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Non-Enzymatic Degradation Pathways of Lactose and Their Significance in Dairy Products

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7.1. Introduction

Milk products are especially sensitive to the effects of heat treatment encountered under conventional process and storage conditions because of an abundance of reactive functional groups: aldehyde group of lactose, ε-amino group of lysine and other reactive N-containing groups (e.g. indolyl group of tryptophan, imidazole group of histidine, guanidino group of arginine and the α-amino group of proteins and free amino acids).

Lactose may isomerize via the Lobrey de Bruyn–Alberda van Ekenstein (LA) transformation, followed by degradation to acids and other sugars. Alternatively, lactose may react with the caseins and whey proteins of milk systems via the Maillard or non-enzymatic browning reaction (also referred to as glycation of proteins, e.g. in the case of lactose, lactosylation). This review examines the chemistry and significance of such reactions in milk products.

Bovine milk contains ~4.8% lactose which is present in the free form (4-*O*-β- D-galactopyranosyl-D-glucopyranose) and lactose-containing oligosaccharides. The concentration of such oligosaccharides is very low and would not be expected to contribute significantly to sugar degradation reactions and Maillard browning in milk systems. In contrast to its LA

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* *The views expressed herein are those of the author and do not necessarily represent the policies of the Food Safety Authority of Ireland*

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Advanced Dairy Chemistry, Volume 3: Lactose, Water, Salts and Minor Constituents.
Edited by P.L.H. McSweeney and P.F. Fox, DOI 10.1007/978-0-387-84865-5_7,

transformation product, lactulose, lactose does not contain furanoid ring structures (Clamp *et al.*, 1961); the formation of such structures is precluded by the (1→4) linkage. In solution, lactose exists as an equilibrium mixture of α- and β-lactose. A third crystalline form, anhydrous α-lactose, may be prepared from crystalline α-lactose hydrate by heating. Although α-lactose hydrate and β-lactose are stable at room temperature, anhydrous α-lactose is unstable and is converted either to α-hydrate (in the presence of water) or to β-lactose (by heating to temperatures greater than 93.5°C). The proportions of α- and β-lactose in an equilibrated solution at room temperature are ~37 and ~63%, respectively. The solubility of lactose in water is low (17.8 g dl^{-1} at 25°C) compared with other sugars, and crystallization is often a problem in concentrated systems.

In addition to its high concentration in dairy products, lactose is still the most widely used excipient in tablet manufacture. Since the majority of drugs have amine functional groups, the role of Maillard reactions between drug molecules and lactose in tablet discoloration is well known. The potential of such reactions in the solid state to limit the shelf-life, efficacy and bioavailability of drug formulations has been studied extensively (Kumar and Banker, 1994; Qiu *et al.*, 2005a). Apart from storage conditions, milling (which influences surface area of reactants) high compression pressure and reactants in the amorphous state have been shown to promote Maillard reactions in tablets (Qiu, *et al.*, 2005b). Whereas browning inhibitors, such as inorganic bisulphites, have been used commonly to stabilize pharmaceutical formulations where Maillard reactions are known to cause a problem, the addition of browning inhibitors is not generally an option for dairy products. However, the detrimental effects of Maillard reactions can be minimized through careful manipulation of processing and storage conditions.

Maillard reactions in dairy products are generally detrimental to the organoleptic, nutritional and functional qualities of the product and are therefore undesirable. However, some dairy processes exploit a controlled Maillard reaction during manufacture. For example, Maillard browning reactions are an integral part of the manufacture of products such as *dulce de leche* in Latin America (also known as *doce de leite* in Portuguese, *confiture de lait* in French or simply 'milk jam' in English) and *khoa* in India in which milk is heated in the presence of sucrose to produce brown products with a pleasant flavour (Pavlovic *et al.*, 1994; Srinivasan and Gopalan, 1994). Maillard reactions also play an important role in the generation of flavour during the manufacture of ghee, or clarified butter, and milk chocolate crumb (Minifie, 1989). Chocolate crumb manufacturing optimally requires high total solids (90–94%), pH 5.5–8.0 and a temperature of 72–77°C for 4–8 h, ideal conditions for Maillard browning.

7.2. Isomerization and Degradation of Sugars

Sugars may undergo five different types of reaction in solution:

- Anomerization
- Enolization/aldo-keto isomerization and epimerization: The Lobrey de Bruyn–Alberda van Ekenstein transformation resulting in the formation of products such as lactulose
- β-Eliminations which may or may not be accompanied by retro-aldol reactions to produce α-dicarbonyl derivatives and compounds such as furfural and hydroxymethylfurfural
- Benzylic acid rearrangements, resulting in the formation of, for example, lactic and saccharinic acids
- Maillard reactions with amino compounds (if present) to give products described above in addition to N-containing products, and S-containing products if sulphur amino acids are present.

Lobrey de Bruyn–Alberda van Ekenstein-type reactions (Figure 7.1) may be catalysed by acid, base or ions of alkaline earth metals (Speck, 1958). In the case of lactulose formation from lactose, the reaction is particularly favoured by increasing pH. In addition to their participation in Maillard reactions, amino acids probably contribute to the catalysis of LA reactions. As in the Maillard reaction (see below), enediols are key intermediates in LA reactions. Enolization is generally accepted to be the rate-limiting step whereas the subsequent β-elimination reactions are rapid in the presence of acid. In the case of 2-ketohexoses, such as fructose, the reaction rate is usually higher than that for aldoses because 1,2-enolization occurs more easily. In addition, the 2-ketoses can form a 2,3-enediol, leading to a wider spectrum of degradation products. Figure 7.1 shows, using lactose as an example, how epimerization among aldoses and ketoses (e.g. glucose $\leftrightarrow$ mannose or fructose $\leftrightarrow$ psicose) may be promoted by alkaline media in addition to aldose–ketose isomerization. At least in the case of [1-^{13}C]-mannose, it appears that epimerization to [1-^{13}C]-glucose and isomerization to [1-^{13}C]-fructose assume equal significance when the system is allowed sufficient time to equilibrate (7 d at 25°C and pH 11.5) (King-Morris and Serianni, 1986).

In the presence of oxygen, the double bond of enediols may be cleaved to produce two corresponding carboxylic acids; for example, formic and arabinonic acids are formed from glucose via this pathway. Retro-aldol cleavage of the double bond is possible at elevated temperatures or in the presence of concentrated alkali to produce hydroxyaldehydes or hydroxyketones. Since enolization is theoretically possible at any part of a molecule, the potential spectrum of products is enormous.

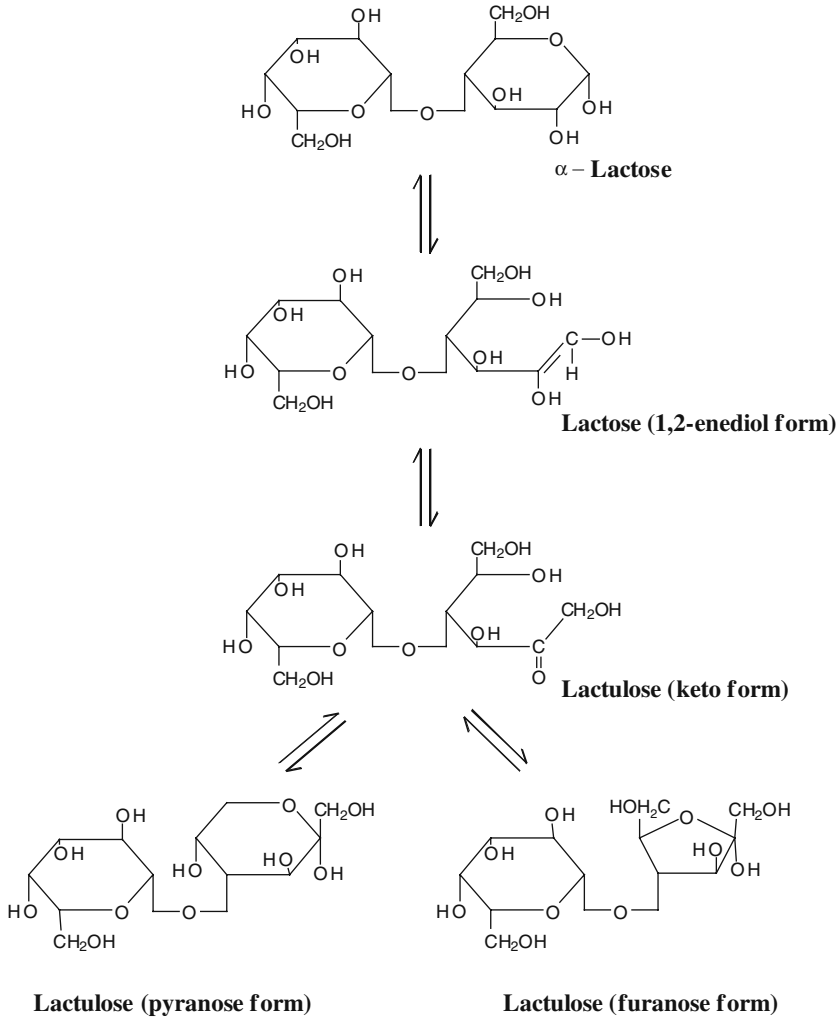

Figure 7.1. Formation of lactulose from lactose via the Lobrey de Bruyn–Alberda van Ekenstein transformation.

Saccharinic acids are products of the benzilic acid rearrangement of the 1,2- and 2,3-dicarbonyl compounds (Figure 7.2) derived from sugar degradation, yielding metasaccharinic acids (from 1,2-dicarbonyls), saccharinic acids (from 1-methyl-2,3-dicarbonyls) and isosaccharinic acids (from 2,3-dicarbonyls). pH has an important effect on such reactions: levulinic acid is characteristic of the oxidation of lactose under acidic conditions

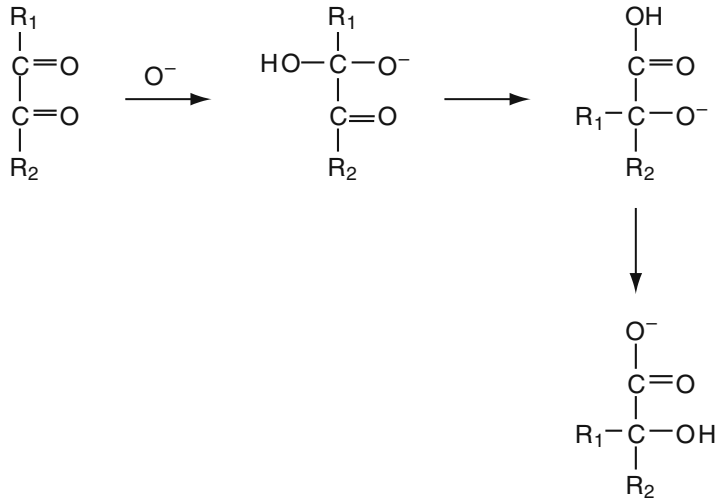

Figure 7.2. Benzilic acid rearrangement of an α-dicarbonyl compound to yield a saccharinic acid.

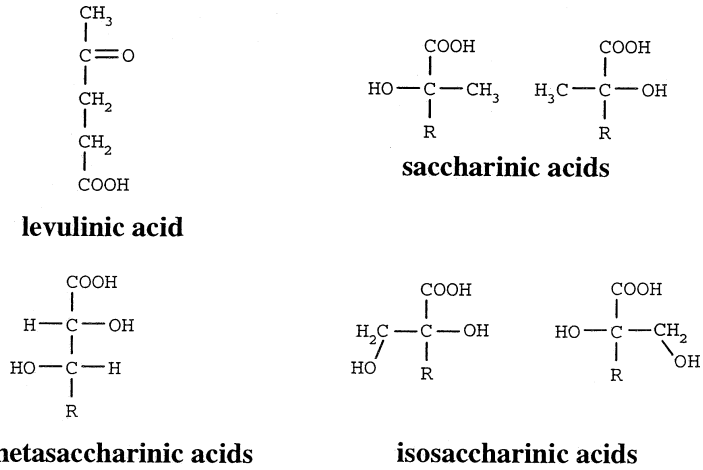

Figure 7.3. Some acid products of sugar degradation.

whereas saccharinic acids are characteristic of oxidation under alkaline conditions (Figure 7.3).

Dehydration of the 1,2-enediol derivatives via a series of β-eliminations is promoted under acidic conditions, resulting in the formation of

2-furaldehyde (e.g. from pentoses or hexoses following cleavage) or 5-hydroxymethyl-2-furaldehyde (e.g. from hexoses). The primary reaction products may, in turn, react via aldol condensations and intramolecular cyclizations (e.g. Cannizaro reactions) to form a range of other products. In the presence of amino acids, α-dicarbonyl compounds may react further in the Strecker reaction which results in the oxidation of the amino acid. In practice, the number of products formed in significant quantities is quite low and depends very much on the severity of heating. However, even a few micrograms of a product with a low odour threshold may be sufficient to have a major impact on the organoleptic quality of a product. In such circumstances, even minor pathways may assume major significance.

7.2.1. Lactulose

Although lactulose was first synthesized in 1929 (Montgomery and Hudson, 1930), its physiological significance was not recognized until 1957 when it was shown to stimulate the growth of bifidobacteria in the intestines of bottle-fed infants. Current figures for the global commercial production of lactulose are difficult to obtain. However, the number of commercial applications of lactulose, lactobionic acid and lactitol is increasing in foods and medical products for use as laxatives and for the modulation of the gut microflora (Mizota *et al.*, 1987; Tamura *et al.*, 1993). Commercial lactulose brand names include 'Cepahalac', 'Constilac', 'Cronolac', 'Constilose', 'Duphalac', 'Evalose', 'Lactogel' and 'Kristalose'. The medical applications of lactulose are now well established and a number of reviews are available (e.g. Als-Nielsen *et al.*, 2004). The production of lactulose for such applications was ~25,000 tonnes in 2006 with a market value of ~US$300m (Horton, 1995; Affertsholt-Allen, 2007). The high lactulose content of some severely heat-treated milks (e.g. in-container-sterilized liquid infant feeds) can lead to a laxative effect which has been shown to require as little as 2 g lactulose fed over 24 h (Andrews, 1986). In addition, some infant formulae have added lactulose for the reasons specified above.

Lactulose has attracted considerable interest as a possible indicator of the temperature/processing history of milk products (International Dairy Federation, IDF, 1993; Pellegrino *et al.*, 1995). The utility of lactulose in this respect was reviewed by Elliott *et al.* (2005). Lactulose content was reported to be on average more than 100-fold higher in sterilized milk samples than in pasteurized milks (Calvo and Olano, 1989). The cut-off currently used by both the IDF and the European Commission is 600 mg l^{-1} lactulose in UHT milk versus a corresponding range for sterilized milk of 600–1400 mg l^{-1}.

Lactulose is formed by the LA transformation of lactose via a 1,2-enediol intermediate. Lactulose is much less stable in solution than lactose and may subsequently degrade via β-elimination to give galactose, tagatose and saccharinic acids and other low molecular weight products (Olano and Martinez-Castro, 1981). Alternatively, lactulose may epimerize via a 2,3-enediol to form epilactose (4-*O*-β-D-galactopyranosyl-D-mannose; a C2 epimer of lactose). Lactulose was reported originally in heated milk by Adachi (1958) but its formation was erroneously attributed to the hydrolysis of the Amadori product, lactulosyl lysine (see below), in addition to the LA transformation (Adachi and Patton, 1961). Heating lactose solutions (pH 6.8) at 120°C for 30 min resulted in the formation of slightly more epilactose than galactose whereas the formation of lactulose was ~50-fold greater than that of epilactose or galactose accounting for up to 20% of the initial lactose content (Martinez-Castro *et al*., 1986). Interestingly, the quantitative differences among lactulose, epilactose and galactose appeared much less in a heated milk ultrafiltrate at pH 6.6. The changing balance in the formation of lactulose, epilactose and galactose in the heated milk ultrafiltrate was attributed by Martinez-Castro *et al.* (1986) to a decrease of pH (from 6.6 to 6.0) as a result of precipitation of calcium phosphate on heating. In the heated milk ultrafiltrate (20 s at 145°C, pH 6.6), the formation of lactulose, epilactose and galactose was reported to be 61.3, 11.3 and 4.4% of total carbohydrate, respectively.

The commercial demand for lactulose has stimulated research into more efficient production methods from lactose or milk ultrafiltrate feedstock. Such research has focused on improving the chemical isomerization of lactose mainly in alkaline media (Montilla *et al.*, 2005a; Aider and de Halleux, 2007). The production of lactulose using enzymatic transgalactosylation using microbial enzymes is also feasible, but may be cost prohibitive (Mayer *et al.*, 2004). Most lactulose sold for medical use is available as concentrated aqueous solutions. Progress has also been made in the manufacture of stable powder forms (Mizota *et al.*, 2004).

More severe treatment of lactose, e.g. acid hydrolysis, may result in the formation of several oligosaccharides by hydrolysis and condensation reactions. The process has been termed 'reversion' in the literature (Clamp *et al.*, 1961). Huh *et al.* (1991) demonstrated the formation of 17 disaccharide derivatives and four anhydro derivatives following acid hydrolysis of a 30% lactose solution. The products included the disaccharides isomaltose, gentiobiose and melibiose, and the anhydro sugar, levogalactosan (Figure 7.4). A number of the disaccharides had (1→4), (1→3), (1→2) and (1→1) glycosidic linkages, but the authors suggested that the preferred linkage appeared to be (1→6), which was present in the majority of the disaccharides characterized.

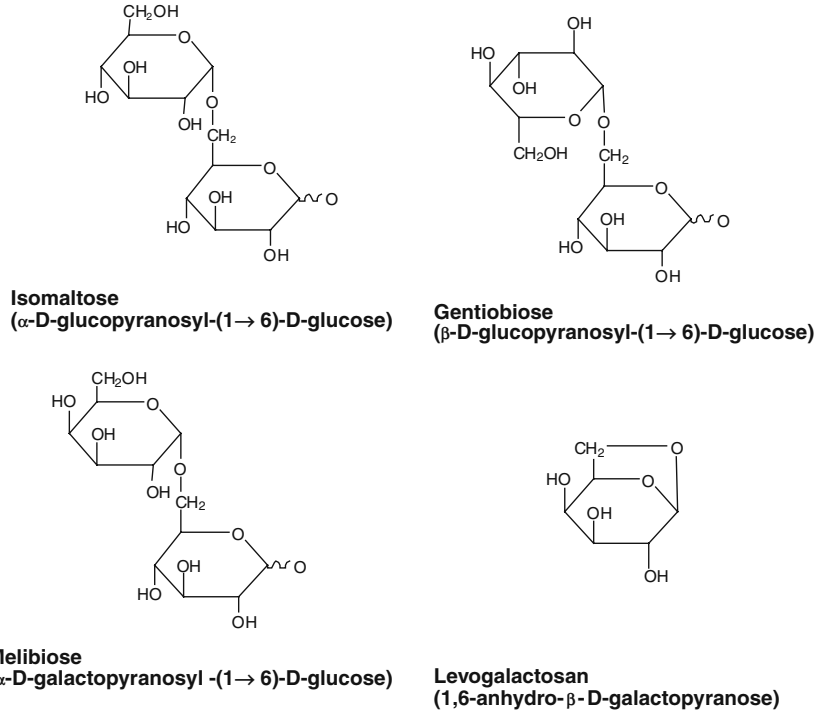

Figure 7.4. Some 'reversion' products of lactose following acid hydrolysis (after Huh *et al.*, 1991).

Although precipitation of tertiary calcium phosphate, hydrolysis of organic casein phosphate and the production of organic acids all contribute to the decrease in pH in heated milk, the most important from a quantitative point of view is the production of organic acids. Of the organic acids, formic is the most important contributor to acidity in heated milks. Based on titratable acidity and HPLC analysis of a heated skim milk (~20 min at 140°C), Berg (1993) concluded that almost all (>95%) of the acid formed is formic acid. The loss of positive charges on protein molecules as a consequence of Maillard reactions is a minor contributor to the decrease in pH under most circumstances. Many workers have noted that the pH decrease in heated milk is strongly influenced by headspace composition (Sweetsur and White, 1975; Fox, 1981). Sweetsur and White (1975) reported that the rate of pH decrease in heated milk was greatest when the headspace contained oxygen, intermediate in the presence of air and least in the presence of nitrogen. It was concluded that oxygen exerts its effect by promoting lactose degradation. In

lactose-free milk, the decrease in pH on heating is dramatically less. pH also decreases during the storage of UHT milk; the extent of the change is proportional to lactose content and to the extent of browning and is higher at elevated temperatures (Venkatachalam *et al.*, 1993). Although formic acid is derived from degradation of sugar moieties, its formation was increased in heated milk at higher casein concentrations, suggesting the involvement of a Maillard reaction (Berg, 1993). Berg (1993) concluded that from a quantitative point of view, direct degradation of lactose is a more important source of heat-induced acidity in milk than the Maillard reaction.

The degradation of lactulose also results in the formation of several C5 compounds. In addition to the formation of furfural and furfuryl alcohol, as discussed earlier, the formation of 3-deoxypentosulose (Troyano *et al.*, 1992a) and deoxyribose (Berg, 1993) has been reported.

The concentration of lactulose in UHT milks has been reported to range from 2 to 25 mg dl^{-1} while the concentration may exceed 75 mg dl^{-1} in sterilized milks (Olano and Calvo, 1989). Interestingly, Klostermeyer and Geier (1983) reported that the lactulose content of UHT milk increased during a production cycle, which they attributed to the build-up of alkaline scale on the heating surfaces. Their observation may offer a partial explanation for the higher lactulose concentration in indirectly heated UHT milks than in those subjected to direct heating. In addition, it is likely that the greater severity of heating during indirect UHT processing contributes to a higher lactulose content (Elliott *et al.*, 2005). Based on the differences between directly and indirectly heat-treated UHT milks, Andrews (1984) suggested that lactulose concentrations could be used to classify such milks according to the processing method.

In UHT milks, the concentration of galactose has been reported to range from 9 to 12 mg dl^{-1} and the concentration exceeds 16 mg dl^{-1} in in-container-sterilized milks, although it is unclear to what extent non-enzymatic degradation of lactose may contribute to such levels. Berg (1993) reported that the amount of free galactose formed in milk at 140°C was more than 60% that of the lactulose formed. Epilactose concentrations up to 5 mg dl^{-1} have been reported for in-container-sterilized milk with values of 1–3 mg dl^{-1} for UHT milks. Tagatose formation appears to require relatively severe heating conditions, such as in-container sterilization (Troyano *et al.*, 1992b, 1994). However, its formation in skim milk powder during storage has been reported; no tagatose was detectable in fresh spray-dried milk powder (Troyano *et al.*, 1994).

Calvo and Olano (1989) reported that the presence of proteins in milk systems reduced the amount of lactulose formed whereas it increased the formation of epilactose and galactose. Furthermore, these authors showed that the galactose did not originate from the degradation of lactose–protein

Amadori products, indicating that the direct degradation of lactose was involved. Studies on the degradation of casein-bound lactose (assumed by the authors to represent the Amadori compound plus Schiff's base) showed the formation of formic acid and galactose, but no HMF or lactulose was detected (Berg, 1993). The yield of galactose appeared to be quite high (~85% of the concentration of the sugar–protein complex after 23 min at 120°C). However, at 140°C, the concentration of galactose began to decrease after 13 min, presumably because of Maillard and degradation reactions of the free sugar. A small amount of lactose was released, presumably from Schiff's base or glycosylamine. Earlier studies (Martinez-Castro *et al.*, 1986; Andrews & Prasad, 1987) suggested that the formation of lactulose in synthetic milk ultrafiltrate was lower than that in milk subjected to similar heat treatment, possibly a result of precipitation of calcium phosphate. Berg (1993) reported that the amount of lactose degraded and the amounts of lactulose and galactose formed increased with increasing casein concentration. The formation of formic acid was also increased at higher protein concentrations. Thus, it is possible that the breakdown of lactulose is promoted at higher protein concentrations. In early papers, this may have led to the erroneous conclusion that protein inhibits lactulose formation. The effect of casein in promoting lactulose formation and degradation is probably related to the buffering capacity of the protein; in the presence of casein the pH decrease on heating is retarded. Furthermore, in the presence of casein, lactulose may be degraded via at least two pathways: via Maillard reaction with casein to form lactosyl lysine adducts and via direct degradation to form galactose, formic acid and other products, the formation of which may be catalysed by the protein.

Protein also affects the levels of HMF and furfural detected. Berg (1993) reported that less free and total HMF were formed in the presence of casein. Since the development of brown colour is predictably greater in the presence of casein, it may be suggested that HMF reacts with casein to produce advanced products of the Maillard reaction. This view is supported by the experiment of Berg (1993) who showed that HMF and furfural were degraded slowly on heating in the presence of casein but appeared to be stable in the absence of protein. Fat content does not appear to influence the kinetics of lactulose formation although the rate of furosine formation appeared to be higher in milk with a higher fat content (Claeys *et al.*, 2003).

Lactulose may react via its keto group with β-lactoglobulin in a Maillard reaction to yield the Heyns rearrangement product, lactosyl lysine (Matsuda *et al.*, 1991). Although lactose is approximately 10 times more reactive in Maillard reactions than lactulose, it appears that the latter is a stronger inducer of protein crosslinking/polymerization on a weight basis than lactose. However, the nature of the actual crosslinking intermediate is

not known. Similarly, the extent of fluorescence development was slightly higher in the lactulose-β-lactoglobulin system than in a lactose-β-lactoglobulin reaction. The release of galactose upon degradation of lactulose is of interest because of its greater reactivity in Maillard reactions than disaccharides or other hexoses (Chavez-Servin *et al.*, 2004). Thus, at least some of the free galactose in heated milks would be expected to react with the ε-NH_2 groups of lysine to form the Amadori product, tagatosyl lysine. The major pathway for the formation of galactose in heated milk is undoubtedly lactulose degradation. The involvement of Amadori product degradation in galactose formation is less clear. Based on studies of the model Amadori product, α-*N*-acetyl-ε-*N*-lactulosyl lysine, Calvo and Olano (1989) concluded that the Amadori product probably did not contribute to the formation of galactose in heated products. However, it is possible that *N*-acetyl-lactulosyl lysine may be more stable than the protein-bound Amadori product. Similarly, Henle *et al.* (1991) could not detect fructosyl lysine in heated milk, suggesting that the disaccharide glycosidic bond is stable.

7.3. Chemistry of the Maillard Reaction

7.3.1. Reaction Mechanisms and Pathways

The first step in the Maillard reaction involves the nucleophilic attack by the nitrogen atom of an amino compound on the electrophilic carbonyl group of an aldehyde or ketone; in food systems, the reactants are predominantly proteins and reducing sugars, although carbonyl products of lipid peroxidation (El Zeany, 1982; Nielsen *et al.*, 1985a; Kaneko *et al.*, 1991), vitamin C (Hayashi *et al.*, 1983), free amino acids and ammonia are also important reactants under some circumstances. The mechanism depends on the ability of the molecule to bear the negative charge on the carbonyl oxygen. Potman and van Wijk (1989) favoured an S_N1 mechanism in which the cyclic pyranose conformation reacts, the leaving group being either the anomeric -OH group (leading to the formation of a cyclic carbocation) or the ring oxygen leading to ring opening. Similarly, the validity of the S_N1 mechanism would depend on the stability of the intermediate carbocation (Isaacs, 1987). The reaction proceeds with the elimination of a molecule of water to form a Schiff's base, which subsequently rearranges to form an *N*-substituted glycosylamine intermediate (Figure 7.5) which has been reported to adopt a β-pyranose structure in the $^4C_1$ conformation (Potman and van Wijk, 1989). The amino acid carboxyl group plays an important role in the catalysis of the Amadori rearrangement. When the carboxyl group is absent (e.g. in aliphatic or aromatic amines) the glycosylamine is more stable and, in many

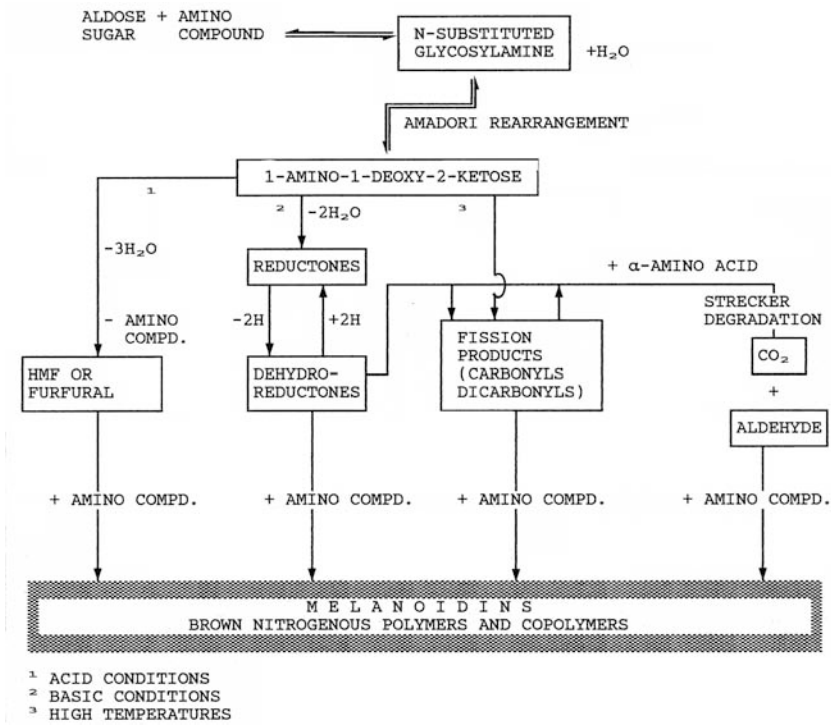

Figure 7.5. Steps in the Maillard reaction.

cases, has been isolated. Thus, *N*-substituted glycosylamines derived from amino acids are inherently unstable and they are either hydrolysed to the parent amino acid and reducing sugar or react via a spontaneous rearrangement to form the corresponding keto-(α-1-amino-1-deoxy-2-ketose) or aldo-(α-2-amino-2-deoxyaldose) derivative, depending on whether the parent sugar is an aldose or a ketose, respectively. The rearrangements are similar chemically to the LA transformation of sugars in heated alkaline solutions. The aldo→keto transformation is referred to as the Amadori rearrangement and the corresponding keto→aldo rearrangement as the Heyns rearrangement. As in the case of LA transformations, such reactions are multistep; for example, in the case of Amadori rearrangements, glycosylamines rearrange first to 1,2-enaminols via a sigmatropic shift followed by ketonization to the Amadori rearrangement product. It appears that the formation of the imminium ion by dehydration during the Amadori rearrangement is rate limiting. Acid catalyses this step, with a maximum rate reported to be between pH 2 and 5 (Yaylayan and Huyghues-Despointes, 1994). Thus, the chemistry of the

reacting carbonyl compound has a major influence on the mechanism of the Maillard reaction because the Amadori and Heyns compounds differ considerably in their reactivity (Pilkova *et al.*, 1990). Heyns products are unstable compared with their corresponding Amadori products, especially in the presence of amino acids. They readily react further to produce Amadori products via an LA-type transformation. However, in spite of their inherent instability, the rate of browning of Heyns products has been reported to be slower than that of corresponding Amadori products.

Although the degradation of the sugar moiety during the early stages of the Maillard reaction is irreversible, some of the amino acids may be recovered by acid hydrolysis of the Amadori product. While Amadori and Heyns products would be expected to brown more readily than mixtures of the corresponding amino acids and sugars (Westphal *et al.*, 1988), such products are relatively stable in food systems and, in many products, are the major or only products of the Maillard reaction.

Glycosylamines may also decompose via a free radical mechanism involving the formation of *N*,*N*′-dialkylpyrazine cation radicals (Hayashi and Namiki, 1981). Such radicals are formed prior to the Amadori rearrangement in a process believed to involve fragmentation of the glycosylamine in a reverse aldol reaction to give 2-carbon enaminols (see review by Rizzi, 2003).

7.3.2. Reactions of Amadori and Hynes Products

The Amadori product of amino compounds is a secondary amine and, as such, may react with a second molecule of sugar to form a diketosyl amine, although this would be expected to be a relatively minor pathway due to both electronic and steric effects. However, once diketosyl Amadori products are formed they are highly reactive and brown more readily than monoketosyl Amadori products. The addition of sugars tends to promote the browning of Amadori products whereas amino acids tend to inhibit browning (Nursten, 2005). The former may be associated with the formation of reactive diketosyl Amadori rearrangement products. The latter is probably due to inhibition of the deamination step (see below).

The extent to which Amadori product degradation contributes to advanced products of the Maillard reaction (including browning) in food and biological systems is contentious and somewhat difficult to quantify. There is evidence that non-Amadori pathways become more significant with increasing pH (>8). Based on observations on the kinetics of Maillard reactions at high and low temperatures, and studies on the fragmentation of sugars by electron impact and Amadori products, Yaylayan (1990) suggested that the direct dehydration of the cyclic forms of Amadori compounds may occur at high temperatures. Similarly, the direct dehydration of the cyclic

forms of the parent sugars may also be possible. It is envisaged that such dehydrations occur via the formation of pyrylium or furylium ions, which may then react with each other to form polymers (Yaylayan, 1990; Yaylayan and Lachambre, 1990). Amadori products may decompose by dehydration reactions or by thermally induced non-hydrolytic scissions (C–C and C–N bond cleavages). The latter become more important at elevated temperatures. Under acidic conditions, the nitrogen atom of the Amadori compound is protonated, and 1,2-enolization (designated the 1,2-E pathway) is promoted by the withdrawal of electrons from C1 of the sugar residue by the positively charged nitrogen atom. As the pH increases, the deprotonation of the nitrogen atom increases the electron density at C1 of the sugar moiety which, in turn, discourages 1,2-enolization. Thus, as pH increases, 2,3-enolization (designated the 2,3-E pathway) becomes more favourable. This effect of pH on the electron density across C1 and C2 is more pronounced with Amadori products derived from basic amino acids. The 2,3-E pathway is a particularly important source of flavour volatiles in food systems, yielding furanones, pyranones and Strecker degradation precursors. The mechanistic similarity between Maillard reactions and caramelization was emphasized by Feather (1981) who used the term 'amine-assisted sugar dehydration reactions'. However, in contrast to caramelization-type reactions, Amadori products can undergo enolization more readily and under milder conditions than the corresponding sugars (as a result of the stabilizing influence of the amino moiety). Thus, the formation of Amadori and Heyns rearrangement products in Maillard reactions can be regarded as low-energy pathways for the decomposition of sugars compared with caramelization.

The hydrolysis of the imminium ion has been reported as the possible rate-limiting step in the decomposition of the Amadori compound to form 5-hydroxymethyl-2-furaldehyde (Yaylayan and Forage, 1991). Similarly, cleavage of the C–N bond in the 2,3-E pathway may be a rate- determining step (Yaylayan and Forage, 1991). There is evidence that amino acids may actually inhibit the decomposition of Amadori products to form brown pigments, possibly due to inhibition of the deamination steps of the 1,2- and 2,3-E pathways or because of reaction with the carbonyl groups of the Amadori compound forming less reactive derivatives (Nursten, 2005). The presence and nature of amino acids have a strong influence on the pathway of reaction of Amadori products and the products formed. Yaylayan and Mandeville (1994) showed a marked increase in the formation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, hydroxymaltol and maltol from the Amadori product, fructosyl proline, when an equal weight of alanine was added. Addition of proline to the fructosyl proline led to significant, though less marked, increases in the yield of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one and hydroxymaltol. Simple methods for following the 1,2-E

and 2,3-E pathways have been developed involving, for example, the determination of products such as 2-furaldehyde (1,2-E) or furanone (2,3-E) derivatives (Feather, 1981). However, relatively little is known of the factors that influence such pathways, despite their importance in flavour chemistry. Many papers have focused on the chemistry of deoxy-dicarbonyl compounds (e.g. deoxyglycosuloses) formed in the Maillard reaction (Feather, 1989). The deoxglycosuloses are formed by the deamination and dehydration of Amadori compounds (Hodge, 1967).

7.3.3. Deoxyglycosuloses

Some of the most reactive intermediates in the Maillard reaction are the deoxydiketoses (systematically referred to as deoxyglycodiuloses) and deoxyaldoketoses (systematically referred to as deoxyglycosuloses; formerly designated as 'osones') derived from degradation of Amadori products. Since 3-deoxyglycosulose is a product of the 1,2-E pathway and 1-deoxyglycosulose is a product of the 2,3-E pathway, their formation is influenced by the pH of the system and the basicity of the amino compound as discussed earlier. As in the case of Amadori and Heyns products, compounds such as 3-deoxyglucosulose are stabilized by the existence of several cyclic forms. Similarly, it has been suggested that 1-deoxyglycodiuloses exist as cyclic hydroxyfuranones and hydroxypyranones in solution (Yaylayan and Huyghues-Despointes, 1994).

In contrast to 3-deoxyglycosuloses in Maillard systems, aqueous solutions of pure 3-deoxyglucosuloses are unexpectedly stable. 3-Deoxyglucosulose also appears to be relatively stable under acidic conditions; the major degradation product is 5-hydroxymethyl-2-furaldehyde with smaller amounts of lactic and formic acids. Labelling studies have demonstrated that the carbon atom of formic acid and the aldehyde carbon atom are derived exclusively from C1 of 3-deoxyglucosulose (Weenan and Tjan, 1992). Under mildly basic conditions (0.1 M K_2HPO_4), a high yield of metasaccharinic acid is formed with complete decomposition of 3-deoxyglycosulose (Weenan and Tjan, 1992).

In the presence of amino compounds, the formation of 5-HMF from 3-deoxyglucosulose tends to be suppressed in favour of nitrogen-containing compounds, e.g. pyrroles and pyridinium betaines. The significance of pyrrole formation from 3-deoxyglucosulose derives from the reactivity of the hydroxymethyl carbon giving rise to the potential for protein crosslinking. The pyrrolealdehyde crosslink pyrraline is formed from the reaction of 3-deoxyglucosulose with the lysine Amadori product.

The 1-deoxy-2,3-glucodiulose compound formed via the 2,3-E pathway is considerably less stable than 3-deoxyglucosulose and has never been

isolated in the pure form although its formation as an intermediate has been inferred mechanistically by several workers. A number of studies have examined the formation of reactive osulose intermediates in the Maillard reaction using the trapping reagent *o*-phenylenediamine, and measuring and characterizing the quinoxaline derivatives formed. Such an approach was used to show that the 3-deoxyhexosulose and 1-deoxy-2,3-hexodiulose intermediates are formed from 1-deoxy-1-propylamino-D-fructose in the ratios 1:20 and 5:8 at pH 7 and 4.5, respectively, after 10 h under reflux conditions (Beck *et al.*, 1988).

Deuterium exchange studies suggest that the free C2 keto group is not necessary for the degradation of 3-deoxyglucosulose to hydroxymethylfurfural (Weenan and Tjan, 1992; Yaylayan and Huyghues-Despointes, 1994). A mechanism for the above reaction involving furylium ion formation has been proposed by Yaylayan (1990) and obviates the need for ring opening.

Yet another reaction pathway of glycosulose intermediates is fragmentation. Weenan and Tjan (1992) suggested retro-aldol fragmentation (3,4-scission and 4,5-scission) of glycosulose intermediates to account for pyrazine formation (via the resulting mono- and di-carbonyl species). Fragmentation reactions are discussed in detail below. As in the case of enediols produced from LA transformations, oxidative cleavage may result in the splitting of glycosuloses between the two carbonyls (Ledl and Schleicher, 1990). Such reactions result in the formation of products such as 2-deoxypentaenoic acid lactone and formic acid from 3-deoxyglycosulose.

Both 1- and 3-deoxyosuloses are products of the decomposition of Amadori products. It has been reported that 4-deoxyosuloses are produced by the treatment of sugars with alkali and are thought to be precursors of isosaccharinic acids (Miller & Cantor, 1952). The acid-catalysed degradation of hexoses and pentoses to 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde, respectively, is also believed to proceed via 3-deoxyosulose intermediates (Feather, 1970). It is now widely accepted that both 1- and 3-deoxyosuloses are important intermediates in flavour production in food systems. In particular, 3-deoxyosuloses are extremely reactive intermediates in the Maillard reaction (Glomb *et al.*, 1991). 3-Deoxyosuloses are powerful protein crosslinking agents in laboratory experiments (Igaki *et al.*, 1990)

In addition to the formation of 3-deoxyglycosuloses and 1-deoxyglycodiuloses, the formation of 1-amino-1,4-dideoxy-2,3-hexodiuloses has been suggested by the results of trapping experiments. This product is formed via the 2,3-E pathway following elimination of the C4 hydroxyl group from the enediol. However, it is not clear what role 1-amino-1,4-dideoxy-2,3-glycodiuloses play in the Maillard reaction (Yaylayan and Huyghues-Despointes, 1994).

7.3.4. Formation of Low Molecular Weight Maillard Products in Dairy Products

As might be expected from the mechanistic considerations discussed above, heating acidified skim milk or a neutral or acidic lactose solution results in the formation of HMF in relatively high yield (Patton, 1950a,b). No furfuryl alcohol was detected under such conditions. In concentrated skim milk and weakly alkaline lactose solutions, both furfuryl alcohol and HMF were formed (Patton, 1950b). Furfuryl alcohol is probably formed on reduction of furfural. Furfural and furfuryl alcohol are also formed in stored casein (Ramshaw and Dunstone, 1969). Patton (1950a) suggested that HMF in heated milk systems is formed largely via the Maillard reaction since it could not be detected on heating lactose solutions in the absence of casein or glycine. By contrast, however, Berg (1993) reported that free HMF and furfural levels were slightly higher in lactose-only model systems (at 140°C) than in systems containing casein. Furthermore, Berg (1993) reported that the levels of HMF formed from a lactose–protein complex at 140°C were only ~1% of those formed in normal skim milk heated under similar conditions. No furfural was detected under such conditions. The formation of furfural, HMF and furfuryl alcohol is an order of magnitude less than that of lactulose and galactose. Although HMF is an important product in heated milk, the concentration of lactulose can be more than 20 times that of HMF in UHT milk (75 d storage at 50°C) (Jimenez-Perez *et al*., 1992). However, the concentrations of HMF and lactulose in UHT milk change differently with time. Jimenez-Perez *et al.* (1992) reported that while the concentration of lactulose decreased after 75 d storage at either 40 or 50°C for all of the commercial lots studied, HMF content increased steadily over the 90 d storage period. In UHT milks, the levels of furfural formed may be more than 20 times lower than those of HMF. By comparison, the levels of furfuryl alcohol and HMF in UHT milk have been reported to be similar (380 μmol kg^{-1} after 23 min at 140°C) (Berg, 1993).

Diacetyl is a major volatile fission product of sugars, especially during heating at alkaline pH values (Hayase and Kato, 1986). Shipe *et al.* (1978) suggested that diacetyl is a major contributor to the rich note of heated milk, with minor contributions from maltol and acetophenone. Fission products, such as methylglyoxal and pyruvic acid, have also been identified in heated milk. Such fission products may react readily with amino compounds to form advanced Maillard reaction products.

The pyranone, maltol, has long been known to be formed in heated milks (Patton, 1950a). Maltol has been suggested as a possible contributor to the flavour of heated milk although it is of secondary importance to the contribution of diacetyl (Shipe *et al.*, 1978). The formation of maltol is an

important feature of Maillard and caramelization reactions in milk systems compared with other foods. In the presence of amino compounds, maltol may be formed from disaccharides or monosaccharides. However, in the absence of amino compounds, monosaccharides do not result in maltol formation. The formation of maltol is promoted under alkaline conditions. Although maltol may be formed via the 2,3-E pathway, Yaylayan and Mandeville (1994) reported that the favoured pathway for its formation is via *ortho*-elimination of the Amadori product. The addition of amino acids to the Amadori product, fructosyl proline, resulted in dramatic increases in the yields of 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one, hydroxymaltol and maltol (Yaylayan and Mandeville, 1994).

There is evidence that carbonyl intermediates of the Maillard reaction may react with sulphur compounds released in heated milk, leading to a decrease in the latter as the intensity of heat treatment is increased (Jaddou *et al.*, 1978). It appears that oxygen may also play a role in this process (Calvo and de la Hoz, 1992). The reactions of sulphydryl compounds formed in milk with Maillard intermediates have not been investigated in detail. However, many sulphur compounds are inhibitors of the Maillard reaction and such interactions may have an inhibitory effect on the reaction in milk systems by making carbonyl compounds unavailable for further reactions and browning. The reactions are also likely to be of organoleptic significance because of the powerful odour of low molecular weight S-compounds.

Strecker degradation reactions involving oxidative degradation of α-amino acids by dicarbonyl intermediates of the Maillard reaction are among the most important reactions in food systems. Such reactions result in the formation of unstable Schiff's bases which easily decarboxylate to form enamines and CO_2. The CO_2 is derived from the amino acid. The enamines subsequently undergo hydrolysis to form an aldehyde from the amino acid (Strecker aldehyde) and an α-aminoketone from the dicarbonyl compound. Dicarbonyl compounds are formed in both the 1,2-E and 2,3-E pathways. The key Maillard intermediate, 3-deoxyglucosulose, has been shown to react with L-phenylalanine in a Strecker degradation (Ghiron *et al.*, 1988). Strecker degradations have long been known to occur during the processing and storage of dairy products. Patton (1955) considered 3-methylbutanal to be a possible Strecker aldehyde of potential flavour significance in milk. Based on studies using ^{14}C-lactose, Dutra *et al.* (1958) suggested Strecker degradation as the major source of CO_2 produced during the sterilization of milk; only $\sim$4% of total CO_2 was attributable to lactose caramelization. Strecker aldehydes have been detected in pasteurized milk (3-methylbutanal, 2-methylbutanal), UHT milk (2-methylbutanal, isobutanal), sterilized milk (3-methylbutanal, 2-methylbutanal) and milk powder (2-methylbutanal, isobutanal) (Calvo and de la Hoz, 1992). In addition, Calvo and de la Hoz (1992)

suggested that Strecker degradation of phenylalanine and methylglyoxal might contribute to the formation of acetophenone in UHT and sterilized milks.

Metabolic products of dairy cultures have attracted attention because of their possible reactivity in Maillard reactions. Kowalewska *et al.* (1985) showed that the Maillard products, 2,5-dimethyl-4-hydroxy-3[2*H*]-furanone and pyrrolidine, could be produced from metabolic products of *Lactobacillus helveticus*. Griffith and Hammond (1989) attempted to model flavour generation in Swiss cheese by reacting carbonyl products known to be produced by *Lactobacillus delbruechii* subsp. *bulgaricus* with amino acids. The Strecker aldehydes, isovaleraldehyde, 2-methylbutanal, isobutanal, phenylacetaldehyde and methional, derived from leucine, isoleucine, valine, phenylalanine and methionine, respectively, were shown to be formed in reaction systems containing methylglyoxal. In addition, volatile Maillard reaction products of lysine and proline with various carbonyls, such as glyoxal, methylglyoxal and dihydroxyacetone, were detected. These included pyrrolidines, pyrrolizines, pyridines and pyrazines. The results suggested that the final stages of flavour formation in Swiss cheese are dominated by Maillard reactions rather than by enzymatic or microbiological processes. Scanlan *et al.* (1968) reported the presence of furfural, phenylacetaldehyde, maltol, acetophenone and diacetyl in milk heated at 146°C for 4 s. Presumably, the phenylacetaldehyde was derived from the Strecker degradation of phenylalanine.

Extraction of spray-dried skim milk powder using a Likens–Nickerson apparatus and ether as solvent resulted in 196 individual GC peaks (Shiratsuchi *et al.*, 1994). Surprisingly, however, no Strecker aldehydes were detected. The Maillard reaction products, furfural and furfuryl alcohol, were the only furans detected. Shiratsuchi *et al.* (1994) suggested that furfural and furfuryl alcohol could be important flavour molecules in condensed milk but concentrations in skim milk powder were probably too low to be important.

Ferretti and Flanagan (1971) detected many potential products of the Maillard reaction in a lactose–casein mixture allowed to brown for 11 d (75% RH; 75°C): acrolein, 5-hydroxymethyl-2-furaldehyde, 2-furaldehyde, 3-hydroxy-2-butanone, acetol, acetol acetate, 2-furfuryl formate, 2,2'-bifuran, 1-(2'-furyl)'-2-butanone, 1-(2'-furyl)'-3-butanone, maltol, methyl-2-thiofuroate, 5-methyl-2-propionylfuran, 2-furfuryl vinylacrylate, methylethylpyrazine, C-4-alkylpyrazine, tetramethyl pyrazine, trimethylpyrazine, *N*-methyl-2-formyl pyrrole and *N*-methyl-2-acetylpyrrole. The formation of several furanones which were probably products of Maillard reactions was also reported. The volatiles present at high concentrations included several Maillard reaction products: acetic acid, acetol, 2-acetylfuran, 5-methyl-2-furaldehyde, furfuryl acetate, furfuryl alcohol and maltol. Many potential Maillard products were also detected in a 2-year-old skim milk

powder (Ferretti and Flanagan, 1972): 2-acetylfuran, 2-furaldehyde, 2-methylpyrazine, 2,5-/2,6-dimethylpyrazine, 2,3-dimethylpyrazine, 2-methyl-5-ethyl/2-methyl-6-ethylpyrazine, 2,3,5-trimethylpyrazine, 2-formylpyrrole, *N*-methyl-2-formylpyrrole, *N*-ethyl-2-formylpyrrole, 2-acetylpyrrole and maltol. The authors suggested that 2-furaldehyde probably contributes to stale flavour development in stored milk powder, along with some of the pyrazines and several other compounds. Since Maillard reactions contributed at least five of the 12 constituents associated with stale flavour in the stored milk powder, the study illustrated the critical importance of control of such reactions in maintaining organoleptic quality.

Sugar fragmentation has been recognized to play an even more critical role in the Maillard reaction than had hitherto been realized. Sugar fragmentation, *per se,* is not significant at low pH but becomes increasingly important as the pH is increased. Hayashi and Namiki (1986) proposed a new Maillard pathway involving cleavage of the sugar moiety of the Schiff's base, forming C2 and C3 fragments. Such products included glycoaldehyde, glyoxal, methylglyoxal, glyceraldehyde or their imine derivatives.

The significance of sugar fragmentation products is highlighted by their rates of browning compared with their parent sugars. At 80°C, the rates of browning with β-alanine of glyoxal, methylglyoxal, glyceraldehyde and glycoaldehyde were, respectively, $\sim$120, $\sim$650, $\sim$2000 and $\sim$2000 times that of glucose. Thus, even a small amount of sugar fragmentation could make a large contribution to browning. By comparison, a mixture of 3-deoxyglucosone and β-alanine browned $\sim$140 times faster than glucose and β-alanine.

5-Hydroxymethyl-2-furaldehyde is formed by the decomposition of Amadori compounds under acidic conditions via the 3-deoxyosulose intermediate. It has a high molar extinction coefficient at 280 nm and is probably a major contributor to the increase in A_{280} that is a characteristic of Maillard reaction systems (Feather, 1989). Furaldehydes are key intermediates in the formation of high molecular weight melanoidin pigments. 2-Furaldehyde has been shown to react readily with glycine via its aldehyde group (Obretanov *et al.*, 1983) and 5-HMF may react with reducing sugars at high temperatures via both the aldehyde and hydroxymethyl groups (Urashima *et al.*, 1988).

Both the Amadori products, lactulosyl lysine and fructosyl lysine, have been shown in heated milks, the latter ostensibly arising from the hydrolysis of the 1$\rightarrow$4 bond of lactulosyl lysine resulting in the release of galactose (Moller *et al.*, 1977a). However, it is unclear to what extent such a mechanism could account for the evolution of free galactose in heated milks compared with the degradation of lactulose. Even in the presence of extensive Maillard reactions in UHT milk, the concentration of fructosyl lysine is only about 10% that of lactulosyl lysine (Moller *et al.*, 1977b). The ratio of lactulosyl

lysine to fructosyl lysine decreases with increasing temperature and duration of storage suggesting that hydrolysis of the glycosidic bond might be taking place. However, it is also clear that hydrolysis of the glycosidic bond is difficult and probably does not occur at the Amadori stage (Pischetsrieder and Severin, 1994).

Compared with the Amadori products of other disaccharides and of glucose, the Amadori product of lactose and the ε-NH_2 of lysine appears to be relatively stable (Kato *et al.*, 1988, 1989). Kato *et al.* (1988) showed that although the decrease in free amino groups of ovalbumin was similar in systems containing lactose or glucose, the rate of browning and polymerization was higher in the presence of glucose. Kato *et al.* (1989), who studied the reaction of ovalbumin with the disaccharides, maltose, cellobiose, isomaltose, lactose and melibiose, showed that the production of advanced Maillard reaction products (brown colour and fluorescent compounds) was weakest in the maltose, lactose and cellobiose systems. The authors concluded that the effect was due to the nature of the non-reducing pyranoside group and the position (1→4 or 1→6) of the bonds; the reducing moiety is glucose in the case of each of the systems studied. Thus, it appears that substitution at the C4 hydroxy position of glucose has a stabilizing effect on the corresponding Amadori product, as confirmed by experiments using 4-*O*-methyl-D-glucose (Kato *et al.*, 1988, 1990). The conclusions drawn from experiments on model systems appear also to be borne out in practice. Burvall *et al.* (1978) reported that although up to 40% of lysine was unavailable in a stored lactose-hydrolysed dried milk, there was no visible browning in the product, suggesting that the Maillard reaction had not progressed beyond the intermediate stages. The (1→4) linkage in lactose and some other disaccharides also influences the profile of Maillard reaction products formed subsequent to the Amadori rearrangement. For example, Patton (1950b) recognized that the structure of lactose favoured the formation of furfuryl alcohol in heated milk whereas those of glucose, galactose, sucrose or methyl-α-glucopyranoside did not. The influence of sugar structure on reaction mechanism is discussed in more detail below.

A variety of flavour volatiles produced in stored or heated milk systems have been identified as putative products of non-enzymatic browning reactions. These include benzaldehyde, methyl furans, acrolein, maltol, diacetyl and acetaldehyde (Ferretti and Flanagan, 1971, 1972; Calvo and de la Hoz, 1992).

In addition to the formation of brown pigments, fluorescent pigments are also formed in heated and stored milk (Patton, 1955). In general, the activation energy for fluorescence development is less than that for browning (Labuza, 1994), although it is not clear if this applies in the case of dairy products.

7.4. Factors that Influence Maillard Reactions

Both the overall rate and product profile of the Maillard reaction in foods are highly dependent on a number of parameters, the most important of which are reactants, pH and temperature. The impact of moisture content and water activity is mentioned briefly below but the physical state of the food system is beyond the scope of the present review. Readers are referred to the papers by Lievonen and Roos (2002), Miao and Roos (2004) and Thomas *et al.* (2004) for further information.

7.4.1. Reactants

Both the nature and the molar ratio of the reacting species have a considerable influence on the rate and mechanism of the Maillard reaction. In general, low molecular weight reactants tend to react more readily than high molecular weight reactants, partially as a result of steric hindrance in the latter. Thus, glucose is more reactive than lactose and contributes to the increased rate of browning in lactose-hydrolysed milks (Lea, 1948; Evangelisti *et al.*, 1994). Colour intensity produced in model systems may be ranked according to the reacting sugars as follows: xylose>arabinose>fructose>glucose>maltose>lactose (Yang and Shin, 1980). Although glucose, mannose (C2 epimer of glucose) and galactose (C4 epimer of glucose) all resulted in a similar loss of free amino groups on reaction with ovalbumin, the browning that developed in the galactose-containing system was two- to threefold higher than with the other sugars (Kato *et al.*, 1986). In addition, ovalbumin stored in the presence of galactose showed a dramatic decrease in solubility (~40% that in the presence of glucose or mannose). Comparisons with talose, which has the same configuration as galactose at C3 and C4, suggested that the configuration contributed to the high rate of advanced reactions preceding browning in the galactose system. It was suggested that the configurations of galactose and talose contribute to a higher browning rate by stabilizing the cyclic chair conformations of the Amadori products by hydrogen bonding (Kato *et al.*, 1986). It is not known whether sugars are released from glycoproteins in heated milk and participate in Maillard reactions. However, even if such reactions occur, their quantitative significance would be negligible relative to the huge excess of lactose in milk.

Steric effects play an important role in determining the reactivity of amino groups on proteins. Thus, it appears that some internal amino groups on globular proteins, such as β-lactoglobulin, may be inaccessible to sugars and unavailable for Maillard reactions. In addition, the pK_a value of amino groups and acid–base catalysis by adjacent proton donor/acceptor groups contribute to the reactivity of individual amino groups. Although all the

major milk proteins have an abundance of reactive groups, the whey proteins and α_{s2}-casein would be expected to show the highest activity in carbonyl-amine reactions based on amino acid composition. Of the protein-bound amino acids, lysine is usually the most reactive, followed by tryptophan, histidine and arginine. Among other factors, the pK_a of the amino group and the distance between the amino and carboxyl groups of free and protein-bound amino acids influence their reactivity in Maillard reactions. In dipeptides, the presence of hydrophobic residues such as isoleucine, leucine or phenylalanine increased lysine reactivity, whereas the basic amino acids decreased reactivity (Mennella *et al.*, 2006).

The indolyl nitrogen atom of tryptophan is less reactive in Maillard reactions than the ε-NH_2 group of lysine because the lone pair of electrons on the nitrogen atom are delocalized about the ring. In practice, protein-bound tryptophan usually reacts with reducing sugars only to a limited extent. The ε-amino group of lysine is reactive and, because of its availability for reaction, is normally the predominant reacting amino function in proteins. Consequently, milk proteins, which are rich in lysine, tend to brown more readily than proteins low in lysine, such as soy proteins (Wolf *et al.*, 1977). In milk, experiments involving the incorporation of ^{14}C from ^{14}C-lactose during UHT processing suggested that casein micelles incorporated most of the radioactivity (5–6 times that of α-lactalbumin or β-lactoglobulin on a weight basis). Of the caseins, the most reactive appears to be κ-casein (Turner *et al.*, 1978). Clearly, this pattern of reactivity conflicts with what might be expected from the amino acid composition of the proteins. It might be expected that ε-NH_2 groups in micellar κ-casein would be more accessible to reacting sugars than NH_2 groups in the other caseins. Such preferential reactivity of κ-casein may have significant implications for the stability of milk proteins during storage. Similarly, it has been suggested that the compact globular structure of whey proteins makes some of their lysine residues inaccessible (Turner *et al.*, 1978), although a weakness in this argument is that heat-induced denaturation of whey proteins should increase the accessibility of such lysine residues. An alternative explanation for the unexpectedly low reactivity of whey proteins in Maillard reactions is that protein–protein interactions of denatured whey proteins in milk could make lysine residues unavailable for reaction with lactose. The study of Turner *et al.* (1978) did not quantify the release of ^{14}C in the form of low molecular weight products.

The reactivities of free amino acids differ dramatically from those of their protein-bound counterparts (Izzo and Ho, 1992). The reactivity of free amino acids with glucose was ranked as follows: lysine>glycine>tryptophan>tyrosine>histidine>arginine>cysteine (Ashoor and Zent, 1984). Such rankings are dependent on the reacting sugar and the pH of the system under study. In contrast, Labuza (1994) asserted that the assumption that lysine is the most

reactive free amino acid is erroneous and that tryptophan is ~30 times more reactive. In a comparative study on the reactivity of amino acids in total parenteral nutrition solutions, Labuza and Massaro (1990) reported that lysine browned the slowest whereas cysteine had the highest rate of browning (~20 times that of lysine). However, paradoxically, in a mixed system containing lysine, tryptophan and cysteine, there was evidence that cysteine acted as an inhibitor of browning (Labuza and Massaro, 1990). Cysteine, *N*-acetyl-L-cysteine and reduced glutathione have been reported to inhibit non-enzymatic and enzymatic browning in both model and real food systems (Friedman and Molnar-Perl, 1990; Molner-Perl and Friedman, 1990). The strong nucleophilicity of such sulphur compounds probably contributes to their activity as browning inhibitors. Among the possible mechanisms for the activity of sulphur compounds, the suppression of free radical formation and the trapping and inactivation of intermediates of non-enzymatic browning, thus preventing further reactions, have been proposed (Friedman and Molnar-Perl, 1990).

Clearly, many unrelated factors influence the reactivity of amino acids in the Maillard reaction. For example, on the basis of p*K*, arginine might be expected to be more reactive than lysine (the p*K* of the guanidino group is ~12 compared with 9.4–10.6 for the ε-NH_2 of lysine). However, the guanidino group has been reported not to undergo the Maillard reaction (Ledl and Schleicher, 1990). Nevertheless, protein-bound arginine undergoes extensive modification during the Maillard reaction by virtue of reactions involving α- and β-dicarbonyl compounds and lysine resulting in the formation of the imidazopyridinium crosslink, pentosidine.

It is generally agreed that an excess of reducing sugar over the amino compound promotes the rate of Maillard browning (O'Brien and Morrissey, 1989). Quantitatively, increasing sugar concentration at a constant amine concentration has a greater effect on the rate of browning than an increase in amine concentration at a constant sugar concentration (Labuza, 1994). Presumably, the latter results in a mass action inhibitory effect on the deamination steps of the enolization pathways. In addition, because of the recycling of amines in the reaction, the concentration of sugar is more likely to be rate limiting than that of the amino compound. Thus, increasing the lactose–protein ratio in an infant formula was shown to increase lysine blockage due to Maillard reaction (Evangelisti *et al.*, 1993).

There are significant quantitative and qualitative differences in the Maillard reactions of disaccharides compared with those of monosaccharides. Degradation of the 1-deoxyglycodiuloses of disaccharides shows major differences from those of monosaccharides, in part because the second sugar residue is a poor leaving group in the subsequent elimination reactions, forcing the selection of an alternative elimination. Thus, whereas the major products of heated monosaccharides (fructose or glucose) are

2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one and 4-hydroxy-2(hydroxymethyl)-5-methyl-3(2*H*)-furanone, the main products of maltose or lactose are 4-(glycosyloxy)-2-hydroxy-2-methyl-2*H*-pyran-3(6*H*)-ones, 4,5-dihydroxy-2-(glycosyloxy)-5-methyl-2-cyclopenten-1-ones and 4-(glycosyloxy)-5-(hydroxymethyl)-2-methyl-3(2*H*)-furanones (Pischetsrieder and Severin, 1994). One such product, 4-(galactosyloxy)-2-hydroxy-2-methyl-2*H*-pyran-3(6*H*)-one, has been detected in heated milk (Ledl *et al.*, 1986). The elimination of the galactosyl residue from the above molecule results in the formation of maltol (a flavour molecule, flavour enhancer and an antioxidant) in heated milk. Similarly, the disaccharide-specific 4-(glycosyloxy)-5-(hydroxmethyl)-2-methyl-3(2*H*)-furanones are strongly reducing and probably contribute to antioxidative activity. Thus, disaccharides, such as lactose, may be useful reactants in Maillard systems since they generate products not known to arise from monosaccharides.

Under mild processing conditions, non-reducing sugars, such as sucrose, do not react in Maillard reactions with amino compounds. At high temperatures and/or at low pH, sucrose may become a major reactant in Maillard reactions due to hydrolysis of the disaccharide. For example, in a comparative study, Lee and Nagy (1990) reported that the rate of 5-HMF formation from fructose was 31.2 times that from glucose whereas the rate from sucrose was 18.5 times that from glucose at pH 3.5 and 50°C. Moreover, casein–glucose and casein–sucrose systems showed similar losses of lysine and arginine when heated in the dry state at high temperatures (200 or 300°C $\times$ 1 h) (Smith and Friedman, 1984). Similarly, the addition of 8% cane sugar to cows' or buffalo milk resulted in severe browning and a decrease in tryptic hydrolysis when milk was sterilized at 104 kN m^{-2} for 20 min (Gothwal and Bhavadasan, 1991). The extent of browning has been reported to be higher in buffalo milk than in cows' milk (Gothwal and Bhavadasan, 1991; Srinivasan and Gopalan, 1994).

7.4.2. pH

The rate of the Maillard reaction has long been reported to increase with increasing pH up to a maximum at ~pH 9–10 (Ashoor and Zent, 1984; Pokorny *et al.*, 1988), depending on the amino acid involved. Bases can catalyse the initial steps of carbonyl-amine reactions by removing a proton from the nucleophile, increasing its nucleophilicity:

$$NuH + B^- \quad \rightleftharpoons \quad Nu:^- + BH$$

The browning of pure Amadori products is also accelerated at alkaline pH values (Westphal *et al.*, 1988). pH exerts a considerable influence on the

mechanism of the Maillard reaction by determining the type of enolization favoured (1,2- or 2,3-enolization) and hence the pattern of Amadori compound degradation. Consequently, the influence of other factors on reaction rate is frequently pH dependent. For example, the addition of D-alanine or L-lysine to an L-ascorbic acid model system had a small or negligible effect on browning rate at pH 5 or 7, whereas an increase in browning rate occurred when the amino acids were added at pH 8.0 (Löscher *et al*., 1991). In contrast, addition of glycine resulted in an increase in browning at all three pH values. The authors attributed this to a reaction of glycine with furfural produced on degradation of ascorbic acid with a resultant increase in brown pigment formation. By contrast with brown colour formation, the development of fluorescence in an epoxyaldehyde–lysine system appeared to be independent of pH above 6.0; brown colour development showed a maximum at pH 9.0 (Hidalgo and Zamora, 1993). At shorter incubation times, development of fluorescence was linear with increasing pH. Increasing pH may have a major, albeit indirect, effect on the Maillard reaction by increasing the rate of mutarotation of both the parent sugar and of hemiacetal and hemiketal forms of intermediates formed in the reaction. Since the pH of a system decreases during the course of Maillard browning (due to the disappearance of basic amino groups and the formation of formic, saccharinic and other acids), the buffering capacity of the system has an important effect on the rate of reaction. The inhibitory effect of supercritical carbon dioxide treatment on Maillard reactions appears to be due to a reduction in pH due to both high pressure and CO_2 (Casal *et al*., 2006).

7.4.3. Mutarotation

Since it is generally accepted that sugars can react only as their acyclic form (at least at low temperatures), the rate of mutarotation is likely to be a significant rate-limiting step because the amount of sugar in the open-chain form is normally limited to a few percent of total sugar. Vogel *et al.* (1988) reported that the rate of glucose mutarotation was minimal at pH 3. As pH is increased, the rate of mutarotation increased rapidly; the rate of mutarotation of glucose at pH 7 was reported to be approximately 8 times that at pH 3 (Vogel *et al.*, 1988). The rate of mutarotation also increases with increasing a_w and temperature (Angyal, 1984; Vogel *et al.*, 1988). The significance of mutarotation rate and the concentration of acyclic forms were studied by Yaylayan and Forage (1992) who reacted tryptophan with glucose or mannose; since they are C2 epimers, both sugars produce the same Amadori product which enables differences in reaction rate due to mutarotation to be determined. Mannose, which has a higher rate of mutarotation and a higher equilibrium concentration of acyclic form than glucose, reacted

1.3 times faster than the latter at 110°C and 1.8 times faster at 140°C. Glucose has one of the lowest equilibrium concentrations of acyclic form of the monosaccharides and one of the lowest rates in Maillard reactions among monosaccharides. Thus, it has been suggested that glucose may have been selected as the universal metabolic fuel during evolution because of its stability (Kaanane and Labuza, 1989). For example, fructose reacted ~7 times faster than glucose with haemoglobin at 37°C (Kaanane and Labuza, 1989)

Little is known of the influence of factors other than pH, temperature and a_w on mutarotation rate. Amino acids have been reported to be weak catalysts for the mutarotation of reducing sugars (Shallenberger, 1984). Depending on the presence of complex-forming configurations, the presence of ions such as Ca^{2+}, Mg^{2+}, Na^+ and K^+ can influence the tautomeric equilibrium of a sugar. It has been reported that Ca^{2+} shifts the tautomeric equilibrium of D-glucose towards the α-anomer. Addition of 1% $CaCl_2$ at a constant initial pH to hydrolysed concentrated whey (50% solids), caused a slight decrease in browning rate (Buera *et al.*, 1990). The authors suggested that a complexation reaction between Ca^{2+} and the sugar resulted in a shift in anomeric equilibrium. The effect was greater in the absence of protein. In contrast, Kaanane and Labuza (1989) reported that $FeCl_2$, $CaCl_2$, $CoCl_2$, KCl or NaCl had no significant effect on the mutarotation of glucose or fructose. However, there was a highly significant difference between systems studied in D_2O or H_2O, illustrating that solvent has an important influence on the kinetics of mutarotation. Addition of KCl, $CaCl_2$ or $MgCl_2$ had no effect on the formation of 5-HMF from glucose, fructose or sucrose in the presence of citric acid (Lee and Nagy, 1990). NaCl and NH_4Cl have been shown to have a significant inhibitory effect on the rate of browning in model food systems, including casein–glucose, when added to a concentration of 0.5% (w/w) (Pham and Cheftel, 1990).

7.4.4. Moisture Content and a_w

Both the moisture content and the a_w of a food system exert a major influence on the Maillard reaction. Water may influence the rate of reactions by controlling the viscosity of the liquid phase and by dissolution, concentration or dilution of reactants (Warmbier *et al.*, 1976; Labuza, 1980). At very low a_w values, the proportion of total reactants in solution is negligible and, therefore, the reaction rate is minimal. As the a_w increases, the concentration of reactants remains constant provided excess solute is available to maintain a saturated solution. However, the total volume in which the reaction takes place increases. Eventually, an a_w value is reached when the solution of reactants is diluted and the reaction rate decreases again. Since water is a product of the Maillard reaction, the a_w may increase with the development of

Maillard reactions, further destabilizing a food system. Conversely, as a product of the reaction, water may inhibit the reaction via a mass action effect (Eichner and Karel, 1972). Indeed where diffusion/dissolution are not limiting factors, as in liquid model systems containing organic solvents, the addition of water can lead to a dramatic decrease in browning rate (Peterson *et al.*, 1994). In studies on liquid cows' and buffalo milk systems, Gothwal and Bhavadasan (1991) reported that a 40% decrease in the concentration of total solids led to a 25.6 and 36.5% decrease in browning rate, respectively. It is generally accepted that rates of Maillard reactions are maximal at intermediate a_w values (0.5–0.7) (Labuza *et al.*, 1970). In milk powder, browning reactions are maximal at $a_w$values of 0.6–0.7 (Loncin *et al.*, 1968; Ben-Gera and Zimmerman, 1972), whereas in whey powders, the maximum occurs at an a_w of 0.44. However, the relative increase in reaction rate with a_w is less as temperature is increased (Malec *et al.*, 2002). The difference is related to the higher concentration of lactose in the latter and differences in physicochemical properties between the dried milk and dried whey systems (Huss, 1974a,b). The moisture content corresponding to maximum browning rates in a spray-dried skim milk powder were 6.5 and 7.5% at 35 and 130°C, respectively (corresponding to a_w values of 0.44 and 0.86, respectively, at those temperatures) (Franzen *et al.*, 1990). In addition, the induction period was shorter in high-moisture systems although the ultimate extent of browning was lower than in the low-moisture systems. In contrast, the addition of water to a liquid model system containing L-proline and D-glucose in propylene glycol led to an increase in the induction period which was more pronounced at lower temperatures (Peterson *et al.*, 1994).

Moisture content also appears to have a significant qualitative effect on the development of Maillard reactions in food systems. For example, in a reaction of casein and glucose in the dry state, the reaction involved mainly basic amino acids, whereas all amino acids were decomposed during solution state reactions (Kato *et al.*, 1981a). In addition, reactions of solution systems led to greater amounts of free amino acids and low molecular weight peptides, suggesting hydrolysis of peptide bonds. The influence of a_w on the mechanism of Amadori product degradation is poorly understood. However, Eichner and Ciner-Doruk (1979) proposed that the 1,2-E pathway assumes increasing importance as the water content increases. Significantly, a_w may influence the site specificity of Maillard reactions involving proteins (Wu *et al.*, 1990) and also the relative reactivity of individual amino acids (Leahy and Warthesen, 1983). Ideally, a_w should be used as a predictor of browning rate and other spoilage reactions in foods only in the absence of moisture sorption–desorption hysteresis (i.e. in fully equilibrated systems) (Franzen *et al.*, 1990; Franks, 1991). In non-equilibrium systems, it has been proposed that the moisture content is a more useful/valid predictor of browning. In addition,

moisture content has an advantage in that it avoids difficulties caused by the dependency of a_w on temperature. However, moisture content as a predictor of browning may be equally flawed if a phase change occurs in the system, which may be possible at constant moisture content.

7.4.5. Miscellaneous Factors

Phosphate, citrate and phthalate buffers have been shown to accelerate the Maillard reaction (Bobbio *et al.*, 1973). However, it is unclear if such anions had a catalytic effect *per se* or if the effect was simply pH related due to differences in buffering capacity. At constant pH in the range 5–7, phosphate has a dramatic effect on reaction rate which increases up to 15-fold over that of a phosphate-free system (Potman and van Wijk, 1989). Chan and Reineccius (1994) showed that the activation energy for the formation of 2-acetylfuran and di(H)di(OH)-6-methylpyranone in a model system was approximately halved by conducting the reaction in phosphate buffer. Potman and van Wijk (1989) concluded that phosphate acts as an acid–base catalyst during the Amadori rearrangement and does not react directly in the Maillard reaction. Increasing the concentration of phosphate or citrate increased the rate of browning in cows' or buffalo milk on sterilization (Gothwal and Bhavadasan, 1991). When citrate or phosphate levels were increased by 50%, there was an increase in the browning index of ~17–23% in buffalo or cows' milk, the increase being slightly greater in the latter. Although the pH values following sterilization were similar, the addition of phosphate or citrate salts led to a change in the initial pH (up to ~0.2 pH units for a 50% increase). It is likely that the increase in initial pH was responsible for the increase in browning. The study of Gothwal and Bhavadasan (1991) illustrates clearly the extreme sensitivity of browning reactions in milk systems to even small changes in pH. Citric acid has a marked catalytic effect on the rate of 5-HMF formation from fructose at constant pH (3.5) (Lee and Nagy, 1990).

The overall Maillard reaction and the rate of browning of Amadori products are accelerated by Fe(II), Fe(III) and Cu(II) (Patton, 1955; Kato *et al.*, 1981b; Pilkova *et al.*, 1990). Some of the effects of metals on the Maillard reaction may be due to pH effects, which is the case for aluminium and zirconium compounds (Powell and Spark, 1971). There is evidence that complex formation between intermediates in the Maillard reaction and the metal ion is a prerequisite for the promoting activity of Fe and Cu on the rate of browning (Kato *et al.*, 1981a). The presence of copper ions (as $CuCl_2$) promoted the rate of browning in glucose–glycine, glyoxal–glycine and 5-HMF–glycine systems (Rendleman and Inglett, 1990). The activity of copper in this study also appeared to be associated with its ability to form

strong complexes with melanoidins. UV absorbance and fluorescence intensity were also increased in the presence of copper. The formation of a range of metal ion complexes with Maillard reaction intermediates was described by O'Brien and Morrissey (1997).

It might be expected that the presence of metal ions would also accelerate the oxidation of Amadori products to form carboxymethyl amino acids which have been used as indicators of the progress of the Maillard reaction in foods (see below). Similarly, the concentration of oxygen may influence both the overall rate of browning and the formation of low molecular weight oxidation products, such as carboxymethyl lysine. Pokorny *et al.* (1988) reported that the replacement of air by nitrogen in model systems containing Amadori products reduced the reaction rate by half.

A variety of other factors, such as tertiary amine salts and acetic acid, have been shown to promote the Maillard reaction in model systems (Yoshimura *et al.*, 1969). However, the effect of all such miscellaneous factors on the rates of Maillard reactions in food systems is likely to be negligible compared with the effect of temperature, moisture and the nature of the reacting species.

Non-thermal energy sources may also influence browning reactions in food systems. For example, treatment of milk with ionizing radiation has been reported to lead to browning on subsequent storage at 4.4°C (Wertheim *et al.*, 1956). Browning was attributed to radiation-induced degradation of lactose, since the removal of carbonyl compounds from irradiated milk as their hydrazone derivatives following reaction with 2,4-dinitrophenylhydrazine markedly reduced the extent of browning. Significantly, the paper by Wertheim *et al.* (1956) was also the first to suggest that browning may be catalysed by free radicals.

The consequences of ultrasound treatment is similar in many respects to that of irradiation treatment. Heusinger (1986) identified malondialdehyde as a product of the ultrasound treatment of glucose or lactose solutions. The effects of such ultrasound treatment appear to be similar to the effect of high-speed stirring operations which have become more common in food manufacturing.

UV irradiation of sugar–amino acid solutions has been reported to produce volatile compounds similar to those found in heated systems, although it appears to be possible to distinguish UV-treated from thermally processed systems based on the volatile profile (Sheldon *et al.*, 1986).

7.5. Interaction of Oxidation and Maillard Reactions

The interaction between oxidation reactions (of sugars and proteins in addition to lipids) and Maillard reactions is now accepted as being of more importance than previously recognized (Zamora and Hidalgo, 2005). The

term 'glycoxidation' recognizes our growing understanding of the Maillard reactions of sugar oxidation products and the oxidation of Maillard reaction products (Goldberg *et al.*, 2004).

Amadori products are more easily oxidized than glucose. The enediol of fructosyl lysine can be oxidatively cleaved to carboxymethyl lysine and erythronic acid under neutral to basic conditions and under slightly acid conditions to 3-(*N*-ε-lysine)-lactic acid and glyceric acid (Figure 7.6). Thus, the rate of disappearance of ε-fructosyl lysine increases in the presence of air. Experiments on model systems have shown that pH has a strong influence on the extent of carboxymethyl lysine formation from fructosyl lysine (Hartkopf and Erbersdobler, 1994); on increasing the pH from 4.0 to 9.0, the yield of carboxymethyl lysine in a lysine–glucose model system heated at 100°C for 3 h increased from 70 to 3170 mg kg^{-1} lysine. The presence of iron, phosphate or nitrate also promotes the formation of carboxymethyl lysine. Zyzak *et al.* (1994) suggested that autoxidative glycosylation may be the major pathway of the Maillard reaction under simulated physiological conditions in vitro. The major product of glucose oxidation was considered to be glyoxal which in

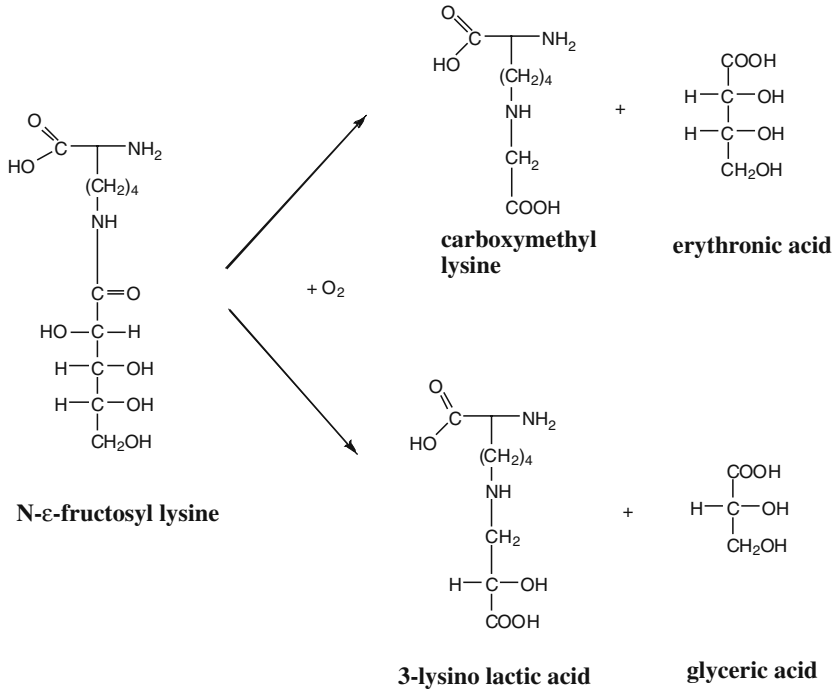

Figure 7.6. Major oxidation pathways of the Amadori product fructosyl lysine.

turn resulted in the formation of carboxymethyl lysine from lysine via an intramolecular Cannizaro reaction. It has been reported that oxidized ascorbic acid reacts rapidly with casein to produce a red coloration (Namiki *et al.*, 1986; Gopalan *et al.*, 1994). Namiki *et al.* (1986) showed that incubation of ascorbic acid in the presence of an air stream was necessary to produce red coloration in the presence of casein. Thus, it appears that the reactivity of ascorbic acid *per se* in Maillard reactions is negligible compared with that of dehydroascorbic acid. It is recognized that volatile aldehyde products of lipid peroxidation react readily with amino acids and proteins in Maillard-type reactions (Okitani *et al.*, 1986; Shibamoto and Yeo, 1992). Hexanal, a major volatile product of lipid peroxidation, reacts with proteins resulting in blocking of lysine and tryptophan residues, and polymerization (Okitani *et al.*, 1986). Aliphatic aldehydes produced on UHT processing of milk have long been known to decrease on storage, presumably due to such reactions (Earley and Hansen, 1982). The major lipid peroxidation product of butterfat, 4,5-epoxy-2-heptenal, has been shown to develop a brown colour and fluorescence on reaction with lysine (Hidalgo and Zamora, 1993). Interestingly, the activation energies for browning and fluorescence development on reaction of 4,5-epoxy-2-heptenal with lysine were comparatively low (66.5 and 50 kJ mol^{-1}, respectively). Products of the Maillard reaction have been shown to have a pro-oxidant effect in food systems (Miyazawa *et al.*, 2005) (see also Section 7.8.1).

Maillard reactions are also responsible for the formation of a host of powerful antioxidant compounds, many of which have not been characterized (Manzocco *et al.*, 2001; Morales and Jimenez-Perez, 2001; Cejpek *et al.*, 2004). The formation of such compounds is now increasingly being exploited industrially to improve the oxidative stability of foods. The inhibitory effect of pasteurization on the oxidation of milk lipids has been attributed to the release of sulphydryl groups during heating (Calvo and de la Hoz, 1992). However, it is possible that products of the Maillard reaction may also have a positive role in this respect. For example, maltol, an important flavour molecule in heated milk, is a powerful antioxidant.

The influence of oxygen on the formation of maltol and other products in a model system was studied by Yaylayan and Mandeville (1994). Although the decomposition of glucose and fructose did not appear to be sensitive to the presence of oxygen, the exclusion of oxygen seemed to promote the formation of maltol and HMF from maltose. In contrast, exclusion of oxygen from a tagatose system shifted the profile of products in favour of HMF, minimizing the formation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one. Oxygen is unlikely to have an important influence on lactose degradation during the heating of milk because of decreasing O_2 solubility at high temperatures. However, the presence of

oxygen may play a significant role in such reactions in stored dairy products, especially in dry systems.

The organoleptic acceptability of stored milk powder is closely related to the formation of volatile lipid peroxidation products and the oxidative stability and shelf-life of whole milk powders may be enhanced by the addition of Maillard reaction products (McGookin, 1991). The addition of Maillard reaction products as an antioxidant to skim milk powder has been reported to have a beneficial effect on the flavour profile (Hall and Andersson, 1985). Such applications are, however, limited by the effect of Maillard reaction products on the organoleptic acceptability of the products.

7.6. Methods for the Analysis of LA-Transformation Products and Degradation Products of Lactose

Although early methods for the identification of lactulose and its degradation products in milk products employed paper or thin layer chromatography or spectrophotometry, the methods most commonly used at present are based on HPLC, GLC or enzymatic reactions. The enzymatic methods of Geier and Klostermeyer (1980) and Andrews (1984) assume that lactulose is the only source of fructose in heated milks. Following the precipitation of fat and protein from milk samples, lactose and lactulose are enzymatically hydrolysed and the fructose is determined enzymatically. This approach is simple and inexpensive but is applicable only to products that have no added source of fructose (Luzzana *et al.*, 2003; Marconi *et al.*, 2004).

The use of capillary gas chromatography has been used to measure lactulose, galactose, epilactose and tagatose in heated milks. Sugars are separated as trimethylsilyl derivatives on a fused silica capillary column coated with, e.g. OV-17 or SPB-17 (Martinez- Castro *et al.*, 1986; Montilla *et al.*, 2005b). Tagatose has been separated from galactose using a similar method where the stationary liquid phase is replaced with AT-1000 (Troyano *et al.*, 1992b).

Perhaps the most straightforward method for the analysis of lactose isomerization products is ion exchange chromatography since no derivatization is required (Verhaar *et al.*, 1979). The international standard method adopted by the International Dairy Federation (1991) for the determination of lactulose in milk samples is based on HPLC separation on an ion exchange column following precipitation of proteins and fat. Lactose, lactulose and galactose may be determined simultaneously by this method using a refractive index detector.

7.7. Methods for Monitoring Maillard Reactions in Milk Products

Many methods are available for evaluating the extent of Maillard reactions in foods (Table 7.1). The objective of such measurements is to determine the nutritional value of the product or the impact of such reactions on the organoleptic quality and functional properties of products (Fayle and Gerrard, 2002; Silvan *et al.*, 2006). Although still of limited value for monitoring the quality of dairy products, the measurement of brown colour, A_{280}, fluorescence and reducing power are crude approaches to monitoring Mail-

Table 7.1. Methods for monitoring the Maillard reaction in foods

Based on chemically reactive lysine
Dye-binding methods (e.g. Acid Orange 12 or Remazol Brilliant Blue R)
Fluorodinitrobenzene (FDNB)
Guanidation
Trinitrobenzene sulphonic acid (TNBS)
Reduction by borohydride
Amino acid analysis, HPLC
Lysine oxidase electrode
Based on biologically available lysine
Rat bioassay (e.g. protein efficiency ratio, net protein utilization)
Microbiological bioassays based
Based on measurement of Amadori products
Direct measurement of Amadori products using HPLC or amino acid analysis following enzymatic hydrolysis
Separation of glycated proteins using capillary electrophoresis or LC–electrospray MS
Measurement of furosine and pyridosine following acid hydrolysis using HPLC, GC or amino acid analysis
Immunoassay of the protein Amadori adduct
Measurement of HMF (colorimetric or HPLC) following its formation from Amadori products by mild acid treatment
Based on the measurement of low molecular weight advanced products of the reaction
Measurement of *N*-ε-carboxymethyl lysine
Determination of hydroxymethylfurfural and/or 2-furaldehyde (HPLC or colorimetric)
Headspace CO_2 measurement
Measurement of volatile products of the reaction
Determination of ε-pyrrole lysine
Miscellaneous methods
Measurement of brown colour (transmission or reflectance spectrophotometry)
A_{280} measurement
Fluorescence measurement
Measurement of reducing power

lard reactions and will not be considered here. In particular, the measurement of advanced products of the Maillard reaction provides limited information about the progress of the reaction because of the existence of induction phases as a result of the formation of intermediates (Fayle and Gerrard, 2002; Nursten, 2005). Similarly, the use of microbiological or rat bioassays has declined as chemical methods have become more refined. Consequently, this review will concentrate on the chemical methods for monitoring the Maillard reaction.

7.7.1. Determination of Available Lysine

Perhaps, the most direct approach to monitoring the Maillard reaction in food systems is to measure the destruction of reactive amino acids. Clearly, because of its abundance and reactivity, lysine is likely to be the most important reactant in milk proteins although the destruction of arginine, tryptophan and histidine may also become significant, depending on the processing and storage conditions. A variety of methods are available for the determination of available lysine in proteins and much experience has been gained on the use of most of the chemical methods. A disadvantage of such an approach is lack of knowledge of the amount of 'blocked' or destroyed lysine obtained by measuring 'available' or reactive lysine. The simplest approach for the estimation of available lysine in proteins is probably the dye-binding methods. A variety of anionic dyes combine at low pH with the basic ε-NH_2, imidazole and guanidino groups of lysine, histidine and arginine residues, respectively, and with the terminal α-NH_2 of proteins. Binding of the dyes, Orange 12 and Remazol Brilliant Blue R, has been reported to correlate well with the fluorodinitrobenzene (FDNB) method for quantifying available ε-NH_2 lysine groups (Hurrell and Carpenter, 1975). However, although the dye-binding methods are very rapid (Hurrell *et al.*, 1979), they suffer from lack of specificity and may also underestimate the damage to lysine due to binding of dyes to some products of the Maillard reaction (e.g. Amadori adducts).

Several methods are available for the chemical estimation of reactive ε-NH_2 groups in proteins, including reaction with trinitrobenzene sulphonic acid (TNBS; Kakade and Liener, 1969), *O*-methylisourea (Mauron and Bujard, 1964), borohydride (Hurrell and Carpenter, 1974), [^{14}C]succinic anhydride (Anderson and Quicke, 1984) and fluorodinitrobenzene (FDNB; Carpenter, 1960; Mottu and Mauron, 1967). Although still used occasionally to measure the destruction of lysine in food systems, the TNBS method is very unsatisfactory for this purpose due to reaction of the reagent with the Amadori adduct (Hurrell and Carpenter, 1974). Of the methods listed above, the FDNB procedure is probably the most satisfactory, although it

is more time-consuming than the TNBS method. In addition, FDNB reacts weakly with the lysine ε-NH_2 Amadori adduct, leading to a slight overestimation of available lysine ($\sim$10%). The original FDNB method has been modified by including a shorter hydrolysis time (4 h), followed by HPLC separation and detection at 254 nm of the *N*-ε-dinitrophenol lysine derivative (Rabasseda *et al.*, 1988). The modified procedure may offer an attractive intermediate-length, intermediate-cost assay for quantifying the extent of Maillard reactions in heat-processed proteins.

Although expensive and time-consuming, acid hydrolysis (in 6 M HCl) and amino acid analysis, followed by reaction with ninhydrin, are still used by some laboratories to determine the nutritional quality of proteins. An advantage of the technique is that amino acid analysis and furosine determination (see below) can be conducted simultaneously. Measurement of lysine in the absence of furosine is of more limited value because of the release of some blocked lysine during acid hydrolysis. Thus, conventional amino acid analysis has long been known to overestimate the amount of unreacted lysine in food products (Moller, 1981). In addition, digestion conditions have a significant effect on the yields of amino acids detected. Rowan *et al.* (1992) suggested that hydrolysis time should, ideally, be optimized for the samples under study because of such variations in amino acid yield.

An addition to the range of methods for the determination of available lysine is the lysine oxidase electrode (Assoumani *et al.*, 1990). However, the method requires acid hydrolysis of samples, which normally releases some of the bound lysine. The authors also reported interference from levulinic acid formed via the degradation of glucose.

7.7.2. Determination of Amadori Products: The Furosine Assay

Acid hydrolysis of the protein-bound or -free Amadori product, ε-*N*-(deoxy-1-D-fructosyl)-L-lysine, results in the release of lysine (50%) and the production of two unique amino acids, furosine (20%) and pyridosine (10%). The Amadori derivative of lactose, ε-*N*-(deoxy-1-D-lactulosyl)-L-lysine, is hydrolysed to 40% lysine and 32% furosine. Over the past decade or so, determination of furosine in acid hydrolysates has been used increasingly to measure the amount of 'blocked' lysine in food proteins, particularly milk proteins. The major disadvantage of the early method for furosine determination was that it required an amino acid analyser. The determination of furosine may now be conducted successfully and reproducibly by GC (Buser and Erbersdobler, 1985) or HPLC (Chiang, 1983; Resmini *et al.*, 1990). Before commercially available furosine standards were available, Resmini *et al.* (1990) described the use of 2-acetylfuran as an external standard in the HPLC separation and detection of furosine at 280 nm.

The study of Hartkopf and Erbersdobler (1994) highlighted the problem of furosine instability during analysis. For example, during ion exchange chromatography, the use of a buffer at pH 6.4 with a temperature programme of 30 min at 60°C followed by 40 min at 80°C, resulted in a 60% decrease in recovery compared with a similar temperature programme at pH 4.7. Temperature alone does not appear to affect recovery but has a dramatic effect at higher pH values. The use of 2-acetylfuran, as proposed by Resmini *et al.* (1990), may also lead to errors; due to differences in the peak areas of equimolar concentrations of furosine and 2-acetylfuran, the use of the latter may overestimate furosine content by as much as 25% (Hartkopf and Erbersdobler, 1994). Hartkopf and Erbersdobler (1994) advocated the use of pure furosine as standard (available from Neosystem, Strasbourg, France) using either HPLC or IEC. Optimum conditions for IEC were reported to be pH 4.0 at a column temperature of 60°C. The advantage of the furosine method over other approaches to monitoring the Maillard reaction in milk systems is that it directly quantifies the concentration of lactose–protein Amadori products. A possible criticism of such an approach is that the furosine method does not detect later products of the Maillard reaction (e.g. 5-hydroxymethyl-2-furaldehyde, melanoidins), a factor that might lead to under-estimation of lysine destruction. However, as outlined above, the stability of the lactose–protein Amadori adduct is such that it would be expected to be the major or only product of Maillard reactions in most milk systems. A serious shortcoming of the early furosine method was lack of sensitivity. Most of the early studies could not detect furosine in conventional UHT or spray-dried milks (Moller *et al.*, 1977a,b; Moller, 1981; Finot *et al.*, 1981). The modified furosine assay used by Erbersdobler *et al.* (1987) gave furosine values between 0.5 and 1.5 mg dl^{-1} in milk samples processed at 135–145°C for 4–16 s. In addition, Erbersdobler *et al.* (1987) observed that UHT milk processed by direct heating contained lower furosine levels (1.5–2.5 mg dl^{-1}) than milk processed by indirect heating (2.6–5.3 mg dl^{-1}), which compares with the trend reported for the production of lactulose in UHT milks. A modification of the furosine procedure involves the enzymatic digestion and dialysis of milk proteins prior to acid hydrolysis and furosine determination, to distinguish between enzymatically available and chemically available lysine (Desrosiers *et al.*, 1989). The authors reported that, of the original lysine present in whey protein concentrate at an a_w of 0.97 heated at 121°C for 5000 s, 93% was chemically available, whereas only 76% was available enzymatically. Differences probably arose due to heat-induced conformational changes and crosslinking, which would have limited the enzymatic digestion without necessarily destroying lysine.

7.7.3. Determination of Amadori Products: Direct Determination of Lactulosyl Lysine

An alternative approach is the direct measurement of Amadori adducts of proteins, for example, the determination of lactulosyl lysine in dairy products. Antibodies have been raised against lactulosyl derivatives of β-lactoglobulin (Matsuda *et al.*, 1985a) and ovalbumin (Matsuda *et al.*, 1985b, 1986). A more promising approach is the determination of lactulosyl lysine in milk products by LC-MS following complete enzymatic hydrolysis. The availability of a standard makes this approach more attractive as a method of detecting low levels of Amadori products in dairy products (Vinale *et al.*, 1999). However, the limitation of this technique is the time-consuming enzymatic hydrolysis step (Henle *et al.*, 1991). In view of the success of the many HPLC approaches for the separation and determination of amino acid–sugar Amadori products in model systems and foods (Reutter and Eichner, 1989; Moll and Gross, 1981), the application of a similar approach to enzymatically hydrolysed proteins offers promise and merits further investigation. In addition, the replacement of the traditional amino acid analysis approach by HPLC, as employed by Henle *et al.* (1991), would greatly reduce the cost of the analysis.

7.7.4. Determination of 5-hydroxymethyl-2-furaldehyde

Determination of HMF, as used in the original method of Keeney and Bassette (1959), is one of the classic methods for monitoring the Maillard reaction in dairy products. It could be classified as an indirect method for the determination of Amadori adducts plus intermediates of the 1,2-E pathway, including preformed HMF. The determination of HMF has long been used as an indicator of temperature abuse of fruit juices, especially citrus juices. Because of the acidic nature of such products, the 1,2-E reaction pathway would be expected to predominate with the result that HMF levels would be a valid and logical indicator of the extent of the Maillard reaction. Both HMF and 2-furaldehyde react with 2-thiobarbituric acid (TBA) under acidic conditions to produce chromophores with absorption maxima at 436 and 414 nm, respectively. Several analytical procedures are available for HMF and 2-furaldehyde in fruit juices, based on the TBA reaction, HPLC methods and combinations of both (Mijares *et al.*, 1986; Poretta and Sandei, 1991; Tu *et al.*, 1992). In the case of milk products, the 1,2-E pathway would not be as predominant as in fruit juices due to the higher pH of the former. Thus, preformed HMF would not be expected to represent as reliable an indicator of the heat treatment of milk products as it is in fruit juices. Keeney and Bassette (1959) attempted to overcome the inherent shortcomings of the

method by including a pre-treatment involving heating samples in 0.3 N oxalic acid at 100°C for 1 h to convert Amadori products and 1,2-E intermediates to HMF. Although the measurement of HMF in dairy products has been criticized on the grounds of lack of specificity (Burton, 1984), it is a cheap and relatively simple method for monitoring Maillard reactions and is still widely in use (De Block *et al.*, 2003). HPLC methods are now available for the determination of free and 'bound' HMF in dairy products exploiting the strong absorption of HMF at 280 nm (van Boekel and Rehman, 1987; Morales *et al.*, 1997).

7.7.5. Fluorescence Spectroscopy

The generation of fluorescent products in Maillard reactions is well described and fluorescence spectroscopy has been used to monitor the development of Maillard reactions in dairy products (Morales *et al.*, 1996; Leclère and Birlouez-Aragon, 2001; Bosch *et al.*, 2007). However, the limitation of classical methods is that they cannot be applied to turbid systems such as dairy products without a sample preparation step. More recently, the application of front-face (surface) fluorescence spectroscopy to dairy products offers potential as a rapid non-destructive means of assessing product quality (Kulmyrzaev and Dufour, 2002; Birlouez-Aragon *et al.*, 2004). The coupling of chemometric methods with fluorescence spectroscopy has been reported to determine accurately the levels of both lactulose and furosine in milk samples (Kulmyrzaev and Dufour, 2002).

7.7.6. ε-Pyrrole Lysine

The advanced Maillard reaction product, ε-pyrrole lysine, has been proposed as a useful indicator of reactions in stored foods because Amadori products tend to decompose over time to other products, limiting their usefulness as indicators of Maillard reactions (Chiang, 1988). Chiang (1988) found that ε-pyrrole lysine was readily detectable in skim milk powder heated at 80°C for 1 or 2 h (6.25 or 20.32 mg kg^{-1}, respectively).

7.7.7. Carboxymethyl Lysine

An alternative to the furosine assay is the measurement of *N*-ε-carboxymethyl lysine (CML; Figure 7.6) formed by oxidation of the protein-bound Amadori product (Erbersdobler and Dehn-Müller, 1989; Badoud *et al.*, 1990; Lüdemann and Erbersdobler, 1990). In the case of milk products, the method involves the oxidation of lactulosyl lysine using periodic acid, followed by acid hydrolysis. The method is reported to have the

advantage that the same oxidation product is formed regardless of the nature of the sugar moiety of the Amadori product. In addition, the method can be extended to the oxidation products of the Amadori products of several other amino acids in addition to lysine; fluorescent detection of carboxymethyl amino acids following HPLC separation affords high sensitivity (Badoud *et al.*, 1990). CML is also formed from fructosyl lysine and lactulosyl lysine in food systems by the action of oxygen on the Amadori product. Its formation is promoted at low a_w values, at high pH and by replacing glucose by maltose or lactose in the food system (Lüdemann and Erbersdobler, 1990). Lüdemann and Erbersdobler (1990) proposed that since CML is more heat resistant than fructosyl lysine, its formation in foods could be a useful indicator of advanced heat damage.

7.7.8. Pyrraline

The determination of the pyrrolealdehyde crosslink, pyrraline, may be a useful marker of heat treatment in that it is independent of the degradation of lactulosyl lysine and increases linearly with heating time of milk powder samples (Henle *et al.*, 1994). However, a dramatic decrease in pyrraline is observed on prolonged heating (100–110°C for >6 h). Levels of pyrraline in food samples varied dramatically. Whereas levels in raw, pasteurized and UHT milks did not exceed 2 mg kg^{-1} protein, levels in skim milk powder and whey powder were as high as 1150 and 3150 mg kg^{-1} protein, respectively. Concentrations in sterilized (<2–260 mg kg^{-1} protein) and evaporated (110–130 mg kg^{-1} protein) milks appeared to be intermediate. In addition to pyrraline, the authors also identified low levels of maltosine (~30-fold less than pyrraline) in samples of severely heated milk powder. It is assumed that maltosine is formed via the 2,3-E pathway while pyrraline is a product of the 1,2-E pathway.

7.7.9. Immunoassays

The use of immunoassays to detect advanced Maillard products has been the subject of several studies. However, a possible disadvantage of most of these assays is that the antigens have not been subjected to full chemical characterization. Kato *et al.* (1994) used monoclonal antibodies for a lactose–protein Maillard reaction product to characterize a number of milk samples. Skim milk powders showed reactivity to the antibody in all milk protein fractions except the κ-casein fraction. Lactoferrin, serum albumin and the immunoglobulins were only weakly reactive in the assay whereas α_{s1}-casein, β-casein, β-lactoglobulin and α-lactalbumin were highly reactive. Packaging of the skim milk powder in N_2 appeared to reduce the formation of the

antigen, suggesting the involvement of an oxidation reaction. Furthermore, the antigen may have been lactose specific as a lactose-free, glucose-containing milk powder was non-reactive.

7.8. Consequences of Undesirable Maillard Reactions in Milk Systems

7.8.1. Nutritional Implications

Maillard reactions may compromise the nutritional value of foods through the destruction of essential amino acids and by limiting the bioavailability of amino acids and other essential nutrients. Clearly, in dairy products, lysine is the most vulnerable amino acid to the Maillard reaction because of its abundance and the reactivity of the ε-NH_2 group. While the free Amadori products, lactulosyl lysine and fructosyl lysine, are absorbed from the intestine, they appear to be excreted rapidly, largely unchanged, in the urine and do not accumulate in the body (Hultsch *et al.*, 2006; Schwenger *et al.*, 2005).

In the developed world, however, the destruction of even 10–20% of lysine in dairy products does not assume great nutritional significance because of the excess of lysine in milk proteins and because of the high intake of proteins by most individuals. In view of the fact that tryptophan, arginine and histidine are not as abundant in milk proteins as lysine, their possible reactivity in Maillard reactions is of nutritional interest. In particular, it has been reported that tryptophan may be even less stable than lysine under some conditions (Leahy and Warthesen, 1983). Dworschak and Hegedüs (1974) reported the loss of 97% of the tryptophan in a milk powder containing 5.7% moisture that was heated at 100°C for 4 h. This conflicts with the results of Nielsen *et al.* (1985b,c), who could not detect a decrease in tryptophan (by chemical analysis or rat bioassays) in a browned milk powder. Nielsen *et al.* (1985b,c) suggested that such differences among studies may be attributable to the different analytical methods used. A study of the nutritional consequences of storage of a dried whey protein concentrate (35% lactose; 52% protein) reported a lysine loss of 23% after 3 months at 40°C and an a_w of 0.41, whereas tryptophan and methionine levels were not significantly affected (Lindemann-Schneider and Fennema, 1989). Nitrogen packing appeared not to affect lysine loss. Based on plasma amino acid levels in adult dogs after a test meal, Longenecker and Hause (1959) reported that arginine was the first limiting amino acid in casein.

Processes such as HTST pasteurization, UHT treatment and spray drying of milk usually result in only slight losses of available lysine. Drying of infant formulae and whey, however, usually results in higher losses due to

the higher concentration of lactose. Roller-dried dairy products are invariably inferior to spray-dried products (see El-Shafei *et al.*, 1988). Roller drying has been reported to lead to available lysine losses of 10–75%, depending on the process (Mauron, 1981; Finot *et al.*, 1981). Lysine losses in fresh UHT-treated milks are generally accepted to be comparable or slightly higher than those in conventionally pasteurized samples. Park and Hong (1991) reported lysine losses of 6.3 and 4.9% for UHT-pasteurized (130°C, 2–3 s; 135°C, 2 s) and UHT-sterilized (135°C, 2 s; 140°C, 2–3 s) milks, respectively, using the TNBS method. Such values are higher than those reported previously. In addition, since the TNBS method has been criticized as underestimating lysine losses, it is possible that the actual losses in the samples of Park and Hong (1991) may be higher. However, ideally, any future conclusions as to the effect of thermal processes on lysine availability should be based on more reliable direct methods of measuring lysine destruction, such as amino acid analysis in combination with the furosine assay. In UHT milks stored at 30–37°C for 6–36 months, 10–30% of lysine residues were blocked as lactulosyl lysine (Moller *et al.*, 1977b).

In contrast to freshly processed UHT milk, stored UHT milk may exhibit relatively high losses of available lysine. Moller (1981) reported lysine losses of 10–30% in UHT milk samples that had been stored for 14 months at 4–30°C. Freeze drying is the most acceptable process for the drying of milk from a nutritional point of view although in practical terms there is a negligible difference between most spray drying processes and freeze drying. Lactose-hydrolysed formulae have the greatest propensity to lysine loss because of the increased number of reactive sugar moieties and because glucose and galactose are individually more reactive than lactose. Burvall *et al.* (1978) reported available lysine losses of 40% for a spray-dried lactose-hydrolysed milk after 6 months storage. Thermal processing of milk in the presence of added sucrose to produce *doce de leite* has been reported to cause significant decreases in lysine (33%), arginine (11%) and histidine (10%) levels (Pavlovic *et al.*, 1994). The available lysine levels, as measured by the FDNB method, were reduced by 50%; the discrepancy between the FDNB method for measuring lysine availability and the results of amino acid analysis highlights the need for extreme care in estimating amino acid bioavailability from heated proteins.

Hypoallergenic infant formulae prepared from protein hydrolysates are more vulnerable to the nutritional consequences of Maillard reactions due to the increased availability of free amino groups for reaction (Finot, 2005). This results in the loss of essential amino acids other than lysine, such as alanine, valine, leucine and isoleucine (Penndorf *et al.*, 2007). Penndorf *et al.* (2007) reported losses of N-terminal amino acids of up to 8.4% in such hydroylsed products which is similar to the losses reported for lysine in conventional

infant formulae. Added iron and ascorbic acid appear to have a particularly destructive effect on tryptophan in dairy systems. It is hypothesized that the main pathway for tryptophan destruction under mild heating conditions is not direct glycation of the indole N but a consequence of oxidation due either to the activity of the Fe-ascorbate system or the pro-oxidant activity of Maillard reaction products following ascorbylation of lysine ε-NH_2 groups (Puscasu and Birlouez-Aragon, 2002; Leclère *et al.*, 2002). The presence of oxygen is particularly detrimental under such conditions (Gliguem and Birlouez-Aragon, 2005). The high levels of blocked lysine in some fortified milks clearly limit their usefulness as dietary supplements (Evangelisti *et al.*, 1999; Rada-Mendoza *et al.*, 2005).

Similarly, the higher extent of the Maillard reaction in liquid-sterilized infant formulae, compared with powdered and UHT counterparts, is well described (Guerra-Hernandez *et al.*, 2002; Puig *et al.*, 2003). The considerably higher lysine losses and furosine levels in follow-on formulae may be related to the processing method but are also related likely to the impact of added nutrients, such as maltodextrin or iron, on the Maillard reaction (Ferrer *et al.*, 2003; Pereyra Gonzales *et al.*, 2003).

Maillard reactions may limit the bioavailability of undamaged amino acids by inhibiting digestive enzymes and/or by inhibiting amino acid transport at the intestinal level. In addition, there is evidence that severely browned proteins are less digestible than native proteins (Dalsgaard *et al.*, 2007). Gothwal and Bhavadasan (1991) reported that although slight browning of milk was accompanied by an increase in the rate of trypsin proteolysis, severely browned milk samples are less readily hydrolysed by trypsin. A stored dried casein–glucose mixture showed a loss of digestibility of 14–88%, depending on conditions (Culver and Swaisgood, 1989). The available chemical evidence on the effect of Maillard reactions on the nutritional value of food proteins is supported by clinical evidence that diets rich in Maillard reaction products have a negative impact on protein digestibility (Seiquer *et al.*, 2006). Moreover, protein-bound lactose is resistant to hydrolysis by β-galactosidase (Morgan *et al.*, 1999a)

Maillard reactions may also contribute to the destruction of vitamins in food systems. Vitamin C can participate directly in the reaction and it has been proposed that vitamins B1, B6, B12 and pantothenic acid may react with pre-melanoidins (Hurrell, 1990). Although vitamin B1 contains an amino group and, therefore, could participate in Maillard reactions, it is unclear to what extent this mechanism contributes to its destruction in food systems. Folic acid is now added in the free form to many manufactured foods and through its free amino group is believed to participate in Maillard reactions.

There is evidence that dietary Maillard reaction products may disrupt mineral homoeostasis in vivo, reducing the bioavailability of some minerals.

Maillard reaction products have been shown to increase the intestinal absorption and urinary excretion of calcium and magnesium (O'Brien and Morrissey, 1989), which resemble the action of poorly digestible carbohydrates, such as lactose and polyols. However, while calcium absorption has been shown to be higher in a bottle-sterilized liquid infant formula, calcium retention was significantly lower in a study on suckling rats (Sarriá *et al.*, 2001). Casein subjected to a Maillard reaction with reducing sugars impairs the uptake of zinc by Caco-2 cells in culture, suggesting a reduction in bioavailability compared with untreated casein (Seiquer *et al.*, 2000). By contrast, Sarriá and Vaquero (2006) reported higher percentage copper absorption and erythrocyte copper concentrations despite lower food intake and body weight in in-bottle-sterilized formula compared with a reconstituted powder formula.

Dietary Maillard reaction products of a glucose–glutamate system have been reported to reduce zinc retention in rats as a result of increases in both faecal and urinary zinc losses (O'Brien and Morrissey, 1989). Furniss *et al.* (1989) reported a large increase in urinary zinc in rats fed a heated casein–glucose mixture. Urinary zinc was also increased in animals fed a reacted casein–lactose mixture, although the effect was not as pronounced as for the casein–glucose system. However, there were no changes in faecal zinc or in zinc retention and the authors concluded that an increased loss of urinary zinc would have little significance for individuals consuming a diet adequate in zinc.

7.8.2. Milk Protein Allergy

There is an increasing volume of scientific literature on the influence of processing, in particular thermal processing, on food protein allergy (Davis *et al.*, 2001). This research has been driven by increased awareness and better risk management of food allergens. However, an additional consideration has been to ensure that novel foods and processes do not increase allergenic risk for sensitized consumers. It was long known that the allergenicity of β-lactoglobulin could be enhanced by the Maillard reaction. Matsuda *et al.* (1985b) found higher antibody titres in mice immunized with a β-lactoglobulin–lactose adduct than animals injected with native β-lactoglobulin. The increased allergenicity of lactosylated β-lactoglobulin was demonstrated by Bleumink and Berrens (1996). Matsuda *et al.* (1990) reported that the lactose-ovalbumin adduct induced a stronger antibody response when injected into mice than the adducts of other sugars (glucose, galactose, melibiose, maltose or cellobiose). The authors suggested that the immunodominancy of the lactose adduct was due to some feature of its stereochemical configuration. The effect of Maillard reactions on the epitopes of native proteins was

illustrated by the work of Öste *et al.* (1990) who showed a decrease in the antigenicity of Kunitz soybean trypsin inhibitor which had been heated with glucose, lactose or maltose.

Recent research on the allergenicity of the major peanut allergens has corroborated the above findings and has suggested a mechanism of action that involved both increased IgE binding and a fourfold increase in trypsin inhibitory activity (Maleki and Hurlburt, 2004), which would potentially allow intact antigens to reach the systemic circulation. As Maillard reactions have the potential to produce trypsin inhibitory compounds and to modify the structure of food proteins, this area is in need of further research. Meanwhile, the effect of processing on a number of allergens has been studied (Berrens, 1996; Wolff *et al.*, 2004).

Research on the lactosylation of milk proteins during heating or storage may help define the structural changes that influence milk protein allergenicity. Electrospray ionization MS and capillary electrophoresis have been used to study the non-enzymatic glycosylation of milk proteins (Leonil *et al.*, 1997; Siciliano *et al.*, 2000; French *et al.*, 2002). Jones *et al.* (1998) demonstrated that capillary electrophoresis can be used to monitor several glycated forms of β-lactoglobulin in skimmed milk powder. Guyomarc'h *et al.* (2000) were able to show several new peaks for β-lactoglobulin, α-lactoglobulin, α_{s1}-casein, β-casein and κ-casein, in capillary electropherograms. Additional analysis by mass spectrometry suggested the increases in mass for the whey protein peaks were approximately multiples of 320 Da consistent with incremental lactosylation reactions. Other studies have demonstrated that lactosylation of β-lactoglobulin can proceed under relatively mild conditions in the absence of browning is site specific (appearing to prefer Lys_{47}), and results in conformational changes and ultimately aggregation via non-covalent interactions (Leonil *et al.*, 1997; Morgan *et al.*, 1999b; Chevalier *et al.*, 2002; French *et al.*, 2002; Czerwenka *et al.*, 2006).

7.8.3. Changes in Functional Properties

In both food systems and biological systems in vivo, there is evidence that Maillard reactions may result in substantial changes in the functional properties of proteins. Such changes include reduced enzyme activity, altered receptor binding (Amaya *et al.*, 1976), altered drug binding to plasma proteins (Tsuchiya *et al.*, 1984) and changes in protein stability (Kato *et al.*, 1983). Kato *et al.* (1981b,c) reported that Maillard reaction products of ovalbumin and glucose were more soluble and heat stable than the native protein. Electrophoretic studies confirmed that such protein–glucose adducts had fewer positive charges. Circular dichroism spectroscopy revealed a more ordered structure in the reacted protein than expected: even though 62% of

the ε-amino groups were blocked, the helix content was 27% compared with 33% in the native protein. The authors suggested that the structure of the reacted protein may be stabilized by intrachain non-covalent interactions between the protein and bound sugar moieties. The ratio of arginine to lysine has been reported to be directly related to the stability of proteins (Kinsella *et al.*, 1989). Since lysine is generally reported to react more readily than arginine (Kaanane and Labuza, 1989) and since there are many more lysyl than argininyl residues, it might be expected that the Maillard reaction would result in increases in the effective arginine: lysine ratio in food proteins, possibly resulting in an increase in protein stability. The selective chemical modification of ε-NH_2 groups has been reported to change the activity, pH optimum and heat stability of several enzymes (Smith *et al.*, 1991; Smith and Yada, 1991). The Maillard reaction of glucose-6-phosphate with β-lactoglobulin has been used as a means of attaching more than 1% phosphorus to the protein (Aoki *et al.*, 1994). The reaction was reported to result in a significant increase in the heat stability and emulsification capacity of the protein. Similar results were reported by Chevalier *et al.* (2001) who used Maillard reactions with several monsaccharides to modify the solubility, heat stability, emulsifying capacity and foaming properties of β-lactoglobulin. The well-described increase in heat stability of milk following preheating may be related to the effect of the Maillard reaction. Such an increase in heat stability might be a consequence of the lowering of isoelectric point which follows Maillard reactions of proteins.

Conjugation of ovalbumin, lysozyme and α-lactalbumin with galactomannan in Maillard reactions resulted in dramatic increases in the emulsifying capacity and emulsion stability of the proteins (Nakamura *et al.*, 1992, 1994). Experiments involving the acetylation of free amino groups of lysozyme showed that the positive charge on the protein (contributing to electrostatic repulsion) is necessary for its emulsifying properties. On the basis of electrophoretic studies and free amino group measurement, the authors suggested that $\sim$3 and $\sim$2 mol of galactomannan bound to α-lactalbumin and lysozyme, respectively (Nakamura *et al.*, 1994). The emulsifying capacity of β-casein was reported to be increased following a Maillard reaction with dextran (Mu *et al.*, 2006).

Studies on the globular proteins, bovine serum albumin, pea protein and soy protein isolate have shown that Maillard reactions increase the gel strength (possibly related to an increased level of crosslinking) on heating and reduce the threshold level of protein required for gelation (Armstrong *et al.*, 1994; Cabodevila *et al.*, 1994). Experiments on glucono-δ-lactone-induced gels showed that the properties of gels formed from proteins subjected to the Maillard reaction could not be explained simply by the pH decrease that accompanies browning.

The presence of lactose during the heating of whole casein has been shown to reduce calcium binding, presumably due to reduced accessibility of

binding sites due to Maillard reactions (Pappas and Rothwell, 1991). The functional consequences, if any, of such a reduction in calcium binding have not been investigated. In view of the reactivity of κ-casein in Maillard reactions, it is possible that such reactions may reduce the stability of casein micelles such as occurs during storage and age gelation of UHT milk. Available evidence suggests that Maillard browning reactions do not contribute to age gelation (Venkatachalam *et al.*, 1993). However, the development of Maillard crosslinks has not yet been examined in UHT milk and, therefore, the possible role of Maillard reactions in age gelation cannot be discounted.

7.8.4. Formation of Toxic Compounds

7.8.4.1. Furan

The formation of furanic derivatives in Maillard reactions is well described and the toxicology of furfural and HMF in particular is well described (see Section 7.3.4). In early 2004, the US Food and Drug Administration (FDA) expressed concern at the presence of furan, a possible human carcinogen, in a number of heat-treated foods (FDA, 2007). The presence of furan and hundreds of furanic molecules in foods has been recognized for at least 30 years. Most of these molecules derive from Maillard reactions (Maga, 1979; Crews and Castle, 2007).

The level of furan found in heated dairy products is relatively low (generally <20 μg kg^{-1} in infant formulas and evaporated milks compared with products such as some soups and canned foods where levels >200 μg kg^{-1} have been reported) (Märk *et al.*, 2006; FDA, 2007).

Maillard products such as furfural and furoic acid have been proposed as furan precursors in heated foods (Crews and Castle, 2007). Systems containing ascorbic acid yield the highest levels of furan, especially in the presence of oxygen (Perez Locas and Yayalayan, 2004; Hasnip *et al.*, 2006). Removal of oxygen or addition of a reducing agent, such as sulphite, results in lower furan levels (Märk *et al.*, 2006). Märk *et al.* (2006) also noted that more complex systems resulted in less furan formation, presumably due to competing reaction pathways; the intermediates 2-deoxyaldotetrose and 4-hydroxy-2-butenal were proposed to yield furan from ascorbic acid/carbohydrate pathways or polyunsaturated fatty acid oxidation, respectively.

7.8.4.2. Acrylamide and Other Vinylogous Maillard Reaction Products

The discovery, in 2002, of high levels of the carcinogen, acrylamide, in some thermally processed plant foods revealed a hitherto unknown Maillard pathway with significant food safety implications (Friedman and Mottram, 2004; Skog and

Alexander, 2006). The most important pathway for acrylamide formation appears to be the decarboxylation of the Schiff's base of asparagine and a carbonyl reactant. Thus, the level of acrylamide in the finished product correlates highly with the level of asparagine, the sugar level and the severity of heat treatment. The levels of acrylamide reported in dairy products are low relative to other foods ($<$10–100 μg hg^{-1}) (Petersen and Tran, 2005). Although this pathway is of little significance for dairy products, the chemistry is of interest as it gives rise to the possibility that vinylogous reaction products may be formed from the decarboxylated Amadori compounds of other amino acids (e.g. styrene from phenylalanine) (Blank *et al.*, 2004; Stadler *et al.*, 2004; Blank, 2005).

7.8.4.3. Other Toxic Compounds

It is now well accepted that the Maillard reaction plays an important role in the formation of the aminoimidazoazarene (AIA) group of food mutagens in muscle foods. Such mutagens are products of the reaction of the Maillard reaction products 2-methylpyridine or 2,5-dimethylpyrazine with creatinine and glycine or alanine (Lee and Shibamoto, 2002). The presence of such mutagens has not been reported in dairy products. Although mutagenic activity has been reported in extracts of severely heated dairy products (Rogers and Shibamoto, 1982; Yen and Lee, 1986), dairy products are not unique in this respect since mutagenic activity develops in a host of severely heat-treated foods (Kato, 1986; O'Brien and Morrissey, 1989). In pure form, HMF and its metabolite 5-sulphooxymethylfurfural have genotoxic properties (Lee and Shibamoto, 2002), but there is no evidence that the levels of HMF found in dairy products pose a health risk. In fact the levels of HMF in dairy products is very low compared with other food categories (Glatt and Sommer, 2006). There is no evidence that dairy products subjected to conventional heat treatments, such as HTST pasteurization, UHT treatment or in-bottle sterilization, are mutagenic (Sekizawa and Shibamoto, 1986; Berg *et al.*, 1990). In fact, there is evidence that mutagen binding to casein may reduce the mutagenicity of food systems (Berg *et al.*, 1990).

7.9. The Future

The discovery of high levels of acrylamide in heated plant-derived food systems in 2002 revealed that there is much yet to be discovered about Maillard reactions in foods. Consequently, Maillard reaction chemistry remains an active area of research aimed at minimizing the detrimental effects of such reactions while enabling preservation of unstable food systems and delivering the organoleptic benefits of thermally processed foods. The

development of functional foods with demonstrated health benefits is an active area of food product development, especially in the dairy industry. Many such products contain unstable active ingredients that may participate in Maillard reactions (e.g. bioactive peptides, vitamins, oligosaccharides, botanical extracts) with consequences for product efficacy (Roscic and Horvat, 2006). There is, therefore, a strong stimulus for continuing research into better understanding and controlling Maillard reactions in dairy products.

Acknowledgement

The author wishes to thank Dr Judith O'Connor for assistance with the references.

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