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# Lipolytic Enzymes and Hydrolytic Rancidity

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## Summary

Lipolysis, the enzymic hydrolysis of milk lipids to free fatty acids and partial glycerides, is a constant concern to the dairy industry because of the detrimental effects it can have on the flavor and other properties of milk and milk products. However, free fatty acids also contribute to the desirable flavor of milk and milk products when present at low concentrations and, in some cheeses, when present at high concentrations.

The enzymes responsible for the detrimental effects of lipolysis are of two main types: those indigenous to milk, and those of microbial origin. The major indigenous milk enzyme is lipoprotein lipase. It is active on the fat in natural milk fat globules only after their disruption by physical treatments or if certain blood serum lipoproteins are present. The major microbial lipases are produced by psychrotrophic bacteria. Many of these enzymes are heat stable and are particularly significant in stored products.

Human milk differs from cows' milk in that it contains two lipases, a lipoprotein lipase and a bile salt-stimulated lipase. The ability of the latter to cause considerable hydrolysis of ingested milk lipids has important nutritional implications.

## 15.1. Introduction

Hydrolytic rancidity in milk and milk products has been a concern to the dairy industry of most countries (Downey, 1975). Although it is not

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considered a serious problem in many countries (IDF, 1983), the potential for problems exists at all times and, therefore, constant vigilance is necessary to ensure effective controls.

Hydrolytic rancidity results from the hydrolytic degradation of milk lipids. The hydrolysis is catalyzed by lipases and produces free fatty acids (FFAs), some of which have a low flavor threshold and can cause unpleasant flavors in milk and milk products. These flavors are variously described as rancid, butyric, bitter, unclean, soapy or astringent. The lipases involved are of two types: indigenous milk enzyme(s) and enzymes of microbial origin.

In the early 1900s, it was recognized that milk contains an enzyme capable of hydrolysing triglycerides and producing rancidity. Considerable research was carried out into the causes and effects of its action in milk. "Bitter milk of late lactation" and the increase in fat acidity following shaking, homogenization and certain temperature manipulations of raw milk were recognized as consequences of milk lipase action. It was also found that some developments in milking and processing methods (e.g., cold storage, mechanisation) could exacerbate the problem. For a review of this early work, see Herrington (1954).

During the 1950s and 1960s, studies focussed on the milk lipase system, the mechanism of its activation and the physico-chemical properties of the enzyme(s) involved. From these studies it was concluded that more than one lipase was present and several attempts to purify a milk lipase were reported. Jensen (1964) reviewed much of this work.

There were several new developments during the 1970s. Of particular importance was the purification and characterization of a lipoprotein lipase (LPL) and the acceptance of the postulate that this was the major, if not the only, lipase in cows' milk (Olivecrona, 1980). Similarly, the elucidation of the lipase system in human milk as consisting of an LPL and a bile salt-stimulated lipase, and the possible role of the latter in infant nutrition, were noteworthy (Fredrikzon *et al.*, 1978). Also, microbial lipolysis assumed substantial significance with the widespread use of low-temperature storage of raw milk and the recognition that heat-stable lipases produced by psychrotrophic bacteria were a major cause of flavor problems in stored dairy products (Law, 1979).

From the 1980s further advances in knowledge of the enzymes and the mechanism of their actions at the molecular level were made (Olivecrona *et al.*, 2003). Bacterial lipases received considerable attention (Fox *et al.*, 1989) and cloning of DNA encoding both milk lipases (Senda *et al.*, 1987; Nilsson *et al.*, 1990; Sbarra *et al.*, 1998) and bacterial lipases and esterases (Chung *et al.*, 1991; McKay *et al.*, 1995; Kojima *et al.*, 2003; Ro *et al.*, 2004) was achieved. Analytical methods for determining FFAs in milk and milk products with increased accuracy using high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC) were also developed

(Stead, 1989). Measurement of lipase activity has received considerable attention because of the defects caused in stored milk and dairy products by low levels of heat-resistant bacterial lipases (Deeth and Touch, 2000; Chen *et al.*, 2003)

In this chapter, the lipolytic enzymes in milk and milk products and the causes, consequences and assessment of their action are discussed. The significance of lipases in human nutrition and in the production of characteristic flavors in certain dairy products is also covered.

## 15.2. The Enzymes

### 15.2.1. Cows' Milk Lipase

Early research on lipolytic enzymes in cows' milk suggested that at least two major lipases were present: a "plasma lipase" in the skim portion and a "membrane lipase" associated with the milk fat globule membrane (Tarassuk and Frankel, 1957) while later research indicated that there might be up to six different molecular species with lipase activity (Downey and Andrews, 1969). However, work by Korn (1962) showed that milk contained a lipoprotein lipase (EC 3.1.1.34) (LPL) with properties very similar to those of post-heparin plasma, adipose tissue and heart LPLs, particularly the enhancement of its activity on emulsified triglycerides by blood serum lipoproteins. It is now accepted that LPL is the major, if not the only, lipase in cows' milk. Its properties have been reviewed by Olivecrona *et al.* (2003).

LPL is synthesized in the mammary gland secretory cells and most is transported to the capillary endothelium where it hydrolyzes triglycerides in circulating lipoproteins to FFAs and 2-monoglycerides. These products are absorbed by the mammary gland and used for the synthesis of milk fat. The LPL in milk appears to be identical with the enzyme in the mammary gland (Askew *et al.*, 1970) and to be the result of a spillover. Its level in milk is low at parturition but increases rapidly during the first few days of lactation and remains almost constant for the remainder of the lactation (Saito and Kim, 1995).

Under normal circumstances, most of the LPL in milk is in the skim milk fraction and the major part of this is associated with the casein micelles (Fox *et al.*, 1967). Some is in soluble form (Anderson, 1982a) and a small amount is associated with the milk fat globule membrane (Deeth and Fitz-Gerald, 1975a). The enzyme is bound to the caseins principally by electrostatic interactions; NaCl (0.75–1M) releases most of it into the serum phase where it is associated with casein in aggregates of molecular weight of *ca.* 500 000 Da (Hoynes and Downey, 1973). The electrostatic binding of lipase in the micelle appears to be *via* positive charges on the enzyme to negatively

charged caseins, (e.g., κ-casein) (Downey and Murphy, 1975). LPL binds strongly to negatively charged heparin, enabling it to be dissociated from the casein micelle by low concentrations of sodium heparin (5 μg/l) (Hoynes and Downey, 1973). Hydrophobic associations may also be involved in the lipase–casein interaction since the lipase can be dissociated from the complex by dimethylformamide (Fox *et al*., 1967).

LPL is a glycoprotein (8% by weight carbohydrate) with a native molecular weight of around 100 000 Da and a monomer subunit of about 50 000 Da (Kinnunen *et al*., 1976). Senda *et al*. (1987) calculated the molecular weight of the unglycosylated form as 50 548 Da based on the cDNA encoding it. LPL has a serine at the active site, located in a beta turn in the enzyme, similar to that at the active site of other serine hydrolases (Reddy *et al*., 1986).

LPL is a relatively unstable enzyme, being inactivated by ultraviolet light, heat, acid, oxidising agents (Frankel and Tarassuk, 1959), and prolonged freezing (Needs, 1992). Even in the mammary gland at body temperature, it is inactivated slowly and, as a consequence, milk contains a mixture of active and inactive LPL (Olivecrona and Bengtsson-Olivecrona, 1991). In milk, it is believed that LPL is stabilized by a factor in the skim milk fraction (Posner and Bermúdez, 1977), possibly a heparin-like glycosaminoglycan (Iverius *et al*., 1972). Caseins (Lebedev and Umanskii, 1979; Anderson, 1982b; Kim *et al*., 1994) and some lipids (Shimada *et al*., 1982) also stabilize it.

High-temperature short-time (HTST) treatment (72°C × 15 s) of milk almost completely inactivates the enzyme (Luhtala and Antila, 1968; Andrews *et al*., 1987; Farkye *et al*., 1995) so that little if any lipolysis caused by milk lipase occurs in pasteurised milk (Downey, 1974). Somewhat higher temperatures are required for cream pasteurization because of the protective effect of the fat (Nilsson and Willart, 1961; Downey and Andrews, 1966). However, some workers have reported that a more severe heat treatment, [e.g., 79°C × 20 s, (Shipe and Senyk, 1981) or 85°C × 10 s (Driessen, 1987)] is required to inactivate completely milk lipase.

The normal substrates for LPLs are long-chain triglycerides in blood chylomicrons and lipoproteins. These particles contain the apolipoproteins (e.g., apo-LP CII), which activate the enzyme (Östlund-Lindqvist and Iverius, 1975). Blood serum, either cows' or human, has an overall activating effect in assays of LPL using emulsified long-chain triglyceride substrates (Egelrud and Olivecrona, 1973). In such assays, a fatty acid acceptor such as bovine serum albumin (BSA) is required because LPL is susceptible to product inhibition by FFAs, which accumulate at the lipid–water interface (Bengtsson and Olivecrona, 1980). LPL is also active on tributyrin, but in this case requires neither serum co-factors nor fatty acid acceptors (Rapp

and Olivecrona, 1978) and a catalytic rate of about 50% of its "lipoprotein lipase activity" (measured on serum-activated long-chain triglycerides) is observed (Egelrud and Olivecrona, 1973). *p*-Nitrophenyl esters (Shirai and Jackson, 1982), Tween 20 and monoglycerides are also hydrolyzed in the absence of serum co-factors (Egelrud and Olivecrona, 1973).

In milk, LPL is not normally active on milk fat because of the protection afforded the fat by the milk fat globule membrane. However, addition of blood serum facilitates the interaction between the enzyme and the fat globule and lipolysis ensues (Castberg and Solberg, 1974; Jellema and Schipper, 1975). The mechanism of this serum-mediated induction of lipolysis is not known, although Bengtsson and Olivecrona (1982) concluded that the activating apo-LP CII performed a dual role of enhancing both binding of LPL to the fat globule and its catalytic efficiency.

Phospholipids also have a role in the LPL-catalyzed hydrolysis of triglycerides. The activator apo-LPs exhibit enhanced activation in the presence of phospholipids such as phosphatidyl choline (La Rosa *et al.*, 1970; Blaton *et al.*, 1974) and in milk there is evidence that apo-LPs in the absence of phospholipids are unable to initiate lipolysis of intact milk fat globules by the indigenous LPL (Driessen and Stadhouders, 1974; Clegg, 1980). The phospholipids are believed to be involved in the reaction through their interaction with the substrate rather than with the enzyme (Blaton *et al.*, 1974).

The discovery of lipolysis-inhibiting glycoproteins in skim milk, particularly proteose-peptone fraction 3 (PP3) (Anderson, 1981; Cartier *et al.*, 1990; Girardet *et al.*, 1993), and in the milk fat globule membrane (Shimizu *et al.*, 1982; Kester and Brunner, 1982; Sundheim and Bengtsson-Olivecrona, 1987b) supports an earlier observation (Dunkley and Smith, 1951) that milk contains lipolysis-inhibiting factors. PP3, a phosphorylated glycoprotein with an apparent molecular mass of 28 000 Da and 135 amino acid residues (Sørensen and Petersen, 1993), has been reviewed by Girardet and Linden (1996). Of particular significance is a C-terminal 38-amino acid residue segment, which binds to membranes (Bak *et al.*, 2000). Shimizu and Yamauchi (1983) considered PP3 to be identical with a major glycoprotein in the milk fat globule membrane, which can be solubilized with dilute NaCl.

Phosphatidyl choline is also hydrolyzed by milk LPL in the presence of serum co-factors (Stocks and Galton, 1980) to yield FFAs and lysophosphatidyl choline (Scow and Egelrud, 1976). The importance of this function of LPL appears to be in facilitating its access to the triglyceride core of particles having a phospholipid-containing membrane. The lysophospholipids have a high affinity for both LPL and lipoproteins (Portman and Alexander, 1976) and are powerful membrane-perturbing agents (Weltzien, 1979) and may aid lipolysis of milk fat in its globular form (Bläckberg *et al.*, 1981a).

LPL exhibits no fatty acid specificity during hydrolysis of mixed triglycerides but does have strong positional specificity (Morley and Kuksis, 1977). It acts on primary ester bonds with some preference for the *sn*-1 over the *sn*-3 position of triglycerides (Somerharju *et al.*, 1978) and can hydrolyze 2-monoglycerides only after their conversion to the *sn*-1 or *sn*-3 isomers (Nilsson-Ehle *et al.*, 1973). It shows phospholipase $A_1$ activity on phosphatidyl choline (i.e., it hydrolyzes the primary ester bond at the *sn*-1 position). This contrasts with most phospholipases A, which exhibit $A_2$ activitiy.

### 15.2.2. Human Milk Lipases

Human milk differs from cows' milk in that it contains, in addition to an LPL similar to that in cows' milk, a bile salt-stimulated lipase (BSSL), which appears to have no counterpart in cows' milk (Hernell and Blackberg, 1994). In addition, a third lipase known as platelet-activating factor acetylhydrolase activity (PAF-AH) has been demonstrated in human milk; the activity is absent, or extremely low in cows' milk (Furukawa, *et al.*, 1994).

The LPL in human milk resembles most other serum-stimulated lipases. While it is capable of hydrolysing triglycerides in the absence of exogenous serum factors, its activity is increased several-fold by blood serum (Hernell and Olivecrona, 1974a). LPL occurs mostly in the skim milk phase of human milk but after freeze–thawing is mostly associated with the cream phase (Neville *et al.*, 1991). In general, human milk contains less than half the LPL activity found in cows' milk (Hernell and Olivecrona, 1974a) but can become rancid on cold storage (Dill *et al.*, 1984; Hamosh *et al.*, 1996) or frozen storage at $\leq -20°C$ (Berkow *et al.*, 1984); freezing and thawing increase the extent of lipolysis. Ultrasonic homogenization at $< 45°C$ stimulates lipolysis but at $> 55°C$ it inactivates the lipolytic activity (Martinez *et al.*, 1992). In contrast to the case of cows' milk (Section 3.2), "spontaneous lipolysis" in human milk is strongly correlated ($r = +0.90$) with the LPL activity of the milk (Castberg and Hernell, 1975).

Human milk is unusual in containing a lipase that is activated by bile salts. BSSL has been found in the milk of only a few other mammals: gorilla (Freudenberg, 1966), cat, and dog, (Freed *et al.*, 1986), and ferret (Ellis and Hamosh, 1992). Ferret milk contains up to 20 times as much BSSL as human milk and constitutes a significant proportion (1–2%) of the total milk protein. BSSL in human milk is present in multimolecular forms, which differ from each other by the extent or quantity of glycosylation in the proline-rich region of the C-terminus of the enzyme (McKillop *et al.*, 2004).

Human BSSL shows immunological identity with the carboxyl ester hydrolase in pancreatic juice, and the two enzymes are very similar in molecular and kinetic properties (Bläckberg *et al.*, 1981b). In fact, Nilsson

*et al.* (1990) have shown from molecular cloning experiments that the two enzymes are identical and encoded by the same gene. However, milk BSSL is, evidently, synthesized in the lactating mammary gland (Bläckberg *et al*, 1987).

BSSL is located mainly in the milk serum and is optimally active at around pH 8 and 37°C in the presence of 8–14 mM sodium taurocholate. In the absence of bile salts, it can hydrolyze soluble esters (e.g., *p*-nitrophenyl acetate) and tributyrin, but is inactive against high molecular weight triglycerides. Bile salts promote the lipolysis of emulsified and micellar substrates, such as triolein and milk fat, and also enhance activity on emulsified tributyrin and soluble esters (Jubelin and Boyer, 1972; Hernell and Olivecrona, 1974b; Hall and Muller, 1982). Unlike LPL, BSSL has no positional specificity, hydrolysing triglyceride to mainly FFA and glycerol, but has a preference for short-chain and polyunsaturated fatty acids (Wang *et al.*, 1983; Wang, 1991).

The activation of BSSL is specific to primary bile salts. Bile salts, secondary as well as primary, protect BSSL against inactivation by intestinal proteinases. BSSL is inactivated by heating at 50°C for 1 h, but sodium taurocholate prevents loss of activity. The enzyme is stable in buffer for 1 h at 37°C between pH 3.5 and 9. It is inhibited by blood serum, 1M NaCl, protamine sulphate, eserine and diisopropylfluorophosphate (Hernell, 1975; Bläckberg and Hernell, 1983).

BSSL is present in high amounts in pre-term and mature milks, and varies little throughout lactation, with activity in the order of 100 times greater than LPL in human and cows' milks (Hernell and Olivecrona, 1974a; Freed *et al.*, 1987). It plays an important role in digestion in infants (O'Connor and Cleverly, 1989) and may provide a defence mechanism against parasites such as *Giardia lamblia* (Reiner *et al.*, 1986; Gillin *et al.*, 1991) (Section 5.2).

### 15.2.3. Milk Lipases of Other Species

Lipase activity has been detected in the milk of many other species and, where characterized, is generally serum-stimulated (e.g., goat, buffalo, horse, guinea pig, rat, rabbit, cat, and dog) (Hamosh and Scow, 1971; Jensen and Pitas, 1976; Freed *et al.*, 1986). With few exceptions, LPL appears to be the only significant indigenous lipase in milk. BSSL occurs in the milk of a small number of species (Section 2.2).

Goats' milk LPL has been studied extensively. In contrast to cows' milk LPL, it is distributed approximately equally between the cream and serum phases, with a small amount (*ca.* 10%) attached to caseins. Total activity is much lower than in cows' milk, and varies widely between individuals

(Chilliard *et al*., 1984). Between-breed and within-breed genetic effects on activity appear to exist (Chilliard, 1982). Physiological variations, such as stage of lactation, also influence activity (Chilliard and Morand-Fehr, 1978). Two distinct LPL forms with molecular weights in the range 55 000–66 000 Da have been isolated and found to differ from cows' milk LPL (De Feo *et al*., 1982).

Like cows' milk LPL, goats' milk LPL is inactivated by pasteurization. However, it is not inactivated by high-pressure treatment (500 MPa, 15 min, 20°C) (Trujillo *et al*., 1999) and appears to contribute to lipolysis in cheese made from pressure-treated milk (Buffa *et al*., 2001).

As with cows' milk (Section 3.1), homogenization, agitation (e.g., during machine milking) and temperature manipulation (cooling–warming–cooling) can initiate lipolysis in goats' milk (Bjørke and Castberg, 1976; Chilliard and Morand-Fehr, 1976; Morand-Fehr *et al*., 1990). Spontaneous lipolysis also occurs in the milk of some goats. In contrast to cows' milk, there is a significant correlation between this lipolysis and LPL activity, perhaps because of the relatively high proportion of LPL associated with the fat globules. The influence of inhibiting and activating factors is also apparent (Chilliard *et al*., 1984). The proteose peptone fraction of goats' milk inhibits mechanically induced and blood serum-activated lipolysis in goats' milk (Arora and Joshi, 1994). Lipolysis is linked with the occurrence of a "goaty" flavor in the milk (Bjørke and Castberg, 1976; Brendehaug and Abrahamsen, 1986).

Buffaloes' milk contains an LPL similar to cows' milk LPL and in comparable quantities. A higher proportion is located in the cream (e.g., 23% compared to 12% for cows' milk; Balasubramanya *et al*., 1988). Bhavadasan *et al*. (1988) found no relationship between the extent of lipolysis and LPL activity in either species. Lipolysis by LPL is inhibited by proteose peptone fractions 3, 5 and 8 from buffaloes' milk, with the PP3 fraction being the most inhibitory (Ram and Joshi, 1989). As in cows' milk, lipolysis can be induced by shaking or homogenization (Sammanwar and Ganguli, 1974).

Guinea pigs' milk contains a high level of LPL (20–50-fold that of cows' milk), with more than 90% in the skim (Hamosh and Scow, 1971). Guinea pig LPL has been purified and found to be very similar in molecular structure and properties to the LPLs of cows' and human milks (Wallinder *et al*., 1982; Bengtsson-Olivecrona *et al*., 1986).

LPL activity in rats' milk is only about 0.2% of that of guinea pigs' milk, probably reflecting interspecial differences in milk secretory processes (Hamosh and Scow, 1971). Activity in dogs' and cats' milk is also low, of a similar order to human milk (Freed *et al*., 1986).

cDNAs have been cloned for guinea pig (Enerbäck *et al*., 1987) and mouse LPL (Kirchgessner *et al*., 1987). Deduced amino acid sequences show considerable homology with human and bovine LPLs and with hepatic and pancreatic lipases, suggesting a common ancestral lipase gene (Olivecrona and Bengtsson-Olivecrona, 1991).

### 15.2.4. Esterases of Cows' Milk

In addition to the now well-documented lipase system, cows' milk contains several other carboxyl ester hydrolases, collectively referred to as esterases. These are distinguished from lipases by their ability to act on ester substrates in solution rather than in an emulsified form (Jaeger *et al*., 1994) and/or by their preference for hydrolysing esters of short-chain rather than long-chain acids (Okuda and Fujii, 1968).

Although several reports concerning esterases in milk have appeared in the literature, little detailed information on the individual enzymes is available. Arylesterase or A-esterase (EC 3.1.1.2), carboxylesterase or B-esterase (EC 3.1.1.1), and cholinesterase or C-esterase (EC 3.1.1.7; EC 3.1.1.8) have been identified (Forster *et al*., 1961; Kitchen, 1971; Nakanishi and Tagata, 1972; Deeth, 1978).

Arylesterase has received considerable attention because of its elevated level in colostrum and mastitic milk (Forster *et al*., 1959; Marquardt and Forster, 1965). Since its level in mastitic milk correlates well with other indices of mastitis (Luedecke, 1964), it has been suggested as a sensitive indicator of the disease (Forster *et al*., 1961; Downey, 1974). The enzyme is believed to originate from blood, where its activity is up to 2000 times that in milk (Marquardt and Forster, 1965).

Carboxylesterase activity is elevated in mastitic milk and colostrum (Fitz-Gerald *et al*., 1981) and may correspond to that of the reported "lipases" from somatic cells (Gaffney and Harper, 1965; Azzara and Dimick, 1985a) and colostrum (Driessen, 1976), respectively. The retinyl esterase activity that co-purifies with, but can be separated from, LPL may also be due to a carboxylesterase (Goldberg *et al*., 1986). It is of interest that the BSSL in human milk that has been shown to be identical with pancreatic carboxylesterase, has retinyl esterase activity (O'Connor and Cleverly, 1989).

Compared with the total lipase activity on emulsified milk fat or tributyrin (0.25–2.5 μmol/ml/min), the esterase activity (on soluble tributyrin) is quite low, about one tenth (Downey, 1974). This may not be so for some abnormal milks where esterase levels are markedly elevated [10–12 times (Marquardt and Forster, 1962) and up to 37 times (Deeth, 1978)]. The significance of these esterases in cows' milk and their relationship to each other, to LPL, and to esterases of other tissues remain to be determined.

### 15.2.5. Lipases of Psychrotrophic Bacteria

Extracellular lipases produced by psychrotrophic bacteria have considerable potential for causing hydrolytic rancidity in milk and milk products. The bacteria principally responsible for these lipases are pseudomonads, particularly *Pseudomonas fluorescens,* and *P. fragi*, Enterobacteriaceae such as *Serratia*, and *Acinetobacter* spp. Other significant organisms include *Achromobacter*, *Aeromonas, Alcaligenes*, *Bacillus, Flavobacterium, Micrococcus,* and *Moraxella* (Stewart *et al*., 1975; Muir *et al*., 1979; Shelley *et al*., 1987; Abdou, 2003). In a study of the lipolytic flora of raw milks showing lipolytic defects, Shelley *et al*. (1987) found *P. fluorescens* to be the species most frequently encountered but *P. fragi* to be associated with the most severe lipolytic defects. Reviews concerning bacterial lipases and their significance in milk and other foods have been published by Cogan (1977), Law (1979), Stead (1986), and Sørhaug and Stepaniak (1997), and by several authors in "*Enzymes of Psychrotrophs in Raw Food*" edited by McKellar (1989).

While many authors have reported that *Pseudomonas* species, particularly *P. fluorescens,* are the dominant lipase-producing psychrotrophs in milk, there appears to be considerable differences in lipase production among these closely related species (Wang and Jayarao, 2001; Deeth *et al*., 2002). Dogan and Boor (2003) reported that patterns of extracellular enzyme activity among *Pseudomonas* isolates appeared to be associated with ribotypes, with ribotypes 50-S-8 and 72-S-3 producing the highest extracellular lipase activity. Ribotyping has been used to identify high lipase-producing ribotypes. However, Wang and Jayarao (2001) found that although the 16S-23S PCR ribotyping technique allowed differentiation between strains, it did not concur with the API 20 NE biotypes and the lipolytic profiles. They concluded that use of biotypes in conjunction with lipolytic (and proteolytic) profiles might have practical value for conducting trace-back studies related to *P. fluorescens*.

Lipases are produced by psychrotrophic bacteria during the late log and early stationary phases of growth, often reaching a peak and then decreasing (Stead, 1987; Stepaniak *et al*., 1987b; Griffiths, 1989; Rowe *et al*., 1990). Little lipase is produced before cell numbers reach $10^6-10^7$/ml. The rate of growth of does not appear to correlate well with lipase production. In fact, Stevenson *et al*. (2003) found that, in most cases, pseudomonads from pasteurized milk with the lowest growth rate produced lipase earlier than those with a high growth rate. The influence of a wide variety of environmental and nutritional factors on the production of these enzymes has been reviewed in McKellar (1989).

Pseudomonad species usually constitute the largest percentage of lipolytic psychrotrophs in raw milk and cream and hence have attracted most attention. Lipases have been purified from *P. fluorescens* (Sugiura *et al*.,

1977; Severina and Bashkatova, 1979; Andersson, 1980; Dring and Fox, 1983; Stepaniak *et al*., 1987a), and *P. fragi* (Lawrence *et al*., 1967; Lu and Liska, 1969). The isolation and molecular characteristics of lipases from psychrotrophic bacteria have been reviewed by Fox *et al*. (1989).

In general, these microbial lipases have a molecular weight in the region 25 000 to 50 000 Da. Sugiura *et al*. (1977) purified a lipase from *P. fluorescens* with molecular weight 33 000 Da and found it to be a single polypeptide chain with no lipid or carbohydrate and without disulphide linkages, while Dring and Fox (1983) and Stepaniak *et al*. (1987a) isolated *P. fluorescens* lipases under dissociating conditions with a molecular weight of around 16 000 Da. On the basis of known amino acid sequences of lipases from *Pseudomonas* and *Burkholderia*, Dieckelmann *et al*. (1998) concluded that there were two major lipase groups, one of molecular weight approximately 30 kDa comprising lipases from *P. fragi, P. aeruginosa, P. fluorescens* C9 and *Burkholderia*, and one of approximately 50 kDa comprising *P. fluorescens* lipases. However, some workers have found lipase activity associated with material of molecular weight >100 000 Da, which probably represents aggregates of subunits as the enzyme associates strongly (Dring and Fox, 1983; Stepaniak *et al*., 1987a); the lipase from *P. fluorescens* SIK W1 has a molecular weight of 52 000 Da by SDS polyacrylamide gel electrophoresis or 48 179 Da as predicted from the isolated gene encoding it. This enzyme contains nine cysteine residues, which may participate in disulphide bridges (Chung *et al*., 1991). A gene coding for the lipase of *P. fragi* that was cloned into *E. coli* had a nucleotide sequence corresponding to a protein of 135 amino acids with a predicted molecular weight of 14 643 Da (Kugimiya *et al*., 1986).

The pH optimum of the lipases is usually in the alkaline region between 7 and 9. They generally show highest activity at 40–50°C, although there are reports of higher or lower temperature optima (Stead, 1986). The apparent optimum temperature may change with the state of purity of the enzyme (Severina and Bashkatova, 1979) and with the assay conditions used (Fitz-Gerald and Deeth, 1983). Many of these lipases show activity at the low temperatures used for storage of dairy products, [e.g., 10°C (Te Whaiti and Fryer, 1978), 1°C (Landaas and Solberg, 1978), and −10°C (Nashif and Nelson, 1953)].

One of the most important properties of these lipases is their heat stability (Stepaniak *et al*., 1995). This varies with the species and strain (Fitz-Gerald *et al*., 1982) and also with the medium in which they are heated (Andersson *et al*., 1979). Many are sufficiently stable to retain at least some activity after pasteurization (Law *et al*., 1976; Fitz-Gerald *et al*., 1982; Kalogridou-Vassiliadou, 1984), and even after UHT treatment (Kishonti, 1975; Mottar, 1981; Christen *et al*., 1986). Milk proteins, except κ-casein,

have been shown to have a thermostabilising effect on these lipases (Kumura *et al.*, 1993). For example, heating a lipase with β-lactoglobulin at 80–90°C caused virtually no change in enzyme activity. Some workers have reported a two-stage inactivation on heating, an initial rapid loss of activity, followed by a slow or even negligible decline (Fox and Stepaniak, 1983; Swaisgood and Bozoğlu, 1984; Driessen, 1987). A recent report suggests that high intensity pulsed electric field treatment may be more effective in inactivating lipases from *P. fluorescens* than thermal treatments; treatment with 80 pulses at 27.4 kV/cm in batch mode caused over 60% inactivation (Bendicho *et al.*, 2002). Similarly, sonication under pressure (650 kPa) at an elevated temperature (110 or 140°C) (manothermosonication) has also been found to inactivate a *P. fluorescens* lipase more efficiently than the corresponding heat treatment alone (Vercet *et al.*, 1997).

Some of the lipases are less stable at temperatures $\leq$ *ca.* 70°C than at higher temperatures (Kroll, 1989) and are thus susceptible to 'low-temperature inactivation' (LTI), which is effective for inactivating the corresponding proteinases (e.g., 55°C for 1 hour; Barach *et al.*, 1976). However, in fat-containing media, considerable lipolysis can occur during prolonged heating at 55°C and hence treatment at this temperature may have limited value for eliminating these lipases from milk products (Griffiths *et al.*, 1981; Fitz-Gerald *et al.*, 1982). Heating at a temperature >70°C (up to *ca.* 120°C) can cause activation of some lipases (Andersson *et al.*, 1979; Fitz-Gerald *et al.*, 1982) and exacerbate lipolysis problems. Bucky *et al.* (1987) combined UHT treatment with a subsequent LTI treatment of 60°C for 5 min in a patented process, which considerably enhances the effectiveness of UHT treatment in reducing lipase activity. Investigations of the mechanism of heat inactivation of these enzymes have concluded that inactivation at high or low temperatures involves different denatured states of the enzyme (Swaisgood and Bozoğlu, 1984) and that proteinases in the environment do not cause LTI, although lipases may exhibit reduced stability in their presence (Kumura *et al.*, 1991).

Unlike the corresponding proteinases, the lipases do not appear to contain metal ions (Nadkarni, 1971; Sugiura and Oikawa, 1977), but do require metal ions, such as $Ca^{2+}$ or $Mg^{2+}$, for activity (Severina and Bashkatova, 1979; Fitz-Gerald and Deeth, 1983). Excess EDTA causes complete inhibition of most bacterial lipases, which can be reversed by $Ca^{2+}$ or $Mg^{2+}$. *Acinetobacter* lipases have been found to be irreversibly inactivated by EDTA, while *P. aeruginosa* lipases are exceptional in being almost unaffected by excess EDTA (Nadkarni, 1971; Fitz-Gerald and Deeth, 1983). Some heavy metals are inhibitory, in particular zinc, iron, mercury, nickel, copper, and cobalt (Fitz-Gerald and Deeth, 1983; Fox *et al.*, 1989). These metals are effective at concentrations of less than 10 mM.

A low level of NaCl (10 mM) may cause activation (Khan *et al.*, 1967), although a high concentration inhibits the lipases. However, more than half of the activity remains in the presence of NaCl (2 M), a level similar to that in the aqueous phase of salted butter (Fitz-Gerald and Deeth, 1983).

Unlike milk LPL, microbial lipases do not require a fatty acid acceptor such as BSA (Bengtsson and Olivecrona, 1980). Blood serum has been found to activate some of these enzymes (Fitz-Gerald and Deeth, 1983), including *P. fluorescens* lipases, and these have, consequently, been designated lipoprotein lipases (Aisaka and Tarada, 1979; Stepaniak and Sørhaug, 1989).

While psychrotrophs produce true lipases that act on emulsified triglyceride substrates, many also produce esterases, which prefer soluble substrates (Stewart *et al.*, 1975; O'Donnell, 1978) or short-chain triglycerides, such as tributyrin, to long-chain triglycerides (Muir *et al.*, 1979; Garcia *et al.*, 1989). Chung *et al.* (1991) elegantly demonstrated that the esterase and lipase activities of a lipolytic strain of *P. fluorescens* were due to two different enzymes. After inserting fragments of the pseudomonad DNA into *E. coli* JM83, 20 of the resulting 12 000 recombinant colonies exhibited activity on tributyrin but only one had activity on longer chain triglycerides (in olive oil). Similarly, McKay *et al.* (1995) constructed highly lipolytic strains of *P. fluorescens* that over-produced, or were specifically deficient in, a lipase (encoded by *lipA*) and an esterase (encoded by *estA*). Analysis of broth cultures showed that the lipase was secreted into the culture medium while the esterase was intracellular and not secreted. They concluded that production of FFAs in milk cultures by this organism was due solely to the single secreted lipase and that the esterase was not involved. They also made the interesting observation that the profile and level of the FFAs accumulated in milk cultures was the result of both the production and degradation of FFA. This may explain the contradictory reports in the literature on the FFA profile in products contaminated with lipolytic organisms.

Lawrence *et al.* (1967) reported some preference for long-chain triglycerides by a *P. fragi* lipase but for short-chain triglycerides by a lipase from *Micrococcus freudenreichii*. Temperature may have an influence on the apparent specificity of lipolysis, with relatively more short-chain and unsaturated fatty acids being released from milk fat at lower temperatures. This appears to be a reflection of the physical state of the substrate (Alford and Pierce, 1961; Sugiura and Isobe, 1975).

Purified *P. fluorescens* lipases have activity against natural vegetable oils and a range of synthetic triglycerides from tributyrin to triolein (Severina and Bashkatova, 1979), and show preference for triglycerides rather than monoglycerides, and for medium-chain-length substrates (i.e., containing $C_8$–$C_{10}$ fatty acids) (Bozoğlu *et al.*, 1984). Most of the lipases have a preference for the primary (*sn*-1 and *sn*-3) positions of triglycerides (Alford

*et al.*, 1964; Nadkarni, 1971; Cooke, 1973). The profile of FFAs released from a given fat may be characteristic of the enzyme (Woo and Lindsay, 1983b; Ren *et al.*, 1988).

The lipases produced in crude cultures are usually capable of hydrolysing the triglyceride in intact milk fat globules (Fitz-Gerald and Deeth 1983), a property not exhibited by the indigenous milk LPL because of the protection afforded by the milk fat globule membrane (Danthine *et al.*, 2000). It is not known whether the lipases *per se* can penetrate the milk fat globule membrane or whether the membrane is first disrupted by other enzymes such as glycosidases, proteases and phospholipases (Mabbitt, 1981; Marin *et al.*, 1984; Alkanhal *et al.*, 1985; Cousin, 1989). Griffiths (1983) showed that a phospholipase C of *Bacillus cereus* increased the lipolytic activity of indigenous LPL in raw milk by making the substrate more susceptible to hydrolysis. However, it did not enhance the activity of lipase from a fluorescent pseudomonad. He considered that addition of phospholipase C had the same effect on lipolysis by lipoprotein lipase as homogenization of raw milk (Section 15.3.1 2).

### 15.2.6. Phospholipases

Phospholipases are potentially important in milk and milk products because of their ability to degrade the phospholipids of the milk fat globule membrane, thereby increasing the susceptibility of the milk fat to lipolytic attack (Fox *et al.*, 1976; Griffiths 1983).

Cows' milk LPL has phospholipase $A_1$ activity (Scow and Egelrud, 1976), but its action on milk phospholipids has not been recorded. Freshly secreted goats' milk has been shown to have phospholipase A activity (Long and Patton, 1978) but it is not known whether this can be attributed to the LPL of that milk. Human milk contains an acid sphingomylinase C, as well as ceramidase activity provided by the bile salt-stimulated lipase present (Nyberg *et al.*, 1998).

Several psychrotrophic bacteria produce extracellular phospholipases, the most prevalent in milk being pseudomonads (particularly *P. fluorescens*), *Alcaligenes, Acinetobacter,* and *Bacillus* species (Fox *et al.*, 1976; Owens, 1978a; Phillips *et al.*, 1981). Most of these produce phospholipase C, some produce phopholipase $A_1$ and some produce both types (Deeth, 1983). *Serratia* spp. have been shown to produce only phospholipase A (Deeth, 1983), while *P. fragi* has been reported not to produce phospholipases (Kwan and Skura, 1985). Phospholipase C from some pseudomonads has been purified and characterised (Doi and Nojima, 1971; Sonoki and Ikezawa, 1975; Stepaniak *et al.*, 1987a; Ivanov *et al.*, 1996). Like the lipases, many of these enzymes have considerable heat stability and are not destroyed by pasteurization

(Owens, 1978b; Stepaniak *et al*., 1987a; Koka and Weimer, 2001). Their heat stability varies with the bacterial strain and growth conditions (Koka and Weimer, 2001)

The phospholipases of *Bacillus* spp., especially *B. cereus*, have been studied because of their association with the "bitty cream" or "broken cream" defect in milk (Stone, 1952). By partially degrading the milk fat globule membrane, they initiate agglutination of the fat globules into cream flakes or flecks (Labots and Galesloot, 1959). The degradation is caused by phospholipase C (Shimizu *et al*., 1980), although *B. cereus* produces a sphingomyelinase (Ikezawa *et al*., 1978), which may be involved also. Phospholipase-producing bacteria other than *Bacillus* species do not appear to cause "bitty cream" (Labots and Galesloot, 1959; Owens, 1978a). The phospholipases of *B. cereus* have been isolated, purified, and extensively characterised (Zwaal *et al*., 1971; Ikezawa *et al*., 1978).

### 15.2.7. Lipolytic Enzymes in Milk Product Manufacture

Most lactic starters used in the manufacture of fermented milk products have weak lipolytic activity due to intracellular lipases and esterases (Fryer *et al*., 1967; Oterholm *et al*., 1968; Paulsen *et al*., 1980). The enzymes, present in the cytoplasm, are released in cheese as the starter cells lyse during maturation. In general, the lipases have pH and temperature optima around 6–7 and 37°C, respectively. They have specificity for short-chain fatty acids and show a preference for partial glycerides over triglycerides (Stadhouders and Veringa, 1973; El Soda *et al*., 1986). The main lipolytic action of starter bacteria in cheese may be to hydrolyze further monoglycerides and diglycerides produced by other lipases. Lipases of adventitious organisms such as yeasts, lactobacilli, and micrococci may also contribute to lipolysis during cheese ripening (Chapman and Sharpe, 1981; Gripon *et al*., 1991).

*Penicillium roqueforti* and *P. camemberti* produce very active extracellular lipases, which are the principal lipolytic agents in mold-ripened cheeses. They preferentially hydrolyze the short-chain fatty acids in milk fat. *P. roqueforti* produces two lipases, one with an alkaline pH optimum and the other most active at pH 6–6.5, with slightly differing fatty acid specificities (Menassa and Lamberet, 1982). *P. camemberti* secretes a single lipase with optimal activity at pH 9 (Lamberet and Lenoir, 1976).

Lipase preparations from numerous microorganisms, including those mentioned above, have been used in the synthesis of "dairy" (buttery or cheesy) flavors from milk fat (Arnold *et al*., 1975; Kilara, 1985) or to enhance flavor development in ripening cheese (Fox, 1988). These include lipases from *Rhizomucor (Mucor) miehei* (Moskowitz *et al*., 1977; Huge-Jensen *et al*., 1987), *Achromobacter lipolyticum* (Khan *et al*., 1967), *Aspergillus niger*

(Fukumoto *et al*., 1963), *A. oryzae* (Arbige *et al*., 1986), *Geotrichum candidum* (Jensen, 1974; Baillargeon and McCarthy, 1991), *Candida cylindracea* (Benzonana and Esposito, 1971), *C. lipolytica* (Alifax, 1979), *Rhizopus delemar* (Fukumoto *et al*., 1964), and *Rh. arrhizus* (Verhaeghe *et al*., 1990). The diversity of properties of these lipases, such as pH optimum and, in particular, specificity, enables the selection of an appropriate enzyme for a specific purpose (Kilara, 1985; Fox and Grufferty, 1991).

One such purpose is to modify milk fat to improve its nutritional properties using a lipase with *sn*-1,3 specificity and fatty acid specificity to reduce its level of long-chain saturated fatty acids (Patel and Thakar, 1994). This has been achieved by using a lipase immobilized onto hydrophobic hollow fibres and carrying out the hydrolysis/interesterification reaction in a solvent-free system under controlled water activity (Balcao *et al*., 1998). In another report, Garcia *et al*. (1998) used immobilized microbial lipases to enrich milk fat with conjugated linoleic acid, an anticarcinogenic fatty acid naturally present in low amounts in milk fat (Parodi, 1994; see Chapters 3 and 13). Safari and Kermasha (1994) used four commercial lipases to alter the positional structure of milk fat and found that three of the lipases enriched the triacylglycerols with palmitic acid at the *sn*-2 position, an important attribute of human milk fat (Innis *et al*., 1994).

Pregastric esterases are used in the manufacture of Italian cheeses to produce the characteristic "piccante" flavors (Fox and Guinee, 1987; Birschbach, 1992). These flavors are due to short-chain fatty acids, especially butyric, which are released preferentially from milk fat by these enzymes (Nelson *et al*., 1977). Pregastric esterases are produced by the salivary glands and can be obtained from the abomasum of milk-fed calves, lambs and kids. They have been isolated in heterogeneous form and have a molecular weight of approximately 172 000 (calf), 168 000 (kid), and 150 000 (lamb) Da (Lee *et al*., 1980). They are optimally active at 32–42°C and pH 4.8–5.5 (Richardson and Nelson, 1967). Pregastric esterases also find use in cheese flavor development and in flavor ingredient manufacture (Kilara, 1985; Fox, 1988).

Picon *et al*. (1997) included phospholipase C with encapsulated proteinases added to milk to stimulate the release of proteinase in Manchego cheese. The phospholipase, acted on the soy lecithin used to form the capsules, accelerated ripening of the cheese.

## 15.3. Causes of Hydrolytic Rancidity in Milk and Milk Products

Raw cows' milk contains a relatively large amount of lipase activity, but seldom undergoes sufficient lipolysis to cause an off-flavor. Under optimal conditions, the lipase (milk LPL) can catalyze the hydrolysis of up to *ca*.

2 μmol of triglyceride/ml/min (Egelrud and Olivecrona, 1972). Since milk with *ca*. 2 μmol FFA/ml has a rancid flavor (Lombard and Bester, 1979), it is of interest to ascertain why excessive lipolysis occurs in milk only under certain conditions.

Milk when freshly secreted from a healthy udder has $\leq 0.5$ μmol FFA/ml (Connolly *et al*., 1979; Bråthen, 1980). These acids result from incomplete synthesis rather than lipolysis. Under proper handling and storage conditions, only small increases in the FFA level should occur. In some cases, however, substantial increases are observed, which result from either "induced" or "spontaneous" lipolysis. "Induced" lipolysis results when the milk lipase system is activated by physical or chemical means. "Spontaneous" lipolysis is defined as that which occurs in milk which has had no treatment other than cooling soon after milking (Tarassuk and Frankel, 1957).

Mastitis and microbial contamination can also contribute to hydrolytic rancidity. In general, lipolysis caused by indigenous milk lipase accounts for most of the rancidity in raw milk and cream; microbial lipolysis is of minor practical importance as little if any lipolysis occurs before the bacterial population reaches $10^6-10^7$ cfu/ml (Suhren and Reichmuth, 1990). However, in stored milk products, lipolysis by microbial lipases is of greatest significance. Short shelf-life products such as pasteurized milks may be affected by pre-pasteurization lipolysis caused by milk lipase but may be affected by bacterial lipolysis at the end of their shelf-life (Deeth *et al*., 2002).

Lipolysis in milk is affected by inhibiting and activating factors. As discussed above, proteose peptone fraction of milk can inhibit milk LPL while apolipoproteins stimulate the enzyme. This is particularly important in spontaneous lipolysis; however, proteose peptone 3 has been shown to inhibit lipolysis induced by homogenization, sonication, and temperature activation (Arora and Joshi, 1994), while protein components of the milk fat globule membrane inhibit lipolysis caused by bacterial lipase (Danthine *et al*., 2000). Several exogenous chemical agents can also inhibit lipolysis (Collomb and Spahni, 1995). For example, polysaccharides such as λ-carrageenan at $\sim 0.3$ g/l effectively inhibits lipolysis in milk activated by mechanical means or temperature manipulation (Shipe *et al*., 1982) and lipolysis caused by the lipase from *P. fluorescens* (Stern *et al*., 1988).

### 15.3.1 Induced Lipolysis

#### 15.3.1.1. Agitation and foaming

Lipolysis in raw milk can be readily initiated by vigorous agitation, causing foaming. Such treatment disrupts the milk fat globule membrane and renders the milk triglycerides more accessible to milk lipase. Incorporation of air (or other gas) and consequent foam formation are essential for damage

to the milk fat globule and for the activation of lipolysis (Tarassuk and Frankel, 1955). The damage results from the high interfacial (liquid–air) tension acting on a small region of the fat globule surface (Mulder and Walstra, 1974).

The amount of lipolysis induced depends on the mode of agitation (e.g., air agitation, pumping, stirring), the severity and duration of agitation, the amount of lipase present, the content and hardness of the fat, and the vulnerability of the milk fat globule membrane (Dunkley and Smith, 1951; Claypool, 1965; Henningson and Adams, 1967; Deeth and Fitz-Gerald, 1977). Milk from cows in late lactation and milk with a tendency to spontaneous lipolysis are quite susceptible to agitation-induced lipolysis (Whittlestone and Lascelles, 1962; Ortiz *et al*., 1970).

The temperature of the milk during agitation has a major influence. In general, activation is greatest at 37–40°C and least at cold storage temperatures (<5°C). However, the relationship between temperature and activation is complex, and depends on the conditions of the mechanical treatment, the characteristics of the milk, and its age and previous thermal history (Deeth and Fitz-Gerald, 1977). Under certain conditions, the amount of induced lipolysis shows maxima at 12–15°C and 30–40°C with a minimum at 20°C (Fitz-Gerald, 1974; Deeth and Fitz-Gerald, 1977; Hisserich and Reuter, 1984). Hisserich and Reuter (1984) found that, at each temperature, there is a threshold mechanical stress intensity above which lipolysis is induced.

The level of agitation-induced lipolysis depends on the nature and extent of fat globule damage. The extent of damage has been estimated by the amount of free fat in the milk (Te Whaiti and Fryer, 1976), the amount of fat in the skim milk (Hlynka *et al*., 1945; Aule and Worstorff, 1975), and by the amount of milk fat globule-associated enzymes (e.g., alkaline phosphatase or Xanthine oxidoreductase) released from the fat globules (Stannard, 1975). While good correlations are observed between these parameters and the amount of induced lipolysis for agitation at a given temperature, poor correlations are found for treatments at different temperatures. This can be explained in terms of the agitation causing either aggregation or dispersion of the milk fat globules, with the predominant effect being determined by the temperature of the milk, and the severity of agitation (Deeth and Fitz-Gerald, 1978). A better indication of fat accessibility can be obtained from the amount of lipolysis, which results on addition of a lipase that does not attack the fat of intact milk globules, [e.g., that of *Candida cyclindracea* (Deeth and Fitz-Gerald, 1978)].

Besides its effect on the integrity of the milk fat globules, agitation redistributes lipase between the skim milk and cream phases (Frankel and Tarassuk, 1959). Agitation of milk at 5–10°C or 37°C results in a severalfold

increase in lipase activity associated with the cream (Deeth and Fitzgerald, 1977). The amount of lipase transferred to the cream is not reflected in the extent of lipolysis in whole milk, but it is in the lipolysis in cream separated from activated milk. Since the transferred enzyme is bound to the milk fat globule membrane and in this form has enhanced heat stability (Frankel and Tarassuk, 1959), the amount of redistribution is of particular relevance in butter manufacture.

Milking machines are a major cause of on-farm lipolysis (Fleming, 1979; O'Brien *et al*., 1998; Evers and Palfreyman, 2001). Agitation-induced activation results from faulty design and installation, and inadequate maintenance of milking machines, and is associated with excessive air intake into the system, causing turbulence and frothing (Whittlestone and Lascelles, 1962; Bakke *et al*., 1983). Features such as elbows, joints, in-line fittings, long and narrow pipes, and vertical risers in the milking line cause turbulence (Kelley and Dunkley, 1954; MacLeod *et al*., 1957; Speer *et al*., 1958). High-line milking machines and single-line systems in which milk and air mix intimately are of particular concern (Downes *et al*., 1974; Rasmussen *et al*., 1988). In a survey of the FFA level in New Zealand farm milk supplies, Evers and Palfreyman (2001) found that milking machine design was the most important factor, as milk with the lowest level of FFAs was obtained from machines in which the milk and air were transported separately. Heuchel (1994) evaluated 10 types of milking machine clusters and found that a significantly higher level of FFAs resulted from two clusters, which were designed with a cyclic air inlet near the base of the teat. An investigation in Ireland showed that a low claw air admission of 6 l/min and float valves in recorder jars were important factors for controlling lipolysis in milk obtained *via* the mid-level recorder system (O'Brien *et al*., 1998). Bulk farm milk tanks are rarely implicated in the activation of milk lipase, although excessive agitation has been reported to increase lipolysis (Hunter, 1966). Similarly, road tankers have been found to cause little activation (Kitchen and Cranston, 1969). However, in factory silos, air agitation of milk can cause activation if the air flow-rate is excessive or if agitation is continuous rather than intermittent (Sjöström, 1959).

Continuous pumping, particularly with aeration, causes damage to the milk fat globule membrane and subsequent lipolysis to an extent dependent on the type of pump (Downes *et al*., 1974; Kirst, 1980). Pumping of raw milk through ultrafiltration membranes (Hicks *et al*., 1990), and sudden release of pressure and the use of a milk exit temperature of $>7^{\circ}$C during concentration of raw milk by reverse osmosis can also induce lipolysis (de Boer and Nooy, 1980; Barbano *et al*., 1983). Factory separation of cream, where the cream is partially homogenized as it leaves the separator bowl at relatively

high pressure, can also promote lipolysis (Downes *et al*., 1974; Anderson *et al*., 1984).

The extent of lipolysis in raw milk or cream following activation is determined by the temperature and duration of storage. Rapid cooling to, and storage at, a low temperature (without freezing) minimizes lipolysis. The rate of lipolysis falls off with time but can be accelerated by further activation treatments (Downey, 1980).

#### 15.3.1.2. Homogenization

Homogenization of raw milk or cream results in the very strong activation of lipolysis. Milk may become perceptibly rancid within 5 min of treatment (Mulder and Walstra, 1974). Homogenization produces a large surface area of vulnerable milk fat and permits ready access for milk LPL. The activity of the enzyme *per se* is not increased by homogenization. Lipolysis in homogenised milk is related to the pressure, time and temperature of homogenization, [i.e., to the efficiency of milk fat dispersal (Parry *et al*., 1966)]. The rate of lipolysis in raw milk is greatest immediately after homogenization, then levels off. A second or third homogenization again promotes rapid lipolysis. The slowing and revival of lipolysis is attributable to the accumulation and dissipation of lipolysis products at the interface (Nilsson and Willart, 1960; Downey, 1980).

Lipolysis proceeds readily when pasteurised homogenised milk is mixed with raw, unhomogenised milk. Here, the amount of lipolysis depends on the ratio of the susceptible substrate to the amount of lipase, and is maximal for an approximately 50/50 mixture of the two milks (Nilsson and Willart, 1960). This phenomenon is particularly important in the dairy factory since any recirculation of pasteurised homogenised milk back into the raw milk during start-up or closedown of processing plants can cause appreciable lipolysis. In commercial practice, the homogeniser is placed immediately before or directly after the pasteurizer so that the milk lipase is heat-inactivated before it can cause lipolysis in the homogenised milk.

#### 15.3.1.3. Temperature Activation

Lipolysis may be induced when fresh milk or cream is subjected to a specific sequence of temperature changes. The optimum amount of ''temperature activation'' is promoted by cooling to $\leq 5^{\circ}C$, warming to 25–35°C, followed by re-cooling to $<10^{\circ}C$ (Krukovsky and Herrington, 1939; Kon and Saito, 1997). Lipolysis proceeds on storage at this low temperature. Rewarming reverses the activation. Milk samples from individual cows vary widely in their susceptibility to temperature activation, the milk from some cows, and even some herds being completely resistant to it. The reasons for

the variability are not clear, although milk susceptible to spontaneous lipolysis is more likely to be affected (Claypool, 1965; Saito and Kim, 1995). The feeding regime of cows may also affect the susceptibility of their milk to "heat-induced" rancidity (Astrup, 1984).

Susceptibility is a property of the cream phase, as has been demonstrated by interchanging cream and skim milk from susceptible and non-susceptible milks (Claypool, 1965; Wang and Randolph, 1978). Kon and Saito (1997) demonstrated that the presence of the MFGM is essential for the phenomenon and suggested that temperature manipulation reduces the protective effect of the membrane and allows the lipase access to the fat. Pasteurised cream can also be activated, and undergoes lipolysis on subsequent mixing with raw (cold) skim milk. The activation treatment facilitates the attachment of the lipase to the surface of the milk fat globules. In contrast to the lipase–milk fat globule membrane interactions that occur in agitation-induced or spontaneous lipolysis, this attachment is reversed by rewarming, but can re-form if the milk is cooled again (Krukovsky and Herrington, 1939; Claypool, 1965).

In practice, temperature activation can occur if a small amount of cooled milk or cream is mixed with a larger amount of warm milk and then recooled (McDowell, 1969; Nielsen, 1978). Separation of previously cooled milk at a temperature around 30°C can lead to lipolysis if the cream is held in cold storage before pasteurization.

#### 15.3.1.4. Freezing

Freezing and thawing of milk that leads to churning of the fat (Mulder and Walstra, 1974) may induce lipolysis (Willart and Sjöström, 1966). Slow freezing and repeated freeze–thawing are most effective, but the extent of lipolysis which ensues is less than that promoted by moderate agitation. Freezing of milk in farm bulk tanks is the most likely cause of this activation. A long period of storage, however, can reduce the susceptibility of milk to lipolysis because of the reduction of lipase activity (Needs, 1992).

### 15.3.2. Spontaneous Lipolysis

#### 15.3.2.1. Characteristics

Milk that undergoes lipolysis without being subjected to any of the treatments described above has been referred to as "naturally active," "susceptible," "spontaneously lipolytic" or "spontaneous" in contrast to "normal" milk in which no lipolysis occurs.

Spontaneous milk can be produced by most, if not all, cows but, because of the individuality of cows and their response to various factors,

only a proportion of cows in a herd produce such milk at any one time. Reported proportions are between 3 and 35% (Downey, 1980). Spontaneous lipolysis is defined here as that which occurs in some individual milks when cooled to < about 15°C soon after milking.

The sooner spontaneous milk is cooled and the lower the temperature to which it is cooled, the more lipolysis that occurs (Tarassuk and Richardson, 1941; Bachman and Wilcox, 1990a); if cooling is delayed, the extent of lipolysis is reduced (Dunkley, 1946; Kitchen and Cranston, 1969). Once the milk is cooled, spontaneous lipolysis proceeds during cold storage and the rate of lipolysis increases if the temperature is raised (Tarassuk and Richardson, 1941). As with induced lipolysis, the rate of spontaneous lipolysis is high initially but levels off later. An FFA level of up to 10 meq/l can be obtained (in extreme cases) after 24 hours storage at 5°C.

A characteristic of spontaneous lipolysis is that it is inhibited by normal milk. Tarassuk and Henderson (1942) reported that mixing normal and spontaneous milk in the ratio of 3:1 before cooling prevented lipolysis. In fact, the ratio required for complete inhibition depends on the properties of both milks and highly susceptible milk requires a high ratio of normal to spontaneous milk to prevent lipolysis (Deeth and Fitz-Gerald, 1975a). Furthermore, admixing of two spontaneous milks can result in more lipolysis than if the two milks are incubated separately. This phenomenon can be explained in terms of the lipase activity in the two milks and the relative levels of inhibitors and activators present (Section 15.3.2.3) (Deeth and Fitz-Gerald, 1975a). Because of the usual predominance of normal milk over spontaneous milk, lipolysis in bulk herd milk is usually low. A high proportion of spontaneous milk, resulting from a coincidence of some of the factors discussed below, can lead to a high FFA level in bulk milk.

#### 15.3.2.2. Factors Affecting Spontaneous Lipolysis

*Stage of lactation*. The variability in propensity to produce spontaneous milk applies to individual cows within a herd, cows in different herds, cows at different stages of lactation and pregnancy, and even to the same cows from day to day and from lactation to lactation (Fredeen *et al*., 1951). Stage of lactation is one of the most important factors responsible for this variability, with cows in late lactation having the greatest tendency to produce spontaneous milk (Fredeen *et al*., 1951; Ortiz *et al*., 1970; Saito, 1983, 1992; Ahrné and Björck, 1985), with the last two months before drying off being critical (Chazal and Chilliard, 1987b). One of the first flavor defects to be linked with lipolysis was known as "bitter milk of late lactation" (Palmer, 1922). However, cows at any stage of lactation can produce spontaneous milk.

Most investigators have found the interval since calving or length of lactation to be more important than the stage of pregnancy, with long lactations being particularly conducive to the production of spontaneous milk (Fredeen *et al*., 1951). However, some reports have suggested that the stage of gestation or a combination of gestation stage and low milk yield may be more significant than the stage of lactation (Bachmann, 1961; Chazal and Chilliard, 1986; Bachman *et al*., 1988).

*Feed and nutrition*. Both the quality and quantity of feed influence the tendency of a cow to produce spontaneous milk (Fredeen *et al*., 1951; Jellema, 1980). The milk of most cows on a low plane of nutrition has an enhanced susceptibility (Gholson *et al*., 1966; Astrup *et al*., 1980). The effect of low energy intake is particularly marked when cows are in late lactation (Stobbs *et al*., 1973; O'Brien *et al*., 1996) but can also be considerable in early lactation (Dillon *et al*., 1997). The cow's body condition has not been found to be a reliable indicator of the susceptibility of her milk to spontaneous lipolysis (Ortiz *et al*., 1970).

Cows fed a dry ration, particularly hay (Tarassuk *et al*., 1962) and high carbohydrate winter feeds (Chen and Bates, 1962; Kodgev and Rachev, 1970) have generally been found to be more likely to produce susceptible milk than cows fed green pasture (Tarassuk and Regen, 1943; Fredeen *et al*., 1951; Jensen *et al*., 1960). This may explain the report of Alkanhal *et al*. (2000) claiming that milk from cows fed a high (70%) level of concentrate exhibited a higher level of spontaneous lipolysis than milk from cows fed a low (40%) level of concentrate. Lipase activity and initial FFA concentration were also higher in raw milk from cows fed high levels of concentrate. However, Chazal and Chilliard (1986) reported higher milk FFA levels for cows at pasture than when housed. Feeding of silage can result in serious problems with spontaneous lipolysis (Johnson and Von Gunten, 1961; Chazal *et al*., 1987). In changeover feeding trials, lag times of 4–5 days have been observed for corresponding changes in the susceptibility of the milk (Tarassuk, 1940; Stobbs *et al*., 1973).

In feeding trials with lipid supplements, Astrup *et al*. (1980) observed that palmitic or myristic acid significantly enhanced spontaneous lipolysis but stearic acid and fatty acids with a chain length shorter than myristic acid had no effect. These workers found that feeding rapeseed oil to underfed cows reduced the susceptibility of their milk to lipolysis, while Chazal and Chilliard (1985) reported that supplementation with non-protected lipids, particularly highly unsaturated oils such as rapeseed, increased the level of FFAs in milk. Protected oil supplements have been found to lead to reduced lipolysis in milk (Astrup *et al*., 1979) or to have little effect on FFA level (Urquhart *et al*., 1984).

An in-depth review of the effects of feed and nutrition on spontaneous lipolysis was published by Jellema (1980).

*Season*. Several authors have suggested a seasonal variation in the incidence of spontaneous lipolysis, with most indicating a higher incidence in the colder months (Dunkley, 1946; Kodgev and Rachev, 1970; O'Brien *et al*., 1999) but others the opposite effect (Hunter *et al*., 1968; Chazal and Chilliard, 1986). In New Zealand, Evers and Palfreyman (2001) found significantly lower FFA levels in spring milk than in autumn milk. However, it appears that the season *per se* is not the determining factor but rather the stage of lactation/pregnancy of the majority of the cows and the type and availability of feed (Speer *et al*., 1958; Hunter *et al*., 1968; Menger, 1975; Chazal and Chilliard, 1986).

*Milk production*. In general, low-yielding cows are more likely to produce spontaneous milk than are high-yielding animals (Hunter *et al*., 1968; Ortiz *et al*., 1970; Jellema and Schipper, 1975; Chazal and Chilliard, 1986; Saito, 1992). The milk yield, like season, cannot be considered an independent variable as it is dependent on other factors such as stage of lactation and quality and quantity of feed (Ahrné and Björck, 1985).

There have been several reports that milk obtained at the evening milking is more susceptible to spontaneous lipolysis than that obtained in the morning (Doody *et al*., 1975; Jellema and Schipper, 1975; Saito, 1983; Ahrné and Björck, 1985). This is attributed to the shorter inter-milking interval before the evening milking, which results in lower milk production (Suhren *et al*., 1981; Saito, 1983). O'Brien *et al*. (1998) examined the effect of milking interval on the FFA level in the milk from cows with an average daily yield of 25 kg and found no difference between a 16:8 h and a 12:12 h interval.

*Other factors*. A cow's hormonal balance can affect the susceptibility of her milk to spontaneous lipolysis (Fredeen *et al*., 1951; Kästli *et al*., 1967; Bachman *et al*., 1988). The oestrus cycle appears to have little effect on spontaneous lipolysis (Fredeen *et al*., 1951) but may affect lipase activity in the milk (Kelly, 1945). In contrast, treatment of cows with oestradiol and progesterone has been shown to lead to increased lipolysis in the milk (Bachman, 1982; Heo, 1983; Bachmann *et al*., 1985) but no change (Bachman, 1982) or a transient increase (Bachmann *et al*., 1985) in total lipase activity. It appears that the increased lipolysis in milk following hormonal treatment, or in milk from cows with ovarian cysts, may not be typical spontaneous lipolysis as cooling is not needed to initiate it (Bachman, 1982); a lipase other than lipoprotein lipase, possibly a bile salt-stimulated lipase, may be responsible for such lipolysis (Heo, 1983; Bachmann *et al*., 1985). Treatment of cows with bovine somatotropin has been reported to have no significant effect on milk lipoprotein lipase activity (Azzara *et al*., 1987).

Research in the Netherlands has indicated that the type of milking machine used can affect the physiology of the cow's udder and thereby influence the susceptibility of the milk to lipolysis (Jellema, 1975). Cows milked with a one-line system produced milk that was more susceptible than milk from the same cows when milked with a two-line system. Shorter times between milking can have a marked effect on the level of spontaneous lipolysis; the milk of some cows changes from normal to spontaneous when milking interval was reduced to 3–4 h (Jellema, 1986). This may be due to the higher LPL activity in milk drawn after a short interval, as the lipase loses activity at body temperature (Olivecrona and Bengtsson-Olivecrona, 1991).

The breed of the cow, generally, does not appear to affect its propensity to produce spontaneous milk (Chilliard, 1982). For example, Chazal and Chilliard (1987a) found no difference between Friesian and Montbéliarde cows in relation to spontaneous lipolysis. Bachman *et al.* (1988), however, found that the milk of Jerseys is more susceptible than that of Holsteins. There also appears to be some within-breed heritability of spontaneous milk production (Deeth and Fitz-Gerald, 1976; Jurczak, 1996).

Bachman *et al.* (1988) reported a low repeatability (0.22) of spontaneous lipolysis in the milk of cows from lactation to lactation. However, according to Chazal and Chilliard (1987c), the repeatability between two successive lactations explained 30–40% of the variation in FFA data. These authors concluded that spontaneous lipolysis evident in late pregnancy is dependent on an intrinsic factor repeatable from lactation to lactation as well as an extrinsic factor, probably linked to diet.

Supplementation of cows' diets with zinc has been found to reduce spontaneous lipolysis significantly (Hermansen *et al.*, 1995). The authors suggested that zinc deficiency may be a potential risk factor for spontaneous lipolysis. However, corroborative evidence is required before zinc supplementation could be recommended for reducing spontaneous lipolysis.

#### 15.3.2.3. Biochemical Aspects

*A priori*, one might expect the extent of spontaneous lipolysis in a milk is determined largely by the amount of enzyme present. All milks have sufficient lipase activity to cause extensive lipolysis (Downey, 1980) but this occurs only when other conditions, as outlined below, are conducive to such lipolysis. With few exceptions (e.g., Hemingway *et al.*, 1970), low correlations between lipase activity and the level of spontaneous lipolysis have been reported (Castberg and Solberg, 1974; Driessen and Stadhouders, 1974; Clegg, 1981; Ahrné and Björck, 1985; Cartier and Chilliard, 1990). However, a universal finding is that there is a high correlation between the

amount of lipase bound to the milk fat globule and the degree of lipolysis (Ahrné and Björck, 1985; Sundheim and Bengtsson-Olivecrona, 1985). Conditions that favor dissociation of the lipase from the casein micelles or attachment of the enzyme to the milk fat globule membrane increase the susceptibility of milk to spontaneous lipolysis (Downey and Andrews, 1966; Deeth and Fitz-Gerald, 1975a; Anderson, 1982a; Sundheim and Bengtsson-Olivecrona, 1985).

The susceptibility of the milk fat globule and the permeability of the milk fat globule membrane have been considered to be important in spontaneous lipolysis (Dunkley and Smith, 1951; Bachmann, 1961; Deeth and Fitz-Gerald, 1975a). However, investigations involving intermixing of skim milk and cream fractions from normal and spontaneous milks have indicated that the extent of lipolysis is dependent more on the skim than on the cream portion (Claypool, 1965; Deeth and Fitz-Gerald 1975a; Murphy *et al.*, 1979). Cartier and Chilliard (1990) obtained a correlation coefficient of +0.57 between spontaneous lipolysis and the susceptibility of the fat to *Candida cylindracea* lipase, a measure of the condition of the milk fat globule membrane (Deeth and Fitz-Gerald, 1978). However, Sundheim and Bengtsson-Olivecrona (1987a) observed only slight differences in the behavior of fat globules isolated from normal or spontaneous milks as substrates for purified lipoprotein lipase. One difference observed was the shorter lag time for spontaneous fat globules before lipolysis commenced. The state of the milk fat globule membrane may be most significant as a factor in spontaneous lipolysis in late lactation milk (Bachmann, 1961; Deeth and Fitz-Gerald, 1975a) where the amount of phospholipid may not be enough for the formation of sufficient membrane to cover the increased surface area (at least twice that of mid-lactation milk) of the fat globules (Kinsella and Houghton, 1975). A further indication of the importance of the condition of the milk fat globule membrane is the greater susceptibility of spontaneous and late-lactation milks to induced lipolysis (Whittlestone and Lascelles, 1962; Jellema and Schipper, 1975).

In spontaneous lipolysis, the milk lipase attaches to the milk fat globule membrane during cooling (Ahrné and Björck, 1985; Hohe *et al.*, 1985; Sundheim and Bengtsson-Olivecrona, 1985). This interaction does not occur if the milk is not cooled, is reduced if cooling is delayed, but is not disrupted if milk is rewarmed after being cooled (Sundheim and Bengtsson-Olivecrona, 1987a). The formation of this enzyme–membrane complex appears to be an essential feature of spontaneous lipolysis as no lipolysis occurs if the interaction is prevented by delayed cooling, addition of NaCl or other inhibitory factors (see below) (Deeth and Fitz-Gerald, 1975a; Sundheim and Bengtsson-Olivecrona, 1987a). The role of the milk fat globule membrane may be a rather complex regulatory one since it contains both activating

lipoproteins (Castberg and Solberg, 1974) and inhibitory proteins (Shimizu *et al*., 1982; Sundheim and Bengtsson-Olivecrona, 1987b). Furthermore, since milk fat globules from both normal or spontaneous milk become receptive to attachment of lipoprotein lipase (and heparin) when cooled in the absence of skim milk, the membrane must undergo some conformational change such that its affinity for lipase (and heparin) increases (Sundheim and Bengtsson-Olivecrona, 1987d).

Following the discovery that the major lipolytic enzyme in milk is a lipoprotein lipase and that lipolysis could be initiated in any milk by the addition of blood serum or serum lipoproteins (Jellema, 1975), it was suggested that spontaneous milk contains activating cofactors derived from the blood (Jellema and Stadhouders, 1974). Some considered that these cofactors may be small (molecular weight *ca*. 10 000 Da) soluble apo-LPs, which could leak into the milk from the blood during times of physiological stress (Olivecrona, 1980). The blood lipoproteins are much larger and are less likely to be transferred to the milk. However, the work of Clegg (1980) and Driessen and Stadhouders (1974) indicate that the lipid component is essential for activating lipolysis. Milk has been shown to contain material that is immunologically cross-reactive with bovine serum lipoproteins (Castberg and Solberg, 1974; Anderson, 1979). Bachman and Wilcox (1990b) found that added HDL, the major lipoprotein responsible for activation of lipolysis (Sundheim *et al*., 1983) redistributes the lipoprotein lipase towards the milk fat globules rather than activating already-bound enzyme, and suggested that HDL in milk could be responsible for spontaneous lipolysis.

The presence of an inhibitory factor (or factors) in milk has been suggested to explain the lack of lipolysis in normal milk and the inhibition of lipolysis when normal milk is mixed with spontaneous milk (Dunkley and Smith, 1951). It has been demonstrated that normal skim milk contains a heat-stable, dialysable inhibitor (Deeth and Fitz-Gerald, 1975a), and that proteose-peptone 3 is an effective non-competitive inhibitor (Anderson, 1981; Cartier *et al*., 1990). The inhibitors prevent lipolysis by blocking the lipase–milk fat globule membrane interaction (Deeth and Fitz-Gerald, 1975a).

The activator–inhibitor balance in the skim fraction of milk appears to be a major determinant of the extent of spontaneous lipolysis (Deeth and Fitz-Gerald, 1975a; Sundheim and Bengtsson-Olivecrona, 1987c; Cartier and Chilliard, 1990). Thus, depending on the relative amounts of activators and inhibitors, skim milk or milk serum can act as an inhibitor or an activator, but are most commonly inhibitory. The inhibiting/activating nature of a skim milk can be assessed by preincubating it, after heating to destroy indigenous LPL, with a substrate emulsion such as Intralipid and

then determining the resulting lipolysis on incubation with purified LPL (Cartier and Chilliard, 1990), or by observing the effect of heated skim milk on the interaction of purified lipoprotein lipase with washed, pooled milk fat globules during cooling, and the subsequent amount of lipolysis (Sundheim and Bengtsson-Olivecrona, 1987c).

Thus, four factors have been shown to contribute to the susceptibility of a milk to spontaneous lipolysis: lipase activity, milk fat globule vulnerability, activating factors and inhibiting factors, with the balance of the last two being most important (Deeth and Fitz-Gerald, 1975a; Sundheim, 1988; Cartier and Chilliard 1990). Sundheim (1988) concluded that these factors could explain 80–87% of lipolysis induced by cold storage.

### 15.3.3. Mastitis

Mastitis has often been considered to be a cause of spontaneous lipolysis (Downey, 1980) because many mastitic milks have an elevated level of FFAs (Tarassuk and Yaguchi, 1958; Tallamy and Randolph, 1969; Fitz-Gerald *et al.*, 1981). These levels tend to increase with increasing somatic cell count (SCC). However, many milks that can be classified as mastitic (SCC $> 0.5 \times 10^6$/ml; Kästli, 1967) do not have an elevated level of FFAs relative to corresponding healthy quarters; the percentage of milks exhibiting lipolysis increases approximately linearly up to 100% at SCC of $\geq 10 \times 10^6$/ml (Fitz-Gerald *et al.*, 1981).

The data of Gudding (1982) suggest that the elevation of FFAs may depend on the cause of mastitis, as relatively higher levels of FFAs were observed in milk from quarters infected with *Staphylococcus aureus*. When mastitis is induced experimentally by intramammary infusion of endotoxins or bacteria, the increases in FFAs correspond closely with the increases in SCC and other indices of mastitis (Salih and Anderson, 1979; Fitz-Gerald *et al.*, 1981; Ma *et al.*, 2000). Murphy *et al.* (1989) concluded that the increased lipolysis in mastitic milk is due to increased susceptibility of the milk fat.

Mastitic milk samples have higher initial levels of FFAs than normal milk (Tarassuk and Yaguchi, 1958; Fitz-Gerald *et al.*, 1981; Murphy *et al.*, 1989; Van den Heever *et al.*, 1990). They also show greater increases in FFAs during storage than milk from healthy quarters (Fitz-Gerald *et al.*, 1981; Van den Heever *et al.*, 1990) but these increases are small compared with those observed in spontaneous milks. For these reasons, mastitis is not considered to be a cause of spontaneous lipolysis as defined here. The relationship between the level of milk lipase and mastitis has been the topic of numerous investigations. Some surveys have shown reduced milk

lipase activity in mastitic milk (Fitz-Gerald *et al.*, 1981; Murphy *et al.*, 1989), an effect attributed to impaired biosynthetic capability of the mammary tissues caused by infection and inflammation or by the action of intracellular proteinases from somatic cells on the milk lipase (Jurczak and Sciubisz, 1982). Other reports have indicated a higher level of lipase in mastitic milk (Tallamy and Randolph, 1969; Azzara and Dimick, 1985b). The lipase in mastitic milk has a different distribution from that of normal milk, with a higher proportion being in non-micellar form, either soluble (Salih and Anderson, 1979) or associated with the cream (Bachmann, 1961).

The leucocytes in milk contain a lipase (Gaffney and Harper, 1965; Azzara and Dimick, 1985a) or carboxylesterase (Deeth, 1978), which may contribute to lipolysis in mastitic milk. When suspensions of these cells are added to milk, the level of FFAs increases, almost linearly up to a cell count of *ca.* $2 \times 10^6$/ml (Salih and Anderson, 1978). More lipolysis is observed if the cells are disrupted prior to addition to milk (Jurczak and Sciubisz, 1981).

Azzara and Dimick (1985a) reported that $2.5 \times 10^6$ macrophages/ml would contribute 1% of total lipase activity on addition to milk, but this value increased to 11.6% after 48 h storage. They suggested that the macrophage lipases may bind to the milk fat globule membrane and activate the substrate to the action of milk lipase.

### 15.3.4. Microbial Lipolysis

Hydrolytic rancidity can occur in milk and milk products as a result of contamination by microorganisms premanufacture or postmanufacture (Lawrence, 1967). The introduction of bulk cold storage has led to the emergence of psychrotrophic (mainly Gram-negative) bacteria as the dominant organisms in raw milk and cream (Thomas and Thomas, 1978; Cousin, 1982). Storage of milk at the farm and factory can extend its age at processing to several days, and allow sufficient growth for spoilage, including lipolysis, to occur. Only a proportion of psychrotrophs (between 0.1 and *ca.* 30%, depending on the composition of the flora of the sample (Chapman *et al.*, 1976; Muir *et al.*, 1978a) produces appreciable amounts of lipolytic enzymes, so that there is not necessarily a good correlation between psychrotroph numbers and FFA or rancid flavor (Muir *et al.*, 1978b). Muir *et al.* (1978a) observed lipolysis in stored farm or factory milks only when psychrotroph counts were $> 5 \times 10^6$/ml.

Lipolysis can occur in pasteurised milk as a result of post-pasteurization contamination by lipolytic psychrotrophs. In whole milk, this is a major cause of spoilage while in skim milk, proteolysis is the major cause

(Deeth *et al.*, 2002). In a study of the bacteriological quality of pasteurized milks from three Australian factories, Craven and Macauley (1992) concluded that the standard of hygiene in the factory (as indicated by the number of contaminants in the milk immediately after processing) and the lipolytic activity of the stains that grow to a sufficient number to cause spoilage are the most important factors determining the keeping quality of the milk.

Lipolysed off-flavor is one of the most common flavor defects in (pasteurised) market cream (Mottar, 1989; Jensen and Poulsen, 1992). Development of flavor defects in pasteurized milk and cream is associated with contamination by high numbers (e.g., $> 10^7$/ml) of psychrotrophs (Bandler *et al.*, 1981). Taints may be evident after 4–5 days at 5°C (Hawney and Royal, 1970) or after a shorter time at a higher temperature (Muir *et al.*, 1978a). In cultures of single lipolytic psychrotrophs, a level of approximately $10^6$/ml (Suhren *et al.*, 1977) or $10^7$/ml (Overcast and Skean, 1959; Shelley *et al.*, 1986) is attained before spoilage is apparent. Psychrotrophic sporeformers can cause lipolytic spoilage in milk heat-treated at greater than 72°C × 15 s but less than UHT (Eibel and Kessler, 1987).

Heat-stable bacterial lipases have been implicated in the development of rancidity during storage of products, [e.g., UHT milk (Mottar, 1981), butter (Nashif and Nelson, 1953; Deeth *et al.*, 1979), and cheese: Cheddar (Law *et al.*, 1976; Cousin and Marth, 1977), Dutch (Driessen and Stadhouders, 1971), Swiss (Pinheiro *et al.*, 1965), Teleme (Kalogridou-Vassiliadou and Alichanidis, 1984) and Camembert (Dumant *et al.*, 1977)] made from milk containing a high number of psychrotrophs (usually $>10^7$/ml) before UHT or HTST treatment. However, Adams and Brawley (1981) reported that UHT milk with counts of $10^4$–$10^5$ cfu/ml before processing lipolysed on storage at 25 or 40°C. Celestino *et al.* (1996) reported that in UHT milk produced from reconstituted powder made from cold-stored (48 h at ~4°C) raw milk, bacterial lipases (and proteinases) were reactivated during storage and that the taste of reconstituted UHT milk was affected more by lipolysis than by proteolysis.

The lipases have been found to be concentrated in the cream on separation and in the curd on rennet-induced coagulation (Kishonti and Sjöström, 1970; Driessen and Stadhouders, 1975; Stead, 1983). They can survive during the manufacture of dried milk (Shamsuzzaman *et al.*, 1987) and cause lipolytic defects in a wide range of fat-containing foods in which milk powder is an ingredient (Stead, 1986).

Psychrotrophic yeasts, molds and bacteria can cause rancidity and surface spoilage in butter (Thomas and Druce, 1971), a problem less significant now than previously due to improved factory methods and hygiene.

## 15.4. Detrimental Effects of Lipolysis in Milk and Milk Products

### 15.4.1. Flavor Defects

#### 15.4.1.1. Milk and Cream

The relationship between the flavor of milk and its FFA content has been examined by numerous workers, and threshold FFA levels for the detection of rancid flavor have been established. Many of these studies have been reviewed (Kuzdzal-Savoie, 1975, 1980; Connolly *et al.*, 1979; Anderson *et al.*, 1991).

The threshold value reported shows considerable variation (Table 15.1). The wide range can be attributed largely to variations in the method (Section 15.6.1) used to obtain the thresholds. Furthermore, individual people differ widely in their ability to detect rancid flavor, according to their natural flavor perception and their degree of training in tasting (Anderson, 1983; Bodyfelt *et al.*, 1988; Duncan *et al.*, 1991). This is particularly evident in studies in which taste panellists have been used (e.g., MacLeod *et al.*, 1957; Connolly *et al.*, 1979). Some authors have reported a low correlation between FFA level and rancidity score. Duncan *et al.* (1991) reported a correlation coefficient of 0.27 for laboratory-prepared rancid samples; however, a much better correlation ($r = 0.93$) was found for farm milk samples. For pasteurized milk, Christen *et al.* (1992) reported a significant correlation between levels of FFAs and sensory evaluation by both a trained panel and experts on day 1 but the correlation was lower on day 15. It was suggested that other flavors may have confounded the results for the stored samples.

**Table 15.1.** Level of Free Fatty Acids (FFA) (meq/100 g fat) in Normal and Rancid Milk

| Normal value | Typical range | Threshold value for off-flavor | Typical range |
|---|---|---|---|
| 0.25–0.4 (Christen, 1993); <1 (Renner *et al.*, 1989) | 0.3–1.0 IDF(1987) | 0.7* (Magnusson, 1974); >1.0 (Azzara and Campbell, 1992); >1.2 (Christen, 1993); 1–1.5 (Allen, 1994); 1.5–2 (Downey, 1980); 2.0* (Bråthen, 1980); >2 (Bodyfelt *et al.*, 1988); 2.0–2.2 Tallamy and Randolph (1969); 2.74 (Kintner and Day, 1965); 1.46–3.62 (Duncan *et al.*, 1991). | 1.5–2.0 IDF (1987) |

* These values are in meq/l

The presence of other flavors may explain the curious situation with UHT milk for which Collins *et al.* (1993) reported a lack of significant correlation between rancidity score and the extent of lipolysis. In other studies, UHT milk samples with an acid degree value (ADV) from 1.2 to 3.0 meq/100 g fat were reported to show no lipolysed flavor (Hansen and Swartzel, 1980; Earley and Hansen, 1982; Rerkrai *et al.*, 1987) while other researchers have shown that when the ADV exceeds 1.5 meq/100 g fat, UHT milk is judged to be rancid (Renner, 1988). One possible explanation is the different patterns of fatty acids released during the storage of raw milk. Choi and Jeon (1993) reported that the fatty acids released were largely long-chain acids which make a low contribution to rancid flavor. Choi *et al.* (1994) subsequently isolated two types of lipase from UHT milk and found that one, associated with the milk fat globules, released predominantly palmitic and strearic acids, while the other from the serum fraction released mostly butyric, followed by caproic and palmitic acids. They suggested that the former activity was due to microbial lipase while the latter was due to milk LPL. The presence of a serum-activated lipase, possibly milk LPL, in UHT milk was also reported by Pande and Mathur (1990, 1992), who found a substantial increase in this activity during storage but no lipolysed flavor was detected during storage. The presence of active milk LPL in UHT milk is unexpected given the heat lability of this enzyme (Section 15.2.1).

The thresholds given by Tallamy and Randolph (1969) (for non-UHT milk) appear to be reasonable and generally acceptable, (i.e., 1.2–1.5 meq/100 g fat for trained experts and 2.0–2.2 meq/100 g fat for the average consumer). A report by the IDF (1987) concluded that rancidity is normally detected between 1.5 and 2.0 meq/100 g fat. Rancidity is detected at approximately the same levels in cream (Dunkley, 1951; IDF, 1991). Similar numerical levels apply to milk where FFA content is measured by an extraction–titration method and expressed as meq/l milk (Bråthen, 1980).

Rancid flavor in milk is not due to a single fatty acid (Bassette *et al.*, 1986; Bodyfelt *et al.*, 1988; Jeon, 1994), but to a mixture of fatty acids, mainly of chain lengths $C_{4:0}$–$C_{12:0}$ (Kolar and Mickle, 1963; Al-Shabibi *et al.*, 1964; Scanlan *et al.*, 1965; Allen, 1994; Jeon, 1994), although some bitterness may be due to partial glycerides (Jensen, 1964). The long-chain acids containing 14 to 18 carbons make little contribution to flavor. Al-Shabibi *et al.* (1964) concluded that although the $C_{4:0}$–$C_{8:0}$ acids constitute part of the rancid flavor, the $C_{10:0}$ and $C_{12:0}$ acids are responsible for most of the unclean, bitter, soapy flavor of lipolysed milk.

The theoretical concentrations of the $C_{4:0}$–$C_{12:0}$ fatty acids in milk with a total FFA content of 2 meq/l are shown in Table 15.2, together with the range for rancid milks found by Kintner and Day (1965) and the threshold levels for these individual acids reported by Scanlan *et al.* (1965).

**Table 15.2.** Concentration (mg/kg) of Free $C_{4:0}$–$C_{12:0}$ Fatty Acids in Rancid Milk

| Fatty acid | Theoretical[a] for milk with 2 meq/l | Found for range of rancid milks[b] | Threshold level of added acids[c] |
|---|---|---|---|
| $C_{4:0}$ | 26.6 | 27.5–85.0 | 46.1 |
| $C_{6:0}$ | 15.1 | 16.0–48.7 | 30.4 |
| $C_{8:0}$ | 9.1 | 8.3–27.9 | 22.5 |
| $C_{10:0}$ | 17.6 | 27.6–78.6 | 28.1 |
| $C_{12:0}$ | 17.6 | 26.7–63.3 | 29.7 |

[a] Assuming an average molecular weight of 228 Da and the FFA profile reported by Day (1966).
[b] The range of concentrations of FFAs from threshold rancidity to extremely rancid (Kintner and Day, 1965).
[c] Amounts added to fresh, pasteurized, homogenized milk to produce a rancid flavor (Scanlan *et al.*, 1965).

It is clear that the levels of the individual acids in rancid milk can be considerably lower than the threshold values reported by Scanlan *et al.* (1965), which do not include the levels contributed by the fresh milk, (i.e., approximately half the concentrations shown in the second column). Thus, the flavor of the combination of the acids in rancid milk is apparently sufficient to exceed the threshold for detection of rancidity.

In milk with a normal pH of $\sim$6.7, most of the acids are in the salt form and have much less flavor than if they were completely in the acid form (Kuzdzal-Savoie, 1980). In fact, acidification of milk greatly enhances the sensitivity of organoleptic detection of lipolysis in milk (Tuckey and Stadhouders, 1967). The detection of rancidity is reduced by the association of the FFAs with certain proteins in milk (Parks and Allen, 1979; Keenan *et al.*, 1982) and by heating of milk (Kintner and Day, 1965). The phase in which the fatty acids are soluble also influences their flavor threshold, since the short-chain acids have much lower thresholds in fat than in water, while the opposite applies to the long-chain acids (Patton, 1964). For example, butyric acid ($C_{4:0}$) has a flavor threshold of 7 mg/kg in water, but only of 0.6 mg/kg in oil (Delahunty and Piggott, 1995).

#### 15.4.1.2. Butter

Hydrolytic rancidity in butter is characterised by off-flavors variously described as "bitter," "unclean," "wintery," "butyric," "rancid" or "lipase." The defect may be evident at manufacture (Störgards and Magnusson, 1966), but is more likely to develop during storage (O'Connell *et al.*, 1975; Bell and Parsons, 1977; Azzara and Campbell, 1992; Champagne *et al.*, 1994).

The concentration of FFAs in butter is usually expressed as ADV in meq/100 g fat (or mg NaOH/100 g fat; 1 meq = 40 mg NaOH). As in the

case of milk, numerous workers have endeavored to correlate FFA level with flavor to establish a threshold for rancidity. In many studies, little or no correlation has been observed between ADV and "lipase" defects (Bell and Parsons, 1977; Connolly *et al.*, 1979). However, Woo and Lindsay (1983a) established a statistical correlation between flavor and individual FFAs. Investigators have reported thresholds ranging from 0.75 to 2.8 meq/100 g fat (Krukovsky and Herrington, 1942a; Downey, 1980; Woo and Lindsay, 1983a). A threshold value of 1.5 meq/100 g fat appears to be a realistic guide for the butter manufacturer (Stadhouders, 1972). Besides lipolytic defects, butter with an ADV $>1.5\,\text{meq}/100\,\text{g}$ fat is likely to have other defects, (e.g., oxidised flavors), and to deteriorate during storage (Deeth *et al.*, 1979). There is some evidence that free fatty acids oxidize more readily than esterified acids (Mukherjee, 1950) and hence may predispose butter to oxidative rancidity (Badings, 1970).

The difficulty in relating rancid flavors in butter to FFA content arises because the short-chain acids, $C_{4:0}$ and $C_{6:0}$, which are the most flavorsome (McDaniel *et al.*, 1969), are water-soluble and hence are mostly lost in the buttermilk and wash water during the manufacture of butter. For this reason, even butter made from cream with an ADV as high as 2.4 meq/100g fat may show little defect while on the other hand, butter with quite a low ADV can be rancid, particularly if lipolysis occurs after manufacture (Deeth *et al.*, 1979; Woo and Lindsay, 1980).

The flavor thresholds for the individual fatty acids are quite different in butter and in milk. Whereas in milk, $C_{10:0}$ and $C_{12:0}$ acids are most significant to rancid flavor, in butter $C_{4:0}$ and $C_{6:0}$ are of most interest since they have much lower flavor thresholds in fat than do the longer-chain acids (Patton, 1964). The reported thresholds of the $C_{4:0}$ to $C_{12:0}$ acids added singly or in pairs to butter are shown in Table 15.3, together with the theoretical amounts for an increase in ADV of 0.1 meq/100 g fat. From these data, it is evident that a low level of lipolysis in butter produces sufficient butyric acid to exceed its flavor threshold and to impart a rancid flavor. Thus, measurement of $C_{4:0}$, $C_{6:0}$ and $C_{8:0}$ gives the best indication of hydrolytic rancidity in butter (McNeill *et al.*, 1986).

The above discussion applies to sweet-cream butter only. Little information is available on cultured butter, but O'Connell *et al.* (1975) found that ripened-cream butter is less prone to the development of hydrolytic rancidity than the corresponding salted or unsalted sweet-cream butter.

#### 15.4.1.3. Cheese

The typical flavor of aged cheese is due to the combination of a variety of flavor compounds, including FFAs (Law, 1984). When excessive lipolysis

**Table 15.3** Concentrations of $C_{4:0}$–$C_{12:0}$ Free Fatty Acids (FFA) in Butter

| | FFA level (mg/kg) | | |
|---|---|---|---|
| | Flavor thresholds[a] | | |
| Fatty acid | Added singly | Added in pairs[c] | Theoretical[b] increase for $\Delta ADV = 0.1$ meq/100 g fat |
| $C_4$ | 11.4 | 4–7 | 11.3 |
| $C_6$ | 51.5 | 16–26 | 6.4 |
| $C_8$ | 454.6 | 148–367 | 3.9 |
| $C_{10}$ | 161.6 | 132–173 | 7.4 |
| $C_{12}$ | 127.9 | 70–84 | 7.4 |

[a] For acids added to good quality butter (McDaniel *et al.*, 1969).
[b] Based on the FFA profile of rancid milk (Day, 1966) and assuming average molecular weight of 228 Da and 82% fat in butter.
[c] Range of thresholds when FFA was tested in pairs with each of the other 4 FFAs in this table
ADV = acid degree value.

occurs in cheese, or in the cheesemilk before manufacture, this balance is upset and rancid flavors result (Woo *et al.*, 1984; Fox and Law, 1991). Pasteurised-milk cheeses have lower levels of FFAs than raw-milk cheeses, for example, 38% lower in Manchego (Gaya *et al.*, 1990) and 50% lower in Cheddar (McSweeney *et al.*, 1993). The difference has been attributed to the higher numbers of non-starter lactic acid bacteria (McSweeney *et al.*, 1993) and the indigenous milk lipase (Grappin and Beuvier 1997) present in raw-milk cheese. Vlaemynck (1992) showed that milk LPL may contribute to lipolysis in raw-milk Gouda-type cheese as it retained some activity at pH 5.3 and in 1 M NaCl. Apart from lipolysis due to milk LPL or contaminant microbial lipases, rancid flavors may arise through the addition of lipases or esterases to accelerate flavor development (Law and Wigmore, 1985) or from lipase-contaminated microbial rennet preparations.

Because of the high total flavor of cheese, the threshold levels of FFAs are higher than for milk or butter. In Cheddar cheese, ADVs of 2.8–3.0 meq/100 g fat are usually attained before rancidity is evident (Deeth and Fitz-Gerald, 1975b). Various studies have shown that rancid Cheddar has 2–10 times more FFAs than good quality cheese (Bills and Day, 1964; Ohren and Tuckey, 1969; Law *et al.*, 1976).

Levels of individual short-chain FFAs or combinations of these have been suggested as superior to total FFA or ADV as indicators of the desirable/undesirable lipolysis status and flavor potential of various cheeses, in particular butyric acid ($C_{4:0}$) and total short-chain FFAs ($C_{4:0} + C_{6:0} + C_{8:0}$) (Woo *et al.*, 1984; Arbige *et al.*, 1986; Lin and Jeon,

1987; McNeill and Connolly, 1989). For some non-Cheddar varieties, Kuzdzal-Savoie (1975) concluded that the concentration of caproic acid ($C_{6:0}$) is the best guide to the lipolysis status of a cheese. She determined the following caproic acid levels (in mg/100 g dry matter) beyond which organoleptic defects was evident: Emmental, 8; Gruyère, 14; and Camembert, 20–25. For good quality Cheddar cheese, Urbach (1993) concluded that less than 0.52% of the fat is lipolysed at manufacture but up to 1.6% after ripening for 20 months. Cheeses that lay above this concentration had off-flavors; many of them were soapy, but oniony and metallic/vomit flavors also occurred. The percentage lipolysis was calculated from the amount of free $C_{16:0}$ that had been released.

### 15.4.2. Technological Consequences

While flavor defects are the most likely result of lipolysis in dairy products, several other practical problems may arise from an elevated level of FFAs. The most common of these is lack of foaming of pasteurised milk for cappuccino-style coffee (IDF, 1987). Reduced efficiency of skimming of raw milk and reduced churning efficiency in cream may be associated with lipolysis, especially where excessive agitation or pumping causes damage to the milk fat globule membrane.

Lipolysis in milk reduces its surface tension (Tarassuk and Smith, 1940) and, as a consequence, lipolysed milk has a low foaming capacity (Brunner, 1950; Dunkley, 1951). This is particularly noticeable during the steam-frothing of milk used for making cappuccino coffee, where stable foam formation is essential (Buchanan, 1965). Milk with an FFA level greater than 1.5 meq/l usually has poor foaming properties, while that with a level greater than 2 meq/l exhibits negligible steam-frothing. Commercial milk-processing operations, pasteurization and homogenization, markedly enhance the steam-frothing capacity of milk (Deeth and Smith, 1983). The reduction in surface tension and the consequent detrimental effect on foaming caused by lipolysis is believed to be due to the partial glycerides, particularly the monoglycerides, formed during lipolysis (Brunner, 1950; Buchanan, 1965).

Difficulties have been experienced in churning cream from spontaneously-rancid milk (Krukovsky and Sharp, 1936). The cream foams excessively and may take several times longer than normal cream to churn. Fat lost in the buttermilk is similar to that for normal milk (Fouts and Weaver, 1936). However, milk in which lipolysis is induced by agitation at a warm temperature gives higher fat losses on separation (Aule and Worstorff, 1975) due to the partial homogenization of the fat. Similarly, excessive pumping of milk has been found to reduce the fat content of cheese due to the loss of fat

in the whey (McDonald *et al.*, 1986). However, Hicks *et al.* (1990) attributed the reduced yield of cheese made from excessively pumped milk to loss in the whey of soluble FFAs produced by lipolysis rather than to the loss of fat. Such a situation is unlikely to arise from normal pumping in factories.

A high level of FFAs may cause minor errors in determination of the fat content of milk (e.g., a decrease of 0.01–0.03% fat per 1 meq/100 g increase in FFA), whether by older methods such as Röse-Gottlieb or turbidimetric or infrared methods (van Reusel, 1975; van de Voort *et al.*, 1987). Lipolysis may also lead to false positives in testing for antibiotic residues in milk (Carlsson and Björck, 1992).

## 15.5. Beneficial Effects of Lipolysis in Milk and Milk Products

### 15.5.1. Production of Desirable Flavor

Lipolysis plays an important role in providing the characteristic flavor of many milk products. In particular, the ripening of most cheese varieties is accompanied by lipolysis due to microorganisms or to added enzyme preparations, and, in raw milk cheese, to the milk LPL. Lipolysis is not extensive, but is more pronounced in some cheeses (e.g., blue-veined and hard Italian varieties), than in others. Excessive lipolysis renders the cheese unacceptable (Fox and Law, 1991; Gripon *et al.*, 1991).

In some mold-ripened cheeses, a very high FFA content (up to 25% of total fatty acids; Gripon, 1987) is acceptable [e.g., >66 000 mg/kg for Blue cheese (Horwood *et al.*, 1981) compared to <4000 mg/kg for good quality Cheddar; (Bills and Day, 1964)]. High levels of butyric acid characterise Italian hard cheeses and certain pickled cheeses (Fox and Guinee, 1987), [e.g., up to 520 mg/kg for Greek Feta (Horwood *et al.*, 1981) and >3000 mg/kg for Romano (Woo and Lindsay, 1984)]. An imbalance in flavor constituents can, nevertheless, lead to undesirably rancid or goaty ($C_{4:0}$–$C_{8:0}$) or soapy ($C_{10:0}$–$C_{12:0}$) flavors in these cheeses (Woo and Lindsay, 1984).

In ripening Cheddar cheese, some of the butyric and most of the higher free fatty acids are formed by lipolysis of the milk fat, mainly by lipases of lactic acid bacteria (Chapman and Sharpe, 1981; Aston and Dulley, 1982). In Swiss varieties, acids can arise through the action of lipases of propionibacteria (Oterholm *et al.*, 1970) or through fermentation (Kleinhenz and Harper, 1997). Italian varieties, such as Provolone and Pecorino cheeses acquire their characteristic flavor from the action of pregastric esterases, traditionally from rennet paste used to coagulate the milk but now in the form of commercially prepared oral gland extracts (Fox and Guinee, 1987; Birschbach, 1992). In Blue cheeses such as Gorgonzola, Roquefort, and Stilton, the FFAs produced by *Penicillium roqueforti* lipase (and milk lipase

when raw milk is used) are important both as flavor agents *per se* and as precursors for the methyl ketones, which provide the peppery taste of such cheeses (Kinsella and Hwang, 1976). A similar function is served by *P. camemberti* lipase in surface-mold ripened cheeses such as Brie and Camembert. Lipase preparations (microbial and pregastric) have been used to enhance the flavor (Arnold *et al.*, 1975; Huang and Dooley, 1976) and accelerate the ripening of cheese, for example Cheddar (Arbige *et al.*, 1986), buffalo milk Gouda (Rajesh and Kanawjia, 1990) and Blue cheese (Jolly and Kosikowski 1975a) (reviewed by Fox, 1988).

Flavor preparations typical of particular varieties of cheese can be produced with the aid of lipases of appropriate specificities (Kilara, 1985). Such flavors are used in processed cheeses, dips and spreads (Jolly and Kosikowski, 1975b). Controlled lipolysis of milk fat is also used to produce creamy and buttery flavors for bakery and cereal products, confectionery (milk chocolate, fudge), coffee whiteners, and other imitation dairy products (Arnold *et al.*, 1975; Fox, 1980; Kilara, 1985).

### 15.5.2. Digestion of Milk Fat

Absorption of fat in the newborn, and particularly in premature infants, is much less efficient than in adults due to the relatively low output of lipase and bile salts from the pancreas of the infant. Intragastric lipolysis by milk BSSL and lingual or salivary lipases, the secretion of which is stimulated by suckling, appears to augment the pancreatic lipase system in the newborn (Hamosh, 1979; Hernell and Blackberg, 1994).

Although LPL activity is commonly present in the milk of mammalian species, its role in the digestion of milk fat by the young animal has not been demonstrated (Olivecrona *et al.*, 2003). Olivecrona *et al.* (2003) speculated that LPL may serve an auxiliary role by binding milk fat globules to the mucosa, binding to intestinal cell surfaces and transferring lipids into the cells; these roles may not be dependent on the hydrolytic activity of the enzyme.

The bile salt-stimulated lipase in human milk acts as a supplementary milk fat digestive enzyme. Its characteristics suit it for such a role (O'Connor and Cleverly, 1989). It is both stable and fully active under the conditions pertaining in the gastrointestinal tract of the newborn and its lack of specificity enables it to hydrolyze a range of acylglycerols and other esters of nutritional significance, such as retinyl and cholesteryl esters (Hernell, 1975, 1985). BSSL has been detected in the gastric and intestinal contents of infants following the ingestion of fresh human milk, and average fat absorption has been found to be higher than after feeding cows' milk formulae or heated human milk (Fredrikzon *et al.*, 1978; Williamson *et al.*, 1978). This

increase is particularly significant in pre-term infants, or those suffering from pancreatic insufficiency (Williamson *et al*., 1978; Hamosh *et al*., 1981).

Bernback *et al*. (1990) found that complete digestion of human milk triglycerides *in vitro* requires the concerted action of gastric lipase, pancreatic co-lipase-dependent lipase and BSSL. While BSSL contributes to the hydrolysis of triglycerides and diglycerides, its unique function is to hydrolyze monoglycerides to FFA and glycerol, which may be absorbed more readily (Hernell, 1975). However, this does not apply to palmitic acid ($C_{16:0}$) which is poorly absorbed as the free acid and is absorbed as the 2-monoacylglycerol; about 70% of this acid is esterified at the *sn*-2 position of human milk triacylglycerols (Innis *et al*., 1994). *In vivo* studies in the suckling dog and mouse suggest, however, a greater role for BSSL in intragastric lipolysis than predicted by *in vitro* experiments (Iverson *et al*., 1991; Howles *et al*., 1999). BSSL also has ceramidase activity, which breaks down the ceramide produced from sphingomyelin after its hydrolysis by an indigenous acid sphingomylinase C (Nyberg *et al*., 1998).

Kittens fed formula supplemented with purified human BSSL grew twice as fast as those on formula alone (Wang *et al*., 1989) and the production and use of BSSL as a dietary supplement has been proposed (Tang and Wang, 1989).

Investigations on the protective effect of breast-feeding against giardiasis in infants have led to the discovery that raw human, gorilla, and grey-cheeked mangabey milk can destroy *Giardia lamblia in vitro* (Gillin *et al*., 1991). The toxic factor has been identified as FFAs produced by BSSL (Reiner *et al*., 1986). Hamosh (1998) suggested that the third lipase in human milk, platelet-activating factor acetylhydrolase, might play a role in preventing inflammatory reactions.

## 15.6. Analytical Methods

### 15.6.1. Free Fatty Acids

The quantification of all FFAs in milk or milk products presents a difficult analytical problem and has been the subject of a large number of research publications. Reviews of the methods have been published by Kuzdzal-Savoie (1980), an IDF/ISO/AOAC Group (Anderson *et al*., 1991), de la Fuente and Juarez (1993), Joshi and Thakar (1994) and Collomb and Spahni (1995).

The FFAs range from the water-soluble, short-chain acids such as butyric ($C_{4:0}$) and caproic ($C_{6:0}$) to the water-insoluble, long-chain acids such as palmitic ($C_{16:0}$) and stearic ($C_{18:0}$). In milk and milk products they are accompanied by relatively large amounts of fat, largely triglycerides,

water-soluble acids such as lactic acid, and phospholipids, some of which are acidic.

The methods usually involve an initial step to isolate the fatty acids, with or without the milk fat, followed by a quantification step, with or without derivatisation of the acids. Direct methods using infrared (Buermeyer *et al*., 2001; Pedersen, 2003) or biosensor technology (Ukeda *et al*., 1992) that do not require a pretreatment step offer some obvious advantages and may be suitable for in-line or at-line FFA monitoring. A summary of the methods used is given in Table 15.4.

The methods involving fat separation, of which the Bureau of Dairy Industries (BDI) methods are most common, measure only fat-soluble FFAs and hence underestimate the total FFA level (Deeth *et al*., 1975; Van Crombrugge *et al*., 1982). For milk, this is of little consequence as the level of the long-chain acids correlates highly with that of the short-chain acids responsible for the flavor. In products such as butter, measurement of fat-soluble FFAs only will not detect small increases in FFA postmanufacture which can have a detrimental effect on flavor due to the short-chain FFAs present (Deeth *et al*., 1979).

Solvent extraction methods estimate a high proportion of FFAs. However, when the acids are quantified by titration, some of these methods can overestimate the FFA level because of the inclusion of lactic acid, acidic phospholipids and other acidic interfering compounds in the extract (Chilliard *et al*., 1983; Selselet-Attou *et al*., 1984). Solvent extraction methods based on mixtures of isopropanol, heptane and sulphuric acid (Dole and Meinertz, 1960; Landaas and Solberg, 1974; Deeth *et al*., 1975; Mouillet *et al*., 1981; Mahieu, 1983) have found widespread use in several countries and are used in routine analysis of large numbers of samples in automated versions (Lindqvist *et al*., 1975; Suhren *et al*., 1977; Bråthen, 1984; Anderson *et al*., 1991).

Adsorption of FFAs on various solid supports, usually after solvent extraction, has led to the development of the best methods for quantifying all the FFAs present in a product. Some of these in which the FFAs are quantified by GC are now regarded as reference methods (Anderson *et al*., 1991). In some earlier solid adsorption methods, hydrolysis of the fat occurred on the support and overestimated the true FFA level (Stark *et al*., 1976). However, methods have been developed using deactivated alumina (Deeth *et al*., 1983) or ion exchange resins (Needs *et al*., 1983; Spangelo *et al*., 1986; de Jong and Badings, 1990) in which fat hydrolysis does not occur.

Capillary gas chromatography of FFAs as either free acids (de Jong and Badings, 1990) or as methyl esters (Chilliard *et al*., 1983; Juarez *et al*., 1992) provides excellent resolution of the component acids. Similarly, well-resolved chromatograms have been obtained in HPLC analyses of milk FFAs as

**Table 15.4.** Summary of Methods Used to Determine Free Fatty Acids (FFAs)

| Type of isolation | Isolation reagent/method | Method of quantification | Product | References |
|---|---|---|---|---|
| Fat separation | Churning | Titration | Milk, cream | Herrington and Krukovsky (1939) |
| | Melting | Titration | Butter, milk fat | Deeth and Fitz-Gerald (1976), IDF(1989) |
| | Detergent demulsification (BDI) | Titration–manual | Milk, cream | Thomas *et al.* (1955) |
| | | Titration–semiautomatic | Milk | Driessen *et al.* (1977) |
| | | Titration–photometric | Cream, whole milk powder | Evers *et al.* (2000) |
| Solvent extraction | Isopropanol–heptane–$H_2SO_4$ (Dole) | Titration–manual colorimetric | Milk, cream | Deeth *et al.* (1975), Mouillet *et al.* (1981) |
| | | Titration–automatic colorimetric | Milk | Suhren *et al.* (1977), Cartier *et al.* (1984) |
| | | Titration–automatic potentiometric | Milk | Bosset *et al.* (1990) |
| | Isopropanol–heptane–$H_2SO_4$–isobutanol (Lipo-R) | Titration–manual | Milk | Mahieu (1983) |
| | Ether–heptane–ethanol–$H_2SO_4$+ weak anion exchanger | Capillary GC of FFAs | Milk, cheese | de Jong and Badings (1990) |
| | Ether–hexane–HCl | Titration | Milk | Salih *et al.* (1977) |
| | Chloroform–heptane–methanol | Colorimetry of copper soaps | Milk, cheese | Koops and Klomp (1977), Shipe *et al.* (1980), Bynum *et al.* (1984) |
| | Acetonitrile–$H_2SO_4$ + $CH_3I$–strong anion exchanger | GC of methyl esters | Milk | Spangelo *et al.* (1986) |
| | Diethyl ether and acid + tetramethylammonium hydroxide | GC of methyl esters after pyrolysis of the tetramethylammonium soaps | Cheese | Juarez *et al.* (1992), Chavarri (1997) |
| | Benzene–K oxalate–$H_3PO_4$ | Colorimetry using Rhodamine 6G | Milk | Nakai *et al.* (1970) |

(*Continued*)

**Table 15.4.** (*Continued*)

| Type of isolation | Isolation reagent/method | Method of quantification | Product | References |
|---|---|---|---|---|
| | Alcohol–ether–petroleum ether | Titration | Milk | Frankel and Tarassuk (1955), Pillay *et al.* (1980) |
| Solid phase adsorption | Silicic acid–$H_2SO_4$–5% butanol in chloroform | Titration–manual | Milk, cream | Harper *et al.* (1956) |
| | Amberlyst 26 | GC of methyl esters | Milk, cream, butter | Needs *et al.* (1983), McNeill *et al.* (1986) |
| | Dowex 1 × 2 | GC of methyl esters | | Spangelo *et al.* (1986) |
| | Aminopropyl | Capillary GLC of FFAs | Milk | de Jong and Badings (1990), |
| | | | Milk, cheese, yogurt | de Jong *et al.* (1994), Chavarri (1997) |
| | Amberlite IRA-400 | GC of methyl esters | Milk, cream, butter | Kintner and Day (1965) |
| | Silicic acid–KOH | GC of FFAs | Milk | Woo and Lindsay (1980) |
| | Alumina–deactivated | GC of FFAs | Milk, milk fat, Butter, cheese, milk powder | Deeth *et al.* (1983) |
| Nil | Enzymatic | Colorimetry–manual, automatic | Milk | Koops *et al.* (1990) |
| | | Fluorimetry | Human milk | Christmass *et al.* (1998) |
| | Derivatization | HPLC of 2-nitrophenylhydrazides | Milk, butter, cheese | Miwa and Yamamoto (1990) |
| | Derivatization | HPLC of *p*-bromophenacyl esters | Butter, milk fat | Reed *et al.* (1984), Garcia *et al.* (1990) |
| | Methyl urea + $BF_3$–methanol | GC of methyl esters | Ghee | Sharma and Bindal (1987) |
| | | Infra Red | Milk | Buermeyer *et al.* (2001), Pedersen (2003) |
| | | Flow injection analysis with microbial electrode | Milk | Ukeda *et al.* (1992) |

GC = Gas chromatography
HPLC = High-performance liquid chromatography

*p*-bromophenacyl esters (Garcia *et al*., 1990) or 2-nitrophenylhydrazides (Miwa and Yamamoto, 1990). Thus, it is now possible to obtain accurate data for the individual FFAs in milk and milk products.

Some methods which do not involve separation of the FFAs from the milk fat or the whole product have considerable appeal because of their simplicity. Sharma and Bindal (1987) exploited the property of methyl urea to complex triglycerides in producing methyl esters with $BF_3$-methanol without first separating the FFAs from the fat, while Spangelo *et al*. (1986) were able to methylate FFAs in an acetonitrile extract of milk with methyl iodide in the presence of an anion exchange resin as catalyst. Miwa and Yamamoto (1990) derivatised the FFAs in milk and milk products for HPLC analysis by direct reaction with 2-nitrophenylhydrazine hydrochloride.

The rapid enzymatic method of Koops *et al*. (1990) involving acyl-CoA synthetase and acyl-CoA oxidase and the colorimetric measurement of the resulting hydrogen peroxide have the potential to become routine procedures. They have been automated and showed good agreement with the BDI method on farm milk samples. In another enzymatic method, Christmass *et al*. (1998) linked acyl-CoA synthetase, UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate-1-dehydrogenase, and the NADH-luciferase to determine FFAs in human milk. The fluorimetric measurement of the resultant NADH overcame the problem of cloudiness due to the added milk, which affects colorimetric measurements.

Several publications have compared various methods for determining FFAs in milk and dairy products (Van Crombrugge *et al*., 1982; Chilliard *et al*., 1983; Suhren, 1983; Bråthen, 1984; Cartier *et al*., 1984; Selselet-Attou *et al*., 1984; Ikins *et al*., 1988; Anderson *et al*., 1991; Joshi and Thakar, 1994). In general, high correlations were found between the various methods although different methods suffered from different limitations such as overestimation due to the inclusion of lactic acid or underestimation of short-chain acids.

Thus, the choice of method depends on the application. For routine analyses of FFAs, the BDI method or modifications of it, and methods based on the Dole extraction procedure appear to be most popular (IDF, 1983), while for accurate determination of all FFAs in a product, the capillary GC (Chilliard *et al*., 1983; de Jong and Badings, 1990; Anderson *et al*., 1991) or the HPLC methods (Garcia *et al*., 1990) are the methods of choice. Solvent extraction using acidified ether (Salih *et al*., 1977) followed by separation of the free acids from fat on an anion exchange resin, [e.g., Amberlyst 26 (Needs *et al*., 1983)], and capillary gas chromatography of the acids or their methyl esters has been suggested by the IDF as a reference method (Anderson *et al*., 1991).

Most of the methods discussed above have been used with milk but the majority can be applied to other products. Some minor variations in extraction procedures, such as the inclusion of anhydrous sodium sulphate to remove water (Deeth *et al*., 1983) or inclusion of an aqueous acid wash step to remove lactic acid (Chilliard *et al*., 1983) may be necessary. Collomb and Spahni (1995) suggested that the above International Dairy Federation reference method could be a universal method if the adaptations made by McNeill *et al*. (1986) for butter and McNeill and Connolly (1989) for cheese were incorporated.

### 15.6.2. Lipase Activity

Numerous methods have been used to measure the activity of lipases from various sources (Jensen 1983; Thomson *et al*., 1999; Deeth and Touch, 2000; Chen *et al*., 2003). They vary considerably in the substrate used, the form of the substrate, additives to the assay mix and the method for determining the extent of hydrolysis.

The natural substrates for lipases are triglycerides but, because of the complexity of these and the fact that they seldom contain a chromophore or other label to enable ready detection of the products, several synthetic substrates have been developed. These enable different detection techniques such as spectrophotometry, fluorimetry, chromatography, or radiometry to be used. It is important to note that, by definition, true lipases are active only on water-insoluble esters while esterases cleave only water-soluble esters (Jaeger *et al*., 1994). Thus, it is important that methods used for milk and milk products use substrates, which detect true lipase but not esterases as lipases play a major role in the hydrolysis of milk fat, while the role of esterases is considered insignificant (McKay *et al*., 1995).

Since lipases act on lipids at lipid–water interfaces, preparation of substrates in a suitable physical form for maximal lipase activity is very important. Preparation methods include: emulsification with an emulsifying agent; incorporation into a gel; dissolution in a water-soluble organic solvent, such as 2-methoxyethanol or tetrahydrofuran, followed by addition to an aqueous reaction mixture; sonication, with or without emulsifier; and formation of a thin film or monolayer.

The most common form of a lipid substrate is as an emulsion, stabilized by a surface-active agent, including bile salts, and gums. Regardless of the method used, the formation of a good emulsion is essential, since the rate of the lipase reaction is dependent on the surface area of the substrate at the substrate–water interface. This can be achieved by vigorous shaking, blending, sonication, or homogenization of the substrate in the aqueous medium. However, although an apparently fine emulsion can be produced by these

methods, the available lipid surface area will differ among methods due to differences in the physico-chemical properties of the substrate system. Accordingly, comparison of lipase activity data from reports using different analytical methods is difficult.

The emulsified water-insoluble substrate is usually incubated with a buffered aqueous enzyme preparation. Lipase activity can be determined by continuous measurement of the reaction products or by incubating for a set time and determining the total amount of product formed, or substrate used. One unit (U) of lipase activity is usually defined as the amount of enzyme required to liberate a certain quantity (e.g., 1 μmol) of product in a certain time (e.g., 1 min) under given conditions.

A current challenge in dairy chemistry is the measurement of low levels of bacterial lipases, which can cause defects in milk and milk products, particularly during prolonged storage (IDF, 1983; Stead, 1989; Deeth and Touch, 2000). To this end, many of the sensitive methods described above are applicable to detecting small amounts of FFAs released during incubation of the enzyme source with triglyceride substrates. In order to maximise the sensitivity of assays for bacterial enzymes, long incubations at temperatures at which the indigenous LPL is not active ($\geq$45°C) can be used (Mortensen and Jansen, 1982).

In addition, colorimetric assays using β-naphthyl caprylate (McKellar and Cholette, 1986; Versaw *et al.*, 1989) or *p*-nitrophenyl esters (Blake *et al.*, 1996; Bendicho *et al.*, 2001) as substrates, as well as fluorimetric assays using 4-methylumbelliferyl esters (Stead, 1983; 1984) have been proposed as sensitive methods for detecting bacterial enzymes in milk or milk products. However, problems such as non-enzymic hydrolysis (McKellar, 1986), interference by milk fat and milk proteins (Stead, 1983), and poor correlations of activities measured with those on milk fat (Fitz-Gerald and Deeth, 1983) have limited their use in predicting the lipolytic stability of milk products during storage. Assays based on umbelliferyl esters (de Laborde de Monpezat *et al.*, 1990) have been shown to have advantages over those using 4-methylumbelliferyl esters and may find application in milk and milk products.

Some progress has been made towards developing a sensitive ELISA method for bacterial lipases (Stepaniak *et al.*, 1987b). However, this technique detects the enzyme protein rather than its activity and hence may be of little practical value to the dairy industry. Further developments can be expected in this field.

The more common methods used for milk and bacterial lipases are summarized in Table 15.5.

Deeth and Touch (2000) evaluated the suitability of various methods for dairy applications. They considered these in two categories: screening

**Table 15.5.** Methods for Determining Lipase Activity in Milk and Milk Products (after Deeth and Touch, 2000)

| Method | Substrate | Assay components/ Conditions | Measurement | Applications | References |
|---|---|---|---|---|---|
| 1. Titrimetric | | | | | |
| (a) pH-stat | | | | | |
| | Tributyrin, olive oil | NaCl | Continuous potentiometric titration | Milk lipase, bacterial lipase | Parry *et al.* (1966), Egelrud and Olivecrona (1973), Andersson *et al.* (1979), Makhzoum *et al.* (1996) |
| | Tributyrin; Triolein | NaCl | - | Bacterial lipase | Tomasini *et al.* (1993) |
| | Olive oil/triolein | Sonication before and during assay | - | Bacterial lipase | Linfield *et al.* (1985), Yang *et al.* (1994) |
| | Tributyrin | Na taurocholate | - | BSSL | Jubelin and Boyer (1972) |
| (b) Solvent-extraction titration methods | | | | | |
| | Milk fat | Blood serum, heparin, $CaCl_2$ | Extraction with isopropanol–heptane–$H_2SO_4$, titration to visual endpoint | Milk lipase | Deeth and Fitz-Gerald (1977) |
| | Milk fat | | Extraction with isopropanol–heptane–$H_2SO_4$, titration to visual endpoint | Bacterial lipase | Fitz-Gerald and Deeth (1983) |
| | Tributyrin | NaCl | Extraction with ether-petroleum ether, titration to visual endpoint | Milk lipase<br>Bacterial lipase | Castberg *et al.* (1975)<br>Fitz-Gerald and Deeth (1983) |
| 2. Colorimetric | | | | | |
| (a) β-Naphthol methods | | | | | |
| | β-Naphthyl caprylate | Thiomersal, bile salts, Ca | Extraction of β-naphthol-Fast Blue complex with ethyl acetate, spectrophotometry at 540 nm | Bacterial lipase | McKellar and Chollette (1986) |

(*Continued*)

**Table 15.5.** (*Continued*)

| Method | Substrate | Assay components/ Conditions | Measurement | Applications | References |
|---|---|---|---|---|---|
| | β-Naphthyl caprylate | Thiomersal, bile salts, Ca | Production of β-naphthol-Fast Blue complex, clarification with ethanol-ethyl acetate, spectrophotometry at 540 nm | Bacterial lipase | Versaw et al. (1989) |
| (b) p-Nitrophenol methods | | | | | |
| | *p*-Nitrophenyl caprylate | Sodium deoxycholate | Acetone used to clarify the solution of substrate, spectrophotometry at 410 nm | Bacterial lipase | Owusu *et al.* (1992), Makhzoum *et al.* (1996) |
| | *p*-Nitrophenyl caprylate | | Reflectance colorimetry (b* color); spectrophotometry at 418 nm | Bacterial lipase | Blake *et al.* (1996), Bendicho *et al.* (2001) |
| | *p*-Nitrophenyl acetate | Na taurocholate, acetonitrile | Spectrophotometry at 418 nm | BSSL | Swan *et al.* (1992) |
| | *p*-Nitrophenyl butyrate | Acetonitrile | Addition of Clarifying Agent®, spectrophotometry at 420 nm | Bacterial lipase | Humbert *et al.* (1997) |
| (c) Indigo methods | | | | | |
| | Indoxyl acetate and caprylate | | Colorimetry of indigo formed | Bacterial lipase | Slack (1987), Allen (1994), Munnich and Haasmann (1999), Brand *et al.* (2000) |
| 3. Fluorimetric | | | | | |
| (a) 4-Methyl umbelliferone methods | | | | | |
| | 4-Methyl-umbelliferyl esters (in 2-methoxy ethanol) | Bile salt, cetrimide | Fluorimetry at 450 nm (emission), 360 nm (excitation) | Bacterial lipase | Stead (1983, 1984), Fitz-Gerald and Deeth (1983), Vercet *et al.* (1997) |

(*Continued*)

**Table 15.5.** (*Continued*)

| Method | Substrate | Assay components/ Conditions | Measurement | Applications | References |
|---|---|---|---|---|---|
| (b) Umbelliferone methods | | | | | |
| | Umbelliferyl myristate | | Fluorimetry at 470 nm (emission), 360 nm (excitation) | Bacterial lipase | de Laborde de Monpezat *et al.* (1990) |
| (c) Substituted triacylglycerol methods | | | | | |
| | 1,2-DPPBA[b] | Na taurocholate | Fluorimetry at 340 (excitation), 396 nm (emission) | Bacterial lipase | Celestino *et al.* (1996) |
| 4. Chromatographic | | | | | |
| | Triacylglycerols (esp. triolein) | | Chloroform-methanol extraction, HPLC with detection at 208 nm; GC of derivatized or free fatty acids. | Bacterial lipase | Veeraragavan (1990), Choi and Jeon (1993) |
| 5. Radiometric | | | | | |
| | Radiolabelled triacylglycerols | | Free fatty acid isolation by solvent partitioning or chromatography, scintillation counting of labelled fatty acids | All lipases | Clegg (1980) |
| | $^3$H-triolein–gum acacia | Na taurocholate, serum albumin, NaCl | Extraction with $CHCl_3$–methanol–heptane, scintillation counting of $^3$H-oleic acid | BSSL | Hernell and Olivecrona (1974b) |
| | Triacylglycerols (e.g., tributyrin) | In agar or as liquid emulsion | Measure size of clear zone or decrease in turbidity (decrease in absorbance at e.g., 450 nm) | All lipases | Lawrence *et al.* (1967), Smeltzer *et al.* (1992), Choi *et al.* (1994) |

[a] BSSL = bile-salt-stimulated lipase.
[b] 1,2-DPPBA = 1(3)-pyrenylbutanoyl-2,3(1,2)- dipalmitoyl-*sn*-glycerol.

tests and confirmatory tests. The former can accommodate large numbers of samples in a relatively short time but may result in some false positives or false negatives while the latter, which involve the use of natural triglyceride substrates, often milk fat, are generally more time-consuming. The most favored methods for each of these categories are as follows:

1. Screening tests:
   - Fluorimetric and colorimetric assays using non-triacylglycerol substrates,
   - Titrimetric assays using tributyrin,
   - Agar diffusion assays using tributyrin, triolein or milk fat.
2. Confirmatory tests:
   - Chromatographic assays based on triolein or milk fat as substrate,
   - Titrimetric assays based on triolein or milk fat as substrate.

## 15.7. Prevention of Hydrolytic Rancidity

Sufficient knowledge exists to enable dairy industry personnel to take precautions to ensure a low incidence of hydrolytic rancidity and associated problems in milk and milk products. Recommendations for prevention have been published (Deeth and Fitz-Gerald, 1976; IDF, 1991; O'Brien *et al.*, 1996). A summary of these is given below.

1. For problems associated with milk lipase:
   - Avoid large numbers of cows in late lactation, especially under poor feed conditions;
   - Provide a constant balanced diet for the cows;
   - Design, install and maintain milking equipment correctly;
   - Avoid excessive air intake at teat cups;
   - Minimize centrifugal pumping, especially of warm milk and with air incorporation;
   - Avoid agitation of raw milk, particularly using air;
   - Never mix raw and homogenized milk.
2. For problems associated with microbial lipases:
   - Do not store milk, either raw or pasteurised, for a long period;
   - Do not store milk or milk products at an inappropriately high temperature;
   - Ensure that all milk handling and processing equipment is properly cleaned and sanitized.

Hygiene on the farm and in the factory is of paramount importance in controlling microbial growth and minimising lipolysis problems. Inadequately cleaned equipment can be a major source of lipolytic psychrotrophic contaminants (Drew and Manners, 1985; Stead, 1987).

It has been suggested that some form of heat treatment, either thermization (Humbert *et al*., 1985; Matselis and Roussis, 1998) or HTST pasteurization (Mogensen and Jansen, 1986), of milk on arrival at the factory should be performed to minimise the incidence or reduce the severity of lipolysis problems. While such treatments have been shown to be effective, they increase the cost of processing and result in double heat treatment of milk, which is not permitted in some countries. Carbonation of raw milk (with 30 mM $CO_2$) has also been reported to reduce the growth of lipolytic psychrotrophs and also to reduce the level of FFAs in cheese made from the carbonated milk (McCarney *et al*., 1994).

Once lipolysis has occurred there is little that can be done to reduce its effects on quality, although, in laboratory trials, Nakai (1983) successfully removed FFAs from rancid milk by adsorbing them on activated charcoal and Takacs *et al*. (1989) by the use of a C18 column. However, such nonspecific adsorption methods are unlikely to find widespread use as other milk components such as proteins and vitamins are also removed.

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