean content of vitamin C in cow's milk is 2.11 mg/100 g (range 1.65–2.75 mg/100 g) (Walstra and Jenness 1984), 5.48 mg/100 mL in goat's milk (Kondyli et al. 2007), 2.49 mg/100 mL in camel's milk (Mehaia 1994), and 3.9 (±1.05) mg/100 mL (summer milk) and 3.02 (±2.01) mg/100 mL (winter milk) in humans (Tawfeek et al. 2002). The mean concentration of vitamin C in human milk is also affected by the stage of lactation and declined from 6.18 ± 0.09 mg/100 mL in colostrum to 4.68 ± 0.1 mg/100 mL at 9 months (Salmenpera 1984). The influence of maternal vitamin C intake and its effect on the vitamin C content of human milk has not been clearly defined. Byerley and Kirksey (1985) observed that the vitamin C level in human milk did not increase significantly in response to increasing maternal intake (up to tenfold) of the vitamin. The authors proposed that a regulatory mechanism may be present in mammary cells to prevent an elevation in the concentration of vitamin C in milk beyond a certain saturation level. On the other hand, when the intake of vitamin C is low, the level in breast milk is sensitive to supplementation. Ortega et al. (1998) observed a relationship between ascorbic acid intake and serum and milk levels. However, the changes in breast milk ascorbate

level were less pronounced than those in serum. The breast milk ascorbate to serum ascorbate ratio decreased with increasing intake, and with serum vitamin C level, which suggests that mammary tissue becomes saturated with the vitamin when intake is high. The plasma concentration of vitamin C in breast-fed infants was generally in the normal range, indicating the exclusively breast-fed infants are well protected against vitamin C deficiency (Salmenpera 1984). Breast-fed infants appear to be capable of maintaining a high plasma concentration of vitamin C independently of maternal and milk concentrations.

There is some evidence that the concentration of vitamin C in bovine milk (Andersson and Oste 1994; Lindmark-Mansson and Akesson 2000) and caprine milk (Kondyli et al. 2007) changes with season. Andersson and Oste (1994) found that, in raw milk sampled in March or August, the concentration of vitamin C was higher (2.0–2.7 mg/100 mL) than in samples collected in October (1.2 mg/100 mL). Lindmark-Mansson and Akesson (2000) reported that mean values for vitamin C were higher in July and September than in January and March. Ascorbic acid is considered to be the first line of defence against oxidizing species and consequently appears to be the vitamin lost most readily in milk and cooking of foods (Bates 1997). Loss of vitamin C is determined primarily by the concentration of O_2 dissolved in milk, and is greatly accelerated by exposure to light and the presence of heavy metals such as Cu and Fe (Scott et al. 1984a, b). Andersson and Oste (1992a, b) observed that, at a low oxygen content (0.6 ppm), ascorbic acid decreased by about 50% after 1–2 weeks storage, with somewhat higher stability at 7 °C than at 23 or 35 °C. At oxygen contents of 3.5–5.4 ppm, the loss was much greater.

10.4 Minerals

10.4.1 Calcium

Calcium is important for the maintenance of healthy teeth and bones, cell signaling, coagulation, muscle contraction, neural transmission and many other functions. Calcium is the fifth most abundant element in the human body. Almost all (99%) of the calcium in the body is located in bones and teeth, mostly as calcium hydroxyapatite. Bone mineral provides structure and strength, and acts as a reservoir of calcium that helps to regulate blood serum calcium concentrations at about 2.5 mmol/L (range 2.3–2.62 mmol/L) (EFSA 2012; IOM 2011).

Calcium is mostly found in dairy foods, but it can also be found in small but important quantities in certain plant foods, mineral waters, and dietary supplements. Milk and dairy products are the most calcium-dense foods in Western diets, typically containing about 300 mg calcium per serving (244 g of milk or yoghurt or 42.5 g of Cheddar cheese). Dairy foods are the main source of calcium during bone growth with between one to two thirds of calcium obtained from dairy, so in its absence an insufficient intake of calcium is likely, unless fortified foods or supplements are consumed. Gao et al. (2006) used data from over 2000 adolescents from

Table 10.3 Average concentrations of minerals and trace elements in human and bovine milks (per 100 mL/g) from the British National Food Composition Database (McCance and Widdowson 2018[a])

	Na (mg)	K (mg)	Ca (mg)	Mg (mg)	P (mg)	Fe (mg)	Cu (mg)	Zn (mg)	Cl (mg)	Mn (mg)	Se (μg)	I (μg)
Milk, condensed, whole, sweetened	90	360	290	29	240	0.23	Trace	1.0	150	Trace	3	74
Milk, evaporated, whole	180	360	290	29	260	0.26	0.02	0.9	250	Trace	3	11
Milk, human, colostrum	47	70	28	3	14	0.07	0.05	0.6	N	Trace	N	N
Milk, human, mature	15	58	34	3	15	0.07	0.04	0.3	42	Trace	1	7
Milk, semi-skimmed, pasteurized	43	156	120	11	94	0.02	Trace	0.4	87	Trace	1	30
Milk, skimmed, pasteurized	44	162	125	11	96	0.03	Trace	0.5	87	Trace	1	30
Milk, whole, pasteurized	42	157	120	11	96	0.02	Trace	0.5	89	Trace	1	31
Milk, whole, UHT	55	140	110	11	87	0.23	0.01	0.4	93	Trace	1	31

N significant amounts present but no reliable indication of the amount

[a] Data from Composition of foods integrated dataset (CoFID)—GOV.UK (www.gov.uk) accessed 12/03/2021

the NHANES study and modeled dietary intakes aligned to the guidelines, with and without dairy foods, and observed that adequate calcium was not achievable with regular food without the inclusion of dairy foods, and was only possible if calcium fortified foods were consumed (Gao et al. 2006). Dairy foods are the main source of calcium during growth, with between one to two thirds of calcium being obtained from dairy (Bates et al. 2014; Cribb et al. 2015). For children aged 2–18 years in the USA, the average calcium intake is ~950 mg/day of which 32–51% is obtained directly from milk and cheese, emphasizing the role of dairy products as the principal dietary source of calcium (O'Neil et al. 2018).

The calcium content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. The calcium concentration in mature human milk is between 250 and 300 mg/L (Lonnerdal 1997), and findings from several longitudinal studies indicate that the calcium concentration in breast milk is constant (Lonnerdal 1997), temporarily decreases (Friel et al. 1999), or decreases significantly within the first 4 months of lactation (Hunt et al. 2005). The amount of

calcium in breast milk is normally unchanged by the mother's diet (Lonnerdal 1986). There is some evidence, however, that calcium levels in breast milk are lower than normal during prolonged lactation (Laskey et al. 1990), and that calcium concentrations in breast milk of lactating young teenage mothers may be lower than those of older women (Laskey et al. 1990). Human milk contains slightly less calcium than bovine milk (1.11 g/L), as well as milk-based (0.50 g/L) formulae (Table 10.3). Since formula-fed babies consume more formula, they get at least twice as much calcium as breast-fed babies (Lonnerdal 1997).

10.4.2 Phosphorous

Phosphorus as phosphate is an essential nutrient involved in many physiological processes, such as cellular energy cycle, regulation of acid-base balance, as a component of cell membranes (phospholipids), in cell regulation and signaling, and in the mineralization of bones and teeth (EFSA 2005). Approximately 80% of the body's phosphorus is present in the skeleton and teeth bound in solid form (hydroxyapatite), with the remainder distributed in soft tissues and extracellular fluid. Phosphorus homeostasis is regulated by a combination of dietary intake, intestinal absorption, exchanges with bone and intracellular compartments and renal excretion and normal concentrations of serum phosphate are in the range of 3.0–4.5 mg/dL (EFSA 2013).

Dietary phosphorus comprises organic and inorganic forms. Intestinal phosphatase enzymes hydrolyze the organic forms facilitating most phosphorus absorption occurs as inorganic phosphate. Foods high in protein, such as dairy products (100–900 mg/100 g), meats (200 mg/100 g), fish (200 mg/100 g), and grain products (100–300 mg/100 g), are high in phosphorus. Habitual dietary intakes in European countries are estimated to be around 1000–1500 mg/day on average, with intakes up to about 2600 mg/day (FSAI 2014). Infants, toddlers, and adolescents derive 32–48% of their dietary phosphorus from milk and milk products, whereas dairy foods provide only 20–30% of phosphorus for most adult age and sex groups (Anderson et al. 2006). Particular attention must be paid to increased risk of hyperphosphatemia in neonates because renal handling of phosphorus is developmentally immature in this age group. Bovine milk or formulae may contain six or three times, respectively, as much phosphorus as human milk. Human milk with its low phosphorus content is both safer and better suited to the growth needs of the infant than bovine milk. The phosphorus content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. Around 15% of the phosphorus in human milk is inorganic, 23% is protein-bound, and 62% is bound to lipids (Renner 1983).

10.4.3 Magnesium

The magnesium (Mg) content of the human body is approximately 25 g (1000 mmol), with 50–60% of that contained in bone (FNB 1997). Mg is a cofactor in over 300 enzyme systems that control a wide range of biochemical reactions, including protein synthesis, muscle and nerve function, blood glucose control, and blood pressure regulation. Magnesium also plays an important role in protein and nucleic acid synthesis and has a stabilizing and protecting effect on membranes. Finally, Mg is considered essential in maintaining Ca, K, and Na homeostasis and has a role in the structural development of bone.

The amount of magnesium in food varies a considerably. Fats, refined sugars, and pure alcohol are low in magnesium. Green leafy plants, such as spinach, legumes, nuts, seeds, and whole grains, are good dietary sources. Cocoa, shrimp, soybeans, and a variety of other beans are also good sources. In general, foods containing dietary fiber provide Mg. Some types of food processing, such as refining grains in ways that remove the nutrient-rich germ and bran, lower Mg content substantially. Other dietary sources of magnesium include tap, mineral, and bottled water, but the amount of Mg in water varies depending on the source and brand (ranging from 1 to 120 mg/L). Laxatives are made from magnesium salts, especially magnesium sulfate ("Epsom salt").

The average magnesium content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. The magnesium concentration of mature bovine milk is ≈100 mg (4.1 mmol)/L (Hunt and Meacham 2001; Volpe 2006). Magnesium content in bovine colostrum is two to three times that of mature milk, but it decreases to the mature milk level within the first 1–3 days of lactation (Hidiroglou and Proulx 1982). The concentration of magnesium in human milk increases dramatically between months 1 and 4 of lactation (Hunt et al. 2005), but remains relatively stable in mature bovine milk (Hidiroglou and Proulx 1982). Since bovine milk contains about three times as much magnesium as human milk, commercial infant formula based on bovine milk have a higher magnesium concentration (40–50 mg or 1.7–2.1 mmol/L) than human milk (Food and Nutrition Board: Institute of Medicine 1997).

10.4.4 Sodium and Chloride

Sodium is the major extra cellular electrolyte and exists as the water-soluble cation. Chloride is also mainly found in the extracellular fluid and exists as the water-soluble anion. The sodium cation and actively regulates osmotic and electrolyte balances. The chloride anion is a passive participant in this regulatory process. Sodium is also involved in nerve conduction, active cellular transport and the formation of mineral apatite of bone. The most common source of sodium in foods is sodium chloride (salt). 35.5 mg chloride and 23 mg sodium are equal in 1 mmol

sodium chloride (approximately 58 mg). Salt or salt additives applied to food during processing and production, as well as during cooking, are the key sources of chloride.

Cereals and cereal products, meat and meat products, and vegetables are the major sources of chloride in the diet. Meat and fish (especially cured/processed meats) and bread account for more than half of the salt consumed by adults in most Western countries. A typical achievable target for the adult populations is a mean intake of 6 g salt (2.4 g sodium) per day. Sodium intakes above this threshold can lead to an elevation in blood pressure in salt-sensitive individuals.

The average sodium and chloride content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. There has been no link established between maternal dietary sodium or chloride intakes and milk electrolyte concentrations (Ereman et al. 1987). The concentration of chloride in bovine milk rises rapidly toward the end of lactation and is unaffected by dietary intake. Sodium and chloride are thought to be almost entirely free ions in milk (Holt 1993). Almost all of the sodium and chloride in milk is absorbed in the gastrointestinal tract, though much of it is lost (Hunt and Nielsen 2009).

Human milk (≈93 mOsmol/L) has a much lower potential renal solute load than bovine milk (≈300 mOsmol/L) (Ziegler and Fomon 1989). Since the kidney excretes a more concentrated urine, the high renal solute load resulting from the ingestion of bovine milk may be of relatively little significance in healthy developing infants without increased evaporative water losses (Hunt and Nielsen 2009). This, on the other hand, decreases the margin of safety against dehydration, which may occur in cases of diarrhea, fever, or insufficient water intake. As a result, the upper limit of possible renal solute load in formulae for young infants is recommended to be about 220 mOsmol/L (Ziegler and Fomon 1989).

10.4.5 Potassium

Potassium is an essential nutrient and the main cation in the intracellular fluid, with potassium concentrations being 30 times higher inside the cell than outside. Potassium, in combination with sodium, regulates cell membrane potential and, as a result, nerve and muscle activity, as well as blood pressure (FSAI 2018). Potassium also plays a role in the acid-base balance and is a cofactor for a number of enzymes. The blood concentration of potassium is tightly regulated and normally 3.6–5.2 mmol/L. Fruits and vegetables, particularly leafy greens, vine fruit (such as tomatoes), and root vegetables, and nuts are all good sources of potassium in the diet. A number of food additives also contain potassium. Salt substitutes, in which part of the sodium chloride has been substituted with potassium salts, can also contribute to potassium intake.

The average potassium content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. The average potassium

concentration in mature human milk is 0.5 g/L, which is significantly lower than in bovine milk (1.5 g/L) (Food and Nutrition Board: Institute of Medicine 2001). Lactating dairy cows have considerably high dietary potassium needs (10 g/kg dry matter). Bovine colostrum has a higher potassium content than mature milk (Ontsouka et al. 2003). There is no evidence supporting an association between maternal dietary potassium and milk potassium concentration. Bovine milk and dairy products can be meaningful contributors to the total dietary intake of potassium (10–20% in typical Westernized diets), because the richest sources, vegetables and fruits, often are not consumed in recommended amounts.

10.5 Trace Elements

10.5.1 Iron

Iron is a metal the roles of which in biological systems are due to its ability to interconvert between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states. It is a vital component of the oxygen-carrying proteins hemoglobin (in red blood cells, comprising almost two-thirds of total body iron) and myoglobin (in muscle). Iron is also a component of the cytochromes (in mitochondria), various other enzymes and several transport and storage proteins. Total adult body iron content is 2.2–3.8 g under iron-adequate conditions. Iron deficiency causes anemia, adverse pregnancy outcomes, impaired psychomotor development and cognitive performance, and reduced immune function. It is reckoned to be the commonest nutritional deficiency worldwide; the highest prevalence being among women of childbearing age and in infants. The richest food sources of iron are red meats, especially offal. Cereals and pulses are moderately good sources, pale meats (pork and poultry), and green vegetables are intermediate sources, while milk and dairy products are poor sources. The amounts in food vary greatly, depending on soil, climate and processing.

The average iron content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. Iron is low in human milk, as well as bovine milk and milk products (Pennington et al. 1987). Most infant formulae are supplemented with iron by 6–9 months of age to avoid iron deficiency and anemia, but at varying concentrations (Lonnerdal 1997). Infants fed typical iron-fortified formula (11.8 mg/L) in usual amounts (0.78 L/day) may have an iron intake of 9.2 mg/day, which is up to 34 times more iron per day than breast-fed infants (0.27 mg Fe/day). The question of whether the iron content of human milk is adequate for breast-fed babies and whether they should be supplemented with iron continues to be debated. The amount of iron in human milk is usually very low, and it drops even more within the first 12 weeks of lactation (Hunt et al. 2004; Kelleher and Lonnerdal 2005). Nonetheless, some studies have shown that the iron status of predominantly breast-fed babies is adequate up to 6 months of age and, in some cases, up to 9 or 12 months of age (Lonnerdal 1997).

10.5.2 Zinc

Zinc is a classified as a group IIB post-transition metal and exists as Zn^{2+} in all body tissues and fluids. Over 300 enzymes involved in the metabolism of proteins, fats, and carbohydrates contain zinc, which serves as a structural, regulatory, or catalytic component (Strain et al. 2019). Zinc is essential for growth and development as well as contributing to the maintenance of optimal functioning of the innate and adaptive immune response. Total body zinc content is between 2 and 4 g. Zinc deficiency is rare; it leads to growth and developmental retardation in children, and diverse effects in adults due to its many roles. Since zinc is linked to proteins in foods, high-protein foods (especially dark-colored meats) and certain shellfish are good sources (due to concentration from seawater). Water and food stored in galvanized containers are also good sources of zinc.

Dairy products such as milk, cheese, and yoghurt are moderately good sources of dietary zinc. The average zinc content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. Twenty-nine percent of zinc in human milk is found in the lipid fraction (bound to the fat globule membrane), 14% in casein, 28% in whey proteins, and 29% in the form of a low-molecular weight compound (probably as citrate) (Lonnerdal et al. 1982; Fransson and Lonnerdal 1983). Zinc supplementation of infant formula is common because of the reported benefits of supplementation doses between 1.8 and 5.8 mg/L in augmenting improved growth in male infants (Walravens and Hambidge 1976). In the United States, the zinc content of bovine milk-based infant formulas ranges from 4.0 to 7.4 mg/L (Hamill et al. 1989; Hunt and Meacham 2001).

10.5.3 Copper

Copper is a transition metal that occurs in biological systems mainly as cupric (Cu^{2+}) and, to a lesser extent, cuprous (Cu^{+}) ions. Copper is a cofactor in a variety of enzymes and proteins. Many copper metalloenzymes act as oxidases, reducing the amount of molecular oxygen in the air. Copper is an essential dietary mineral for infant development, immune function, bone strength, red and white cell maturation, as well as iron, cholesterol, and glucose metabolism (FSA 2014). Between 50 and 150 mg of copper are found in the human body, the majority of which is bound to proteins. Anemia, nutropenia, and bone defects are common symptoms of copper deficiency. Organ foods, fish, nuts, and seeds are the best sources of copper in the diet. Wheat bran cereals and wholegrain goods are good sources of dietary copper. In acid and soft water areas, copper-piped water can add 1.0 mg/day to intakes (0.1 mg/day in hard water areas). In the EU, the maximum allowed concentration in drinking water is 2.0 mg/L (EU Directive 98/83).

The average copper content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. The concentration of copper in

human milk is highest during early lactation and declines over time (Hunt et al. 2004). For the first 6 months of lactation, the average copper concentration in human milk is about 250 mg/L, then drops to between 100 and 200 mg/L between 7 and 12 months post-partum (Food and Nutrition Board: Institute of Medicine 2001). The mean concentration of copper is higher in human milk than in bovine milk (60–90 mg/L) (Hunt and Meacham 2001). During the first 3 days of lactation, the concentration of copper in bovine milk drops by up to 50% (de Maria 1978), although it can be increased by dietary copper supplementation (Murthy and Thomas 1974) or interaction with copper-containing containers and processing equipment (Roh et al. 1976). The copper content of commercial infant formulae replicates that in human milk.

10.5.4 Selenium

Inorganic forms of selenium include selenate, selenite, and selenide. It is an important component of selenoproteins and is found in organic forms as seleno-cysteine (SeCys) and selenomethionine (SeMet). There are about 30 mammalian selenoproteins identified, which are involved in thyroid, neural, immune, and gastrointestinal function but the major selenoproteins of relevance to human nutrition include glutathione peroxidase (GPx), iodothyronine deiodinase and selenoprotein P. Selenium nutritional status is closely linked to soil Se content, with low Se soil in countries like Denmark, Finland, New Zealand, and parts of China and Russia, and high Se soil in countries like the United States, Canada, and parts of Colombia (FSAI 2018). The selenium content of food is determined by the selenium content of the soil in which the animal or plant was raised or grown. Furthermore, supplementing animal feed with selenium can boost the selenium content of animal-derived products (e.g., eggs). A North American report on selenium content of foods shows the following composition: meats and seafood 0.4–1.5 μg/g; muscle meats, 0.1–0.4 μg/g; cereals and grains, less than 0.1 to greater than 0.8 μg/g; dairy products, less than 0.1–0.3 μg/g; and fruits and vegetables, less than 0.1 μg/g (Food and Nutrition Board, 2000). Brazil nuts are a particularly rich source of selenium, containing 18–12 μg/g.

The average selenium content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. Selenium concentrations in human milk are typically between 12 and 30 mg/L (Arnaud et al. 1993; al-Saleh et al. 1997; Bianchi et al. 1999; Hunt et al. 2004; Yamawaki et al. 2005). The mean selenium concentration in breast milk ranges from about 10 mg/L (collected in Finland with low soil selenium) (Kumpulainen et al. 1985), to about 22 mg/L (collected in St John's, Canada) (Hunt et al. 2004). Selenium concentrations in infant formulae manufactured in the United States vary from 13 to 18 mg/L (US Department of Agriculture Agricultural Research Service 2018). During the first 12 weeks of lactation, the concentration of selenium in human milk decreases (Levander et al. 1987; Hunt et al. 2004). The majority of selenium in bovine milk whey is incorporated as selenomethionine into the two main whey proteins, α-lactoglobulin and α-lactalbumin.

10.5.5 Iodine

Iodine is needed for the production of the thyroid hormones thyroxine (T4) and triiodothyronine (T3). Thyroid hormones play a role in maintaining metabolic rate, cellular metabolism, and connective tissue integrity. Low iodine consumption is compensated for by a number of mechanisms, including thyroid gland enlargement (goiter). Symptoms of hypothyroidism (elevated levels of thyroid stimulating hormone (TSH)) appear only when these mechanisms fail. Lethargy, fatigue, weight gain, impaired concentration, edema, myalgia, dry skin, delayed tendon reflexes, and a slow heart rate are the main symptoms of hypothyroidism. During pregnancy, iodine deficiency is linked to an increased risk of miscarriage, stillbirth, and congenital abnormalities. Iodine deficiency in the developing fetus can result in cretinism. Natural goitrogens (which impair thyroid hormone synthesis) may be present in foodstuffs such as soybeans and walnuts or formed from foods such as corn and maize. Sea fish, shellfish, marine algae, seaweed, and sea salt all contain high levels of iodine. The iodine content of cereals and grains is variable, as the level is dependent on the iodine content of the soil. In most European countries, iodine intake is maintained by the use of iodized table salt (15–25 mg/kg).

Iodine is also found in large amounts in milk and dairy products. Average Iodine content of bovine and human milk from the British National Food Composition Dataset is shown in Table 10.3. The iodine concentration in both human and bovine milks reflects maternal intake and thus are highly variable (Hunt and Nielsen 2009). In the United States, the average concentration of iodine in breast milk is 146 mg/L. Breast milk iodine concentrations in countries with endemic IDD are usually less than 50 mg/L. Bovine milk is a good source of iodine in the diet (Hunt and Nielsen 2009). The use of iodine-enriched feeds in dairy herds has resulted in a general rise in the iodine content of milk in recent years. Iodine can also be found in milk due to the use of iodophor disinfectants in sanitization and teat dipping. The iodine content of milk varies by season, with winter milk containing significantly more iodine than summer milk, owing to dairy herds' increased reliance on iodine-rich artificial feeds during these months (Nawoor et al. 2006; Philips 1997). Furthermore, organic milk has been found to be lower in iodine content than conventional milk, which is believed to be a result of general mineral restrictions in organic farming (Bath et al. 2012; Rey-Crespo et al. 2013).

10.5.6 Manganese

Manganese is a common transition metal that exists in a variety of oxidation states. In biological systems, Mn(II) is the most common type. Manganese is a catalytic cofactor for a variety of enzymes, as well as an activator for a number of others. Manganese deficiency has only been seen in laboratory settings. Manganese can be found in a variety of foods, including green vegetables, nuts, pasta, and other cereals (FSAI 2018). Milk and milk products are considered poor manganese sources in

humans (Table 10.3). Average manganese concentration in mature bovine milk is 30 mg/L (Lonnerdal et al. 1981). Bovine colostrum has a manganese concentration between 100 and 160 mg/L, although it drops by more than half within the first 3 days of lactation (de Maria 1978). The manganese content of bovine milk can be increased by long term oral supplementation with large doses of manganese (Archibald 1958). Manganese content in formulae has raised concerns due to the fact that infants' homeostatic regulation of manganese is not completely established. Supplementation of formulas in the early 1980s resulted in some formulas containing 100–1000 times the amount of manganese found in human milk (Lonnerdal et al. 1983; Stastny et al. 1984).

10.5.7 Molybdenum

Molybdenum is a transition metal that exists in five oxidation states, with +4 and +6 being the most common. Molybdenum is a cofactor in a variety of enzymes. All of the molybdoenzymes are oxidoreductases. Humans require the molybdoenzymes xanthine oxidase, sulfite oxidase, and aldehyde oxidase (FSAI 2018). Molybdenum is considered an essential dietary element for mammals though the clinical signs of dietary molybdenum deficiency in otherwise healthy individuals have not been described. Milk and dairy products are good dietary sources of molybdenum for humans (Nielsen 2006). The concentration of molybdenum is much higher in bovine milk and human infant feeds, with reported concentrations between 50 and 100 mg/L (Hunt and Meacham 2001; Hunt and Nielsen 2009). The concentration in milk can be increased substantially by ammonium molybdate supplementation (Archibald 1951). Ammonium molybdate supplementation can significantly increase the molybdenum concentration in milk (Archibald 1951). Much of the molybdenum present in human (Zeise and Zikakis 1987) and bovine (Hart et al. 1967) milks is associated with the enzyme xanthine oxidase.

10.5.8 Fluoride

Fluorine is a halogen gas. Since it is the most electronegative and reactive of all the elements, it only exists in ionic forms (fluorides) in nature after reaction with metallic elements or hydrogen. Fluoride is found in nature and is mostly associated with calcified tissue (bone and teeth) in the human body due to its high affinity for calcium. Fluoride has no known essential function for human growth and development and no signs of fluoride deficiency have been identified (FSAI 2018). The mean concentration of fluoride in mature human milk is approximately 18 mg/L (Hunt and Nielsen 2009). Around 46–64% of the fluoride in bovine milk is present as free fluoride ions, with the remainder bound to proteins (Esala et al. 1982). In a fasted state, the gastrointestinal tract absorbs approximately 100% of fluoride ingested as fluoridated water, and 50–80% of fluoride ingested with food (Nielsen 2006).

10.5.9 Chromium

While elemental chromium does not occur naturally, its compounds are abundant in water, soil, and biological systems, with the most stable valence states being 0 (metals and alloys), +3 (trivalent, found in food and supplements), and +6 (hexavalent). Chromium compounds with oxidation states less than +3 are reducing, whereas those with oxidation states greater than +3 are oxidizing. Chromium is considered to be an essential nutrient (Strain et al. 2019; FSAI 2018). It improves glucose tolerance and may have beneficial effects on lipid metabolism by potentiating the action of insulin. Human deficiency is uncommon. Meats, oils and fats, bread, nuts and cereals, as well as some beers and wines, are all high in chromium. Whole grains are a good source, while refined cereals are a poor source. Acidic foods may acquire chromium during processing from stainless steel vessels or utensils (FSAI 2018). The average chromium concentration in human milk has been calculated to be 0.25 mg/L (Casey et al. 1985). There is very little chromium in bovine milk; according to one study, the amount of chromium in whole or skim milk was less than 0.5 mg/L (Anderson et al. 1992). As a result, milk and dairy products are poor sources of chromium in the diet.

10.5.10 Boron

Boron occurs in foods as borate and boric acid. It is not considered to be an essential nutrient for humans, no specific biochemical function for has been identified, and there are therefore no recommended intakes. There is some evidence that it may influence mineral metabolism, particularly calcium, and have a beneficial effect on bone calcification and maintenance, possibly via vitamin D and estrogen (Strain et al. 2019). Fruit-based beverages and products (including wine, cider and beer), tubers and legumes have been found to have the highest concentrations of boron. Chocolate powder and pecan nuts are among the foods highest in boron. The main contributors in the US diet (accounting for 27% of dietary boron) are reported to be coffee, milk, apples, dried beans and potatoes (FSAI 2018). The boron content of bovine whole milk is around 280 mg B/L. The mean concentration of boron in human milk from full term mothers over the first 12 weeks of lactation is 28 mg/L which is far less than that reported in ready-to-eat infant formulae (120 mg B/L) (Hunt and Meacham 2001).

10.5.11 Other Trace Minerals in Milk

In addition to the elements discussed above, there are 12 other dietary elements consumed in food which are not believed to be essential for humans; aluminum, arsenic, bromine, cadmium, germanium, lead, lithium, nickel, rubidium, silicon, tin, and vanadium (Strain et al. 2019). A recent global systematic review on the

potential toxicity of heavy metals in raw bovine milk showed that the highest levels of Ni (833 mg/L), Pb (60 mg/L), and Cu (36 mg/L) were observed in raw bovine milk collected in granite-gneisses regions in India, while the highest level of Cd (12 mg/L) was reported in a barite-mining region in India (Boudebbouz et al. 2021). The highest Al level was (22.50 mg/L) reported for raw cow's milk was collected in Turkey (Boudebbouz et al. 2021). The Target Hazard Quotients (THQ), defined as the ratio of exposure to the toxic element and the reference dose which is the highest level at which no adverse health effects are expected, were below 1 for Hg, suggesting that milk consumers are not at a non-carcinogenic risk except in Faisalabad province (Pakistan) where THQ values were 7.7 (Boudebbouz et al. 2021). For the other heavy metals, the THQ values were >1 for Pb (10 regions out of 70), for Cd (6 regions out of 59), for Ni (3 out of 29), and for Cu (3 out of 54). The above data highlights the importance of regular screening of milk for heavy metal content in specific 'high' risk regions of the world.

10.6 Conclusions

Micronutrients are required by humans in small quantities for normal growth, development and on-going wellbeing. The World Health Organisation recommends exclusive breastfeeding for the first 6 months of life, because human milk protects against gastrointestinal infections and supplies balanced and adequate nutrient contents to the infant. While reliable data on micronutrient concentrations in human milk are sparse, the nutritional content of bovine milks is generally well understood. Bovine milk is a significant dietary source of many micronutrients and is the major dietary source of calcium, riboflavin, vitamin B_{12}, Iodine, and vitamin D (especially when fortified) for many people around the world. The levels, location, chemical forms of micronutrients and their interactions with other ions or organic molecules in milk are relatively well defined and understood from a physicochemical standpoint. The micronutrient values for human and bovine milks are very similar throughout a large range of geographical areas, with a few variations (e.g., iodine and selenium). The vitamin concentrations in milk vary depending on a variety of factors, including biosynthesis, animal feeding practices, physicochemical conditions (oxygen, heat, light and oxidizing agents), and analytical procedures used to determine them.

There have been huge strides over the last few decades in developing analytical approaches to determine bovine milk micronutrient content, including chromatography, nuclear magnetic resonance or inductively coupled plasma spectroscopy, microbiological and competitive protein-binding assays. Important considerations for the acquisition of accurate and reliable data on human milk micronutrient composition include ensuring suitable quality control and assurance, as well as guidance for pre-analytical considerations, particularly in light of the complexity of the human milk matrix.

In Memoriam This chapter is dedicated to the memory of Professor Patrick Morrissey, Emeritus Professor of Nutrition, University College Cork, a cherished mentor and colleague.

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Chapter 11
Water in Dairy Products

Eoin Murphy

11.1 Introduction

Milk is, on average, constituted of 87–88% water, with the exact content being a function of a combination of seasonal, genetic and dietary factors (Holland et al. 1991; Timlin et al. 2021). Processing of milk into various products and derivatives involves numerous operations which change both the content of water in systems (see Fig. 11.1) and influences how water interacts with constituent dairy solids. Across products of all moisture contents, from liquid milks to powders, water remains a key constituent, the unusual properties (compared to similar molecules) of which have important effects on microbial and overall physical characteristics of such products. As a result, significant focus has been placed on understanding water in dairy products over the last 60 years.

11.2 Chemical and Physical Properties of Pure Water

Water is one of the most abundant, and probably the most recognisable, molecules on earth. However, this most commonplace of molecules, which encompasses over 70% of the planet's surface, is actually quite unusual in terms of its chemical and physical properties. As water is the most important solvent in dairy, insight can be gained through a study of its basic properties, helping dairy scientists to understand, predict and control how interactions of water with dairy components may affect both manufacture and storage properties of products.

E. Murphy (✉)
Teagasc Food Research Centre, Co. Cork, Ireland
e-mail: eoin.murphy@teagasc.ie

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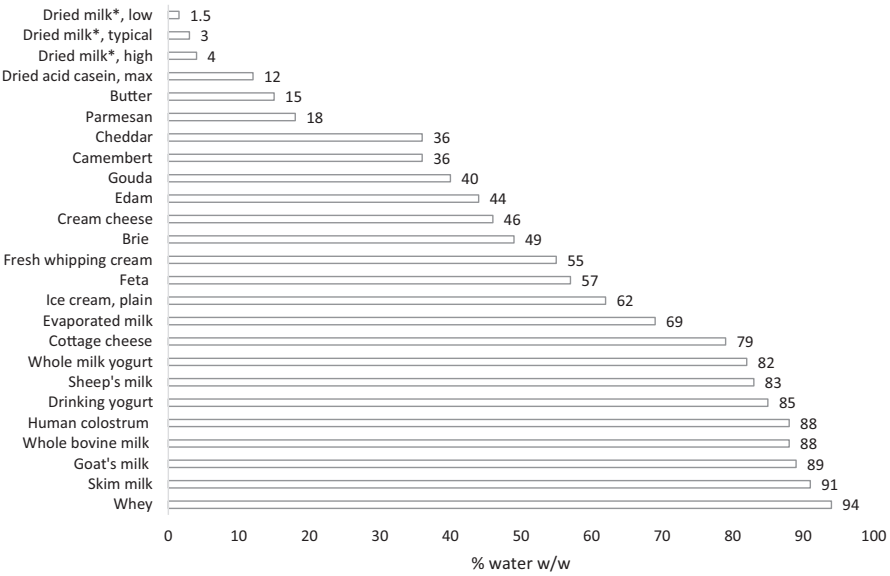


Fig. 11.1 Moisture content of a variety of dairy products. Adapted from Holland et al. (1991). *Dried milk used here as a generic term for all powders derived from milk (whole milk, skim milk, whey, etc.)

11.2.1 Polarity and Hydrogen Bonding

Water is a polar molecule, due to the high electronegativity of oxygen. Electronegativity is the attraction that an atom exerts on shared electrons. In water, one oxygen atom is covalently bonded to two hydrogen atoms, and while there is no net charge on the molecule, the relatively low electronegativity of hydrogen compared to oxygen results in a slightly positive charge on each of the hydrogen atoms, counteracted by a slightly negative charge on the oxygen atom. Polar molecules are also known as permanent dipoles, due to the presence of poles of local negative and positive charge. This results in a high level of intermolecular, or dipolar, forces which can significantly alter physical properties. Table 11.1 shows properties of water compared to other polar and non-polar molecules of similar molecular weight. It is evident that, in general, polar molecules have higher melting and boiling points, compared to non-polar molecules. This is due to the greater energy required to overcome inherent intermolecular forces. However, water stands out even among the polar substances as having exceptionally high melting and boiling points. This is because water, or rather the atomic structure of oxygen when covalently bonded to hydrogen, results in a high occurrence of another, stronger intermolecular force known as hydrogen bonding.

Hydrogen bonding can occur in molecules where hydrogen is covalently bonded to nitrogen, oxygen or fluorine. As described above, these highly electronegative

Table 11.1 Properties of water and similar molecules, adapted from Weast (1986)

Molecule	Mol. wt.	H-bonds	Melting pt.	Boiling pt.	Critical T	Critical P
	Da	No.	°C	°C	°C	bar
Methane	16.04	0	−182.6	−161.4	−82.1	46.4
Ammonia	17.03	2	−77.7	−33.4	132.5	114
Water	18.02	4	0	100.0	374.2	221.5
Hydrofluoric acid	20.02	2	−83.1	19.54	188	64.8
Hydrogen sulphide	34.08	0	−85.5	−60.7	100.4	90.1
	Correlation with H-bonds (Pearson's r)		0.84	0.91	0.91	0.84

H-bonds Hydrogen bonds, *T* temperature, *P* pressure

elements can form polar molecules. The local positive charge on hydrogen atoms will lead to an attraction between the net negative charge on the other molecule (N, O or F), specifically the electrons in the outer shell which are not involved in the covalent bond. Oxygen, for example, has 6 electrons in its outer shell, two of which are shared with separate hydrogen atoms to form covalent bonds, leaving four electrons, or two pairs of electrons (lone pairs) in the O atom of a water molecule. Therefore, each water molecule (H_2O) can potentially form four hydrogen bonds with adjacent water molecules, i.e., two bonds formed from the slightly positive H atoms and two bonds formed from the slightly negative lone pairs in the O atom. Hydrogen bonding in HF, in contrast, is limited by the shortage of positively charged H atoms, similarly NH_3 is limited by a shortage of lone pairs. Therefore, water is the ideal molecule for hydrogen bonding, which is manifested by increased transition temperatures (Table 11.1). Indeed, the transition properties of all molecules in Table 11.1 are reasonably well correlated with the number of H-bonds the molecule is capable of forming ($r > 0.84$ in all cases).

11.2.2 Vapour Pressure

Vapour pressure is a measure of the tendency of a liquid or solid to transition into a gaseous state. The concept of vapour pressure is particularly important for understanding the water activity of dairy products (see Sect. 11.3.1). Vapour pressure is directly proportional to water temperature, and boiling will result at temperatures where vapour pressure equals or exceeds atmospheric pressure. Likewise, ice will exist at temperatures where the vapour pressure of the solid form is lower than that of the liquid, i.e., below the melting point. At 0.0099 °C and a pressure of 0.6104 kPa, the vapour pressures of all three states of water are in equilibrium, allowing for co-existence of ice, liquid water and water vapour and this is known as the triple point.

11.3 General Properties of Water in Dairy Products

11.3.1 Water Activity

Water activity (a_w) is a temperature dependent parameter used to determine the equilibrium of water within a multi-component system. It is widely used to characterise the availability of water and predict how this can affect biological, chemical and physical reactions in foods. In the measurement of water activity, equilibrium should be taken to mean that the chemical potential of all phases in the system is equal. In dilute systems, chemical potential of water (μ_w) is given as:

$$\mu_w = \mu_w^{pure} + RT \ln a_w \tag{11.1}$$

where μ_w^{pure} is the chemical potential of pure water, R is the ideal gas constant and at a constant temperature. Assuming ideal gas conditions the chemical potential of water vapour ($\mu_{w,gas}$) should be taken to be:

$$\mu_{w,gas} = \mu_w^0 + RT \ln \frac{p_w}{p^0} \tag{11.2}$$

where μ_w^0 is the chemical potential of water at a reference pressure p_o and p_w is the vapour pressure of water in solution. At equilibrium, the μ_w^{pure} is equal to that of the gaseous phase, so it can be expressed by a similar equation:

$$\mu_w^{pure} = \mu_w^0 + RT \ln \frac{p_w^{pure}}{p^0} \tag{11.3}$$

where p_w^{pure} is the vapour pressure of pure water. Equating 11.1 and 11.2 to each other and substituting the μ_w^{pure} with 11.3 yields an expression which reduces to:

$$\ln a_w = \ln \frac{p_w}{p_w^{pure}} \tag{11.4}$$

$$a_w = \frac{p_w}{p_w^{pure}} = \frac{ERH}{100} \tag{11.5}$$

Therefore, the water activity is equal to the equilibrium relative humidity (ERH) directly above the material, which is a logical conclusion if the assumption of equilibrium is valid. In general, a_w will increase concomitantly with water content of the material, as is given by Raoult's law; however, this can only be considered likely when the system is dilute and/or small solutes are present. Therefore, a correction factor (γ) is required when equating water activity to molar concentration of water in a material (X_w):

$$a_w = \gamma X_w \tag{11.6}$$

Solute–water interactions and the presence of large solutes compared to water result in $\gamma < 1$; solute–solute interactions result in $\gamma > 1$. Consequently, it is important to note that while water content plays a central role in water activity, the nature of the system, its solutes and their interactions also contribute significantly.

While the concept of a_w is centred on the assumption that materials are at equilibrium, which may only be true for solutions of small solutes, it is still a useful and widely employed tool for characterisation of dairy systems. Other parameters such as pH, nature of constituents and molecular mobility are important to describe the behaviour of many food systems. However, water activity has been shown to be an especially useful marker for describing behaviour such as microbial growth, physical stability and aroma (Chirife et al. 1996).

In the case of dehydrated dairy products, what is measured is the relative humidity of the atmosphere in contact with the product. If the dehydrated product, which has a low chemical potential due to a reduced a_w, is in contact with humid air (high chemical potential), water will transfer from air to product. In such cases, while the dynamic changes in the product over time are not in keeping with the assumption of equilibrium, water activity is still a useful tool as it can be used to determine ideal storage conditions for products, to reduce water transfer and subsequent changes in product quality (see Sect. 11.5).

11.3.2 Water Mobility

It is evident from Table 11.1 that water content in common dairy products is highly variable. In general, the water in these products is often described as being "free" or "bound". A broad definition of bound water is water that does not freeze at −40 °C, with the proximity of solutes resulting in low molecular mobility compared to free water. In addition, bound water is not available for chemical reactions, cannot act as a plasticiser (see Sects. 11.3.4 and 11.4.2) and has a significantly higher enthalpy of vaporisation than free water, which can have significant effects when drying or modelling drying operations (Schuck et al. 2009). However, it must be noted that the term "bound" was coined based on physical observations such as altered dehydration behaviour, while in reality, water is never permanently associated with the dairy components.

A more detailed description of water in food products is given by Hills multistate theory (Hills 1999; Hills et al. 1996) which considers three states of water:

1. structural and tightly bound water bound to cavities and grooves within the solutes, the maximum extent of which corresponds to the monolayer value, i.e., the point where all hydrophilic molecules have an associated water molecule. This water is not immobilised but has relatively long exchange times with bulk water (in the nano- to millisecond order),

2. multilayer or surface water which extends for a number of layers from the surface of the solute and is more mobile than the tightly bound water with exchange times of nanoseconds or picoseconds,
3. bulk or free water which is uninfluenced by the solutes and has similar properties to pure water.

There are a number of different approaches to describing water mobility in food systems. The a_w based approach describes the macroscopic movement of water due to differences in chemical potential. This approach is particularly relevant for low to medium moisture systems whereby water is readily sorbed by the material from the atmosphere (see Sect. 11.3.3). The second is the polymer science or glass transition approach which seeks to determine the transition of the material as a whole from a low-mobility glassy state to a rubber state of higher mobility. This is usually characterised by a glass transition temperature (T_g) which can be measured at mesoscopic scale by methods such as differential scanning calorimetry, or at macroscopic scale using thermo-mechanical methods such as the rheology-based method of Hogan et al. (2010) (see Sect. 11.3.4). Lastly, techniques such as nuclear magnetic resonance may also be employed to detect the mobility of water itself at a molecular level (see Sect. 11.3.5). For further information, the reader is referred to the review of Schmidt (2007). However, while each approach can be applied to describe water mobility, selection of the most appropriate technique should take into account the matrix in question and the information required (Schmidt 2007).

11.3.3 Water Sorption

Dairy products, under the correct conditions, are excellent matrices for sorption of water. Water sorption in milk products generally refers to the adsorption or desorption of vapour phase water and is due mainly to high carbohydrate and protein contents. Water sorption increases water activity, which can affect microbial growth; however, water sorption can also propagate significant structural changes in components, such as protein conformational changes and crystallisation of lactose.

Water sorption isotherms are used to characterise water adsorption and desorption properties of materials. These are generally constructed by exposing materials to various atmospheric humidities at constant temperature and recording the equilibrium moisture content reached (or % change in mass). To construct an absorption isotherm, a low moisture material is exposed to atmospheres where the vapour pressure of water (or relative humidity) is higher than in the product, resulting in moisture transfer from the air. This can be performed stepwise, whereby equilibrium (or constant weight of product) is reached at certain humidity before moving to a higher humidity. Similarly, a desorption isotherm is generated by starting with a wet material and exposing it stepwise to a series of decreasing humidities. Adsorption isotherms are particularly useful for characterising storage stability and designing tailored packaging solutions. Desorption isotherms are widely used to characterise dehydration operations, such as spray drying (Lin et al. 2005; Schuck et al. 2009).

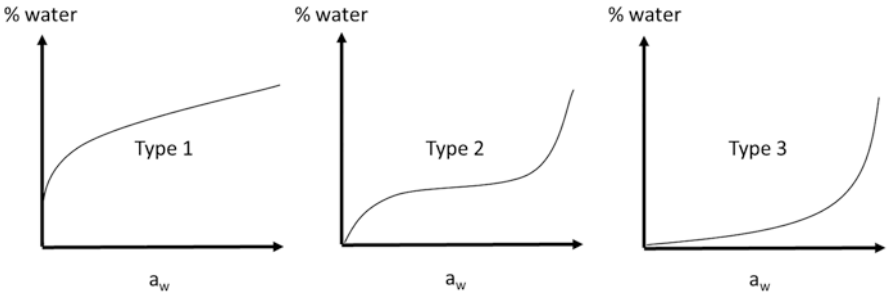


Fig. 11.2 Types of water sorption isotherms found in food materials

Typical water sorption isotherms of dairy products exhibit a sigmoidal shape (Fig. 11.2, Type 2 isotherm). However, Brunauer (1945) described five general types of isotherm on the basis of non-polar gasses adsorbing onto non-polar solids. Of those five, only Types 1, 2 and 3 are applicable to food/dairy systems (Labuza and Altunakar 2007) (Fig. 11.2). Type 1 isotherms are typically not applicable to dairy foods; however, they are worth mentioning here as they apply to anti-caking agents which are sometimes added to dairy products. As indicated by the shape of the isotherm, Type 1 materials hold large quantities of water at low a_w. Once binding is complete, further increases in humidity result in limited adsorption, as the content of dissolvable solutes is low. As mentioned, Type 2 isotherms are the most commonly found isotherms in dairy materials. The characteristic sigmoid shape is a result of a combination of Raoult's law, surface effects and capillary interactions. Behaviour in the lower region of the curve is mainly due to monolayer bonding and subsequent multilayer growth, whereas, in the upper region swelling of constituents, filling of large pores and, ultimately, dissolution are dominant. Type 3 isotherms generally describe sorption behaviour in crystalline solids, such as α-lactose monohydrate powder (Bronlund and Paterson 2004). Due to their crystalline nature, such materials do not readily adsorb moisture at low humidities. Moisture content remains low until a certain critical point whereby humidity becomes high enough to solubilise the portion of crystals in contact with the air; this is referred to as deliquescence. Bronlund (1997) found that liquid bridges between lactose crystals were formed at humidity >85%, which if allowed to dry could themselves crystallise and induce caking in powders. Furthermore, as deliquescence is largely a surface phenomenon, factors such as crystal size, surface amorphous lactose and spatial packing of the crystals are important factors (Bronlund and Paterson 2004).

In many dairy powders, lactose is present in amorphous rather than crystalline state. Amorphous lactose is thermodynamically unstable and can exist in glassy or rubber states (Sect. 11.3.4). As humidity increases, transition to the rubbery state occurs, allowing crystallisation of lactose to arise. This results in rapid release of previously adsorbed moisture and a significant departure from the typical sigmoidal shape (Fig. 11.3). This type of behaviour can be useful in determining the optimum storage conditions to avoid lactose crystallisation in various dairy systems (O'Donoghue et al. 2020).

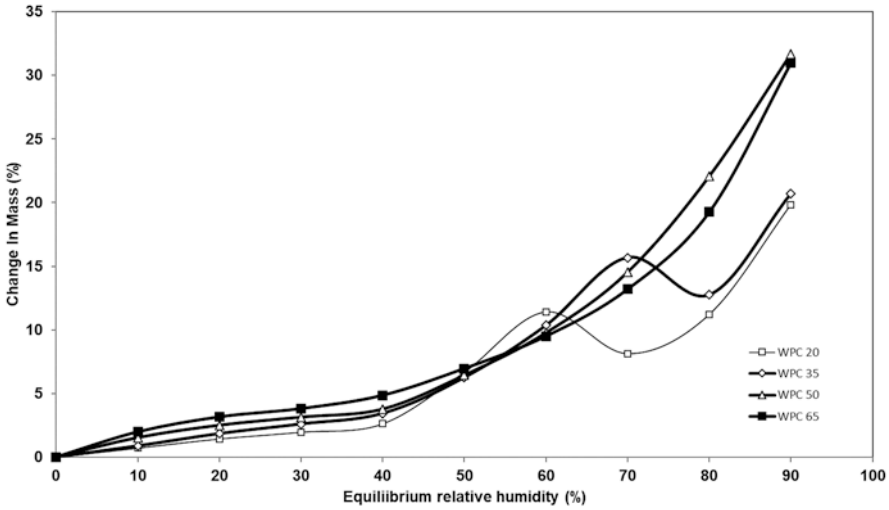
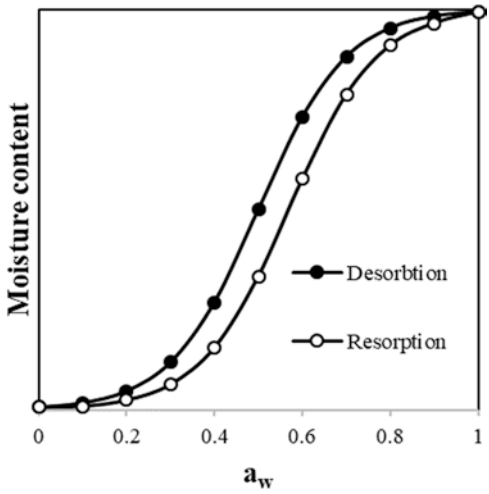


Fig. 11.3 Moisture sorption isotherm for model whey protein concentrate (WPC) powders with protein content in the range of 20% to 65%. Decrease in mass observed for WPC 20 and WPC 35 powders is indicative of lactose crystallisation. Redrawn from (O'Donoghue et al. 2020)

Fig. 11.4 Moisture sorption isotherm exhibiting hysteresis (Fennema 1985)



Hysteresis is commonly observed in sorption isotherms (Fig. 11.4). Typically, the equilibrium moisture content at a given a_w is higher in desorption curves, as compared to adsorption curves. It is difficult to determine the exact mechanism for hysteresis in food and dairy materials due to their complex nature (Iglesias and Chirife 1976). However, various theories have been proposed, including differences in contact angles between liquid and solids during adsorption and desorption, capillary condensation during adsorption and structural changes (Al-Muhtaseb et al.

2002). A practical illustration which supports the existence of hysteresis was published by Labuza et al. (1972) who found that lipid oxidation in intermediate moisture foods prepared by adsorption was 3–6 times lower than corresponding foods prepared by desorption.

Water sorption of dairy powders has been the subject of many studies and a number of mathematical approaches have been proposed to predict such behaviour (Basu et al. 2006). Mathematical modelling of sorption isotherms has a number of useful applications, including controlling of drying processes, determining the correct formulation to obtain a desired a_w and optimising design of packaging environments. One of the most widely used models is the Brunauer–Emmett–Teller (BET) equation (Brunauer et al. 1938) which can be expressed in the following linear form:

$$\frac{a_w}{(1-a_w)M} = \frac{1}{M_0 C} + \frac{C-1}{M_0 C} a_w \tag{11.7}$$

where M is moisture content (g/100 g), M_0 is monolayer moisture value (g/100 g) and C is a dimensionless constant related to heat of sorption. An example of a linear BET plot is shown in Fig. 11.5. Simple manipulation of the slope (S) and intercept (I) values allows for calculation of M_0 and C with:

$$M_0 = \frac{1}{S+I} \tag{11.8}$$

$$C = \frac{S+I}{I} \tag{11.9}$$

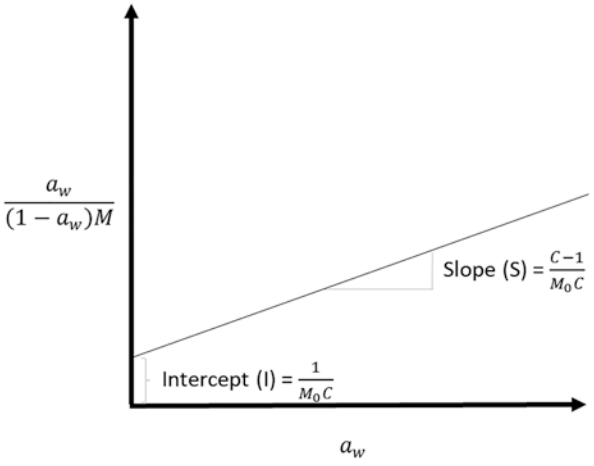


Fig. 11.5 Linear representation of the BET sorption model

The applicability of the BET isotherm is limited to a_w values between 0.1 and 0.5, with Jouppila and Roos (1994b) reporting that the model successfully fitted sorption data for milk powders up to 44% RH. However, estimation of the BET monolayer value is a particularly useful tool for low moisture foods, providing a target moisture content for optimal stability (Sect. 11.5).

The Guggenheim, Anderson de Boer (GAB) model was subsequently developed as an improvement on the BET model (van den Berg 1984). An additional dimensionless parameter, K, is introduced, which is related to the heat of sorption of the multilayer region. The model can be written in the form of a second-order polynomial (Blahovec and Yanniotis 2008):

$$\frac{a_w}{M} = \alpha a_w^2 + \beta a_w + \gamma \tag{11.10}$$

where

$$\alpha = \frac{K}{M_0}\left(\frac{1}{C} - 1\right) \tag{11.11}$$

$$\beta = \frac{1}{M_0}\left(1 - \frac{2}{C}\right) \tag{11.12}$$

$$\gamma = \frac{1}{M_0 KC} \tag{11.13}$$

α, β and γ can be obtained through regression analysis. Further manipulation of the above equations generates the following:

$$\gamma K^2 + \beta K + \alpha = 0 \tag{11.13}$$

allowing for the calculation of K as the solution of the quadratic equation. Subsequent rearrangement of the equations yields the following expressions:

$$C = \frac{\beta}{\gamma K} + 2 \tag{11.14}$$

$$M_0 = \frac{1}{\gamma KC} \tag{11.15}$$

The GAB equation has been shown to produce model isotherms which fit experimental data for a_w values from 0 to 0.95. It is possible to further refine the GAB model and its applicability to the high moisture region by addition of a third sorption parameter as per the work of Timmermann and Chirife (1991) and Viollaz and Rovedo (1999). However, it should be noted that while both BET and GAB models

are based on the central premise of monolayer moisture sorption, the underlying mechanisms of sorption may be more complex. This is well illustrated by the fact that inclusion of additional sorption terms to BET and GAB isotherms has been shown to increase sensitivity. It is also possible to achieve excellent fits through a variety of empirical models, such as the four parameter model developed by Peleg (1993). While these models are useful, a limitation is that, unlike BET and GAB models, they are not supported by a theoretical mechanism of sorption (Labuza and Altunakar 2007).

11.3.4 Glass Transition

Glass transition is a concept from polymer science which is widely applied to dairy systems to describe molecular mobility and mechanical properties of non-crystalline solids. The central premise of the transition is a transformation of solids from a "supercooled" rubbery state where molecular mobility is relatively high, to a solid-like glass state where molecular mobility is low. The transition is strongly mediated by both temperature and water content, whereby an increase in either tends to push materials towards a rubbery state. At a given moisture content, the temperature at which the transition occurs is called the glass transition temperature (T_g). Given the wide range of temperatures and moisture contents observed in the manufacture and storage of dairy products, T_g can be applied to a wide range of operations such as freezing, drying and packaging. Particular attention must be paid to glass transition during drying due to the inherent high temperatures and humidities. Above T_g, increased molecular mobility can induce stickiness in powders, resulting in significant fouling or, in extreme cases, blocking of dryers.

T_g can also be related to relaxation time, i.e., the time taken for a material to come to equilibrium after exposure to a perturbation. Below T_g, relaxation times are high due to its highly viscous glass state. At $T > T_g$, increased molecular mobility allows for shorter relaxation times with physical manifestations that can be readily observed in products. The effect of temperature changes on relaxation times (and viscosity) is often described using the Williams–Landel–Ferry (WLF) equation which can be used to determine the relative relaxation time at an experimental temperature (T) compared to a reference (T_r):

$$\log \frac{\tau}{\tau_r} = \log \frac{\mu}{\mu_r} = \frac{-C_1 \left(T - T_r\right)}{C_2 + \left(T - T_r\right)} \tag{11.16}$$

where τ and τ_r are the relaxation times at the experimental and reference conditions. Likewise, μ and μ_r are the experimental and reference viscosities. C_1 and C_2 are constants which are sometimes taken to be "universal" with values of 17.44 and 51.6, respectively. It should be noted that these values may not be applicable to all food systems and sometimes it is appropriate to use constants generated to fit experimental data. One current opinion is that the universal value for C_1 may be realistic, while the value for C_2 varies (Liu et al. 2006; Ubbink and Dupas-Langlet 2020).

Using T_g as the reference and the "universal" constants, Fig. 11.6 illustrates that changing outlet temperature of a spray dryer can have a significant effect on relaxation times. At higher moisture contents, T_g is reduced to below typical dryer outlet temperature, resulting in relatively lower relaxation times, which can ultimately manifest as fouling and blocking of equipment. It should be noted here that, in reality, changing of outlet temperature also results in a change in moisture content; however, the central point observed in Fig. 11.6 still applies, moisture content and temperature are the key determinants of T_g, relaxation time and hence stickiness of dairy powders (Sect. 11.4.2).

The applicability of WLF kinetics is closely related to the concept of fragility of amorphous materials, a term which describes the extent of deviation from Arrhenius type kinetics around T_g (Angell 2002). In the vicinity of T_g, fragility (m) can be expressed as (Ubbink and Dupas-Langlet 2020):

$$m = \frac{E_a}{RT_g \ln 10} = \frac{C_1 T_g}{C_2} \tag{11.17}$$

where E_a is activation energy and R is the universal gas constant. Fragile materials exhibit higher values of m, indicating greater deviation from Arrhenius behaviour, resulting in large changes in relaxation time and viscosity in the vicinity of T_g. An alternative approach was proposed by Fan and Roos (2017) and Maidannyk and Roos (2018), which used temperature dependence of structural relaxation as a basis. Strength (S) can be written as:

$$S = T_d - T_g = \frac{C_2 d}{C_1 - d} \tag{11.18}$$

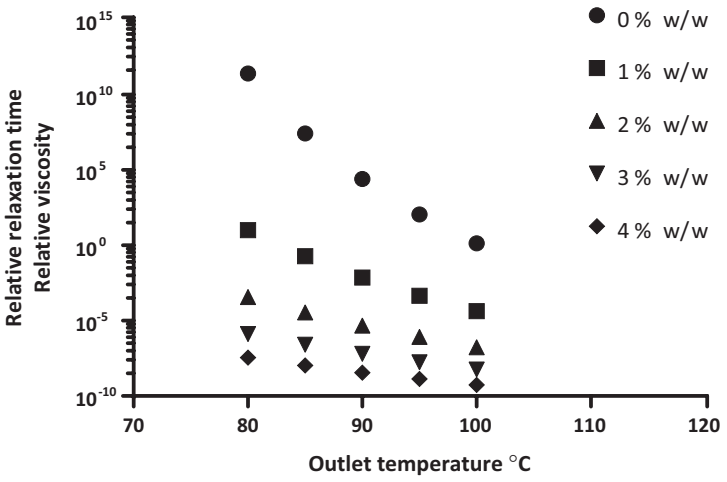


Fig. 11.6 Relative relaxation times of a model first age infant formula as a function of outlet temperature and moisture content. Each relative relaxation time was calculated using the Williams–Landel–Ferry equation using glass transition temperature as the reference temperature

where T_d is the temperature at which relaxation is d decades shorter than T_g. By convention, d is set to 4 (Fan and Roos 2017; Maidannyk and Roos 2018). As S is dependent on the temperature increase above T_g required to bring about a certain reduction in relaxation time or viscosity, higher values indicate greater resistance to flow.

While the water content may be indirectly taken into account in WLF when using T_g as the base temperature (Fig. 11.6) the standard equation is a function of temperature only. Therefore, it has limitations for use in modelling relaxation times where both temperature and moisture content are variable. Attempts have been made by authors to include the effect of water and these include modification of C_2 using a similar expression to the Gordon–Taylor expression (Dupas-Langlet et al. 2019), linear variation of C_2 with water content (Ubbink and Dupas-Langlet 2020) and modification of the WLF expression to include a water term (Kasapis 2001; Kasapis et al. 2000). Ubbink and Dupas-Langlet (2020) assessed the former approaches, finding that fragility increased with water content for maltopolymer/maltose blends.

As a material undergoes glass transition, its enthalpy and specific volume change, which is often measured as a change in heat capacity (ΔC_p) by differential scanning calorimetry (DSC). T_g and ΔC_p values for a range of dairy components are shown in Table 11.2. The physical manifestation of the transition may also be measured by thermo-mechanical techniques such as dynamic mechanical analysis (DMA) or the rheological method developed by Hogan et al. (2010). Glucose and galactose are included in Table 11.2 to show the effect of molecular weight on T_g, although it is worth noting they may also be present at high concentrations in dairy products containing hydrolysed lactose. As a general rule, T_g increases with increasing molecular weight, which can be seen in Table 11.2.

The presence of low molecular weight components can significantly affect the T_g of dairy products. Increasing the quantity of these materials in, for example, a formulated product, or through hydrolysis, increases free volume and hence decreases T_g; this effect is called plasticisation. In powders, at a constant a_w, changes to the carbohydrate fraction can have a significant plasticisation effect; Fenelon et al. (2020) calculated that substitution of lactose (342 Da) with DE20 Maltodextrin

Table 11.2 Glass transition temperatures (T_g) and ΔC_p values for dairy components

Component	T_g (°C)	ΔC_P (J/g °C)
Casein	132	0.26
Whey	127	0.09
Lactose	98	0.38
Glucose	31	0.24
Galactose	30	0.24
Water	−135	1.94

(~900 Da) increased T_g of a first age infant formula by ~20 °C. Similarly, (Jouppila and Roos 1994a) found that hydrolysis of lactose in skim milk powders reduced T_g by approximately 40 °C. Proteolysis may be expected to yield a similar reduction in T_g of dairy powders (Netto et al. 1998); however, this was not found in DSC measurements on powders made from hydrolysed infant formula and sodium caseinate–lactose powders (Kelly et al. 2016; Mounsey et al. 2012). This indicates that, in such materials, the T_g is dominated by the continuous carbohydrate component. However, it should be noted that T_g, as determined using a thermo-mechanical method, was lower in hydrolysed materials (Mounsey et al. 2012), thereby indicating the complexity of the phenomena and the benefits of employing numerous measurement approaches.

Despite the effects of low molecular weight solids on T_g, water is the most significant plasticiser in dairy products (Slade et al. 1991). The effect of water (and other components) on T_g is often modelled using the Gordon–Taylor equation:

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \tag{11.19}$$

where w_1 and w_2 are the weight fractions of components 1 and 2 in the system. When applied to water plasticisation, component 1 is often the dry solids, with component 2 being the water present. T_{g1} and T_{g2} are the glass transitions of the individual components, and k is a constant which can be derived from T_g experiments over a variety of water contents. The plasticisation effect of water can be easily visualised using the Gordon–Taylor equation; the generally accepted T_g of pure water is −135 °C, which when substituted into the equation has a significant effect on the calculated T_g, especially at high weight fractions. The Gordon–Taylor equation can be considered to be a simplified version of the Couchman–Karasz expression, which allows for the expansion of the prediction beyond binary systems and takes into account the T_g and ΔC_p of each component (see Table 11.2):

$$\ln T_g = \sum_{i=1}^{n} \frac{w_i \Delta C p_i \ln T_{gi}}{w_i \Delta C p_i} \tag{11.20}$$

11.3.5 Water Mobility

The mobility of water in dairy systems is often characterised using nuclear magnetic resonance (NMR) spectroscopy, which is based on the ability of nuclei to absorb or emit electromagnetic radiation when placed in a strong magnetic field. The broader application of NMR in dairy products is reviewed by Belloque and Ramos (1999) and Maher and Rochfort (2014) who detail the application of, in particular, the ^{1}H,

^{13}C and ^{31}P nuclei to analyse various milk components. Water mobility is deduced from the fading of NMR signals with time due to two types of reaction: (1) longitudinal relaxation with is characteristic time T_1 and (2) transverse relaxation, characterised by T_2. T_2 and its inverse R_2 (relaxation rate) are associated with mobility. When water molecules are in close contact with solutes, mobility is reduced, which in NMR manifests as a decrease in T_2 or an increase in R_2. Water mobility has been studied using all possible nuclei (^1H, ^2H and ^{17}O); however, it is now clear that ^{17}O relaxation is the best option for tracking water mobility due to the tendency of ^1H and ^2H to undergo cross-relaxation with protons and deuterons in the solid phase (Mariette 2008).

Interpreting relaxation times and rates in foods can be challenging compared to other systems. Dairy products are always multi-component and often heterogeneous which adds a great deal of complexity compared to pure systems. Relaxation time in such cases is multi-exponential and cannot be described by a single component. Water relaxation time is especially dependent on the physical state of the system. This was illustrated by Hills et al. (1990) using various matrices derived from skim milk powder. For rehydrated skim milk, isolated casein micelles and a coarse paste of compacted skim milk powder, it was observed that multi-exponent relaxation can be expected if the following were true:

$$\frac{a^2}{D}\Delta y \geq 1 \tag{11.21}$$

where α is the characteristic dimension of the heterogeneity, i.e., casein micelle diameter for rehydrated skim milk and isolated casein micelle dispersions, or, powder particle size for the coarse paste; D is the diffusion coefficient, assumed to be that of bulk water $\approx 2 \times 10^{-5}$ cm^2/s, and Δy is the difference between the relaxation rate in the two areas. For this condition to be met in rehydrated skim milk, in which α was measured to be 0.05 μm, the relaxation time of water in the casein micelle would be unfeasibly short (~10 μs). This is in keeping with Mariette et al. (1993) who determined relaxation time of micellar water to be in the range of a few milliseconds. Therefore, the single exponent relaxation time observed agrees with the conditions of Eq. 11.21. However, in the coarse paste, the characteristic dimension was 400 μm, meaning the condition for multi-exponent relaxation (Eq. 11.21) could easily be met; this was confirmed by the data which consisted of a three component relaxation.

Water retention by gels and cheese may also be described in terms of Eq. 11.21. Tellier et al. (1993) observed that upon curd formation, relaxation time remained mono-exponential, concluding that relaxation mechanisms, or system heterogeneity, did not change significantly upon gelation. However, after onset of syneresis, bi-exponential relaxation times were observed: (1) a short T_2 associated with exchange of water and macromolecular protons within the curd and (2) a long T_2 which was associated with water expelled from the curd.

^{17}O transverse relaxation times, along with molecular dynamic simulation, have been widely used by the group of Halle (Denisov et al. 1995; Halle 2004; Halle

et al. 1981; Mattea et al. 2008) to gain insights into protein hydration. Using this approach, correlation times (τ) relating to water relaxation can be generated, which give the mobility of water in bulk phase and protein hydration layer. Correlation time for bulk water (τ_{bulk}) is approximately 2 ps, indicating a high degree of mobility. Denisov et al. (1995) reported a highly mobile hydration layer for globular protein solutions, with the majority of water molecules in the hydration layer having a τ of 20 ps, i.e., 10 times less mobile than water. Only a very small number of water molecules buried deep within the protein exhibited extreme reduction of mobility as indicated by a τ in excess of 1 ns. This is supported by the work of Mattea et al. (2008) who found that the majority of hydration water was weakly perturbed, with mobility only reduced by a factor of 2 for 90% of the water. In milk systems, relaxation of water due to serum components can be expected to behave similarly.

Overall, application of NMR relaxation is a useful non-destructive approach for determining water mobility in dairy studies. In addition to the cheese and protein hydration applications outlined above, it has also been used to investigate a number of other dairy systems, such as yoghurts (Hinrichs et al. 2003; Laligant et al. 2003), whey protein concentrates (Hinrichs et al. 2004) and gels (Hinrichs et al. 2003; Mariette et al. 2002). However, it is important to note that it is best applied along with complementary techniques, such as compositional and structural analysis, which help with interpretation of sometimes complex data. Furthermore, it may be used in conjunction with other techniques to measure water mobility in foods. Several authors have had success in measuring glass transition of food products by monitoring T_1 and T_2 changes associated with solid phase nuclei (i.e., ^{1}H and ^{13}C) (Farhat 2004; Ruan and Chen 1998; Ruan et al. 1998; Van Den Dries et al. 2000). The ability of NMR to detect changes in molecular mobility over a very small range (1–2 nm) can potentially provide even more detailed information than glass transition methods based on thermal or mechanical methods (Rahman 2010).

11.3.6 Ice Formation

Freezing and melting of systems are often characterised using a T_m curve (Fig. 11.7) which represents the concentration-dependent temperature at which ice starts to separate when the system is cooled under equilibrium conditions. At freezing point, the vapour pressure of ice is equal to that of water. Therefore, the lower vapour pressure in milk systems (due to the presence of solutes) reduces the freezing temperature, as a lower ice vapour pressure is also needed. As the system becomes more concentrated, lower and lower temperatures are required due to the increased effect of solutes on the vapour pressure; this is known as freezing point depression.

More often than not, milk systems are cooled rapidly, resulting in non-equilibrium ice formation. The exact physical condition of ice and dairy solids is a function of the conditions employed during freezing, which can be better understood through the application of a state diagram (Roos 1997; Vuataz 2002).

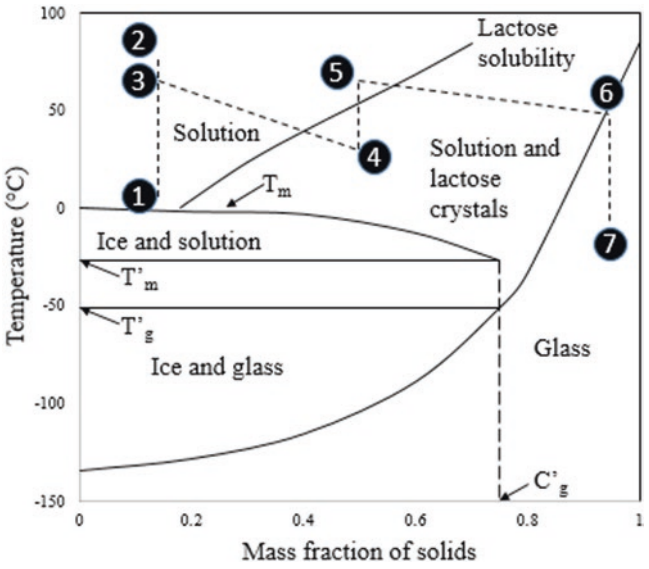


Fig. 11.7 State diagram of a typical dairy product, based on water and lactose transitions (Modified from Roos (1997)). Also shown are typical processing points in the manufacture of a dairy powder: (1) dilute milk under cold storage, (2) heat treatment, (3) before evaporation, (4) after evaporation, (5) feed to spray dryer, (6) hot dry powder, (7) cooled powder

11.4 Effect of Water on the Physical State of Dairy Products

11.4.1 State Diagrams

State diagrams are useful tools which describe the interactions of food materials with water over a range of dry matter contents and temperatures. They combine data on melting point and solubility, more often relevant to high moisture products, with T_g data relevant to low moisture products. Additionally, processing operations may be represented on the diagram and are particularly useful for determining effects of temperature modulating operations, e.g., heat treatment, homogenisation and concentration operations such as evaporation and spray drying.

The T_m curve (Fig. 11.7) represents the concentration-dependent temperature at which ice starts to separate when the system is cooled under equilibrium conditions. It may also be used to determine weight fraction in the freeze concentrated liquid phase as temperature decreases. The solubility line T_s for milk systems generally represents the solubility limit for lactose. The eutectic point is where the T_m and T_s curves meet. For some solutions, such as binary mixtures of NaCl and water, eutectic solutions are formed where both solvent and solute crystallise at the same point (Ludl et al. 2015). This is not observed in milk systems as super-saturated lactose solutions often solidify in a glassy state, thus limiting crystallisation.

The glass transition (T_g) line may also be represented on the state diagram. As the T_m curve approaches T_g, maximal freeze concentration of the system is observed. This is often considered to be a particularly desirable state, as it maximises the amount of water in a stable crystal form. DSC thermograms for annealed systems can be used to determine concentration-independent values for onset of ice melting (T'_m) and glass transition (T'_g) in a maximally concentrated state. These concentration-independent values can be depicted as straight lines on the state diagram, with the intercept of T'_g and the T_g curves giving the maximal freeze concentration (C'_g). At temperatures above T'_m the equilibrium amount of ice is formed. The additional unfrozen water associated with this can cause a decrease in viscosity and lead to increased rates of diffusion-controlled reactions (Levine and Slade 1988; Roos 1991). At temperatures between T_g and T'_g, a non-equilibrium amount of ice is formed, which is commonly the case in frozen food systems (Singh and Roos 2007). Under these conditions, ice and glass are formed and the viscosity is high enough to limit reaction rates.

Super-imposing typical production conditions in the manufacture of milk powders onto a state diagram can be helpful to understand and predict transformations which occur during processing. Milk systems start as dilute solutions below the solubility limit of lactose; heating and evaporation take the solution beyond the solubility limit, which in some cases may lead to lactose crystallisation (as in the case of whey and permeate). Drying transforms materials into "supercooled" rubbery states, which may lead to stickiness during processing. However, sufficient water removal, and cooling of the powder, transforms the material into a stable glass which can be stored for long periods of time under appropriate conditions.

11.4.2 Plasticisation of Powder Surfaces

Surface properties of dairy powders are key determinants of their processability and final functionality. This is because many of the interactions during drying and storage are surface-controlled. It is well established that drying of powders results in powder surface compositions which differ significantly from that of the bulk powder (Fäldt and Bergenståhl 1994; O'Donoghue et al. 2019; O'Donoghue et al. 2020). In general, drying results in powder surfaces where proportions of fat and protein are higher than the bulk composition of the solids. Water induced plasticisation and viscosity of these surfaces are important properties, having significant effects on stickiness, caking and general flowability.

Stickiness is a surface phenomenon occurring when powder particles interact with each other (cohesion) or external surfaces (adhesion) (Boonyai et al. 2004; Downton et al. 1982). The extent of cohesive or adhesive behaviour is related to the viscosity of the surface, which is sometimes modelled by the WLF equation (Eq. 11.16) for temperatures above T_g. As can be seen in Fig. 11.6, the relative surface viscosities of high temperature and water content materials are low. If viscosity is low enough, surface energy-driven viscous flow occurs, allowing liquid bridges to

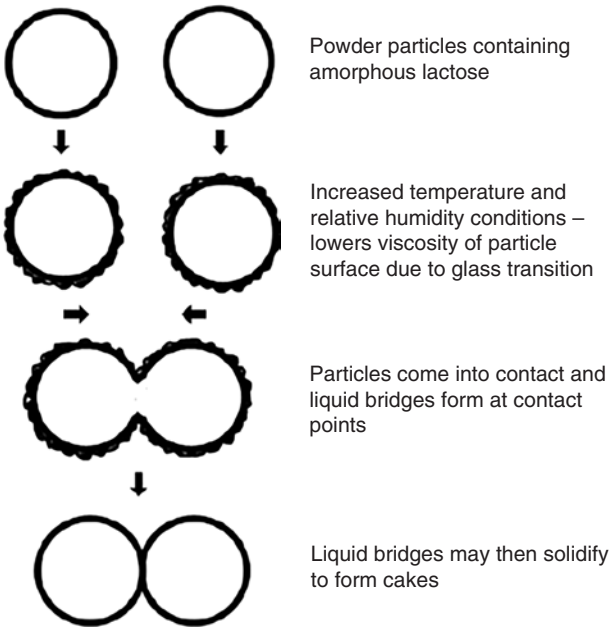


Fig. 11.8 Mechanism of stickiness in dairy powders containing amorphous lactose (O'Donoghue 2019)

form between particles and/or with external surfaces. Stickiness occurs when the liquid bridges formed are strong enough to resist mechanical deformations which may arise due to particle velocities and trajectories, for example. Therefore, stickiness is an extremely important consideration during drying, because both high temperatures and humidities prevail, which can cause significant thermal and water plasticisation of powder surfaces. The stickiness mechanism is presented in Fig. 11.8.

Many authors have successfully applied T_g based approaches to describe stickiness in dairy powders (Hogan et al. 2010; Mounsey et al. 2012; O'Donoghue et al. 2019; O'Donoghue et al. 2020). In general, it is agreed that temperature must exceed T_g for stickiness to occur, i.e., molecular mobility must be high. Under these conditions, viscosity is decreased which reduces the contact time required for stickiness to within the range that can be observed under industrial/laboratory conditions. Downton et al. (1982) reported that the critical viscosity required for induction of stickiness was nearly independent of water content and ranged from 0.3×10^6 and 4×10^7 Pa s. Further decreases in viscosity through thermal or water plasticisation result in increased stickiness. This is in keeping with several reports which found rate of stickiness development is dependent on the extent of $T - T_g$ (Foster et al. 2006; Murti 2006; Paterson et al. 2005; Paterson et al. 2007). Most observers note that a certain $T - T_g$ value must be exceeded before stickiness is observed. This temperature is often referred to as the sticky point temperature and is likely the temperature at which the surface viscosity approaches a critical value in the range

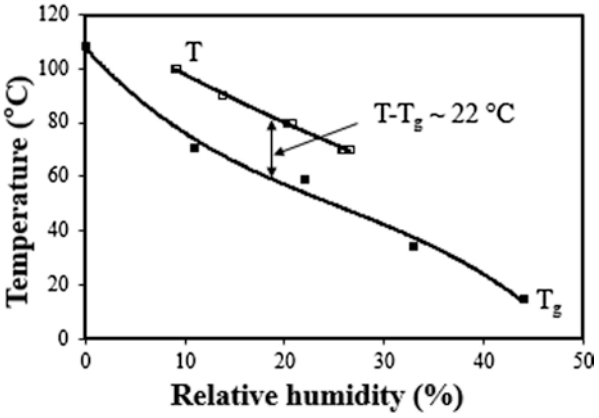


Fig. 11.9 Typical relationship between sticking temperature (T, open symbols) and glass transition temperature (T_g, closed symbols). Data taken from Hogan and O'Callaghan (2010) where T_g was estimated using the Couchmann–Karasz equation and sticking temperature was measured using a fluidised bed apparatus for a powder comprising of 27% protein and 59% lactose (w/w)

of 10^7 Pa s as reported by Downton et al. (1982). Figure 11.9 shows a typical stickiness curve and its relationship to T_g. However, it should be noted that the $T - T_g$ values observed in the literature are extremely variable due to the lack of a standardised testing protocol. For example, stickiness in skim milk powder has been measured using a particle gun (Murti 2006), fluid bed apparatus (Hogan and O'Callaghan 2010; Hogan et al. 2009), thermo-mechanical method (Ozmen and Langrish 2002) and direct agitation (Hennigs et al. 2001), resulting in $T - T_g$ values ranging from 33.6 °C to 14 °C. In practice, the most reliable stickiness data is obtained directly from industrial operations rather than from laboratory methods that cannot adequately recreate real-life particle trajectories, residence times, etc. From the perspective of water plasticisation of the particle surface, dynamic methods which expose particle surfaces to air streams of controlled temperature and humidity (i.e., the fluidisation method) may be more favourable than methods which rely on pre-conditioning bulk powders at fixed a_w.

In the absence of physical data, as may be the case during product development, a modelling approach could be undertaken to estimate stickiness in powders. If we assume viscosity in the glass (i.e., below T_g) to be 10^{12} Pa s (Roos 1997) and the viscosity at the sticky point to be 10^7 Pa s (Downton et al. 1982), we can estimate the temperature required to induce stickiness using a combination of the Couchman–Karasz and WLF equations (with "universal" constants). This approach is shown in Fig. 11.10 for a model IMF formulation. While this can be a useful tool and clearly shows the plasticisation effect of water, a number of weaknesses are associated with the approach, meaning it can only ever be used as an indicator:

- universal constants of the WLF equation are not always applicable (Paterson et al. 2005),
- bulk composition is used to estimate T_g when surface composition may be significantly different,
- the presence of other potentially sticky surface materials, such as free fats, is not considered,
- the effects of particle trajectories and residence times cannot be factored into this approach.

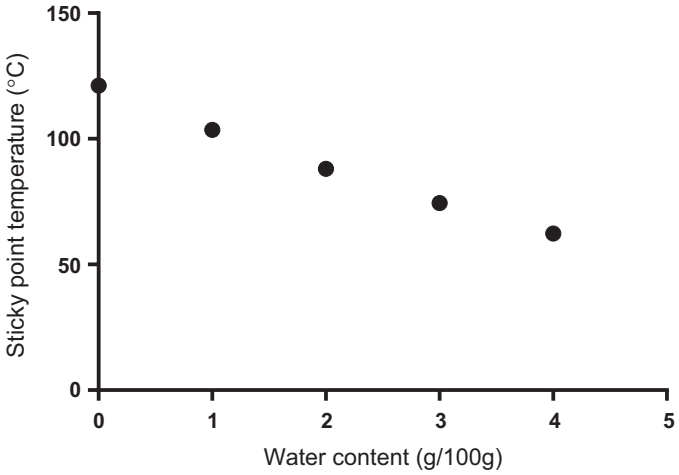


Fig. 11.10 Modelling the effect of water content on sticky point temperature for infant milk formula. Glass transition values were obtained using the Couchman–Karasz equation. Sticking point temperature was then obtained using Williams–Landel–Ferry equation

Caking is a related phenomenon which results in undesirable formation of lumps, varying in size and hardness. As caking is generally observed during storage, it is discussed in more detail in Sect. 11.5.

11.4.3 Lactose Crystallisation

Lactose crystallisation is one of the most important transformative steps in the manufacture of dairy products, the effects of which are reviewed in detail by Huppertz and Gazi (2016). Water is a key factor in crystallisation of lactose, both in high and low moisture environments. In high moisture products, crystallisation is governed by lactose solubility (Fig. 11.7), whereby lactose crystallises out of solution at >2.1 times the solubility limit (approximately). The solubility limit of lactose in water (C_{max} g lactose/100 g water) can be described as a function of temperature T (Butler 1998; Huppertz and Gazi 2016):

$$C_{max} = 10.9109 \times e^{0.02804T} \tag{11.22}$$

It should be noted that solubility of lactose in water will be different to solubility in dairy systems. Ions present as a result of the mineral content of milk products may be described as structure breaking or structure making in terms of their effect on water (Marcus 2010); for example, phosphate is classified as a structure making ion,

meaning dipolar water molecules become oriented around the ion, which causes decreased solubility. Many authors have reported significant effects of ionic environment and certain mineral salts in increasing and decreasing lactose solubility (Bhargava and Jelen 1996; Smart 1988; Smart and Smith 1992) some of which may be explained by water-structuring. Therefore, the output of Eq. 11.22 should be taken only as a general indication of lactose solubility in dairy systems.

More detail on lactose solubility and crystallisation can be found in Chap. 4; however, it is pertinent to note here that crystallisation is often purposely induced during manufacturing of whey/permeate powders to reduce stickiness during drying of high lactose products. The process involves concentration of lactose in the dairy streams to very high levels (>110 g lactose /100 g water in whey, for example) followed by cooling to induce crystallisation. When carried out at typical temperatures (i.e., <93.5 °C) α-lactose-monohydrate crystals are formed. As the name suggests, α-lactose-monohydrate consists of one lactose molecule chemically bound to one molecule of water. Therefore, each lactose crystal is approximately 5% water, which is typically not measurable by standard oven based gravimetric procedures. As a result, total moisture content in whey and permeate powders may be 3–4% higher than indicated by free moisture methods (O'Donoghue et al. 2019), with the difference between the two values indicating the extent of crystallisation in the product (typically 70–80% of total lactose for well crystallised wheys and permeates). In addition to reducing stickiness during drying, pre-crystallisation also reduces the potential for subsequent crystallisation in powders. Lactose crystallisation in dairy powders occurs as a result of thermal or water plasticisation with detrimental effects on quality and shelf life. Although crystallisation of lactose in powders may occur during processing as a result of poor handling, it is more often a factor during storage. Therefore, crystallisation of lactose in dairy powders is discussed in Sect. 11.5.

11.4.4 Ice Crystallisation

As mentioned previously, freezing results in the formation of a freeze concentrated, liquid serum phase, the viscosity of which plays an important role in ice crystal formation. Viscosities lower than 10^8 Pa s are likely to be sufficient to allow for growth of ice crystals, as outlined by Luyet and Rasmussen (1967) for polyvinylpyrrolidone systems. The effective temperature limit for the formation of ice crystals is generally reported to be T'_g (Levine and Slade 1989; Roos 1997; Slade et al. 1991), with the critical viscosity limit for ice formation likely to be in the region of 10^{12} Pa s (Franks 1985). The size of ice crystals formed during freezing is dependent on rate of freezing; slow freezing results in the formation of a small number of large crystals, whereas rapid freezing results in a large number of small crystals (Cook and Hartel 2010). The crystals formed during initial freezing are unstable and tend to grow during storage in what is known as recrystallisation. Recrystallisation can have significant effects on the storage stability of ice cream and is discussed in more detail in Sect. 11.5.

11.5 Water during Storage

Water content and a_w are key determinants of a product's shelf-life stability, affecting the rate of deteriorative physical, chemical and microbial changes over time. To conceptualise these effects, stability maps are often employed to show the relative rates of various reactions over the a_w range (Fig. 11.11). In general, at low a_w the rate of lipid oxidation is high; increasing a_w initially reduces oxidation rate until an intermediate a_w whereafter the rate increases. At intermediate a_w, structural transformations related to the transition from glass to rubber state begin to occur, e.g., stickiness and caking. Similarly, the increased molecular mobility of the system allows for increased rates of (potentially) diffusion-controlled reactions such as enzymatic browning, lactose crystallisation and enzymatic activity. At high a_w, rates of microbial growth increase with implications for product safety.

11.5.1 Physical Stability

11.5.1.1 General Physical Stability

The effects of water and thermal plasticisation on T_g and molecular mobility are well known (see Sects. 11.3.4 and 11.4.2). Where possible, storage conditions are generally maintained below T_g to avoid deleterious physical changes over storage. This is particularly relevant for low moisture foods such as dairy powders which may easily exceed T_g if exposed to atmospheric conditions. However, it is also pertinent to add that sub-T_g amorphous lactose is thermodynamically unstable,

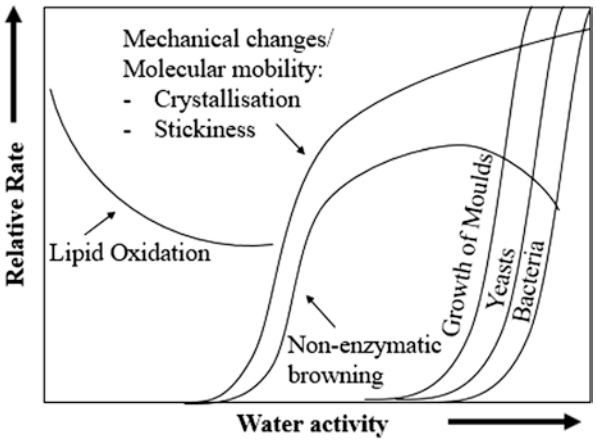


Fig. 11.11 Food stability map showing the general effect of water activity on relative rate of deleterious reactions in amorphous foods (Roos 1997)

meaning that during long-term storage its structure may begin to relax towards a state of equilibrium. This is known as physical ageing and is associated with a decrease in the enthalpy and volume of glassy materials over time (Chung and Lim 2003; Chung and Lim 2006; Haque et al. 2006). The concept of physical ageing is not widely applied to dairy powders, perhaps indicative of the fact that the changes above T_g are more easily observable with obvious impacts on product quality. However, Haque et al. (2006) did study physical ageing in amorphous lactose glass stored below T_g and enthalpy relaxation times were observed to range from a few minutes at temperatures close to T_g to a few months when stored at 77 °C below T_g. This implies that sub-T_g relaxations may occur in dairy powders. While little information is available on the effects of physical ageing on dairy powders, it could be expected to cause changes in texture and physical strength, as observed in other food materials (Badii et al. 2006; Chung et al. 2005).

11.5.1.2 Caking and Lactose Crystallisation

Plasticisation of powder surfaces, as discussed in Sect. 11.4.2 in relation to its impact during processing, also plays an important role during storage. Caking, for example, occurs when sticky powder surfaces are given sufficient time to interact. At $T > T_g$, liquid bridges form between powder particles, which can then solidify if the temperature is reduced below T_g, resulting in large aggregated masses of powder (Huppertz and Gazi 2016). Therefore, as with stickiness during drying, the tendency to cake may be reduced by crystallisation of lactose prior to drying. It should be noted that fat induced caking is also possible, due to the formation of liquid bridges between liquid surface fat which subsequently solidifies after cooling. This is mostly of concern for high fat powders, e.g., whole milk or fat filled milk powders; surface fat composition of industrial whole milk powder samples has been reported to be as high as 95% (Kim et al. 2009). Similarly, surface composition of fat in skim milk (9.6%) and demineralised whey (28.4%) powder surfaces was found by O'Donoghue et al. (2019) to be significantly higher than the respective bulk composition (~1%). However, as observed by Özkan et al. (2002), caking behaviour of low fat powders is likely to be dominated by water plasticisation as the majority of the surface remains available to undergo glass transition.

During storage, crystallisation of lactose may also occur as a result of water sorption and/or high temperature. At ambient storage conditions, pure amorphous lactose will typically crystallise at an a_w of between 0.3 and 0.4. This is observed as a break in the moisture sorption isotherm (Fig. 11.3). Several authors have shown that protein and minerals present in most dairy products can be expected to delay crystallisation of lactose compared to pure amorphous lactose (Haque and Roos 2004; Hogan and O'Callaghan 2013; Jouppila and Roos 1994a, 1994b; Mounsey et al. 2012). Roos (1997) related crystallisation rates in powders to water content/activity of powders. At ~0.6 a_w, crystallisation time was 2 min, which was significantly increased at lower a_w, due to decreased $T - T_g$ values.

The main physical effect of crystallisation is to exacerbate the caking phenomena. Crystallisation also results in increased rates of deleterious chemical reactions (Sect. 11.5.2) such as Maillard browning, as the non-crystalline components are concentrated within the matrix, thereby increasing reaction rates (Buera et al. 2005). In contrast to pre-crystallisation, where the type of lactose crystal formed is mainly temperature dependent (i.e., α-lactose <93.5 °C), water content or activity is a key determinant of crystal form in powders. At room temperature, lactose crystallisation occurs as α-lactose monohydrate at $a_w > 0.57$ and as β-lactose anhydride at low water activities (Roos 1997; Vuataz 1988).

11.5.1.3 Ice Recrystallisation

Ice crystal size in ice cream is important as it plays a central role in development of unwanted grainy textures. An average size of <40 μm is acceptable for ice crystals; however, during storage this may be increased by a process called recrystallisation, resulting in graininess (Blanshard and Franks 1987). Recrystallisation is mainly affected by ice-cream formulation and storage conditions, with temperature fluctuations during storage being particularly important (Cook and Hartel 2010; Donhowe and Hartel 1996a, 1996b; Hartel 1998). The mechanisms of ice recrystallisation are reviewed in detail by Hartel (1998). These include:

- Migratory crystallisation, where size-dependent stability causes small crystals to melt and large crystals to grow.
- Isomass rounding, where rough surfaces are smoothed during storage.
- Accretion, where crystals in close proximity initially form an interconnecting liquid bridge before finally growing into a single crystal.
- Melt-refreeze crystallisation, where crystals become smaller or melt completely when temperature is increased. When temperature is subsequently decreased, conditions do not favour nucleation, therefore growth of the existing crystals is favoured.

Over long periods, recrystallisation can be modelled by the following equation (Cook and Hartel 2010; Hartel 1998):

$$r = r_0 + Rt^{\frac{1}{n}} \tag{11.23}$$

where r is the crystal size at time t, R is the recrystallisation rate and n is a variable related to the mechanism of recrystallisation. Hartel (1998) provides a summary which relates value of n to mechanism of recrystallisation relevant in ice-cream systems; for example, $n = 3$ relates to recrystallisation that limited by non-convective diffusion. Donhowe and Hartel (1996b) determined recrystallisation rate followed Arrhenius type behaviour with $n = 3$ for hardened ice cream. WLF kinetics have also been applied with some success to recrystallisation behaviour (Donhowe and Hartel 1996a); however, this approach has also been found to predict

recrystallisation rates which are higher than experimental values (Hartel 1998). It must be noted that recrystallisation (and general behaviour of ice-cream systems) is a complex phenomenon, with other factors such as composition and crystal size distribution playing key roles. The reader is referred to the comprehensive reviews of Hartel (1998) and Cook and Hartel (2010) for more detail.

11.5.2 Chemical Changes

Rates of chemical reactions in foods are quantified as a function of reactant concentration and time:

$$\frac{\mathrm{d}[C]}{\mathrm{d}t} = k[C]^n \tag{11.24}$$

where $[C]$ is the quantity of reactant, $\mathrm{d}[C]/\mathrm{d}t$ is the rate of change of the reactant, k is reaction rate constant and n is the order of reaction. It is clear from the expression that there is no term directly related to water. However, water and many other factors such as pH, oxygen, light, and especially temperature can affect rates of reactions. As seen previously, the effects of water on chemical reactivity can be considered from numerous perspectives, e.g., water content, a_w, molecular mobility and solubilisation reactants. Indeed, the effect of water can rarely be considered in isolation. Temperature and water content of many materials are in many cases interdependent, e.g., the effect of spray dryer outlet temperature on water content. The effect of temperature on chemical reactions is often modelled using the Arrhenius equation:

$$k = A\mathrm{e}^{\frac{-E_a}{RT}} \tag{11.25}$$

where A is a pre-exponential factor, E_a is the activation energy of the reaction, R is the universal gas constant and T is absolute temperature (in Kelvin). While there is no moisture term in the equation, indirect effects have been shown to influence both inactivation energy and temperature dependence of rate of reaction (Miao and Roos 2004a; b).

From perhaps the simplest perspective, the water content influences reactions by acting as a solvent, providing the means for solute dissolution and subsequent reactions. Furthermore, inspection of Eq. 11.24 reveals that, for first-order reactions ($n = 1$), reaction rate is directly proportional to concentration of reactant $[C]$, therefore increasing the moisture content to the point where $[C]$ becomes dilute, results in a decrease in the reaction rate. A potential benefit of using a_w to describe the role of water in chemical reactions is that, compared to water content, it gives a better indication on the ability of the water to act as a solvent or reactant. Modelling of a_w and moisture sorption isotherms using the BET or GAB methodology (Sect. 11.3.3) to obtain the monolayer, M_0, value can be a useful exercise to determine storage

conditions that minimise potential reactions. At M_0, each molecule of water is theoretically associated with a polar group, meaning that there is no "free" water to participate in reactions. In addition, below M_0 the molecular mobility required for reactions to take place is not detectable (Bell 2007). Once the a_w corresponding to the monolayer value is exceeded, increased dissolution and molecular mobility result in higher reaction rates. However, as described above, at high a_w, reactant dilution may become significant, resulting in reduced reaction rates.

11.5.2.1 Arrhenius vs. Diffusion-Controlled Kinetics

Rates of bimolecular reactions, such as Maillard browning, may be controlled by diffusion and therefore the following equation applies (Karel and Saguy 1991; LeMeste et al. 2002):

$$k = \frac{k_0}{1 + \frac{k_0}{\alpha D}} \tag{11.26}$$

where k is the observed reaction rate constant, k_0 is the reaction constant observed in well-stirred solutions, D is the diffusivity of the reactants and α is a constant relating to the collision distance. If the well-stirred reaction rate k_0 is much higher than the diffusion and collision (αD) term in the equation, then it follows that the temperature dependence of the reaction is governed by diffusion. Therefore, viscosity changes associated with T_g (and hence water content) may be expected to play an important role, with WLF kinetics applying above T_g. This was observed by Karmas et al. (1992) as a break in Arrhenius plots for Maillard browning of food systems in the vicinity of T_g. Conversely, in reactions with high activation energies, reaction rate is controlled by Arrhenius type temperature dependency, with k remaining low until a certain temperature is exceeded. In these cases, T_g and WLF kinetics are not significant factors (Sect. 11.3.4).

11.5.2.2 Maillard Reaction

The Maillard reaction is significant in the manufacture of many foods, including dairy products. Depending on the product application, it can have favourable or unfavourable effects on sensory properties, such as browning, taste and aroma. In simple terms, the reaction is bimolecular in nature, resulting from the interaction between amino groups and reducing sugars. In dairy products, this typically manifests as a reaction between the lysine residues of milk proteins and lactose. From a nutritional point of view, the association of lactose with lysine renders them unavailable for digestion. In reality, the bimolecular reaction is far from simple and includes a number of stages and pathways, resulting in the formation of many compounds (Van Boekel 1998).

While several authors report Maillard browning at temperatures below T_g, it is at $T > T_g$ where the reaction becomes significant. Miao and Roos (2004a, b) reported higher reaction rates at higher moisture contents in lactose–trehalose matrices. Additionally, E_a for the reaction was found to decrease from 156 to 118 kJ/mol as moisture content increased from 3.8 to 6.9 g/100 g. The difference was attributed to increasing diffusion rate and solubility at higher moisture contents. Ultimately, this manifests as an increased temperature dependence at lower moisture contents, which is particularly relevant for drying operations. This is in general keeping with Labuza and Saltmarch (1981) who, on review of kinetic data, concluded that temperature had a much more significant effect on browning in low moisture foods compared to solutions.

11.5.2.3 Lipid Oxidation

Lipid oxidation is a significant concern in fat containing dairy products, whereby unsaturated fatty acids undergo a variety of complex reactions which result in a deterioration of sensory properties. In brief, lipid oxidation begins with formation of a lipid free radical, a step which can be catalysed by the presence of trace metals. The lipid free radical reacts with oxygen to form a peroxyl free radical, which then reacts with a fatty acid, resulting in a hydrogen peroxide and another lipid free radical. Breakdown of the hydrogen peroxide results in the generation of off-flavours and aromas, while the new free radical can be oxidised by the same pathway. However, two free radicals may also interact to form a non-radical product, resulting in termination of the reaction (Bell 2007).

Figure 11.11 shows the general effect of a_w on lipid oxidation. Oxidation in dry materials is relatively high and increasing water activity initially results in reduced oxidation rates, with a minimum rate typically occurring near the monolayer; further increases of a_w result in higher oxidation rates. At low a_w, water acts as an antioxidant by limiting the activity of trace metal catalysts and promoting interaction of free radicals. In addition, hydration of hydrogen peroxide alters its decomposition mechanism and reduces the rate of free radical formation (Karel 1980). Water may also reduce oxidation rate at low a_w through displacement of air from pores and capillaries within the food (Bell 2007). At water contents and a_w above the monolayer, the observed increase in oxidation rates is generally attributed to greater hydration of solutes and/or higher molecular mobility. The influence of glass transition and water activity on lipid oxidation is reviewed in detail by Nelson and Labuza (1992); in keeping with theories of molecular mobility presented above, it can be generally concluded that rates are low in glasses and high in rubbery matrices. However, structural collapse of rubbery materials may result in altered rates depending on how it affects porosity or liberation of fat from the matrix (Bell 2007; Labrousse et al. 1992). Crystallisation of lactose in dairy powders stored above T_g has been shown to increase surface free fat (Masum et al. 2020) which facilitates high levels of oxidation.

11.5.2.4 Enzyme Stability and Activity

Milk is naturally rich in enzymes such as alkaline phosphatase, lipase, proteinase and lactoperoxidase. Furthermore, exogenous enzymes such as β-galactosidase may be added to products for various purposes, with an example being hydrolysis of lactose in the case of β-galactosidase. Water can affect both the stability and activity of enzymes in foods. As regard thermal stability, Burin et al. (2002a) studied the rate of thermal inactivation of β-galactosidase in model dehydrated dairy systems, concluding that inactivation was more dependent on water content than glass transition phenomena. However, in non-dairy systems, conflicting data exists on the storage stability of enzymes in model polyvinylpyrrolidone (PVP) systems, with Chen et al. (1999a) reporting minimal influence of glass transition on invertase stability; this conflicted with findings from the same group of authors (Chen et al. 1999b) who reported glass transition influenced tyrosinase stability during storage.

Enzyme activity relates to the ability of the enzyme to perform its specified purpose. For this, some amount of water is required for molecular mobilisation, substrate solubilisation and/or optimal enzyme configuration. In terms of molecular mobility, glass transition has been shown to affect the hydrolysis of starch by α-amylase, with distinct differences in reaction observed between $T < T_g$ and $T > T_g$ (Chaudhary et al. 2017) with the rate of reaction being negligible at $T < T_g$. However, in the same study, amylosis of maltodextrin was observed to occur at sub-T_g conditions. Likewise, Burin et al. (2002b) found that β-galactosidase activity in whey powder was more related to a moisture dependent pH change than T_g. The apparent variability in the success of T_g, molecular mobility and diffusion to describe enzymatic stability and activity is confusing. However, describing the variability in findings relating to sucrose hydrolysis, Bell (2007) concluded that a number of competing factors such as enzyme configuration, dissolution, diffusion and sufficient water for hydrolysis could all play a role. Furthermore, depending on specific conditions the dominant factor(s) may vary, perhaps explaining the variability.

11.5.3 Microbiological Stability

Many factors affect microbial growth and stability in dairy products. These include temperature, oxygen, nutrient availability and pH. Water, however, is a pre-requisite for growth. More specifically water must be available; therefore, a_w has been the predominant method of describing the effect of moisture on microbes in dairy products, of which bacteria, yeast and mould are the primary organisms of interest. In general, bacteria require high a_w (>0.95) to grow; however, certain species, such as *Staphylococcus aureus* (aerobic), can grow at a_w as low as 0.87. At lower a_w values down to 0.61, growth of yeasts and moulds predominates. The majority of non-dried products have a_w values of >0.90. Therefore, in such products (e.g., cheeses and dilute fresh milks) bacterial growth is of great concern. In cheese, bacterial growth and localisation of colonies within the matrix are extremely important during

ripening (Hickey et al. 2015). Bacterial growth may be inhibited through addition of salt to reduce a_w, an example being the generally lower growth in salted butter (a_w~0.92) compared to un-salted butter ($a_w \sim 0.99$). However, certain halophilic bacteria can grow at a_w as low 0.75. Haastrup et al. (2018) studied the microbiota of Danish brines, finding high prevalence of moderately halophilic bacteria such as *Tetragenococcus muriaticus* and *Psychrobacter celer*, concluding that such species may be responsible for surface inoculation of cheese with potential effects on organoleptic properties.

While microorganisms cannot grow without sufficient water, vegetative cells and spores of certain bacteria can survive for long time intervals in low a_w environments. Species of Cronobacter, in particular, have been shown to survive in powdered dairy products such as infant formula and skim milk powder for extended periods (Barron and Forsythe 2007; Beuchat et al. 2013; Gurtler and Beuchat 2007). In such products, survival of bacteria may be exacerbated by the end-user who will typically rehydrate to a_w levels where growth can occur. Therefore, every attempt must be made by processors to reduce levels in products prior to drying through effective microbial reduction steps and hygienic design of manufacturing lines.

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Chapter 12
Physical and Physicochemical Properties of Milk and Milk Products

M. J. Lewis

12.1 Introduction

Milk is a colloidal dispersion, and its components span a range of sizes. Its main component is water and dissolved in this aqueous phase are sugars, salts, free amino acids, organic acids, free fatty acids, peptides, polypeptides, proteins, whey proteins, soluble casein and enzymes. Suspended in the aqueous phase are casein micelles, fat globules, somatic cells and microorganisms, although it is claimed that milk is sterile before it is expressed. One objective might be to remove one or more of these components or to inactivate specific microorganisms or enzymes—in doing so, the size of these different components is important, as is their density. We would normally think of milk as a low viscosity fluid, with its characteristic "milky" appearance. However, the products produced from milk are multifarious and may have quite different properties to milk. These include evaporated and sweetened condensed milk, milk powder and other powders derived from cheese whey and casein, in addition to fermented products such as cheeses and yoghurts. Within the cheese category, there is vast variety, with fresh cheeses of high moisture which are consumed within days of production, through to harder and drier cheeses which may be matured and eaten 2 years or longer after production. Finally, there are products with more fat, cream products, butter and frozen products such as ice cream, and more specifically dairy ice cream, where the fat is butterfat. Much milk is consumed as liquid milk in its various heat-treated forms and these heat treatments have been described in detail by Deeth and Lewis (2017). The remainder is converted into a range of products: some of these will involve coagulating the milk; for example, cheesemaking and yoghurt manufacture or preventing coagulation, for example, in concentration processes, heating processes and drying. This chapter will

M. J. Lewis (✉)
Department of Food and Nutritional Sciences, University of Reading, Reading, UK
e-mail: m.j.lewis@reading.ac.uk

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focus on the factors affecting the physical and physicochemical properties of milk and some of its products, together with examples of why it is important to know these properties. It will also briefly review some of these properties, as they might affect the quality attributes of some important milk products and to show how knowledge of them will help to improve food processing operations and finished product quality. This milk is most likely to be cow's milk but it may originate from buffalo, goat, sheep or horse and of course human milk for infant nutrition. There are situations where one can resort to literature values and situations where it may be best to measure these properties, especially for quality assurance purposes, for example, rheological measurements. Most products will show a range of values, so it is important for the manufacturer to establish what this range might be. If the product is outside this range, the consumer will start to notice that it is not normal and may thus register a complaint; for example, by observation on pouring the product or from tasting the product. This is a sensible approach to take, considering the complexity of milk products, their day-to-day variations in composition, the complexity of the protein and fat and the many minor components in milk that can also influence how milk behaves and how it is perceived by the consumer.

12.2 Size Range of Components in Milk

A diagram showing the range of sizes to be encountered for the different components in milk is given in Fig. 12.1. Milk contains lactose, minerals and vitamins in solution and all are much smaller than 1 nm; whey proteins, individual casein molecules and many other enzymes of approximately 1–2 nm are also in true solution. Most of the casein exists in micelles and the casein is cemented together by calcium phosphate nanoclusters; the size range of the micelles is 30 to 300 nm diameter. Casein micelles and milk fat in the form of globules will be dispersed in this aqueous phase. The fat globules in raw milk range from 1 to 10 μm in raw milk and this will be discussed in greater detail later in the context of emulsion stability. There may also be sediment and an associated microbial flora suspended in the milk, with somatic cells typically being in the size range 8.5 to 10 μm. The number of fat globules might exceed the number of somatic cells by a factor of 5000, but their size ranges overlap. This is useful practical information for processes that might involve recovering specific components from milk, such as filtration, centrifugation or crystallisation; some of these components may also separate under gravity. Whether a specific component will separate is influenced by both its particle size and density, but also the fluid viscosity and factors affecting separation rates (terminal velocity) can be determined from Stokes equation.

In this case the terminal velocity of a particle can be calculated from:

$$v = \frac{D^2\left(\rho_f - \rho_s\right)g}{18\mu} \tag{12.1}$$

v = terminal velocity (m s^{-1}); D = particle diameter (m); ρ_s and ρ_f are the solid and fluid densities, respectively (kg m^{-3}) and μ is the fluid viscosity (Pa s).

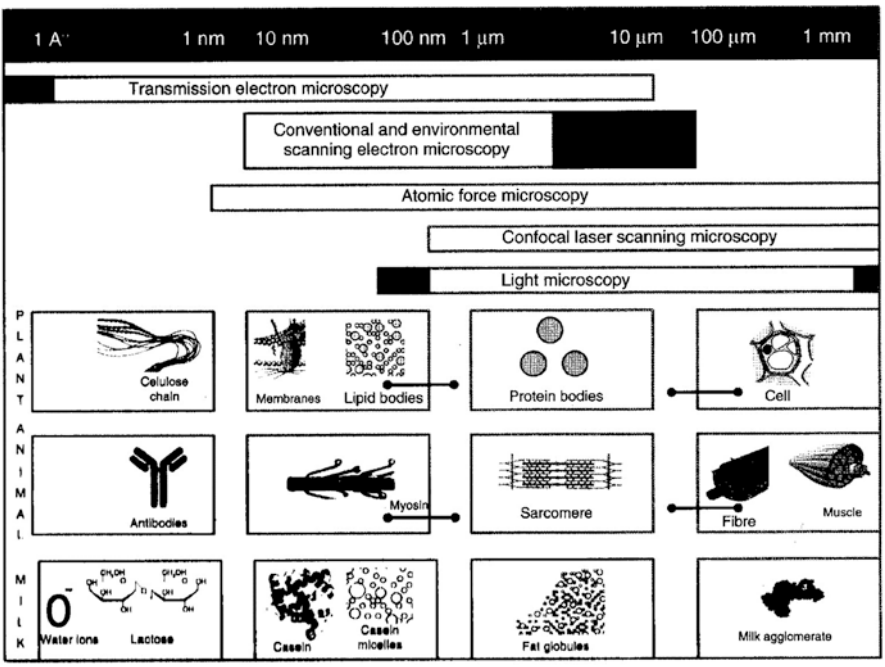

Fig. 12.1 Particle size range for some food structures and components in milk and other food products together with size ranges measured by different microscopic methods

This equation has some important applications in dairy technology. For emulsion stability, it shows the factors influencing how quickly a dispersed fat globule will rise in a liquid. Particle diameter is extremely important; if the diameter is reduced by a factor of 10, then the terminal velocity will be reduced by a factor of one hundred. Stokes equation can also be used for investigating sediment formation in beverages, for measuring the dynamic viscosity of a fluid and for calculating the velocity of bubbles in liquids. Centrifugation is used to accelerate the natural process of gravity; centrifugal forces are used for separating the fat phase in milk for cream production and higher centrifugal forces (>50,000 g) will cause casein micelles to sediment out. This is described for partitioning milk in Chap. 9. Some of the particles in milk can be visualised using microscopy, with the simplest method being light microscopy. A stable milk emulsion would be observed as a mass of very small oil droplets, while observing even a small number of larger droplets, or clumping of the droplets, would suggest that there is some product quality/process issue. Typically, the fat globule size of an emulsion should be reduced below 1 micron and there should be no large droplets or clumps observed. For particles smaller than the wavelength of light, other forms of microscopy will be needed for their visualisation. Some of these methods are shown in Table 12.1 and described by de

Table 12.1 Microscopy methods for visualising some of the components in milk (taken from Lewis 2021)

Technique	Characteristics
Light microscopy	Surface structures, sizes, shapes, magnification 10–1500x, visible light illumination, size dictated by wavelength of light
Confocal laser scanning microscopy	Laser beam replaces light source, can produce optical sections of a three-dimensional specimen, distinguishes lipid particles from proteins, combined with staining procedures for identification purposes
Scanning electron microscopy	Uses high speed electrons, surface structures; looks at electrons that are reflected or scattered from the surface. <1 nm to 10^{-1} mm
Transmission electron microscopy	Similar principle to light microscopy but uses electrons: Resolution can be 1000 times better than light microscope, well suited for food applications, thin internal sections; <1 nm to 10^{-1} mm
Atomic force microscopy	Typography, nanoscale structural information, some food applications are macromolecular and polymer imaging, surface topography

Jesus-Flores et al. (2012). Confocal microscopy is extremely useful for investigating the microstructure of milk products and staining techniques can identify the fat and protein components individually and how they interact in creating food texture. Particle size analysis is widely practised using light and laser scattering methods, both for looking at size distributions for casein micelles and for fat globules in milk and dairy products.

12.3 Milk Acidity and Buffering Capacity

Milk acidity is measured by its pH, which in effect is a measure of hydrogen ion concentration, or activity. It is the most effective way to measure food acidity. The pH of a material is defined as the logarithm (base 10) of the reciprocal of the hydrogen ion activity. For most food systems the hydrogen ion activity is the same as the hydrogen ion concentration.

$$\mathrm{pH} = \frac{1}{H^{+}} \tag{12.2}$$

Thus, pH is measured on a scale of 1 to 14, with 7 being the point of neutrality where.

$$\mathrm{H}^{+} = \mathrm{OH}^{-} = 10^{-7}$$

Water is thought to be a neutral component, although you would be unlikely to find a sample of water which has a pH value of seven and it is much affected by the presence of dissolved ions and dissolved carbon dioxide. Most dairy products fall on the acidic side of neutrality, as can be seen in Table 12.2. Milk pH is normally measured at room temperature. The pH of milk is usually taken as around 6.7, and in this sense, it is one of the least acidic of all food categories. However, it is

Table 12.2 Range of pH values for a selection of milk and milk products

Product	pH
Cow's milk	6.4 to 6.8
Evaporated milk	5.9 to 6.3
Cream, 20% fat	6.50 to 6.68
Cottage cheese	4.75 to 5.02
Cheddar cheese	5.9
Hard cheeses	5.6 to 6.2
Yoghurt and white cheese	4.0 to 4.5

important to stress that there will be day-to-day variations in the pH of milk from individual cows and also in bulk milk delivered to dairies. Chen et al. (2014) found bulk milk from the same farm at pH 6.73 to 6.87 over the period of 1 year (25 samples). Similar variations can be found for all other products; for example, the range for commercial evaporated milk samples was found to be from 6.06 to 6.33 (Table 12.9). All microorganisms have their optimum pH for growth but many will grow (albeit slowly) over a wide range of pH values. Controlling microbial activity is fundamental toward making food safe for consumption.

Milk pH is most easily determined by using a pH probe, which is the most common of the selective ion electrodes. Probes should be handled with care and checked regularly with standard buffer solutions of known pH, usually pH 4 and 7 for dairy products. This is a potentiometric measurement and is measuring the flow of hydrogen ions through a selective ion membrane, which sets up a small potential difference, which is measured by an accurate millivoltmeter. For hydrogen ions, a ten-fold change in concentration will increase the potential difference by approximately 58–59 mV. Thus, if using pH 7 and pH 4 buffers for calibration purposes, then a difference in potential of about 176 mV should be recorded. Note that this represents an increase in concentration of 1000 fold. This is a worthwhile procedure for checking whether a pH probe is in good working order. The pH of milk decreases as temperature increases, although this receives much less attention in the literature. For example, milk has a pH of about 6.7 at 20 °C, but there is evidence that it will fall to about 5.6, when it is heated at 140 °C during ultra-high heat treatment (UHT) processing. However, when it is then cooled it reverts to almost its original value when it reaches 20 °C. These changes are largely due to calcium phosphate precipitation. This is largely reversible and if the heating has not been too severe, the calcium phosphate will redissolve on cooling and the pH will approach its initial value. Heating and other processing operations may also change the pH of foods, with one reaction responsible for this being the Maillard reaction. Such changes may occur during more severe heating processes and also when storing foods at temperature above 30 °C. Many pH probes can be used up to a temperature of 80 °C and some can also be used up to 120 °C, claiming to be steam sterilisable. However, pH is very rarely measured in foods at high temperatures. Deeth and Lewis (2017) have described alternative procedures of dialysis and ultrafiltration to measure the pH of milk up to 140 °C and these are further discussed in Chap. 9. A reduction in the pH

of raw milk will have a detrimental effect on its heat stability. It is important to appreciate that when milk pH is reduced (by whatever means) its ionic calcium concentration will increase and this is described in more detail by Deeth and Lewis (2017).

12.3.1 Strong and Weak Acids

It is relevant to ask which is the main acid in milk. The answer to this question would be lactic acid and in most milk products, this would be true. However, an interesting discussion point is provided by Cronshaw (1947) who states that freshly expressed milk contains no lactic acid and any found in milk, results from microbial activity from lactic acid bacteria. Nuclear magnetic resonance (NMR) technology is a fantastic tool which has identified several other organic acids in milk and also the many other free amino acids in milk, which are rarely discussed. Some data for free amino acids in milk and their concentrations are provided by Foroutan et al. (2019). A selection is presented in Table 12.3. for some of the acidic metabolites found in milk, as determined by NMR, and indeed this data would also suggest that lactic acid is not the main acid in milk. NMR is a very powerful analytical tool for examining a wide range of non-protein nitrogen compounds and organic acids in milk and I am sure that more will be heard about compounds such as orotic acid, hippuric acid and other minor milk components in future years as important milk metabolites.

The degree of dissociation into positive and negative ions is an important characteristic of any acid. The dissociation constant represents the situation when equilibrium is achieved between the undissociated acid HA and the dissociated ions H^+ and A^-

$$\mathrm{HA} \Leftrightarrow \mathrm{H}^+ + \mathrm{A}^- \tag{12.3}$$

and

$$\mathrm{K_a} = \frac{\left[\mathrm{H}^-\right]\left[\mathrm{A}^-\right]}{\left[\mathrm{HA}\right]} \tag{12.4}$$

It is commonplace to use a logarithmic measure of acid dissociation pK_a, where

$$pK_a = -\log_{10} K_a \tag{12.5}$$

$$\mathrm{p_H} - pK_a = \log\left[\frac{\mathrm{A}^-}{\mathrm{AH}}\right] \tag{12.6}$$

Strong acids are characterised by low pK_a values and are largely dissociated, to the extent that the undissociated form becomes undetectable. The three most common strong acids are hydrochloric, sulphuric and nitric acids. As such they are in a

Table 12.3 Principle buffering groups in milk (taken from McCarthy and Singh 2009)

Group	Approximate concentration (mM)[a,b]	Expected pK_a[c,d]	pK_a (in milk)[a]
Salts			
Inorganic phosphate	21.0[e]	2.1, 7.2, 12.3	3, 5.8, 6.6[f]
Citrate	9.0–9.2	3.1, 4.7, 5.4	3, 4.1, 4.8[f]
Organic phosphate esters	2.5–3.5	1.4, 6.6	1.7, 5.9[f]
Carbonate	2.0	6.4, 10.1	6.4, 10.1
Lactic acid	<0.4	3.9	3.9
Formic acid	0.2–1.8	3.6	3.6
Acetic acid	0.05–0.8	4.7	4.8
Various amines	1.5	~7.6	7.6
Protein-bound residues	Titratable group[c]	Expected pK_a[c,d]	pK_a (in milk)[a]
Aspartic acid	β-COOH	4.6	4.1
Glutamic acid	γ-COOH	4.6	4.6
Histidine	Imidazole	6.1	6.5
Tyrosine	Phenol	9.7	–
Lysine	ε-NH_3^+	10.4	10.5
Phosphoserine	Phosphate	1.5, 6.5	2, 6
N-Acetylneuraminic acid	COOH	2.6	5.0
Terminal carboxyl	α-COOH	3.6	3.7
Terminal amino	α-NH_3^+	7.9	7.9

[a]Data from Walstra and Jenness (1984)
[b]Data from Jenness (1988)
[c]Data from Tanford (1962)
[d]Data from Edsal and Wyman (1958)
[e]About 10 mM colloidal phosphate. 11 mM in solution (at pH 6.6)
[f]pK_a values from titration with $Ca(OH)_2$

dissociated form. as H^+ and Cl^-, SO_4^{--} and NO_3^- over the complete pH range. These anions are also found in most foods, with many of the other acids that are found in foods being weak acids. Where the pH is equal to the pK_a value, then the acid is 50% dissociated, whereas when the pH is much below pK_a, the acid remains largely undissociated. It is stated that benzoic acid is more effective as a preservative in its undissociated form, hence at low pH values for inhibiting yeasts and moulds. Some pK_a values for some food acids are given here: acetic acid 4.75; ascorbic acid 4.17; benzoic acid 4.19; citric acid 3.12; lactic acid 3.86; malic acid 3.40; propionic acid 4.87, sorbic acid 4.75 and benzoic acid 4.19. The latter three acids are used as preservatives. Peracetic acid, which is used as a disinfecting agent has a pK_a of 8.2 and is a very weak acid. Phosphoric acid is of particular interest to dairy scientists; it is tribasic and exists as PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$ and H_3PO_4 (undissociated phosphoric acid). Thus, its three pK_a values are 3, 5.8 and 6.6. Phosphates play a role in buffering milk and are known as either stabilising salts or buffering salts. Citric acid also has three pK_a values, 3, 4.1 and 4.8. It is precipitation of calcium phosphate that is responsible for the reduction in pH when milk temperature increases on thermal processing. Calcium phosphate is an intriguing salt; it is exceptional because its

solubility decreases as temperature increases. In addition, its solubility increases dramatically as pH is reduced, especially in the pH range over which milk is converted to yoghurt, e.g., pH 6.8 to pH 4.5. Some calcium phosphate precipitation will occur when milk is heated. Calcium citrate may also precipitate out in evaporated milk, especially if trisodium citrate is used as a stabiliser and for this reason it is best avoided. It is interesting that milk will decrease in pH both when it is increased in temperature and also when subjected to the pressures reached in high pressure processing and this may cause some disruption of the casein micelle. This is discussed further in Chap. 9. As well as measuring pH, selective ion electrodes can also be used for measuring Na, K and Ca ions. Calcium is a divalent ion and for divalent ions, a ten-fold change in concentration will change the electrical potential by a factor of 29 mV. For further information, ionic calcium in milk has been recently reviewed by Lewis (2011).

12.3.2 Buffering Capacity and Titratable Acidity

Buffering capacity tests relate to measuring the change of pH brought about by additions of a strong acid or strong base, with the pH plotted against the amount of acid that is added. A simpler method is to measure the change of pH resulting from a standard addition of acid and alkali. The buffering capacity in milk is again a complex subject, as many components contribute to this (Table 12.4). More information on the buffering capacity of dairy products is provided by Salaun et al. (2005). Of most practical interest would be buffering capacity over the range 7.0 to 4.5 in fermentation processes and for acidified milk products down to about 3.0. Very rarely does the pH of milk exceed pH 7 when it is being processed. Buffer solutions are used as a means of preventing changes in pH. Citrates and phosphates are natural buffers in milk, as are amino acids and protein. Additives with buffering capacity or for modifying the pH of a formulation are termed acidity regulators.

In the dairy sector, titratable acidity (TA) is often determined. It is frequently used with dairy products for monitoring raw milk quality (which should be done with caution) and acid development after addition of starter bacteria in cheese, yoghurt and other fermented products, which can be most informative. Despite its name, TA is not measuring acidity (in the case of milk). It is measuring the buffering capacity of the milk. This is still not fully understood and is often misrepresented in textbooks. TA is determined by titrating 10 mL milk (sample) with 0.11 M (0.11 N) caustic soda solution, using phenolphthalein as indicator. This, according to many texts, allows lactic acid (%) in the sample to be determined by dividing the titre value (mL) by 10. Although TA is expressed in terms of lactic acid, what is being measured in reality is the buffering capacity of the sample, i.e., the amount of alkali required to increase the pH of milk from its starting value to about 8.4, which is the pH at which the indicator colour changes from colourless to pink. A better more explicit name would be total absorbing capacity. Some typical values for the TA of milk are shown in Table 12.5. If the TA value is 0.14% lactic acid, then the

Table 12.4 Some interesting titratable acidity values in milk processing (from Cronshaw 1947)

Below 0.10	May be normal, but may be adulterated with alkali
0.12 to 0.15	Normal milk
0.18 to 0.19	An arbitrary maximum between satisfactory and unsatisfactory milk
0.22	Practical limit for milk pasteurisation
0.25	Milk clots on boiling
03 to 0.4	Start to detect a sour taste
0.32	Milk begins to clot at pasteurisation temperature
0.5	Milk clots at room temperature

Table 12.5 Some metabolites associated with organic and free amino acids in milk (compiled from data in Foroutan et al. (2019)

Metabolite	Range in skim milk to 3.25% fat milk (μM)	Literature values (μM)
Lactate	55 to 61.4	0–167
Citrate	4793–5808	3692–7435
Formate	15–16	3.3–46
Malate	141–151	55–122
Glutamate	259–271	111–740
Hippurate	65–72	79–267
Orotic acid	511–565	208–1002

contributions of different components in milk to this value have been listed as follows: CO_2, 0.01; citrate, 0.01; casein, 0.07; whey P, 0.01 and phosphates, 0.3. Thus, decisions should not be made about rejecting raw milk on TA results alone. Although this is appreciated in many quarters, it still happens. China has specifications for minimum and maximum levels for TA of imported UHT milk. The TA should range between 12 and 18°Th (0.108–0.162% LA). The lower limit may have been introduced to prevent adulteration of milk with alkali, which would decrease the TA value (but would also change the Na or K concentrations and most probably freezing point depression). This is important for companies exporting milk to China, since milk having a TA value below the minimum can be rejected. Some significant TA values for milk are shown in Table 12.5, which is compiled from information in Cronshaw (1947).

Two common beverages that milk is added to are tea and coffee. Both these are acidic beverages and they are also hot when the milk is added. The pH range of brewed coffee could be from 4.8 to 5.8 and tea is also acidic. On occasions, a defect known as feathering may occur, which is an unsightly white deposit on the surface of the coffee. It is assumed that this is caused by faulty milk, but it may in fact be caused by the tea or coffee being more acidic or the coffee temperature being higher than normal or even by the water quality. It is important that coffee houses understand the complexity of their own ingredients, e.g., the coffee and the water used to

make the coffee (for example, is it hard or soft). For further information, a description of feathering is provided by Davis (1955).

12.3.3 Redox Potential

Redox potential is another example of a potentiometric reading which is now not encountered as much as it was in the past, when redox indicators such as methylene blue and resazurin were used for monitoring raw milk quality. Although not widely discussed, milk often comes almost saturated with dissolved oxygen. Although only sparingly soluble, it remains in solution, even during pasteurisation and sterilisation. However, dissolved oxygen is just one factor which will contribute to the redox potential.

The oxidation reduction potential or redox potential (E_h) of a system at 25 °C is given by

$$E_h = E_0 + 0.059 \log \frac{\text{Ox}}{\text{Red}} - 0.059\,\text{pH} \tag{12.7}$$

where E_h = redox potential (V); E_o = standard redox potential where reactant and product are both at unit activity, Ox and Red are molar concentrations of oxidised and reduced forms, respectively. The equation also shows how E_h is affected by pH, showing that it increases as pH is reduced. A loss of electrons is termed oxidation and leads to an increase in E_h. A gain of electrons is reduction and leads to a decrease of E_h. The more positive the redox potential, the greater is its affinity for electrons.

E_h of milk is influenced by a variety of factors. E_h will give information regarding bacterial contamination or susceptibility to oxidation. Fresh milk drawn anaerobically has a redox potential of approximately +130 mV. On exposure to air, it absorbs oxygen whereupon it rises to a characteristic value for fresh milk of +200 mV to +300 mV. Removal of oxygen by an inert gas lowers the value to +50 mV. Dye reduction tests, using dyes which change colour as the redox potential changes have been used as rapid indicators of raw milk quality in earlier times. The two most common ones have been resazurin and methylene blue. The tests are simple and involve mixing 1 mL of the dye solution with 9 mL of milk and incubating at 37 °C. The dyes change colour as the redox potential is reduced and results can be obtained in 10 min for the rapid resazurin test and between 2 and 6 h for methylene blue. Colour changes for resazurin are from blue (excellent), lilac, mauve (good), pink-purple, purple-pink, pink (poor) and colourless (bad). Methylene blue goes from blue to colourless. The assumption is that the more quickly the colour change takes place, the poorer is the hygiene quality of the milk. The tests and their drawbacks are described in great detail by Davis (1955), at a time when they were widely used. These tests were part of the procedure for detecting poor quality raw milk when it arrived at the dairy in churns. Each churn was first smelt and churns with a suspicious smell were further tested. It is noteworthy that at that time microbial

testing would have taken at least two days, whereas now the rapid analytical techniques will measure microbial counts much more quickly. It is also noteworthy that in the 1950s, raw milk counts were often in the 100,000 s/mL, whereas now they typically average about 25,000/mL.

The principle systems that determine the E_h of milk are dissolved oxygen, ascorbate and riboflavin (Walstra and Jenness 1984), who also provide further information on these systems. The range of E_h at which different groups of microorganisms grow is: aerobic bacteria between +500 and + 300 mV; anaerobic bacteria between +100 and −250 mV; facultative anaerobes between +300 and −100 mV. The presence or absence of oxygen and the E_h of food determine the growth of a particular microbial group in foods. This is important in microbial spoilage of foods and in desirable characteristics of fermented foods. In general, aerobic microorganisms show a preference for positive E_h values and anaerobic bacteria prefer negative E_h values. The redox potential is measured in reference to a standard hydrogen electrode, which is given a value of zero. Anaerobic bacteria, such as clostridia, may be present in raw milk, but recent high-throughput DNA sequencing methods suggest that they will not flourish in raw milk and will even diminish in numbers (McHugh et al. 2020). Thus, raw milk provides an environment which is not conducive to proliferation of clostridia and thus milk derived products should only in rare circumstances be subject to issues arising from clostridia. I never had to measure redox potential and dye reduction tests were going out of fashion when I started at the university. It is now sometime since redox indicators were used but because of their potential simplicity, it is perhaps time for a comeback. For those with an interest in the practical aspects of measuring redox potential, these have been recently discussed by Alwazeer (2020). He has provided examples were there may be opportunities for using it more routinely for quality assurance purposes, for example, as a means of detecting whether anaerobic bacteria such as clostridia are not capable of growing.

12.4 Colligative Properties

Concentrations are often expressed in terms of molarity, where a molar solution is the molecular weight of the substance (kg) dissolved in 1 L. Thus, the molarity of a solution is the mass in 1 L divided by the molecular weight of the substance. The mole fraction of a component in a mixture is the number of moles of the component compared to the total number of moles in the system, and as such, it has no units. When a non-volatile solute such as sugar or salt is added to water, the presence of the solute will have some important effects. Firstly, it will elevate the boiling point of the solution; secondly, it will depress its freezing point; and thirdly, it will increase its osmotic pressure. These three properties are known as colligative properties, so called because they will depend upon the number of particles present in the solutions. The number of particles present can be measured by osmolarity. Avogadro stated that equal volumes of different gases at the same temperature and

pressure contain the same number of molecules. One kilomole (kmol) of any gas (or vapour) is the molecular weight expressed in kg and will contain 6.02×10^{26} molecules and occupies 22.4 m^3 at normal temperature and pressure (NTP). Essentially, since molarity is equal to (c/M), it is the molecular weight (M) and the concentration (c) that will have the greatest effects when it goes into solution. For example, when sodium chloride is added to water it dissociates and forms two particles, but when calcium chloride is added to water it dissociates and produces three particles. It is worth mentioning that the molar concentrations of some sugars and salts that we encounter in foods might be quite high, but the molarities of proteins and other macromolecules will be very low, because of their high molecular weights. Thus, it will be the sugars and salts and other low molecular weight components that will most influence colligative properties. The simple theory of colligative action is claimed to apply only to dilute solutions, but it is less clear from much of the literature what a dilute solution is.

Freezing point depression is very important for detecting whether milk has been watered down, and osmolarity is used in medical applications; for example, blood products and in many fluids prescribed for medical purposes and infant formulations, where it should be as close as possible to human milk. It is also much used in dealing with biological fluids in general. Both these properties are measured using a cryoscope, which is one of the most fascinating instruments that I have used. It can measure the initial freezing point of a solution to 1/000th of a degree C. The reading is recorded as a freezing point or a freezing point depression. Thus, milk would typically have a freezing point of −0.520 °C or a freezing point depression of 520 m °C. Milk is secreted from the mammary cell. All the components in milk are derived from blood and in osmotic terms, milk is in equilibrium with blood. As a lactating animal has mechanisms to keep the osmotic pressure of its blood constant, it follows that the osmotic pressure of milk will be constant. As the factors affecting osmotic pressure are the same as those affecting freezing point depression, the freezing point of milk will remain constant as it leaves the udder and will only change if it is watered down, or if any of its components are broken down, for example, the conversion of lactose to lactic acid being the main one. In this case one molecule of lactose is converted to three molecules of lactic acid. Thus, osmotic pressure and freezing point depression are two of the least variable of all the properties of milk.

As mentioned, a cryoscope can also be used to measure osmolarity. It can be done by using standards of known osmolarity, which are supplied by the instrument manufacturers. Note that a 0.9%, w/v, sodium chloride solution has an osmolarity value of approximately 308 mOsmol/L (calculated from 9 g/L × 2/58.9). The normal range of human serum osmolarity is 285–295 mOsmol/kg, and breast milk might also be expected to be in this range. The osmolarity of infant formulae should match that of breast milk, although values for breast milk have not been widely reported; the range given is 300–400 mOsmol/L. For both applications, standard solutions of sodium chloride, or other fluids provided by the instrument manufacturers, can be used to calibrate the cryoscope. Thus, molarity is based on number of moles, whereas osmolarity is based on the number of particles. Some osmotic

pressure values for some solutions are 6.9 bar for both milk and whey, as opposed to 23.2 bar for sea water. Other applications for a cryoscope include checking batch to batch consistency of formulated milk drinks, checking the quality of permeate from dairy processing, assessing the consistency of any milk formulation and checking the quality of RO and UF permeates/water quality, e.g., from evaporators, distilled water, deionised water and the local water supply. Care should be taken to distinguish between osmolarity and osmolality. Osmolarity is based on the number of particles per litre, whereas osmolality is the number of particles per kg water, often abbreviated to Osmol kg^{-1}. Similar factors will affect boiling point elevation, which will be encountered in the finishing (high total solids) section of evaporators and will reduce the overall temperature driving force and reduce the evaporation rate.

12.5 Density

Density is defined as mass/volume and as such is a very simple concept. Water is the main component of milk, with its density of 1000 kg m^{-3}. Density values for the main components of milk are given in Table 12.6. Thus, the density of milk will also depend upon its composition, and to a much lesser extent, the amount of air in the milk. Of its main components, fat is the least dense, whereas all the other components are more dense, apart from air, which will be discussed later. Milk, for the purpose of many engineering calculations is assumed to be incompressible, that is its density is not affected by moderate changes in temperature and pressure. However, this is not strictly true, as the density of milk will increase slightly when its temperature increases. It is these density differences in heated milk that will cause circulation and redistribution of heat by natural circulation.

How milk density changes with temperature is given by the following equations, over the temperature range 0–150 °C, where Θ = temperature °C.

whole milk:

$$p = 1033.7 - 0.2308\,\theta - 2.46\times10^{-3}\,\theta^2 \tag{12.8}$$

cream (20% fat):

$$p = 1031.8 - 0.3179\theta - 1.95\times10^{-3}\,\theta^2 \tag{12.9}$$

Bertsch et al. (1982) provide information on the density of milk and cream over the temperature range 65 to 140 °C.

The following are some equations for estimating the density of milk and milk products, from knowledge of their composition.

$$\text{Two component systems}: \rho = 1/\left(m_w/\rho_w + m_s/\rho_s\right) \tag{12.10}$$

Table 12.6 Density values (Kg m^{-3}) for different components in milk

Component	Walstra and Jenness (1984)	Peleg (1983)
Lactose	1780	
Protein	1400	1400
Fat	918	900–950
Salt	1850	2160
Water	1000	998.2
Sucrose		1590
Glucose		1560

Example: What is the density of an emulsion with 40% fat, $\rho = 950$; 60% water, $\rho = 1000$.

$$\rho = 1/\left(0.4/950 + 0.6/1000\right) \therefore \rho = 979.4\,\mathrm{kg\,m^{-3}}$$

The equation for a multi-component system is

$$\rho = \frac{1}{\frac{m_1}{\rho_1} + \frac{m_2}{\rho_2} + \frac{m_3}{\rho_3} + \frac{m_4}{\rho_4}} \tag{12.11}$$

An example is shown below for milk given its composition:
(water 87.5%, $\rho = 1000$), (sugar 4.7%, $\rho = 1590$), (fat 4.0%, $\rho = 925$), (protein 3.2%, $\rho = 1400$), (minerals 0.6%, $\rho = 2160$)

$$\rho = \frac{1}{\frac{0.875}{1000} + \frac{0.047}{1590} + \frac{0.04}{925} + \frac{0.032}{1400} + \frac{0.006}{2160}}$$
$$= \frac{10^{-3}}{0.875 + 0.0295 + 0.0432 + 0.0228 + 0.0027} = 1027.5\,\mathrm{kg\,m^{-3}}$$

Density can be measured accurately with simple instruments, for example, a density hydrometer or specific gravity bottle, or equipment that will measure it to five places of decimals is also available.

Note that specific gravity may also be used, where specific gravity (SG) is defined in comparison to water (ρ_w):

$$\mathrm{SG} = \frac{\text{density } \rho}{\text{density of water } \rho_w} \tag{12.12}$$

A narrow-range and specific hydrometer for milk is known as a lactometer, with a density range from 1025 to 1035 kg m^{-3}, which is the normal density range for most milk samples. In fact, in earlier times, knowledge of the fat content of milk and its density value was sufficient to determine the solids-not fat content and hence the total solids content of the milk.

$$T = 0.25D + 1.21F + 0.66 : 1937\,\text{equation} \tag{12.13}$$

$$T = 0.25D + 1.22F + 0.72 : 1959\,\text{equation} \tag{12.14}$$

where T = total solids (w/W); D = 1000 (density − 1), where density = g/mL and f = fat percentage.

Thus, milk with a fat content of 3.5% and a density of 1.0320 would be corrected to a value of 1.0322 at 20 °C and have a total solids of 12.95% if using the 1937 equation or 13.05% if using the later equation. One criticism of the 1959 equation is that it overestimates total solids, but only by a small amount. This methodology has now largely been replaced by rapid near infrared (NIR) analysis for measuring protein, fat and lactose, with more recent machines including other measurements such as urea, freezing point depression and somatic cell count. One of the main sources of error in interpreting density results is the role of dissolved or entrained air. Air has a density of 1.27 kg m^{-3}. Such air, when it is present, is also compressible, unlike the remainder of the milk. Raw milk has been estimated to contain, on average, 6% dissolved gases by volume. Some milk tankers are equipped with deaerators to remove air to obtain a more accurate measure of volume. However, much of the dissolved gases in milk is removed by procedures in the milking parlour and in the reception area on arrival at the dairy (TetraPak 2003). Deaerators in heat treatment equipment work by subjecting the milk to a vacuum flash cooling process, typically from 68 °C down to 60 °C. This is effective in removing dissolved gases but also removes a small amount of water, as vapour. Air leaking into liquids maintained under vacuum conditions may also cause excessive foaming. The presence of air in milk and milk products complicates predicting food density from its composition. This air may pose problems in milk processing if ignored and is removed before processing. Air is approximately 79% nitrogen and 21% oxygen. It may be the air itself or more specifically its dissolved oxygen that may cause problems. There is also a small amount of carbon dioxide in air, which when it dissolves may increase the acidity of the product. Air is sparingly soluble in water and hence in milk and other beverages. However, it becomes less soluble as temperature increases and any dissolved air will come out of solution. Flash cooling is used in direct UHT processes; not only does it reduce dissolved oxygen, thereby reducing susceptibility to oxidation reactions during storage, but it also removes undesirable sulphur volatiles from UHT milk (Deeth and Lewis 2017).

12.5.1 Particulate Materials

For particulate systems we need to distinguish between bulk density and particle density. Milk powders will contain a substantial amount of air between the particles, which is known as the porosity or internal porosity. Solid density is the density of an individual unit; for example, an individual particle of the milk powder. It will dictate whether the powder will sink or float when immersed in water. In principle it is measured by measuring the mass and the volume. Volume can be conveniently measured by displacement. Bulk density is based on the volume occupied by the powder, where bulk density (ρ_b) is equal to the mass of powder divided by the volume of powder. It can be determined simply by weighing a known amount of the solid and pouring it into a measuring cylinder and tapping the measuring cylinder until the volume remains constant. This compacted volume will include the air which is trapped between the particles. Bulk density is extremely important for determining the total volumes required when transporting and storing powdered products.

$$\text{Volume required} = \frac{\text{mass}\left(\text{kg}\right)}{\text{bulk density}\left(\text{kg m}^{-3}\right)} \tag{12.15}$$

Porosity (ε) is the fraction of the total volume occupied by air (i.e., the volume fraction)

$$\varepsilon = \frac{\rho_s - \rho_b}{\rho_s}\left(\text{external porosity}\right) \tag{12.16}$$

Example: if $\rho_s = 1250$ and $\rho_b = 700$, then $\varepsilon = 0.44$ (44% by volume).

Bulk density and particle density of milk powder cover a wide range and depend very much on the methods of manufacture and especially the solids content of the feed entering the drier, with density values increasing as total solids increases. Lewis (1993) provides a table of values where bulk density ranges from 390 to 660 kg m^{-3}, particle density from 802 to 1150 kg m^{-3} and porosity from 42% to 70% for dairy powders. Less obvious is the air in food powder particles, where there may be substantial air trapped within the structure of the powder, again making them more compressible. On the whole, powders which are compressible are more difficult to handle and may cause problems when transporting, sorting and using these powders. Tests for compressing powders are described in more detail in Sect. 12.13.

12.5.2 *Foams and Aerated Systems*

There are products where we wish to incorporate air into foods, for example, whipped cream and ice cream. Overrun is a term used for aerated products, such as whipped cream and ice cream and other foams. Usually, air is incorporated into the liquid by beating or some other form of agitation.

Overrun may be calculated as follows:

$$\text{Overrun} = \frac{\text{increase in volume}}{\text{original volume}} \times 100 \tag{12.17}$$

The standard method for measuring overrun is to weigh a container full of the original liquid (before aeration) and full of aerated product.

$$\text{Overrun} = \frac{\text{weight of fluid} - \text{weight of aerated product}}{\text{weight of aerated product}} \times 100 \tag{12.18}$$

Typical overrun values are shown in Table 12.7. Aerosol creams which use nitrous oxide as the propellant can have overruns between 400% and 500%. They may look impressive for short periods, but do not have long term stability. Ice cream is another product where the overrun will have a pronounced effect on the texture and mouthfeel of the product and may range from below 20% to over 100%. More information is provided by Arbuckle (1977).

Table 12.7 Some typical overrun values for frozen desserts

Product	Overrun (%)
Ice cream (packaged)	70–80
Ice cream (bulk)	90–100
Sherbert	30–40
Soft ice cream	30–50
Ice milk	50–80
Milkshake	10–15

12.6 Rheological Properties of Milk and Milk Products

Rheology is the study of the deformation of materials, subjected to applied forces. A distinction is often made between fluids and solids; fluids will flow under the influence of an applied force, whereas solids will stretch, buckle and even break. Sometimes this distinction is not clear. Milk is of great interest because we would

recognise it as a low viscosity fluid, but it can be used to make products which embrace the entire rheological spectra. Practical applications include using viscosity for pressure drop calculations and for determining whether flow is streamline of turbulent, for determining pressure drops when pumping such fluids and for predicting heat transfer rates and residence time distributions. Measuring rheological properties of dairy products is important for evaluation of texture. For many products the aim would be to control rheological properties between limits, and some examples will be given throughout the text.

12.6.1 Viscosity

The rheology of fluid dairy products is covered by viscosity measurement. Fluid viscosity is a measure of the internal friction. If a force is applied to a fluid, then this will give rise to a deformation. The deformation is known as flow.

We usually express the force applied as a shear stress:

$$\text{shear stress}\left(\tau\right) = \frac{\text{force}\left(F\right)}{\text{area}\left(A\right)} \tag{12.19}$$

This gives rise to a deformation or shear rate (dv/dy). This can be likened to velocity gradient or how quickly the fluid will flow.

Viscosity is defined as the ratio of shear stress to shear rate:

$$\text{viscosity}\left(\mu\right) = \frac{\text{shear stress}}{\text{shear rate}} = \frac{\tau}{\frac{dv}{dy}} \tag{12.20}$$

The SI units for viscosity are Pa s, Nsm^{-2}, kg m^{-1} s^{-1}. The corresponding centimetre-gram-second (cgs) unit is the Poise: g cm^{-1} s^{-1}.

Viscosity values are still often given as centipoise (cP), where 1 cP = 1 mPa s. Water is a low viscosity fluid and has a viscosity of 1.002 mPas at 20 °C. Milk and some of the simpler fluids derived from milk processing operations, such as skim milk, cheese whey and UF permeate are relatively low in total solids and have a low viscosity. In my opinion, the viscosity of these fluids is best measured by capillary flow viscometer, which measures the kinematic viscosity. Milk has about twice the viscosity of water, i.e., ~2 cp. Some representative values at 20 °C are: 5% lactose solution, 1.15 mPas; rennet whey, 1.25 mPas; skim milk, 1.79 mPas and whole milk, 2.127 mPas. Chen et al. (2014) reported the viscosity values of bulk milk collected on 25 occasions over a complete year to be in the range 1.53 to 2.36 mPas, with an average value of 1.93 and standard deviation of 0.21 mPas. For most engineering calculations, these fluids can be considered to be Newtonian fluids. However, milk at low temperatures (i.e., below 40 °C) has been observed to follow shear

thinning behaviour occasionally, which was attributed to cold agglutination of the fat globules (McCarthy and Singh 2009).

The viscosity of all fluids decreases as temperature increases. When measuring viscosity, the temperature should always be recorded. How the viscosity of some dairy products changes with temperature is shown in Fig. 12.2. Data for viscosity of fluids is scarce above 100 °C, as the fluid has to be pressurised while its viscosity is being measured. On average, fluids decrease in viscosity by about 2% for each degree Celsius increase in temperature, although some fluids will be more temperature dependent; for accurate measurement of viscosity it should be controlled to ±0.1 °C and for more routine quality assurance work to ±1 °C. Bertsch and Cerf (1983) used a capillary viscometer to measure the viscosity of a variety of milk products in the range 70 to 135 °C; fat contents ranged from 0.03% to 15% and some milks were homogenised. Some equations for estimating the viscosity of Newtonian milk products at different temperatures and compositions have been compiled in Table 12.8. During pasteurisation or sterilisation, viscosity will decrease

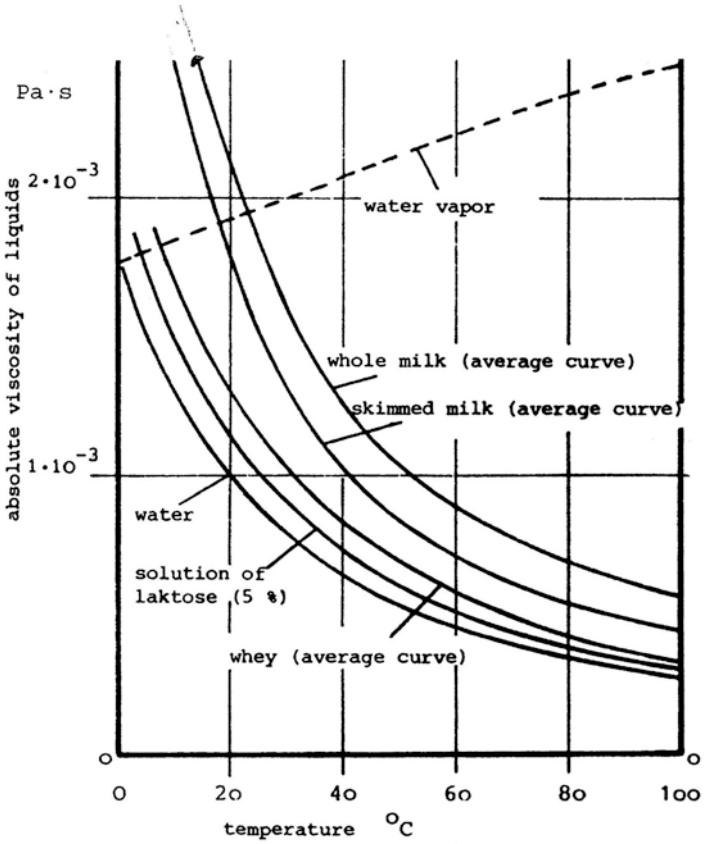

Fig. 12.2 The viscosity of some milk products at different temperatures (from Kessler 1981), with permission

Table 12.8 Technologically useful relationships between the viscosity of Newtonian milk products and temperature and composition; viscosity ($\acute{\eta}$) is in mPa s, Θ = temperature in °C; TS = % total solids; F = % fat and P = % protein (taken from McCarthy and Singh 2009)

Product specifications	Relationship
Milk	$\log\eta = 0.249 - 1.3\times10^{-2}\theta + 5.2\times10^{-5}\theta^2 + \left(2.549\times10^{-2} - 9.8\times10^{-5}\theta + 4\times10^{-7}\theta^2\right)(TS) + \left(5.43\times10^{-4} - 1.39\times10^{-5}\theta + 1.117\times10^{-7}\theta^2\right)(TS)^2$
8–30% TS	
0.07–7.4% fat	
Fat to solids-not-fat ratio:	
0.01–0.4	(Fernández-Martin 1972b)
0–80 °C	
Milk	$\ell n\eta = 3.92\times10^{-5}\theta^2 - 1.951\times10^{-2}\theta + 0.666 + F\left(-9.53\times10^{-6}\theta^2 + 1.674\times10^{-3}\theta - 4.37\times10^{-2}\right) + F^2\left(9.75\times10^{-7}\theta^2 - 1.739\times10^{-4}\theta + 9.83\times10^{-3}\right)$
0.03–15% fat	
70–135°C	
	(Bertsch and Cerf (1983))
Milk of normal composition	$\eta = 0.96 + 0.058F + 0.156P$
25 °C	(Rohm et al. 1996)

Milk and cream 0.1–30% fat	$\ell n\eta = \left(\frac{2731.5}{(273+\theta)}\right) + 0.1F - 8.9$
0–30 °C	(Bakshi and Smith 1984)
Milk and cream	$\log\eta = A(F + F^{5/3}) + \log \eta_0$
0–40% fat	where $A = 1.2876 + 11.07 \times 10^{-4}\theta$
40–80 °C	and $\eta_0 = 0.7687\left(\frac{10^3}{273+\theta}\right) = 2.437$
	(Phipps 1969)

substantially during the heating period and increase substantially during the cooling period. One challenging situation is heat treatment of starch-containing materials, for example, milk custards. These will have a low viscosity when they are being heated, but when gelatinisation occurs the viscosity will increase substantially and there will be a further increase during cooling. This could be disastrous in the cooling section of a plate heat exchanger, where gaskets have been blown due to excessive viscosities at that location in the process, with tubular heat exchangers being the preferred technological option for heat exchange with such products.

Kinematic viscosity can be measured directly with a capillary flow viscometer, where kinematic viscosity is defined as:

$$\text{kinematic viscosity} = \frac{\text{dynamic viscosity}}{\text{density}} \tag{12.21}$$

with SI units of $m^2\ s^{-1}$.

An alternative semi-empirical approach for dispersions is outlined by Walstra and Jenness (1984). It assumes that the increase in viscosity results from hydrodynamic interactions only:

$$\mu = \mu_o \left(1 + \left(\frac{1.25\varphi}{1 - \frac{\varphi}{\varphi_{max}}}\right)\right)^2 \tag{12.22}$$

where μ represents viscosity of the suspension (milk) and μ_0 represents viscosity of water (solvent) (note that this equation does not include a term for the concentration of salts), φ represents volume fraction of the dispersed particles; φ_{max} is the hypothetical volume fraction giving close packing. This is assumed to be 0.9 for fluid milk products, but it may be somewhat higher for evaporated milk and somewhat lower for high fat cream. Thus, the total volume fraction is given by

$$\varphi = \varphi_c + \varphi_{wp} + \varphi_l + \varphi_f \tag{12.23}$$

φ_c = hydrodynamic volume of casein, calculated using volume factor of 3.9 ml/g
φ_{wp} = hydrodynamic volume of whey protein, calculated using volume factor of 1.5 ml/g
φ_l = hydrodynamic volume of lactose calculated using volume factor of 1.0 ml/g
φ_f = hydrodynamic volume of fat calculated using volume factor of 1.11 ml/g

Note that hydrodynamic volume for each fraction is obtained from the product of the volume fraction and the concentration, as shown in the example below.

Thus, for milk containing 2.9% casein; 4% fat, 5% lactose and 0.6% whey protein (w/v)

$$\varphi = 3.9 \times 0.029 + 1.5 \times 0.006 + 1.0 \times 0.05 + 1.11 \times 0.04 = 0.2165 \quad (12.24)$$

Substituting this value into the above equation, using a value of φ_{max} of 0.9, this gives a viscosity of 1.68 mPas; inspection of this equation shows that the amount of casein is the most influential factor affecting the viscosity.

12.6.2 Newtonian and Non-Newtonian Fluids

Newtonian fluids show a linear relationship between the shear stress and the shear rate. Therefore, the viscosity is independent of the shear rate. As mentioned, milk is usually assumed to be a Newtonian fluid. Fluids flowing through tubes are subject to a shear rate, whereby the shear rate is approximately equal to 8 v/D, where v is the average velocity and D is the pipe diameter. In mixing equipment, the shear rate is proportional to the rotational speed of the mixer. Some shear rates encountered in pumping liquids through pipes are 1 to 1000 s^{-1} and for mixing and stirring applications, between 10 and 1000 s^{-1}, while shear rates encountered for other unit processes are given by Figura and Teixeira (2007) and Lewis (2022).

Cream, with fat contents ranging from 12% to over 50% and concentrated milk products may show more complex behaviour, as will ice cream mixes and fermented products such as yoghurt and other soft cheeses. Often the main aim from a quality assurance perspective is to achieve a product of constant consistency, and for such products, the manufacturer might well establish a range of values, between which the consumer will notice no changes. The range of Newtonian and non-Newtonian behaviour is illustrated in Fig. 12.3. These can be sub-divided into time-independent and time-dependent behaviour. The simplest type of non-Newtonian behaviour is time-independent, while the most commonly encountered is pseudoplastic, also known as shear thinning behaviour, whereby the viscosity of the fluid decreases with increasing shear rate. A lesser encountered behaviour is dilatant or shear thickening behaviour; I have observed this type of rheological behaviour rarely, but one occasion was with single cream (18% fat) which had been rapidly cooled.

Non-Newtonian behaviour can be characterised for many fluids by the Power Law equation:

$$\tau = k\left(\frac{dv}{dy}\right)^n \quad (12.25)$$

where n represents power law index and k is the consistency index ($Nm^{-2}\ s^n$).

For pseudoplastic fluids $n < 1$, while for dilatant fluids $n > 1$. A third type of behaviour is plastic flow, whereby materials will not flow at low shear stress. However, as shear stress is increased, a point is reached where they start to flow, and this is termed the yield stress. Above this yield stress value, a Bingham plastic will give a linear relationship between the shear stress and the shear rate and the plastic

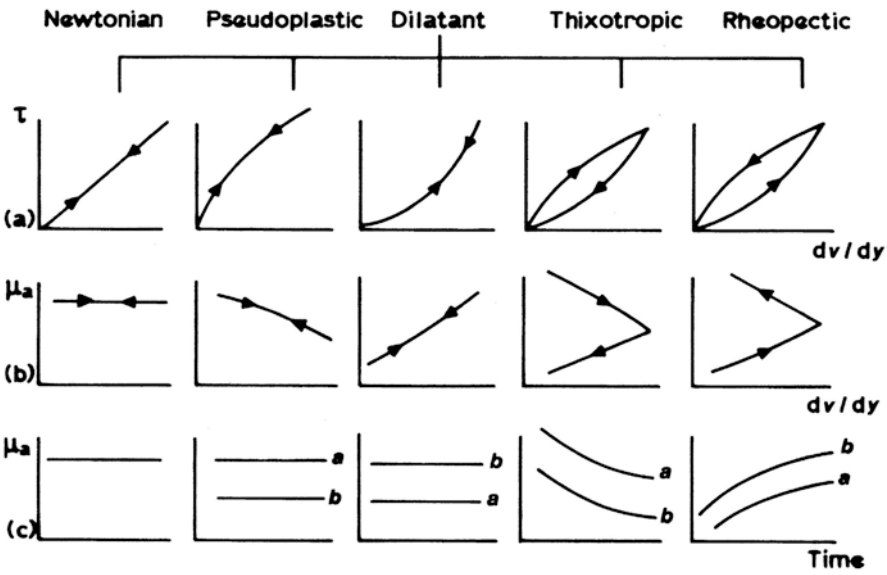

Fig. 12.3 Rheograms for Newtonian and non-Newtonian fluids; shear stress (τ) against shear rate (dv/dy); *b*) apparent viscosity μ_a against shear rate; and *c*) apparent viscosity against time at two different shear rates *a* and *b*, where $b > a$) from Lewis (1993) with permission

viscosity of such a fluid is given by the gradient of the straight line. One equation is the Herschel–Bulkley, which is similar to the Power Law equation, but with an additional yield stress (τ_o) term:

$$\tau = k\left(\frac{\mathrm{d}v}{\mathrm{d}y}\right)^n + \tau_o \tag{12.26}$$

A Casson fluid gives a parabolic relationship, which can be transformed to a straight line when the square root of the shear stress is plotted against the square root of the shear rate. Examples of plastic fluids are butter, some soft cheeses, chocolate and pastes such as tomato and toothpaste. Time-dependent fluids vary with shear rate and with time and are more difficult to characterise. The most common type of time-dependent behaviour is of a shear thinning nature, known as thixotropy. When measured at a constant speed, such fluids will show decreasing viscosity; an equilibrium may be eventually reached. Note that a time-independent fluid will show a constant value. Thixotropic fluids may also recover their viscosity when rested. They show hysteresis when subject to increasing followed by decreasing shear stress. When measuring viscosity of a non-Newtonian fluid, one consideration is to measure it at a shear rate close to those it is likely to experience during processing. There are a number of rotational viscometers available for measuring fluid viscosity with different measuring systems, such as concentric cylinder or cone and plate. The Brookfield viscometer with a simple spindle of attachments for low and

high viscosity fluids has been widely used for quality assurance purposes in the dairy processing sector.

12.6.3 Viscosity of Creams and Other High Fat Products

The starting point for cream manufacture is separation of raw milk. Milk can be separated cold at 10 °C or lower or at higher temperatures up to 50 °C. One important difference is where the agglutinin proteins will reside. Above 40 °C they will be predominantly in the skim milk but at low temperatures they will remain in the cream. Agglutinins are claimed to promote aggregation of fat globules and are said to account for why raw milk or raw cream will separate more quickly than might be expected. However, they are inactivated at higher temperatures, e.g., pasteurisation and in heat-treated creams should play no further part in fat globule coalescence. Because hot milk is less viscous than cold milk, it can pass faster through the separator, enabling higher throughput, and is often preferred for industrial processing for this reason. Separators working on hot milk are typically integrated with a milk pasteuriser, enabling cream separation, in line fat content standardisation and pasteurisation in a single integrated process. Nevertheless, cold milk separation has other benefits. It may, for example, allow longer production run time by avoiding heat-induced fouling. It also reduces the potential growth of thermophilic thermoduric bacteria, capable of surviving high temperatures. Commercial cream products may range in fat content from 12 to over 50%. Common types of cream are single cream at 18% fat, whipping cream at 35% fat and double cream at 48% fat. A cream manufacturer will aim to make each product to a constant consistency. Although the fat content of these creams is very different, the composition of the non-fat solids will not be so different. Some typical values for the kinematic viscosity ($m^2\ s^{-1}$) of cream at 20 °C are given by Kessler (1981) as: 20% fat, 6.2×10^{-6}; 35% fat, 14.5×10^{-6} and 45% fat, 35×10^{-6}. However, cream can show viscosity characteristics across the whole range, from Newtonian, shear thinning behaviour through shear thinning with yield stress. They may also exhibit time-dependency when they are measured as well as showing further age-thickening during storage. As the fat content increases, the rheological properties will be much influenced by the size and nature of the fat globule and that will include not only the total amount of fat, how much is crystallised at the relevant temperature, but also which of the crystalline forms are produced. Most creams are also homogenised to reduce fat separation during storage, with homogenisation also having a pronounced effect on viscosity. To summarise, there are many factors that affect cream viscosity, the main ones being cream separation temperature, fat content, fat globule size and distribution, how it was heated and cooled and its temperature. Cream cooled quickly sometimes shows dilatant behaviour but this may change during storage to pseudoplastic behaviour. Rewarming cream which has cooled down to 5 °C, to 20 to 25 °C and cooling it down will also increase the viscosity of the cream and is known as rebodying. UHT cream may have a shelf life of 6 months to 1 year and it is important that

it does not change too much during storage. Some of the more fundamental aspects affecting viscosity of creams have been discussed by Walstra and Jenness (1984) and McCarthay and Singh (2009).

12.6.4 Concentrated Milk

Some notable viscosity changes will also occur during milk concentration, for example, in evaporation, reverse osmosis and ultrafiltration. Most of these concentrates will be spray dried, and to reduce energy costs, the aim is to remove as much water as possible during these concentration processes prior to drying. In practical terms, this may be limited by viscosity or solubility limits (e.g., lactose). Another benefit of removing as much water as possible is that it will increase both particle density and bulk density of the product and most probably make the product less compressible, more free flowing and easy to handle. In the early stages of concentration, these fluids may exhibit Newtonian behaviour, changing to non-Newtonian behaviour at some point during the concentration process. Some general points are:

- Compositionally, there will be some differences between concentrates produced by heat, reverse osmosis or ultrafiltration, or between whole milk and cream concentrates on one hand and skim milk concentrates on the other. So, a wide range of rheological experiences may be encountered. For example, whey concentrates concentrated by thermal evaporation will have higher viscosity at the same total solids whey concentrates concentrated by membrane technology (nonthermal).
- Non-Newtonian behaviour is found at lower total solids in UF concentrates than in evaporated milk because the proportion of protein is higher and lactose lower.

It is found at lower total solids in skim milk concentrates than whole milk concentrates, again because the proportion of protein on a dry weight basis is higher. Newtonian behaviour was found to persist to higher TS% at higher temperatures. Since viscosities (at a specified shear rate) are more related to compositional factors, Eq. 12.1 can be used for predicting the viscosity of milk concentrates; one modification was that the voluminosity factor for denatured whey protein was 1.07 for native whey protein, but was 3.09 for denatured whey protein. Changes from Newtonian to non-Newtonian behaviour were observed for whole milk; concentrates were Newtonian up to 20% total solids, obeyed the power law equation from 20% to 34% solids and obeyed the Herschel–Bulkley equation (plastic behaviour) at higher total solids. The precise ranges over which these different behaviours are found will depend upon numerous factors such as dry solids composition, temperature, shear rate conditions, pre-treatment of milk prior to evaporation and evaporation method and conditions. Generally, the power law constant k will increase as total solids increase, whereas n does the opposite. It should be stressed that even in evaporated products, the effects of heat on viscosity are generally minimal, as evaporation systems are normally operated under vacuum.

Table 12.9 Some comparisons of evaporated milk products purchased from different supermarkets in the UK

Commercial Samples	Viscosity (cSt)	Density (g/ml)	pH	Ca^{2+} (mM)	Total solids (%)	Sediment (%)
Sainsbury	27.6 ± 0.5	1.082 ± 0.01	6.25 ± 0.02	0.74 ± 0.03	27.7 ± 0.0	0.18 ± 0.02
Tesco	36.1 ± 2.2	1.083 ± 0.01	6.26 ± 0.01	0.76 ± 0.04	27.9 ± 0.1	0.06 ± 0.02
Delicious desserts	42.8 ± 1.2	1.082 ± 0.01	6.25 ± 0.01	0.71 ± 0.05	27.6 ± 0.1	0.07 ± 0.03
Milbona	45.5 ± 1.3	1.081 ± 0.01	6.33 ± 0.02	0.72 ± 0.10	27.7 ± 0.0	0.05 ± 0.03
ASDA	46.1 ± 5.4	1.082 ± 0.00	6.03 ± 0.02	–	27.8 ± 0.7	0.05 ± 0.03
Morrison	30.0 ± 3.7	1.081 ± 0.00	6.28 ± 0.02	0.71 ± 0.05	27.7 ± 0.4	0.08 ± 0.02
Nestle	43.0 ± 3.8	1.083 ± 0.01	6.06 ± 0.01	–	27.5 ± 0.2	0.04 ± 0.04

A product of a very different nature is evaporated milk, where the final stage of production involves stabiliser addition and sterilisation of the product. In this situation, the viscosity increases substantially as a result of the sterilisation process, typically at 115 °C to 120 °C for 10 to 20 min. The evaporated milk product may become extremely viscous, or even coagulate, if the process is not properly controlled. In this case the aim is to prevent excessive thickening and even coagulation during the sterilisation process. This is facilitated by an intensive forewarming process and by addition of stabilisers such as trisodium citrate (TSC) and disodium hydrogen phosphate (DSHP) with DSHP appearing to be the stabiliser which is most used. TSC is also effective, but it may give rise to production of calcium citrate crystals during storage. This may impart a gritty mouthfeel and the crystals may also block nozzles when used in coffee machines. Some examples are shown for evaporated milk in Table 12.9. Some fluid dairy products are kept for a substantial time before consumption and further viscosity changes may occur, one example being gelation during storage of UHT milk.

12.6.5 Viscoelasticity

A viscoelastic material is one that exhibits both viscous and elastic properties, but at the same time. Thus, they are different to plastic materials, which also exhibit viscous and elastic properties, but *not* at the same time. Viscous properties are observed above the yield shear stress, where the product will flow, while elastic properties are observed below the yield shear stress. A further property of a viscoelastic material is that when the shear stress is removed, the strain in the material is not immediately reduced to zero. Many dairy products are viscoelastic, including cheeses and many other gelled products. The behaviour of viscoelastic materials is more complex than that for viscous or elastic materials. There are four major approaches to analysing their behaviour, which have been described in more detail by Lewis (2021), consequently, this section will focus on oscillatory methods.

12.6.5.1 Oscillatory Methods

Oscillatory methods involve subjecting the sample to a harmonic shear strain and measuring the corresponding shear stress that is set up in the sample. The common equipment geometries are cone and plate or concentric cylinder viscometers, with the fluid placed in the gap. It is also possible to vary the frequency of the harmonic signals. The harmonic shear strain is set up in one of the elements and the resulting harmonic shear rate is detected by the other element. By measuring the phase angle and the values of the harmonic shear stress, and its resulting harmonic shear strain, the material can be characterised by defining a storage modulus and a loss modulus.

The storage modulus (G') is given by

$$G' = \frac{\tau_o \cos\theta}{\gamma_o} \tag{12.27}$$

This is high for substances showing substantial elastic behaviour.

The loss modulus, also known as *G* double prime (G'') is given by

$$G'' = \frac{\tau_o \sin\theta}{\gamma_o} \tag{12.28}$$

This is high for materials showing substantial viscous behaviour.

Note that

$$\tan\theta = \frac{G''}{G'} \tag{12.29a}$$

The frequency of the sweep may range typically for 0.10 to 1 Hz. These rheometers are capable of generating a considerable amount of data and provide a variety of approaches for measuring rheological properties (Lewis 2022). One approach is to perform an amplitude sweep, at constant frequency, to look at the behaviour of samples in the non-destructive deformation range and to determine the upper limit of this range. It may also be worthwhile to characterise behaviour, with increasing deformation if this upper limit is exceeded, where the internal structure gets softer, starts to flow or breaks down in a brittle way. A second approach is a frequency sweep to describe the time-dependent behaviour of a sample in the non-destructive deformation range. The range is usually 0.01 to 1 Hz. High frequencies will simulate fast motion on short times scales, whereas low frequencies simulate slow motion on longer time scales or even when the product is at rest. Using frequency sweeps, the oscillation frequency is increased or decreased step-wise from one measuring point to the next while keeping the amplitude constant. There are different ways to present the results. The loss factor, tan δ, can be plotted in addition to the curves of G' and G''; this is very useful when there is a phase transition in the

sample, for example, a sol/gel transition or simply a point of gelation. Usually, for practical applications, a liquid is called ideally viscous if tan $\delta > 100{:}1 = 100$, while a solid material is called ideally elastic if tan $\delta < 1{:}100 = 0.01$. So, when inspecting literature data on G' and G'' values, one should establish the frequency range used, the temperature used and the amplitude used. In conclusion, G' and G'' values can provide some very useful insights into the behaviour of a range of dairy products and changes in that behaviour induced by different processing conditions. Two important areas of application have been monitoring gelation processes and how quickly gelation proceeds and secondly monitoring the final strength of the gel. It is a procedure which is widely used in academic research and looking at fundamental issues in gelation and also as a quality assurance procedure for monitoring the quality and consistency of gel formation.

12.6.6 A Practical Example

An example of a dairy product where a range of rheological approaches are often used is yoghurt manufacture. Yoghurt technology involves the fermentation of milk under controlled conditions and is described in excellent detail by Tamime and Robinson (1999). The main processing parameters influencing the texture of yoghurt are the materials used and their fortification level, fat content and homogenisation conditions, starter culture, incubation temperature, pH at breaking, cooling conditions and any additional handling of the product after manufacture. Milk for yoghurt is usually heated above 90 °C to improve product consistency and stabilisers may be added. There are many different starter cultures that will vary in the amount of acid, volatile components and polysaccharides they produce. Where skim milk is used, it is usually fortified to about 14% to 16% solids. The two main types of yoghurt are set yoghurts and stirred yoghurts. Thus, it is not surprising that yoghurt can exhibit a variety of non-Newtonian effects, such as shear thinning, yield stress, viscoelasticity and time-dependency. One of the most important attributes of yoghurt quality is its texture. For quality assurance, the main interest would be to ensure a product of consistent quality, so the method of measurement in the factory would be specifically chosen for the products being selected, but might include penetrometers, falling sphere viscometers or a very simple protocol with a rotational viscometer. Most yoghurts show shear thinning behaviour and some time-dependency. Controlled stress rheometry would most likely be used in a research environment to understand some of the more fundamental aspects of the gelation process. In this case, values of G' and G'' and frequency sweeps would be more appropriate. Such methods might also be more appropriate for troubleshooting operations, for example, looking at cases where consistency is poor or samples show syneresis, where a clear liquid forms on the surface as the gel shrinks during storage. Some examples are shown in Lucey (2016).

12.7 Surface and Interfacial Properties

12.7.1 Surface Tension

Surface tension is concerned with the forces acting within a fluid. Molecules at the surface of fluid will be subject to an imbalance of molecular forces and will be attracted into the bulk of the fluid, and consequently, the surface is under a state of tension. The surface tension of a fluid can be regarded in two ways, either as a force per unit length acting on a given length of surface or as the work done in increasing its surface area under isothermal conditions. The SI units of surface tension are Nm^{-1} or Jm^{-2}. It is the surface tension forces which cause finely dispersed liquids to form spherical droplets, the shape having minimum surface area to volume ratio. Methods for determining surface tension are discussed by Levitt (1973). Water has a surface tension of 72.6 mN m^{-1} at 20 °C, although it is easy to contaminate with surface active agents. Milk has an approximate surface tension of about 50 m Nm^{-1} at 20 °C (Jenness et al. 1974). The surface tension is lower than that of water due to the presence of milk proteins and phospholipid material, which are all surface active. Bertsch (1983) measured the surface tension of whole milk containing 4% fat in the temperature range 18 to 135 °C using a drop weight method. Surface tension was found to decrease in an almost linear fashion as temperature increased. There was very little difference between whole milk and skim milk and the temperature dependence of both types of milk could be represented by the following equation:

$$y = 1.8 \times 10^{-4} \theta^2 - 0.163\theta + 55.6 \tag{12.29b}$$

where γ = surface tension (m Nm^{-1}) and Θ = temperature (°C).

Jenness et al. (1974) made some further interesting observations, in that surface tension of milk decreased slightly with fat content up to about 4%, thereafter remaining constant. Sweet cheese whey was reported to have a similar value to skim milk. Homogenisation was found to increase the surface tension slightly. Heat treatment had little effect on surface tension, but lipolysis and liberation of free fatty acids decreased surface tension. The presence of detergent in milk would drastically reduce its surface tension. Surface tension is an important property in spray drying when dispersing a concentrated milk into fine droplets. In contrast to milk, information on surface tension of concentrated milk products is limited. This would be important for atomisation in the spray drying process. Williams et al. (2005) found that surface tension was affected more by temperature than by fat content or solids concentration. The surface tensions for concentrates correspond to published values for standard milk below 60 °C, but above 60 °C, the surface tension increased markedly, which was attributed to changes in the milk chemistry.

12.7.1.1 Surface Tension and Milk Processing

Surface tension plays a subtle role in food processing operations, involving dispersing a gas into very small bubbles or dispersing a liquid into fine droplets. A dimensionless group known as the Weber Number (We) gives the ratio of the inertial forces to the surface tension forces in these situations.

$$\mathrm{We} = \frac{pv^2 L}{y} \tag{12.30}$$

where ρ = fluid density, v = fluid velocity, L = a characteristic length and γ is the surface tension.

The main aim in these processes is to promote heat and mass transfer processes by obtaining high interfacial area.

The break-up of liquids falling through an immiscible liquid or gas is as follows:

$D_{\max}$ is proportional to $\left(\frac{\gamma}{\Delta\rho}\right)^{0.5}$, where $\Delta\rho$ = density difference.

Again, a reduction in the surface tension will lead to a reduction in the diameter of the droplet. This approach is applicable to the production of droplets in a spray drier for pressure nozzles, two-fluid nozzles and rotary atomisers by Masters (1991) and with homogenisation processes by Loncin and Merson (1979).

12.8 Emulsion Stability and Foaming

There are many surface active components in milk and these components tend to accumulate at interfaces. Two important interfaces will be considered in more detail. The first is the fat–water interphase and what components will accumulate there to prevent fat globules coming together. The second is the air–water interface, which is relevant in foaming of milk and milk products. Practical situations where foaming is an integral part of the process include foaming of milk for coffee drinks, foaming of cream for whipped cream and aerosol creams. A more complex situation is ice cream freezing, which involves the simultaneous formation and freezing of a foam.

12.8.1 Emulsion Stability

Milk is a natural emulsion and comes with its own milk fat globule membrane material. However, as expressed from the cow, it does not have long term stability as the fat droplets are between 1 and 10 micron in size and will separate naturally. Sixty years ago, milk was supplied in glass bottles and was not homogenised, so it was natural to see a cream layer form in the product. Now homogenisation is widely practised, which reduces the size of most of the fat globules to below 1 micron and

such separation is rarely observed. Homogenisation is widely practised to reduce fat globule separation; on milk, cream products, evaporated milk and ice cream. Emulsifiers may be used in ice cream products. UHT milk is homogenised and may have a shelf life of up to 1 year, so the emulsion must be stable for this time. Ideally, a UHT milk producer would like to evaluate emulsion stability before the product is released for sale, to know in advance what the emulsion would be like at the end of shelf life. Centrifugation will be useful for this (Deeth and Lewis 2017). Milk products can be centrifuged at about 3000 × *g* for 30–60 min in clear tubes, typically of about 50 mL capacity. One can observe how much fat and how much sediment have formed. If a sample is taken from the middle of the tube below any separated fat layer and its fat content determined, this can be compared with the fat content of the original sample (well mixed), this is the basis of the NIZO method. The ratio of the fat contents of the centrifuged sample to the non-centrifuged sample is a measure of the stability of the emulsion and this value should be close to 1. Note that the rate of fat globule separation is also influenced by the product viscosity, in accordance with Stokes Law. The website below gives further details (TetraPak, 2013).

If particle size measuring equipment is available, information can be obtained on the fat globule size distribution. However, there is a scarcity of information to help interpret particle size information, especially in terms of predicting emulsion stability during storage and what it might be like in 12 months. Hooi et al. (2004) recommended that the D(0.9) value for homogenised milk should be less than 1.7 μm, whereas that for raw milk it is 5 to 6 μm. They claim that a properly functioning homogeniser should give a value below 1.3 μm; note that a D(0.9) value of 1.3 μm indicates that 90% of the fat in globules (by volume) is below that size. Two instruments that are used to measure and predict emulsion stability are the LumiSizer and Turbiscan. The LumiSizer combines centrifugation with light scattering for measuring emulsion stability and sedimentation, allowing accelerated stability testing results to be obtained. Optical properties are measured along the length of the sample in a polycarbonate tube throughout the centrifugation process using a near infrared light source. The movement of the phase boundary can be used to calculate sedimentation rate which can be converted into a sedimentation rate under normal gravity conditions. The instrument can be used in a reflectance (back scattering) or transmission mode. A second type of instrument is the Turbiscan or the Coulter Quickscan. These instruments are also equipped with a near-infra-red light source (860 nm) which scans the length of a sample held vertically in a flat bottomed tube and detects the degree of creaming and sedimentation at a point in time or changes over time in the light scattering profiles. One application is to detect rennet induced coagulation in milk and it has demonstrated ability to detect coagulation at a much earlier stage than could the human eye. These latter instruments do not use centrifugation, but still permit more rapid detection of the early stages of fat separation or sediment formation at the bottom and the top of the tubes.

12.8.2 Foaming of Milk

Considerable amounts of milk are used by the coffee industry for making products such as cappuccino. Appliances are also available for frothing milk in the kitchen, so its foaming ability is becoming more obvious to the general public. Those with frothing machines are starting to notice differences in the frothing capacity of different milk samples. I recently purchased a frothing machine and when testing different types of milk to my surprise found that the best foam was from full cream milk. Skim milk produced a reasonable foam but my semi-skim milk hardly produced any foam. It was interesting that the machine showed differences in foaming capacity of different milk, making it potentially useful for those supplying milk to coffee manufacturers. I also observed differences in the foam stability and also the foam structure. Since then, most of the semi-skim milk samples I have tested have produced much better foams than the first sample.

Some general principles are that proteins encourage foam formation, whereas fat, lipolysis and free fatty acids tend to suppress foam formation. Huppertz (2010) reviewed the foaming properties of milk, and some of his conclusions were that "skim milk foams could be extremely stable, particularly when formed at 40–50 °C. The presence of lipid can be detrimental to the formation and stability of milk foams. The presence of phospholipids, free fatty acids and partial glycerides strongly impairs foaming of milk". Although this information might provide ideas for improving the foaming capacity of a milk sample, it is less clear what compositional factors may be used to assess the foaming capacity of a milk.

12.9 Optical Properties of Milk

There are a number of optical properties of milk that are important, its light scattering properties, refractive index, colour and general appearance. The first thing that one notices about milk is its milkiness, in contrast to water and many other fluids that are clear; this is due to the light scattering properties of particles in milk, mainly the casein micelles and the fat globules. In fact, UHT milk is whiter than raw milk immediately after production, with this lightening effect first described by Rhim et al. (1988). In contrast, sterilised milk is always brown and will also have a reduced pH following heat treatment. Milk can lose its turbidity if micelles are disrupted, for example, by removing calcium from milk by ion exchange or by using compounds which will sequester calcium, such as trisodium citrate and sodium hexametaphosphate. The colour and appearance of milk are also important; those who buy milk regularly would have expectations about its appearance and would not expect any abnormalities to be present, especially its milkiness and also its colour.

12.9.1 Measurement of Milk Colour

We all perceive colour slightly differently since colour is a sensory or psychological property. This perception of colour will depend upon three factors, namely the colour source itself, the nature of the illumination source and the observer. For the observer, factors such as age, health and visual abilities are extremely important. In fact, different people will perceive the colour of the same object differently and some people may experience difficulties distinguishing between different colours. Ideally, to make colour measurement less ambiguous, it would be useful to have a technical measurement (set of parameters) for colour, which is independent of the nature of the illuminant and the vagaries of the observer. Many of the systems used to define and measure colour are based on three variables. The aim is to uniquely define or describe a colour which is immune from variation in human perception and subjective judgement. It may well be dependent upon other factors such as illumination systems. The physiological basis of vision has been described in greater detail by MacDougal (1988). The colour (or chromatic attributes) of food depends upon three factors:

lightness: whether the colour is closer to black or white

hue: the perceived colour, e.g., red-green

saturation: the vividness or purity of the system

These factors depend upon the type of light that has been used to view the food. A number of instruments are available that determine the colour on the spectral signal that results from light transmitted through or reflected by the sample when light is directed at the sample. A tristimulus colorimeter can be used or it can be measured over the visible spectrum (i.e., 380–750 nm using a spectrophotometer). They use three filters (red, green and blue), each with a transmission curve duplicating the response of the three types of cone, and the signals produced from the three filters can be used to measure the amount of each of the primary colours making up the colour being measured. These signals can be used to evaluate the CIE X, Y, Z tristimulus values. Although not corresponding precisely to the primary colours, X is associated with red, Y with green and Z with blue. Y is also defined as the luminous reflectance or transmittance.

Another type of colour solid widely used are the HunterLab and the CIELAB systems. The sample is illuminated according to the manufacturer's instructions. The first system was the Hunter *L*, *a*, *b*, but the CIELAB system (*L**, *a**, *b**) has now largely replaced the Hunter System. The difference between the two systems is explained in Hunterlab (2012). Several (≥5) readings are recorded for each sample and the mean values are calculated.

The CIELAB values are:

*L** (degree of lightness): 100 is perfect white and 0 is black

*a** (red-green hues): +100 to −80: zero is grey or neutral

*b** (yellow-blue hues): +70 to −80: zero is grey or neutral

They are obtained using a colour meter such as a Minolta Chromameter. This system is now much used on different types of milk and milk products. One example is to use the *L**, *a** and *b** values for measuring browning in milk. Table 12.10

Table 12.10 L^*, a^* and b^* values for UHT milk stored for four months at different temperatures. The ΔE value is the colour difference between the sample stored at 4 °C and those stored at other temperatures

Temperature (°C)	L^*	a^*	b^*	ΔE
4	100.6	−0.53	1.52	
20	103.0	−0.14	1.50	2.43
35	86.8	7.71	13.9	20.3
50	63.1	8.94	20.1	43.0

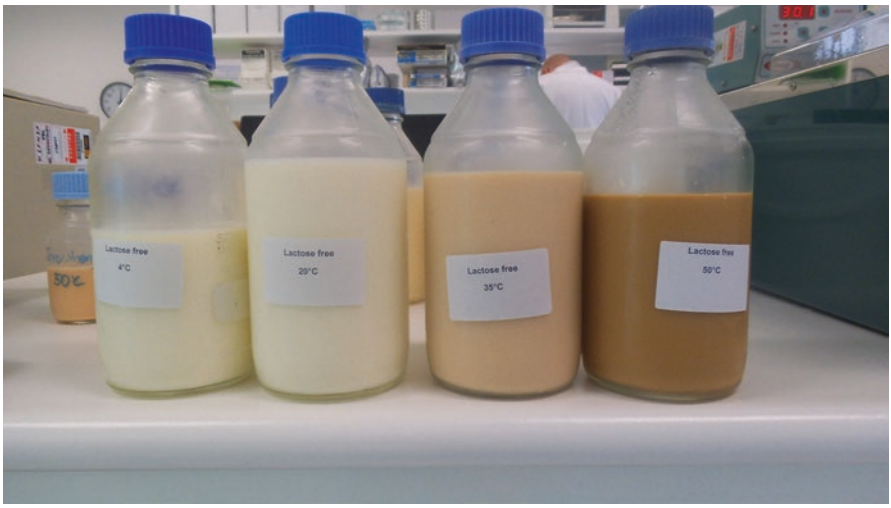

Fig. 12.4 Photographic images of selected browned milk samples

shows L^*, a^*, b^* values for samples of UHT milk which were stored for 4 months at temperatures of 4, 20, 35 and 50 °C. The values in the table most probably do not convey any sense of the colour of these samples, while Fig. 12.4 shows what these milk samples look like, and it can be seen that significant browning occurs at storage temperature of 30 °C.

However, to measure the total colour difference between samples or between samples and a colour reference such as a white tile, a colour difference parameter is needed. One that is widely used is the colour difference parameter delta E (ΔE) or ΔE^*, according to the equation below:

$$\Delta E = \left\{ \left(L_1^* - L_2^* \right)^2 + \left(a_1^* - a_2^* \right)^2 + \left(b_1^* - b_2^* \right)^2 \right\}^{0.5} \tag{12.31}$$

Deeth and Lewis (2017) reviewed some of the information on colour difference measurement. "ΔE values between 0 and 0.5 are impossible to detect by eye,

between 0.5 and 1.5 are difficult to detect by eye, between 1.5 and 3.0 are detectable by trained people and between 3 and 6 are detectable by most people. A value of 2.3 has been reported as the JND (just noticeable difference), while Pagliarini et al. (1990) showed that a minimum ΔE of 3.8 should be attained before there is a visual perception of milk browning". Looking at the samples in Fig. 12.4, and comparing them with the one stored at 4 °C, there appears to be very little difference between the sample stored at 4 °C and that stored at 20 °C, but notable differences between that stored at 4 °C and those stored at 35 and 50 °C. There are very obvious differences between samples stored at 35 and 50 °C. The appropriate ΔE^* values are recorded in Table 12.10. In a second storage trial reported by Deeth and Lewis (2017), some UHT samples were stored at −18 °C and with disastrous consequences; frozen milk samples lost their milkiness due to disintegration of the casein micelles and a great deal of sediment was produced in the product. These results are relevant as UHT milk is described as an ambient stable product and ambient temperature may range from below −20 °C to above 50 °C in various locations on our planet. This type of instrumentation provides a rapid method to measure the colour of foods.

12.9.2 Refractive Index

When light passes from one medium to another, such as air to glass or air to water, it is bent or refracted. A measurement of this property is refractive index. The refractive index of a transparent material is defined as the ratio of the velocity of light in air to that in the medium. One resulting effect is that light bends when it moves from one medium to another, such as from air to water or from air to glass. A refractometer is a simple device for measuring refractive index, which can be quickly and easily measured to four places of decimals. Refractive index values are usually measured using light of constant wavelength of 589.3 nm, which corresponds to the sodium D line and are normally quoted at a constant temperature of 20 °C, as the value is affected by both temperature and wavelength.

Refractive index of water is 1.3330 and that for bovine milk is in the range 1.3440 to 1.3485 and for buffalo milk in the range 1.3461 to 1.3500. Refractive index is very useful for measuring lactose content of UF permeates. However, components which are greater in size than one quarter wavelength (about 0.1 micron) have no effect. Some examples in milk are fat globules, air bubbles and lactose crystals and some casein micelles. Butterfat has a refractive index of 1.4620. Thus, RI measurement can be used as a simple method to measure the purity of butterfat, as potential fat contaminants have different refractive index values.

12.10 Thermal Properties of Milk

Heating and cooling, and occasionally freezing, of milk and milk products are very common operations. Different types of information may be required when dealing with these operations. Some examples are:

- How much heat is required to process a particular material and what methods are available for conserving energy. Simple heating and cooling operations will involve only sensible heat changes, whereas evaporation and drying or freezing will also involve latent heat changes.
- What size heat exchangers are required for pasteurisation and sterilisation duties or evaporation processes. Even on farm, what size coolers are required for chilling milk quickly after milking? Answering these questions will involve understanding the mechanisms of heat transfer.
- What are the heating times and cooling times for different operations or freezing times, for example, for milk? This involves unsteady-state heat transfer principles.
- What are the requirements for steam, hot water, refrigerants, electricity and compressed air. This involves knowledge of the thermodynamic properties of these fluids.
- To answer these questions, it is important to understand and have knowledge of the thermal properties of milk and milk products relevant to these processes. These will now be discussed in more detail.

In fact, energy utilisation in food processing has now become one of the major environmental and sustainability considerations, with the dairy industry having done a great deal of work in this area. If the amount of energy used in a dairy process is known, along with the volume of milk being processed, then the amount of energy used to process 1 L of milk can be established; some data is presented by Rad and Lewis (2014). It is obvious from the literature that different dairies are using different amounts of energy to complete the same task and the comparisons can be staggering, with similar principles also applying to water use and wastewater generation.

12.10.1 Specific Heat

Supplying energy to any food product will usually result in an increase in its temperature. Such changes are known as sensible heat changes. The important property that describes sensible heat changes is specific heat.

Specific heat (c; J kg^{-1} K^{-1}) is the amount of energy required to raise unit mass by unit temperature unit. Water has a high value compared to other food components. The contextual importance is that water is the main component in most foods. Thus, water is the major compositional factor affecting the specific heat of food. Some specific heat values for the major components of food are given in Table 12.11.

Table 12.11 Specific heat values for components of milk and milk products

Component	Specific heat (*a*)	Specific heat (*b*)
Water	4.18	4.18
Carbohydrate	1.4	1.22
Protein	1.6	1.9
Fat	1.7	1.9
Ash	0.8	

If the proximate analysis of the milk product is known, for example, from chemical analysis, or alternatively from food composition tables, then the specific heat can be estimated. The simplest approach is to consider the food as a two component system, i.e., water and solids, whereby:

$$c = m_w c_w + m_s c_s \tag{12.32}$$

with c_s taken as 1.46 kJ kg^{-1} K^{-1} and c_w = 4.18 kJ kg^{-1} K^{-1}, where m_w and m_s are the mass fractions of water and solid and c_w and c_s are the specific heat values of water and solid.

Miles et al. (1983) make a further distinction based on water (w), fat (f) and solids-not fat (snf), which is an approach often used for dairy products:

$$c = \left(0.5m_f + 0.3m_{snf} + m_w\right)4.18 \tag{12.33}$$

A more thorough approach would be to consider all the major components, i.e., a multi-component system; specific heat values for the different components of foods are shown in Table 12.11:

$$c = \Sigma m_i c_i \text{, i.e., the sum of } m_i c_i \text{ for each component} \tag{12.34}$$

The following example shows a calculation for the specific heat of milk:

Its composition is: water: 87.5%, c = 4.18 kJ kg^{-1} K^{-1}; sugar: 4.7%, c = 1.4 kJ kg^{-1} K^{-1}; fat: 4.0%, c = 1.7 kJ kg^{-1} K^{-1}; protein: 3.2%, c = 1.6 kJ kg^{-1} K^{-1} and minerals 0.6%; c = 0.8 kJ kg^{-1} K^{-1}).

The calculated specific heat = 0.875 × 4.18 + 0.047 × 1.4 + 0.04 × 1.7 + 0.032 × 1.6 + 0.006 × 0.8 = 3.847 kg^{-1} K^{-1}. These values and this approach will provide a more than adequate starting point for performing heat transfer calculations.

Table 12.12 shows some specific heat values for a range of dairy products. Also, the specific heat of ice is about half the value for liquid water. So, it follows that the specific heat of frozen food is much less than its fresh counterpart and for foods with high moisture content as much as 50% lower. Bertsch (1982) showed that the specific heat of milk changes over the range 50 to 140 °C: the relationship for skim milk is as follows:

$$c = 2.814\theta + 3942 \tag{12.35}$$

and for whole milk:

$$c = 2.976\theta + 3692 \tag{12.36}$$

over the temperature range 53–143 °C, where Θ = temperature (°C).

Both these equations show that the specific heat increases slightly with temperature; for whole milk the values are 3.87 and 4.11 kJ kg^{-1} K^{-1} at 60 °C and 140 °C, respectively.

Fernandez-Martin (1972a, b) described the specific heat of milk concentrates (8–30% TS) over the temperature range 40–80 C as:

$$c = \left(m_w + \left(0.328 + 0.0027\theta\right)m_s\right)4.18 \tag{12.37}$$

Thus, the specific heat of milk concentrate at 30% TS and 60 °C is 3.54 kJ kg^{-1} K^{-1}.

For a sensible heat change, e.g., supplying or removing energy:

$$\text{heat added or removed}\left(\text{kJ}\right) = \text{mass}\left(\text{kg}\right) \times \text{specific heat}\left(\text{kJ kg}^{-1}\,\text{K}^{-1}\right) \times \text{temp. change}\left(\text{K}\right) \tag{12.38}$$

Note: temperature change 1 K = 1 °C.

Over the temperature range 45 to below 0 °C, milk products containing fat will be subject to some fat crystallisation, and energy will be given out as the fat crystallises. The percentage crystalline fat at any temperature will depend upon the fatty acid profile of the butterfat, with typical figures discussed further in Sect. 12.10.3.

Table 12.12 Specific heat and latent heat values for some dairy products, compiled from data in Polley et al. (1980) and ASHRAE (1985)

	Specific heat (kJ kg^{-1} K^{-1})		
	Above freezing point	Below freezing point	Latent heat (kJ kg^{-1})
Cheese (37–38% moisture)	2.09	1.30	125.6
Roquefort	2.72	1.34	183.8
Cheese, low-fat	2.68	1.47	
Cream, 15% fat	3.85		
Cream, 40% fat	3.56	1.68	209.3
Ice cream (34–42% solids)	3.27	1.88	222.3
Milk	3.85		
Skim milk	3.98	2.51	305.0
Butter	2.05		53.5

12.10.2 Latent Heat

Water is the main component of many dairy products and as such may change its phase during the processing of dairy products. Energy must be supplied to convert ice to liquid water or liquid water to water vapour. Conversely, energy is released when water vapour condenses or when liquid water freezes, and these amounts of energy are generally substantial. The latent heat of fusion (and vapourisation) is the energy required to change the state without a change in temperature.

$$\text{The total heat evolved}\left(\text{kJ}\right) = \text{mass}\left(\text{kg}\right) \times \text{latent heat}\left(\text{kJ kg}^{-1}\right) \quad (12.39a)$$

Water has a very latent heat of vaporisation, as can be seen from steam tables (Lewis 2022).

12.10.2.1 Latent Heat of Fusion

The latent heat of fusion of water is 335 kJ kg^{-1}. Since it is the water in a dairy product which will freeze when reduced below −1 °C, it is reasonable to assume that the latent heat of fusion for milk or other milk products will be 335 m_w (kJ kg^{-1}), where m_w is the mass fraction of water in the product. Latent and sensible heats can be used to estimate the amount of energy that needs to be removed in a freezing process, by considering the process as two sensible heat changes and one latent heat change, with such an approach giving a reasonable estimation of the energy to be removed. How much energy must be removed to reduce 200 kg of ice cream mix from 25 to −20 °C?

sensible heat change$(25 \text{ to } -1)$	$= mc\Delta\theta$	$= 200 \times 3.27 \times 26$	$= 17,004$ kJ
latent heat change$(\text{water} - \text{ice})$	$= mL$	$= 200 \times 222.3$	$= 44,460$ kJ
sensible heat change$(-1 \text{ to } -20)$	$= mc\Delta\theta$	$= 200 \times 1.88 \times 19$	$= 7,144$ kJ
		TOTAL	$=$ **68,608 kJ**

Note that 64.8% of the total energy to be removed is involved with removing the latent heat component. The assumptions made are that the ice cream freezes at −1 °C; all water is converted to ice at this temperature. This latter assumption is discussed in more detail later. Some latent heat of fusion values for different dairy products is given in Table 12.12.

12.10.3 Enthalpy and Specific Enthalpy

Enthalpy is an extremely useful thermodynamic function. Enthalpy data, where it is available can be extremely helpful for calculating heating and cooling loss, especially those involving phase changes. Where it is available, the calculation can be completed in one step, rather than having to consider latent heat and sensible heat values separately, with examples provided later in the chapter.

12.10.3.1 At Constant Pressure, Enthalpy Changes Are Equivalent to Heat Changes

Enthalpy is defined as the sum of the internal energy, plus the product of the pressure and volume

$$\text{Enthalpy}\left(H\right) = U + PV \tag{12.39b}$$

where U = internal energy; P = pressure; V = volume.

It can be shown that enthalpy change is equal to the heat change for reactions taking place at constant pressure

$$\Delta H = q\left(\text{i.e., } \Delta H = \text{final enthalpy} - \text{initial enthalpy} = \text{heat absorbed or released}\right) \tag{12.40}$$

An **exothermic** reaction is one that gives out heat, that is ΔH is negative (−ve); Steam condensing and water freezing are also examples of exothermic reactions, as is the burning of methane.

An **endothermic** reaction is one that absorbs heat, that is ΔH is positive (+ve). Examples are heating of foods and evaporation of water.

An **isenthalpic process** is one that takes place at constant enthalpy; for an isenthalpic process: $\Delta H = 0$ (e.g., rapid expansion of a gas or a throttling expansion); an example of such a process is found in the vapour compression refrigeration cycle.

Specific enthalpy is enthalpy per unit mass (kJ kg^{-1}). It is this parameter that is used in most thermodynamic charts and tables.

$$\underset{\left(\text{kJ}\right)}{\text{Total heat absorbed or released}} = \underset{\text{kg}}{\text{mass}} \times \underset{\text{kJ kg}^{-1}}{\text{specific enthalpy change}} \tag{12.41}$$

In situations where crystallisation accompanies cooling, the enthalpy change takes into account both the cooling and the crystallisation processes.

Enthalpy data for foods and fluids used in food processing can be found in tables and charts, e.g., specific enthalpy/temperature/percentage crystalline solids (α) for fats.

Table 12.13 Melting characteristics and specific enthalpies of some oils and fats (taken from data in Lewis (2022). H = specific enthalpy kcal kg^{-1}; α = amount of crystalline solids (%)

	Butterfat		Sunflower Oil		Lard	
Temperature (°C)	H	α (%)	H	α	H	α
−40	3	100	4	100	3	100
−20	11	98	11	94	10	100
−10	17	90	25	18	15	94
0	24	75	37	0	22	82
10	32	56	42	0	33	59
20	45	20	47	0	39	50
30	54	10	52	0	50	33
40	60	0	57	0	60	10
50	65	0			67	2

Table 12.14 Apparent specific heat of butterfat at different temperatures

Temperature (°C)	−40	−20	−10	0	10	20	30	40	50
Apparent specific heat (kJ kg^{-1} K^{-1})	1.59	1.84	2.01	3.34	4.39	5.35	3.34	2.09	2.01

12.10.3.2 Enthalpy Tables

Table 12.13 shows data for milk fat and other fats for comparison purposes. Also included in this data is the amount of crystalline fat at each temperature, which shows the melting behaviour for these fat:

As an example, cooling butterfat from 20 to −20 °C is considered.

The enthalpy change is ΔH = 11–45 = −34 kcal kg^{-1} = −142.12 kJ kg^{-1}.

The negative sign indicates that heat is given out.

If 200 kg of butterfat is cooled, then the total heat evolved = $m\ \Delta H$ = −34 × 4.1 8 kJ kg^{-1} × 200 = 28,424 kJ

This will give the refrigeration requirement for this application.

The amount of crystalline solids increases from 20% to 98% (Table 12.13). Thus one simple calculation will take into account both the sensible and latent heat terms in this cooling application. For butterfat, a**pparent specific heat** data is available, which is useful for materials containing substantial amounts of fat, accounting for crystallisation as well as temperature changes (Table 12.14). The highest value is at 20 °C, which indicates that considerable crystallisation/melting is taking place about this temperature. Note that the apparent specific heat in this example above would be $\Delta H/\Delta\theta$ = 142.12/40 = 3.553 kJ kg^{-1} K^{-1}.

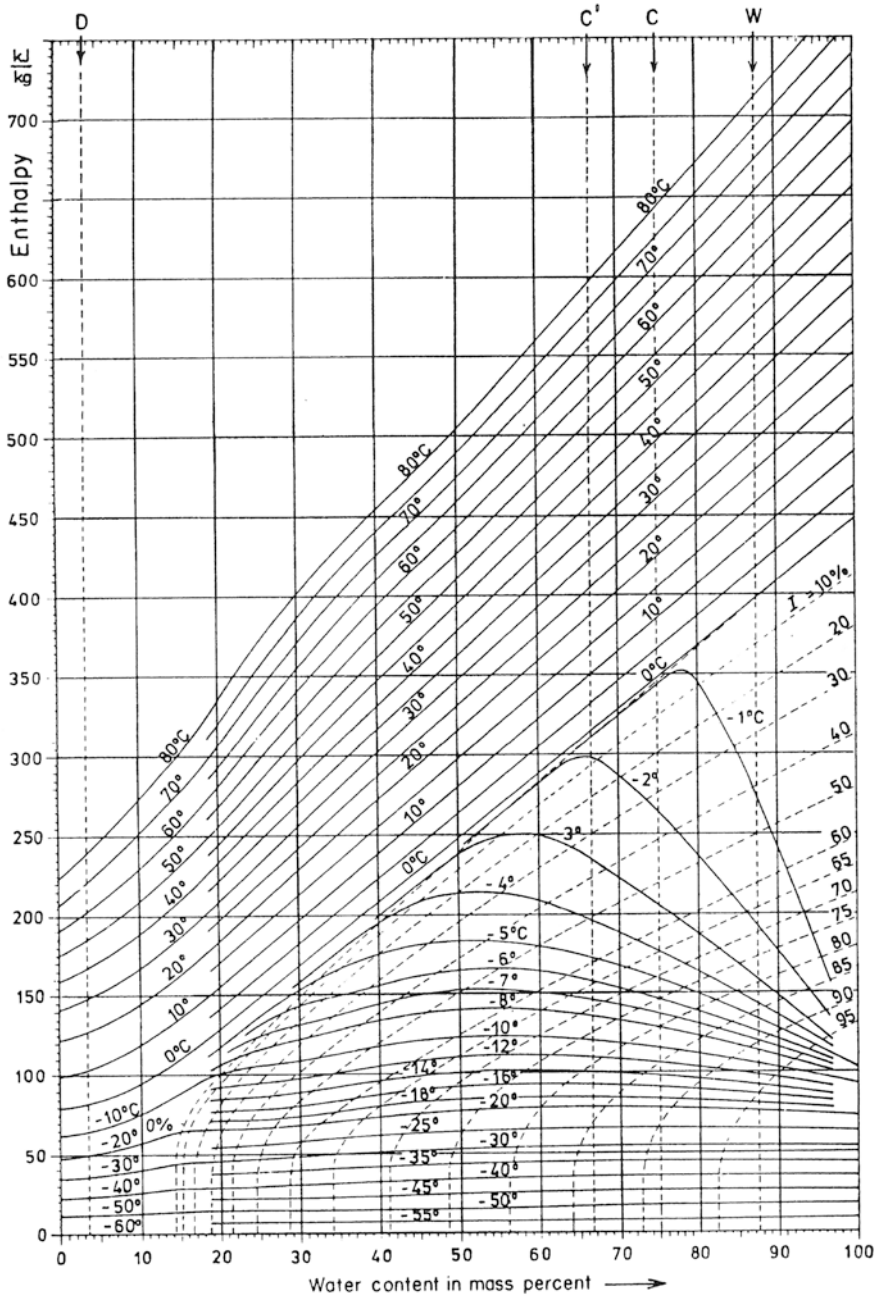

Fig. 12.5 Enthalpy-composition diagram for the system dry/whole milk/water. The dry solids contain 30% fat on a dry weight basis. The value of the enthalpy is 0 at −60 °C. I is the percentage of water frozen; from Riedel 1976

Table 12.15 Examples for when milk at 80 °C is taken down to −20 °C, for milk at 12% TS, 30% TS and 60% TS

Milk (TS)	H 80 °C (kJ kg^{-1})	H −20 °C (kJ kg^{-1})	ΔH (kJ kg^{-1})	α −20 °C
12	670	90	−580	95
30	615	85	−530	85
60*	455	75	−380	66

Assuming this level of total solids could be reached

12.10.4 Enthalpy Charts

Figure 12.5 shows an enthalpy chart for milk. The specific enthalpy for the food is plotted against its moisture content, with two main regions. The first deals with temperatures above the initial freezing point of the food, for sensible heat changes, and the second looking at temperatures below the initial freezing point. In this region, another important parameter is the amount of unfrozen water. It also shows lines of constant temperature. Thus, this chart can be used to estimate enthalpy changes for a wide range of moisture contents; for milk, it can range from skim milk, almost to milk powder. Table 12.15 shows examples for when milk at 80 °C is taken down to −20 °C, for milk at 12, 25 and 50% TS. Table 12.15 provides examples for when milk at 80 °C is taken down to −20 °C, for milk at 12, 30 and 60% TS. As milk becomes more concentrated, the enthalpy change decreases. Also, the amount of frozen water at −20 °C also decreases with increasing solids concentration. Thus, calculations are simple and involve not having to consider sensible and latent heat terms separately.

12.10.5 How Water Freezes in Milk and Dairy Products

When water in a typical food product starts to freeze, the initial freezing point of food might typically be between −1 and − 5 °C. This will depend upon the food composition, especially its level of sugars and salts. As ice crystals separate, there will be an increase in concentration of these solutes which will further depress the freezing point and the amount of ice will increase as the temperature is reduced and for many foods there will still be a small amount of unfrozen water at a temperature of −20 °C, although most of the water freezes over the range −1 to −10 °C. If the temperature is plotted against the amount of water frozen, the relationship is shown in Fig. 12.6. Every food will have its own individual characteristic curve. Figure 12.6 shows such a curve for two ice cream mixes with slightly different compositions (Mitten and Neircinckx 1993).

There are two interesting discussion points. The curve represents a dynamic situation. As the temperature of the frozen ice cream fluctuates, the amount of frozen water will also be changing and some ice crystals will melt and then refreeze. In thermodynamic terms, the smallest ice crystals melt preferentially and when that

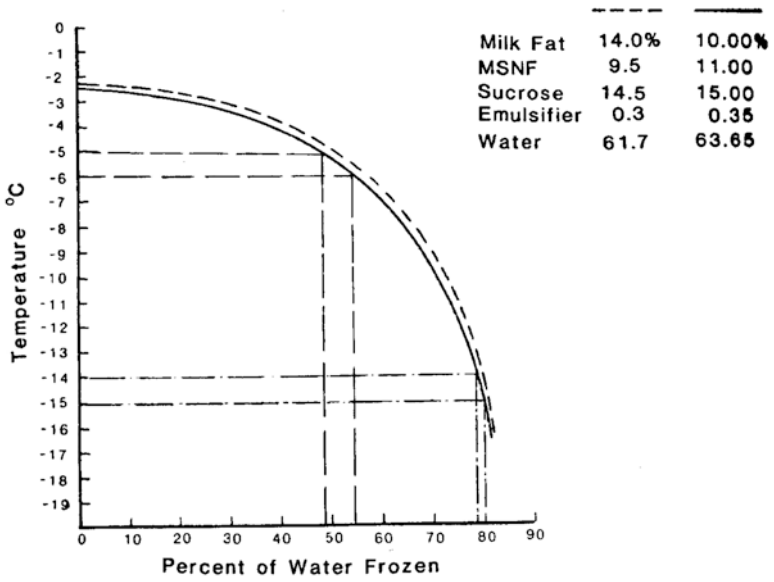

Fig. 12.6 Percentage of frozen water in two different ice cream mixes at different temperatures (taken from Goff (2016))

melted water refreezes, larger ice crystals will form. If this happens many times, ice crystal size will further increase and result in a product which is icy in appearance and not very attractive. This phenomenon is called recrystallisation and is a sign of poor temperature control during storage of frozen products. This will have an adverse effect on the mouthfeel of the ice cream.

Also, as the amount of ice in the product increases the product will become harder. This is also important for ice cream and other products which are eaten in their frozen form. For example, by manipulating the formulation, the amount of frozen water present at any temperature can be controlled and this will affect the texture or softness of the ice cream. Typically, ice cream is dispensed from the freezer at −5 to −7 °C, at which point it is pumpable and soft. Ice cream is subsequently hardened at −18 °C, and in most cases kept at this temperature until used. It may be difficult to scoop out at his low temperature and the consumer may have a preference for a product that appears easier to dispense.

12.10.6 Thermal Conductivity

Thermal conductivity is a measure of heat transfer through a material, when conduction is the controlling mechanism. It is applicable for solids but it can also be measured for fluids when convection is eliminated. Thermal conductivity is defined as the steady-state rate of heat transfer through an area of 1 m^2, when a temperature

driving force of 1 K is maintained over a distance of 1 m. It can be measured for materials under steady-state or unsteady-state conditions. Methods for measuring the thermal conductivity of foods have been described by Mohsenin (1980) and Jowitt et al. (1983). Metals are good conductors of heat with values for copper (403 $Wm^{-1}\ K^{-1}$), silver (428) and aluminium (218); in comparison, stainless steel has a lower thermal conductivity of 16 to 20 $Wm^{-1}\ K^{-1}$. Compared to metals, foods are poor conductors of heat and the thermal conductivity of foods is affected by its moisture content. Lamb (1976) gave the following equation for predicting the thermal conductivity of a food from its moisture content:

$$k = 0.0841 + 0.568\, m_w \tag{12.42}$$

which would give values for full cream milk and skim milk of 0.578 and 0.061 $Wm^{-1}\ K^{-1}$, respectively. This would provide a good starting point, although it was claimed that there are large discrepancies below 50% moisture content. Values for frozen foods would be higher than fresh foods as ice has a thermal conductivity about four times higher than fresh foods. It is not so straightforward to predict the thermal conductivity from food composition as it depends whether the components are considered to be in parallel or series; this is discussed by Miles et al. (1983) and more recently by Lewis (2022). Thermal conductivity also changes slightly with temperature and in all cases increases as the temperature increase, with some examples available in Lewis (1993). McCarthay (1984) measured the effective thermal conductivity of skim milk powder, with values ranging from 0.036 to 0.109 $W\ m^{-1}\ K^{-1}$ in the temperature range 11.8–49 °C for bulk densities between 292 and 724 $kg\ m^{-3}$; the effective thermal conductivity increased with temperature and with bulk density.

12.10.7 Thermal Diffusivity

Thermal diffusivity (α) is an unsteady-state property, defined as:

$$\alpha = \frac{k}{\rho c} \tag{12.43}$$

where k = thermal conductivity; ρ = density and c = specific heat.

It has SI units of $m^2\ s^{-1}$. It should be noted that a frozen product has a much higher thermal diffusivity than its unfrozen counterpart, of about eight times, due to its thermal conductivity being about four times higher and its specific heat being about two times lower. Thermal diffusivity is a measure of how quickly a product temperature will change with time during heating process and is used for solving unsteady-state heat transfer problems for estimating heat and cooling times. Some examples are described by Jackson and Lamb (1981), Lewis (1987) and Cleland and Earle (1982) and more recently by Lewis (2022).

12.11 Electrical Properties

12.11.1 Electrical Conductivity

When electricity is flowing through solutions, the term conductance is more widely used than resistance. The specific conductance (or conductivity) (K) is the inverse of resistivity.

Therefore

$$K = \frac{1}{\rho_r} = \frac{L}{R_a} = \Omega^{-1}\,\mathrm{m}^{-1} \tag{12.44}$$

The reciprocal ohm (Ω^{-1}) is also known as the mho or the Siemen (S).

Therefore SI units of conductivity are Sm^{-1}. Some care is required as specific conductance most commonly encountered are mho^{-1}/cm, and the conversion factor is 1 mho cm^{-1} = 100 Sm^{-1}. Physical chemists often use the term molar conductivity (Λ), which is the conductivity per mole of electrolyte. If the concentration of the solution is c (mol/m^3)

then:

$$\Lambda = \frac{K}{c} \tag{12.45}$$

There is much more information about electrical conductance of liquids in the literature, compared to resistivity values. Methods for measuring specific conductance have been described by Levitt (1973). Where the dimensions of the cell are not accurately known, they can be calibrated using a solution whose conductivity is accurately known, often using a solution of potassium chloride. Conductivity measurements are used by physical chemists for determining the solubility of sparingly soluble salts and rates of reactions where changes in conductivity occur. Table 12.16 shows some conductivity values for milk at different temperatures. It can be seen from this table that electrical conductivity increases as temperature increases. The main contributor to electrical conductance will be its salt content. Note that these values can only be regarded as average values, as any of the components listed will

Table 12.16 Electrical conductivity of some liquids at different temperatures (Sm^{-1}) taken from data in Zhang (2015)

	4 °C	22 °C	30 °C	40 °C	50 °C	60 °C
Skim milk	0.328	0.511	0.599	0.713	0.832	0.973
Whole milk	0.357	0.527	0.617	0.683	0.800	0.973
Lactose free milk	0.380	0.497	0.583	0.717	0.817	0.883
Chocolate milk, skim	0.532	0.558	0.663	0.746	0.948	1.089
Chocolate milk, 3% fat	0.332	0.433	0.483	0.567	0.700	0.800

be subject to biological variability. The values for water also show considerable variations; for example, deionised water and distilled water will have very low conductivity. Some values are distilled water 3–5 × 10^{-4} S m^{-1} and deionised water has a value of approximately 10^{-5} S m^{-1}. Tap water will be extremely variable, depending upon its mineral content. Conductivity meters are often supplied with water treatment equipment to determine the quality of the distilled or deionised water and when to intervene to maintain quality. Conductivity measurement could be applied to any process which removes ions from solution, such as ion exchange, electrodialysis and nanofiltration. Most dairy products are poor conductors of heat and bovine milk as a conductivity in the range 0.4 to 0.55 Sm^{-1}. The main contributors are sodium, potassium and chloride ions. The presence of fat tends to decrease the conductivity of milk products. Milk fat has a specific conductance of less than 10^{-14} Sm^{-1}, as do other oils and fats. The conductance of skim milk as it was concentrated was found to increase to a maximum at about 28% solids and then decrease, explained by the complicated salt balance and the reduction in pH that results when milk solids concentration increases. It is possible to heat milk by some less conventional methods, involving resistance (Ohmic heating) or by microwave heating and the properties discussed in this section will influence how quickly the temperature increases in these processes.

12.11.2 Capacitors and Dielectric Properties

Capacitors are able to store electric charge. The simplest form is represented by two parallel metal plates separated by an insulating material, known as a dielectric. Dielectrics are materials which allow more charge to be stored. Methods that are available for measuring capacitance can be adapted to measure the dielectric constant. The dielectric properties of foods are currently receiving more attention, mainly because of dielectric and microwave heating processes. The two properties of interest are the dielectric constant and the dielectric loss factor. The dielectric constant (ε') is a measure of the amount of energy stored and it is the ratio of the capacitance of the material being studied to that of a vacuum (or air) under the same conditions. The term relative is sometimes introduced, to illustrate that the values are determined relative to air or a vacuum, making them dimensionless, but the "relative" is often omitted. The dielectric constant of food depends upon a number of factors; such as temperature, moisture content and the frequency at which it is measured.

12.11.2.1 Dielectric Loss Factor

The relative dielectric loss factor (ε'') is a measure of how much energy a component will dissipate when it is subjected to an alternating electrical field. In an AC circuit containing an ideal capacitor, the current will lead the voltage by 90°. When a dielectric material is introduced between the capacitor, this angle may be reduced. The loss angle, δ, is a measure of this reduction and is usually expressed as a loss tangent (tan δ), which may be regarded as introducing a resistance (in parallel) to the circuit, with the capacitor. This will lead to dissipation of energy and as the loss tangent increases, the amount of energy dissipated increases within the dielectric material.

The dielectric loss factor is related to the dielectric constant as follows:

$$\varepsilon'' = \varepsilon' \tan\delta \text{ or } \tan\delta = \frac{\varepsilon''}{\varepsilon'} \tag{12.46}$$

During microwave and dielectric heating, the power (P) dissipated is given by

$$P = 55.61 \times 10^{-14} f E^2 \varepsilon'' \tag{12.47}$$

where P is power absorbed (W cm^{-3}), f = frequency (Hz), E = electric field strength (V cm^{-1}).

The frequency range for dielectric heating is selected values in the range 13.56 to 40.68 MHz and for microwaves 896 to 22,125 MHz. Both these properties are affected by moisture content and temperature and frequency of the electric field (Table 12.17). Munoz et al. (2018) measured the dielectric properties of three types of milk (raw, skimmed and concentrated non-fat) at high frequencies between 10 and 2450 MHz for producing temperatures between 20 and 150 °C. The dielectric constant (ε') was found to decrease with frequency at all temperatures but increase with temperature at low frequencies and decrease with temperature at high frequencies. The dielectric loss factor (ε'') decreased with frequency and increased with temperature in almost the entire range of frequencies. Ionic conduction was the dominant mechanism across most of the frequency range.

Table 12.17 Some values for dielectric constant and dielectric loss factor for milk in the microwave region (taken from McCarthy and Singh 2009)

Sample	Temp (°C)	Frequency (GHz)	ε'	ε''	Source
Skim milk	25	3	*68	*18	
Skim milk	55	3	59	16	Mudgett et al. (1974)
1% fat milk	20	**2.45	70.6	17.6	
2% fat milk	20	2.45	69.4	17.8	Kudra et al. (1982)
3.25% fat milk	20	2.45	67.9	17.6	
Water	20	2.45	80.2	13.4	Lide and Frederikse (1996)

Zhu et al. (2015) measured the values of ε' and ε'' of raw cow's milk with protein content of 3.21–7.12% over the frequency range 10–4500 MHz at temperatures from 25 to 75 °C using a vector network analyser and an open-ended coaxial-line probe. The results showed that the ε' decreased with increasing either frequency or temperature. ε'' decreased linearly with frequency in a log–log plot at the low frequency end and had minima at 2000–3500 MHz, with the minima increasing with temperature. Below about 600 MHz, ε'' increased with increasing temperature and decreased above 1000 MHz. ε' increased linearly with an increase of protein content below about 150 MHz and decreased linearly above 600 MHz, and ε'' increased linearly with increasing protein content over full investigated frequency range. If the dielectric properties and temperature of milk can be obtained, its protein content could be sensed. Guo et al. (2010) reported a tendency of the loss factor being inverse to pH during milk storage, with the best linear correlation ($R^2 = 0.983$) at 1100 MHz and proposed that loss factor could be an indicator in predicting milk concentration and freshness.

12.12 Water Activity and Moisture Absorption

Water is the main component of most dairy products. The availability of water to act as a solvent for chemical, enzymatic and microbial reactions in food is measured by the water activity of a food. Some food components will bind water more than others and make it less available to participate in different reactions, especially microbial activity. In effect such components will lower the water vapour pressure at any temperature. Water activity is the ratio of the water vapour pressure exerted by the food compared to that of pure water at the same temperature.

$$a_w = \frac{p}{p_s} \tag{12.48}$$

Milk and most fresh dairy products with a high moisture content have a high water activity of almost 1.0 and for evaporated milk it is about 0.98 and ice cream mix at approximately 40% solids a value of 0.97, which alone is not low enough to suppress microbial activity. Sweetened condensed milk with its added sugar content has a value between 0.85 and 0.89, whereas dried milk powder lies between 0.02 and 0.2. The water activity of a selection of cheeses is shown in Fig. 12.7. One method to determine water activity involves an equilibrium procedure in which a large amount of food is allowed to come to equilibrium with the air that it is in contact within a sealed chamber. The equilibrium relative humidity is determined and the water activity is determined from:

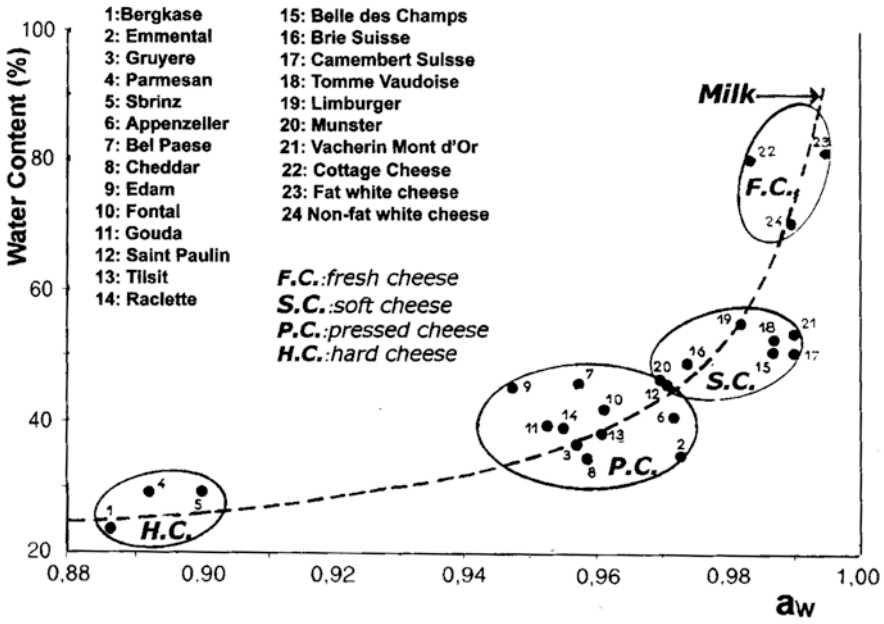

Fig. 12.7 Water activity values for a range of cheeses (taken from Simatos et al. 2009)

$$a_w = \frac{RH}{100} \tag{12.49}$$

A sorption isotherm shows how the equilibrium moisture content changes with relative humidity.

They can be performed at any temperature and are sometimes performed at a range of temperatures; for milk powders they will show whether the powder will be susceptible to adsorbing moisture if storage conditions are poor. There are a number of different equations relating water activity to moisture content, with one of the most useful being the Brunauer–Emmett–Teller (BBET) isotherm:

$$\frac{a}{m(1-a)} = \frac{1}{m_1 c} + \frac{c-1}{m_1 c} a \tag{12.50}$$

where a = water activity, m = water content (% dry weight), c = a constant and m_1 is the monomolecular layer water content, which is a measure of the amount of water that is strongly bound to the material. Iglesias and Chirife (1982) have compiled sorption isotherms for a wide variety of foods and a selection for dairy products is given in Table 12.18, together with the two-parameter equations that best fit the data (from Lewis 1993).

Table 12.18 Summary of sorption isotherm data for a range of dairy products (taken from Lewis 1993)

	Temperature (°C)	Type[a]	a_w range	Equation[b]	B_1	B_2	Monomolecular layer per cent (dry weight)
Cheese Emmental	25	A	0.1–0.8	1	1.1889	5.9967	3.3
	25	D	0.1–0.8	1	1.4435	11.9777	3.7
Cheese Edam	25	A	0.1–0.8	1	1.0668	4.9692	3.3
	25	D	0.1–0.8	1	1.2540	8.5716	3.5
Casein	30	—	0.1–0.8	2	2.1510	0.0044	7.6
β-Lactoglobulin[c]	25	A	0.1–0.8	2	1.5211	0.0166	6.6
Non-fat dry milk[c,d]	30	D	0.1–0.8	1	1.9684	72.3080	6.5
	37.8	D	0.1–0.8	1	1.9927	67.8072	6.1
Skim-milk[c,d]	20	A	0.1–0.8	4	–3.0113	1.3983	2.8
	34	D	0.1–0.8	1	2.0544	54.3870	4.7
	34	A	0.1–0.8	1	1.7764	23.8439	4.0
	14	D	0.1–0.8	1	2.6527	290.2579	
Whole milk[c,d]	24.5	D	0.1–0.8	4	–1.4503	3.1356	3.1
	24.5	A	0.1–0.8	1	2.1884	37.9004	3.5
Sweet whey[d]	24.5	D	0.1–0.8	3	3.1279	3.0619	—
Whey protein concentrate[c]	24.0	A	0.12–0.86	1	1.4806	17.6165	4.8
Yoghurt[c]	25.0	A	0.1–0.8	1	1.0529	6.4806	4.1
	45.0	A	0.1–0.8	4	–3.6752	0.2732	3.0

Compiled from data in Iglesias and Chirife (1982)

[a]Type of isotherm: A, adsorption; D, desorption

[b]The following equations are relevant:

(1) Halsey's equation $a_w = \exp(-B(2)/X^{B(1)})$

(2) Henderson's equation $1 - a_w = \exp(-(B(2)X^{B(1)})$

(3) Iglesias and Chirife's equation $X = B(1)[a_w/(1 - a_w)] + B(2)$

(4) Kuhn's equation $X = \dfrac{B(1)}{\ln a_w} + B(2)$ X = moisture content (% dry weight basis)

[c]Isotherms are also given at other temperatures, or alternatives are given

[d]Broken isotherms

12.13 Powder Properties

There are many variants of milk and whey powders now available. Each consignment of powder would normally be accompanied by a specifications list, which describes the chemical composition, microbial flora and a range of other physical properties. Some of the main physical properties that could be included in such specifications are briefly summarised below, although the thermal properties are rarely included: More information is provided by Lewis (2022) and Niro (1978)

- solubility, wettability, dispersibility, sinkability
- particle density, bulk density, compressibility, flow characteristics
- heat designation; low, medium high and very high heat powders, based on whey protein denaturation
- heat stability, when reconstituted, especially in evaporated milk
- functional properties, such as emulsification, foaming and gelation
- thermal properties: specific heat, thermal conductivity
- storage stability, water activity, glass transition temperature
- other aspects; dust hazards and potential to be explosive, microbial quality

12.13.1 Some Engineering Properties of Powders

The behaviour of the collective mass of particles is important in operations involving transportation, distribution and storage. Spray drying can produce fine powders, which are not always easy to use. Therefore, conditions are often manipulated, especially with respect to controlling the particle size, for improving some of the properties described above. The bulk properties of powders are dependent upon factors such as particle size, particle shape, surface characteristics, chemical composition, moisture content and processing history. For those producing powders, it is crucial that their product is easy to handle. Such ease-of-use would be main requirement for those involved with dry blending of powders. The term cohesive is used to describe the behaviour of powders as they are influenced by the forces of attraction (or repulsion) between particles. For powders that are cohesive, the ratio of the interparticle force to the particles own weight is large. This ratio is also inversely proportional to the square of the particle size. This explains why small particles adhere much more closely to each other than large particles. The more cohesive a powder is, the more difficult it is to handle. Schubert (1987) states that the majority of food particles are non-cohesive (and hence free flowing) when their particle size exceeds 100 microns. This is one good argument for agglomeration. If the moisture content of a powder increases it also makes powders more cohesive and increases the particle size at which the transition from cohesive to non-cohesive takes place.

12.13.1.1 Powder Compressibility

Powders can be compressed, thereby increasing their bulk density, either by tapping or by more strenuous compression, for example, when sacks of powder are stacked on top of each other, or when powders are tableted. The ratio of the tapped bulk density to the loose bulk density is referred to as the Hausner ratio.

This can be used to predict how free flowing the powder is, with typical classifications as follows:

1.0 to 1.1	Free Flowing
1.1 to 1.25	Medium Flowing
1.25 to 1.4	Difficult
>1.4	very difficult

Peleg (1983) suggested that the Hausner ratio is a useful indicator of flowability where friction is the major obstacle to flow. However, there is no evidence that it is useful for cohesive powders.

12.13.1.2 Flowability

Flowability of powders is very important property, especially in their transportation or when dispersing them into liquids. Generally, flowability increases with increasing particle size and decreasing moisture content. As well as compressibility, other factors can be used to assess flowability, with two such empirical factors including slide angle and angle of repose. Slide angle is measured by placing powder samples on a flat smooth horizontal surface, which is slowly inclined until the powder begins to move. Angle of repose is also useful in the design of powder handling systems, and its value depends upon the method of determination which is normally by allowing the powder to form a heap. According to Carr (1976), angles up to 35° indicate free flowability; 35–45° indicates some cohesiveness and 45–55° indicates cohesiveness or loss of free flowability and >55° indicates very high cohesiveness and very limited or zero flow. These parameters are empirical in nature and often results are not applicable when conditions are changed, Peleg (1977).

12.13.1.3 Jenike Flow Cell and Function

A more fundamental method was described by Peleg (1977) and Schubert (1987) and more recently by Lewis (2022). The powder is subjected to a compressive force (F) and the shearing force (S) at different values of bulk density and these readings are converted to a normal stress (σ) N/A and a shear stress (τ). This compressional data can be used to define the unconfined yield stress F_c and the major consolidation stress (σ_1). The ratio (σ_1/F_c) is termed the Jenike flow function, and its value corresponds to the following characteristics:

<2	very cohesive, non-flowing
2–4	cohesive
4–10	easy flowing
>10	free flowing

Measuring these properties will help to determine whether a powder will be problematic in terms of its flow characteristics and general ease of use. A useful form of presentation is a plot of unconfined failure stress against major principle consolidating stress. Such a plot can be obtained using a powder flow tester (Brookfield) and clearly shows the different regions, which are free flowing, easy flowing, cohesive, very cohesive and non-flowing. Crowley et al. (2014) have presented some data for milk protein concentrates ranging from 35% to 95% protein, with the paper giving more detail of the methodology. It should be noted that these powders not only had different protein contents, but also different surface area to volume ratios.

12.13.1.4 Hydrodynamics of Powders

The hydrodynamics of powders are different to those for liquids. When height of material in the hopper increases (height increases), the pressure at the base does not increase linearly with height and in some cases it is almost independent of height. Powders may be stored in hoppers and when required will be discharged from that hopper. The particulate material will exert both a vertical stress and also a lateral or horizontal stress. The horizontal stress needs to be considered as this will reduce the vertical stress. This horizontal stress may even cause mechanical damage and buckling of the silo in worst-case situations. Predicting the lateral stress can involve complicated mathematics. One of the worse situations that can occur is a large horizontal stress during powder discharge, which can severely damage the silo, so being aware of these lateral stress lateral values is important when designing silos

When powders are discharged from hoppers, the ideal flow characteristics are known as mass flow. The rheological properties of the powder should be controlled to ensure that the product is discharged under these conditions. Some situations which can occur when powder properties are non-ideal are *arching,* which is the formation of a stable arch that impedes flow from the hopper. *Rat-holing* is the build-up of a stagnant region close to the hopper walls and *flooding* is the uncontrollable fluidisation of powder during discharge. Knowing the flow characteristics of your powders will help you to minimise these situations. Products in sacks will also be stacked and there could be large stresses on the sacks toward the bottom of the pile. This may compress the powder sufficiently to make it more difficult to handle. Another fault in powders includes time consolidation, with induced impairment of flow. This occurs when powders have been stored for too long under compression, for example, at the bottom of powder stores. A second fault is segregation, which is a stratification of particles due to physical differences. This will occur when a milk powder is mixed with other particles of different sizes and densities are shaken or

agitated. It may be a problem, both in terms of getting a well-mixed product into the retail packet and keeping the product well mixed in the packet during storage.

12.14 Some Closing Remarks

Many of the properties described in this chapter are essential for both designing processes and ensuring product quality. Much of the information on physicochemical and physical properties of milk and milk products has been compiled over a long time period. Some of these properties can be estimated from knowledge of the composition of the product but others need to be determined experimentally. A wide range of analytical methods are available, costing anything from tens of pounds to upwards of £100,000. A quality assurance laboratory in a small dairy is unlikely to contain some of the sophisticated equipment discussed in this chapter, being more commonplace in research laboratories in larger companies or at universities. For example, viscosity can be measured simply and accurately using a capillary flow viscometer, and products can be viewed by a simple light microscope, but it may be necessary to resort to more complex equipment to solve problems and gain a fuller understanding of the mechanisms responsible for processing performance and quality issues. Thus, many companies will not have direct access to equipment such as particle size analysers, oscillatory viscometers and powder rheometers to help with their quality assurance programmes. Neither will they have access to methods such as NMR which provide information on a wide range of free amino acids, organic acids and non-protein nitrogen compounds in milk and milk products.

It should not be forgotten that both milk and milk products are variable in their composition, and that milk can be used to make a wide range of very interesting products. Manufacturers will aim to produce their products to a common specification, and in theory this should not present any difficulties. To do this, they would follow standard production procedures, but despite doing this the products they produce will vary from day to day. Manufacturers of products such as Cheddar cheese, whipping cream, dairy foamers, evaporated milk and the wide range of powders will be well aware of this. The properties discussed in this chapter are fundamental to product quality, especially in terms of the sensory characteristics such as appearance, texture and mouthfeel. The consumer demands uniformity in its end products, but the milk is variable in its composition, so herein lies the challenges and opportunities that milk processing throws at us.

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