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Review

Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge

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Abstract

The progress of lipolysis and its effect on flavour development during cheese ripening is reviewed. The review begins by describing the structure and composition of milk fat and thereafter discusses current knowledge regarding the role of various lipolytic agents and their influence on lipolysis in various cheese varieties. While free fatty acids (FFA) liberated during lipolysis directly affect cheese flavour, they are also metabolized to other highly flavoured compounds, including methyl ketones and lactones. The pathways of FFA catabolism and the effect of these catabolic products on cheese flavour are discussed. Finally, the current methods for the quantification of FFA in cheese are reviewed and compared.
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Keywords: Lipolysis; Cheese ripening; Catabolic products; Flavour

Contents

1. Introduction . . . 841
   1.1. Milk fat: chemistry and structure . . . 842
2. Agents of lipolysis in milk and cheese . . . 843
   2.1. Milk lipase . . . 844
   2.2. Rennet paste . . . 844
   2.3. Microbial lipolytic enzymes . . . 845
3. Catabolism of fatty acids . . . 848
4. Contribution of lipolysis and metabolism of FFA to cheese flavour . . . 852
5. Patterns of lipolysis in various cheese varieties . . . 854
6. Measurement of lipolysis . . . 855
7. Conclusion . . . 860
References . . . 860

1. Introduction

Lipolysis is an important biochemical event occurring during cheese ripening and has been studied quite

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extensively in varieties such as Blue and hard Italian cheeses where lipolysis reaches high levels and is a major pathway for flavour generation. However, in the case of cheeses such as Cheddar and Gouda, in which levels of lipolysis are moderate during ripening, the contribution of lipolytic end products to cheese quality and flavour has received relatively little attention. FFA are important precursors of catabolic reactions, which produce compounds that are volatile and contribute to flavour; however, these catabolic reactions are not well understood (McSweeney & Sousa, 2000). The aim of this work is to review the existing knowledge of the way in which lipolysis and FFA catabolism proceed during cheese ripening, how lipolysis may be measured and monitored and also how this biochemical event contributes to cheese flavour.

1.1. Milk fat: chemistry and structure

Bovine milk typically contains, ca. 3.5–5 g fat $100\,mL^{-1}$ in the form of emulsified globules ranging from 0.1 to 10 μm in diameter (McPherson & Kitchen, 1983; Jensen, Ferris, & Lammi-Keefe, 1991). Milk may therefore be described as an oil-in-water emulsion with the fat globules dispersed in the continuous serum phase. Fat globules are surrounded by a thin membrane called the milk fat globule membrane (MFGM) and this interfacial layer lends stability to the fat globule (Brunner, 1965, 1969; Huang & Kuksis, 1967; Prentice, 1969; Bauer, 1972; Anderson, 1974, 1977; Anderson & Cawston, 1975; Magino & Brunner, 1975; Diaz-Maurino & Nieto, 1977; McPherson & Kitchen, 1983). Milk fat has a complex fatty acid composition, which is reflected in its melting behaviour. At room temperature (20°C), milk fat is a mixture of oil, semi-hard fat and hard fat. Melting begins at −30°C and is only complete at 40°C (Banks, 1991a; Boudreau & Arul, 1993). The range of fatty acid chain lengths and degree of unsaturation, as well as the stereospecific distribution of fatty acids, are responsible for the particular melting behaviour of milk fat (Boudreau & Arul, 1993). Ruminant milk fats contain a wide range of fatty acids and 437 distinct acids have been identified in bovine milk fats. The major FFA found in milk fat are butanoic ($C_{4:0}$), hexanoic ($C_{6:0}$), octanoic ($C_{8:0}$), decanoic ($C_{10:0}$), dodecanoic ($C_{12:0}$), tetradecanoic ($C_{14:0}$), hexadecanoic ($C_{16:0}$), octadecanoic ($C_{18:0}$), *cis*-9-octadecenoic ($C_{18:1}$), *cis*, *cis*-9,12-octadecadienoic ($C_{18:2}$), and 9,12,15-octadecatrienoic acids ($C_{18:3}$) (Jensen, Gander, & Sampugna, 1962; Banks, 1991a; Jensen et al., 1991). Hexadecanoic and octadecanoic are the most abundant FFA (Banks, 1991b; Gunstone, Harwood, & Padley, 1994), comprising ~25% and ~27% of total lipids, respectively (Jensen et al., 1962). Some notable features of the fatty acid profiles of bovine milk lipids include the high level of butanoic acid and other short chain fatty acids, the low levels of polyunsaturated fatty acids and the fact that these lipids are rich in medium chain fatty acids (Oba & Wiltholt, 1994).

The principal lipids of milk are triacylglycerides, which may represent up to 98% of the total lipids (Christie, 1983; Jensen et al., 1991; Gunstone et al., 1994); the structure of triacylglycerides is illustrated in Fig. 1. Triacylglycerides have molecular weights ranging from 470 to 890 Da, corresponding to 24–54 acyl carbons (Boudreau & Arul, 1993; Balcao & Malcata, 1998). Triacylglycerides are esters of glycerol composed of a glycerol backbone with three fatty acids attached (Stryer, 1988). Positioning of fatty acids on the triacylglyceride is non-random; the *sn*-position of a fatty acid denotes its position on the triacylglyceride. Fatty acids may be esterified at positions 1, 2 or 3 as shown in Fig. 1. $C_{4:0}$ and $C_{6:0}$ are predominately located at the *sn*-3 position and the *sn*-1 and *sn*-3 positions, respectively. As chain length increases up to $C_{16:0}$, an increasing proportion is esterified at the *sn*-2 position. $C_{18:0}$ is generally located at the *sn*-1 position, while unsaturated fatty acids are esterified mainly at the *sn*-1 and *sn*-3 positions (Balcao & Malcata, 1998).

While phospholipids represent < 1% of total lipids, they play an important role in the MFGM. Phospholipids are amphipolar in nature and are strongly surface active. These properties enable them to stabilize both oil-in-water and water-in-oil emulsions (Banks, 1991a). On average, phospholipids contain longer and more unsaturated fatty acids than triacylglycerides (Banks, 1991a; Jensen et al., 1991). The principal phospholipids found in milk fat are phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin (Christie, 1983; Grummer, 1991; Gunstone et al., 1994). Trace amounts of other polar lipids have also been reported in milk fat, including ceramides, cerobrosides and gangliosides. Cholesterol is the dominant sterol of milk (>95% of total sterols) (Anderson & Cheesman, 1971; Christie, 1983; Jensen et al., 1991) and accounts for ca. 0.3% of total lipids. The MFGM itself consists of a complex mixture of proteins, phospholipids, glycoproteins, triacylglycerides, cholesterol, enzymes, and other minor components, and acts as a natural emulsifying agent enabling the fat to remain dispersed in the aqueous

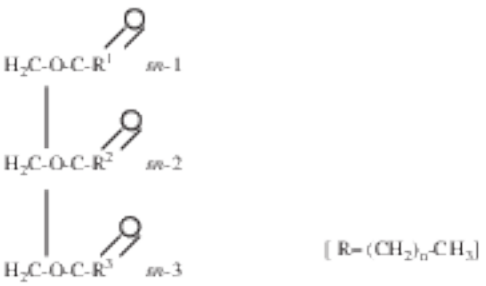


Fig. 1. Triacylglyceride structure (Fox et al., 2000).

phase of milk (Anderson, Cheeseman, Knight, & Shipe, 1972; Kinsella, 1970; Mather & Keenan, 1975; Mather, 1978; Kanno, 1980; Keenan, Dylewski, Woodford, & Ford, 1983; McPherson & Kitchen, 1983).

Microstructural studies of fat present in cheese indicate the presence of globules of varying size and shape. Using confocal laser scanning microscopy (CLSM), Gunasekaran and Ding (1999) examined the three dimensional characteristics of fat globules in one month old Cheddar of varying fat contents ($\sim$40–340 g kg^{-1}). For all cheeses, most globules were $<2\mu$m in diameter. However, globule size was smallest at lowest fat content, but at lowest fat content more globules were noted. The average globule size appeared to be inversely related to the total fat content of the cheese. Overall, increasing fat content in the cheese was reflected by the presence of larger, less circular globules. These workers noted that the nature of protein matrix in low fat cheese may influence fat globules by preventing changes in their size and shape. Guinee, Auty, and Fenelon (2000) also examined the microstructure of Cheddar cheeses of fat contents, in the range $\sim$70–300 g kg^{-1} using CLSM. Reduction of fat content of cheese was accompanied by dispersion of discrete globules without clumping, while increasing fat content of cheese resulted in progressive clumping and coalescence of the globules. Guinee et al. (2000) attributed the clumping and coalescence of globules to the destruction of the MFGM during processing and also to heating of curds during cheesemaking, respectively. Dufour et al. (2000) showed that fluorescence and infra-red spectroscopy could monitor and differentiate patterns of triacylglyceride phase transition in cheeses of varying composition. Spectral changes of triacylglycerides, indicating partial crystallization, were noted over ripening. These changes, were evident in two distinct intervals, 1–21, and, 21–82 days of ripening, and were accompanied by an increase in viscosity. Microstructural and physico-chemical dynamics of fat globules in cheese also appear to influence the localization and retention of starter lactococci in cheese (Laloy, Vuillemard, El Soda, & Simard, 1996). Full fat Cheddar cheese retained higher cell populations in the curd compared to 50% fat reduced Cheddar. Lactococci, visualized using electron microscopy, were shown to be located on the periphery of the fat globule. As ripening progressed, lactococci became more intimately associated with the fat globule such that non-viable cells appeared to become integrated into the fat globule membrane. The potential influence of proteolysis on this progressive association between starter cells and fat globule membrane was raised in this study; whereby hydrolysis of the protein matrix may reduce pressure on the fat globule influencing starter localization within cheese (Laloy, Vuillemard, El Soda, & Simard, 1996). While this is a very interesting theory, to date, no detailed scientific investigation has been undertaken to elucidate the mechanism of accessibility of fat in cheese for lipolysis.

2. Agents of lipolysis in milk and cheese

It is well established that milk fat is essential for the development of correct flavour in cheese during ripening. This was demonstrated in studies with cheeses made from skim milk, or milk in which milk fat had been replaced by other lipids; such cheeses did not develop correct flavour (Foda, Hammond, Reinbold, & Hotchkiss, 1974; El-Safty & Isamil, 1982; Wijesundera, Drury, Muthuku-marappan, Gunasekaran, & Everett, 1998).

Lipids present in foods may undergo oxidative or hydrolytic degradation (McSweeney & Sousa, 2000). Polyunsaturated fatty acids are especially prone to oxidation, which leads to the formation of various unsaturated aldehydes that are strongly flavoured and result in the flavour defect referred to as oxidative rancidity (Fox, Guinee, Cogan, & McSweeney, 2000). Lipid oxidation does not occur to a significant extent in cheese, probably because of its low redox potential (-250 mV) (Fox & Wallace, 1997; Fox et al., 2000; McSweeney & Sousa, 2000) and the presence of natural antioxidants (e.g., vitamin E) (Fox & McSweeney, 1998); its contribution to cheese flavour development is considered to be of little importance (Fox & Wallace, 1997; Fox et al., 2000; McSweeney & Sousa, 2000). However, enzymatic hydrolysis of triacylglycerides to fatty acids and glycerol, mono- or diacylglycerides (lipolysis) is essential to flavour development in some cheese varieties (McSweeney & Sousa, 2000).

Lipolysis in cheese is due to the presence of lipolytic enzymes, which are hydrolases that cleave the ester linkage between a fatty acid and the glycerol core of the triacylglyceride, producing FFA, and mono- and diacylglycerides (Deeth & Touch, 2000). Lipolytic enzymes may be classified as esterases or lipases, which are distinguished according to three main characteristics: (1) length of the hydrolysed acyl ester chain, (2) physico-chemical nature of the substrate and (3) enzymatic kinetics. Esterases hydrolyse acyl ester chains between 2 and 8 carbon atoms in length, while lipases hydrolyse those acyl ester chains of 10 or more carbon atoms. Esterases hydrolyse soluble substrates in aqueous solutions while lipases hydrolyse emulsified substrates. The enzymatic kinetics of esterases and lipases also differ; esterases have classical Michaelis–Menten type kinetics while lipases, since they are activated only in the presence of a hydrophobic/hydrophilic interface, display interfacial Michaelis–Menten type kinetics (Chich, Marchesseau, & Gripon, 1997). Unfortunately, the terms "esterases" and "lipases" are often used interchangeably in the scientific literature.

FFA are released upon lipolysis and contribute directly to cheese flavour, especially short- and intermediate-chain FFA (Bills & Day, 1964). The proportions of free $C_{6:0}$ to $C_{18:3}$ in Cheddar cheese appear to be similar to those in milk fat. However, free butanoic acid ($C_{4:0}$) occurs at a greater relative concentration in cheese than in milk fat (Bills & Day, 1964), suggesting its selective release by lipases present in cheese or its synthesis by the cheese microflora (Bills & Day, 1964; Fox et al., 2000; McSweeney & Sousa, 2000). In general, lipolytic enzymes are specific for the outer ester bonds of tri- or diacylglycerides (i.e., *sn*-1 and *sn*-3 positions) (Deeth & Touch, 2000). Initially, triacylglycerides are hydrolysed to 1,2- and 2,3-diacylglycerides and later to 2-monoacylglycerides; butanoic, as well as the other short- and medium-chain acids, is located mainly at the *sn*-3 position and is preferentially released by lipolytic enzymes (Parodi, 1971; Christie, 1995).

Lipases in cheese originate from six possible sources: (1) the milk, (2) rennet preparation (rennet paste), (3) starter, (4) adjunct starter, (5) non-starter bacteria and, possibly, (6) their addition as exogenous lipases (Deeth & Fitz-Gerald, 1995; Fox & Wallace, 1997; McSweeney & Sousa, 2000).

2.1. Milk lipase

Milk contains a very potent indigenous lipoprotein lipase (LPL), which normally never reaches its full activity in milk (Fox & Stepaniak, 1993; Fox, Law, McSweeney, & Wallace, 1993). The enzyme is present in milk due to leakage through the mammary cell membrane from the blood where it is involved in the metabolism of plasma triacylglycerides. Bovine milk contains 10–20 nM L^{-1} lipase which, under optimum conditions (37°C, pH 7) with addition of an apolipoprotein activator, apo-CII, could theoretically release sufficient FFA acids within 10 s to cause perceptible hydrolytic rancidity. Hydrolysis of as little as 1–2% (w/v) of the milk triacylglycerides to fatty acids gives a rancid or "lipolysed" flavour to the milk (Olivecrona & Bengtsson-Olivecrona, 1991). This does not occur under normal circumstances as LPL and fat are compartmentalized; ca. 90% (w/v) LPL in milk is associated with the casein micelles and the fat, occurring in globules, is surrounded by a lipoprotein membrane (MFGM). If the MFGM is damaged, e.g., due to agitation, foaming, homogenization, inappropriate milking or milk-handling techniques, significant lipolysis may occur resulting in off-flavours in cheese and other dairy products (Darling & Butcher, 1978; Deeth & Fitz-Gerald, 1978; Fox et al., 2000). LPL displays a preference for hydrolysis of medium-chain triacylglycerides (MCT) with a 2 fold increase in the rate of hydrolysis of MCT emulsions containing $C_{6:0}$, $C_{8:0}$, $C_{10:0}$ or $C_{12:0}$ esterified FA compared to long chain triacylglyceride (LCT) emulsions containing esterified $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, or $C_{20:0}$ (Deckelbaum et al., 1990). This difference in rates of hydrolysis was attributed to the greater solubility and mobility of MCT in emulsion systems which allows a more rapid hydrolysis compared with LCT. Interestingly, the actual affinity of LPL was shown to be higher for LCT than for MCT emulsions which may reflect differences in the "quality" (composition and physical properties) of the enzyme-substrate interface. Hence, the differences noted in hydrolysis rates were attributed to higher concentrations of MCT present at the emulsion surface (Deckelbaum et al., 1990). LPL has been shown to be relatively non-specific for fatty acid type, but is specific for the *sn*-1 and *sn*-3 positions of mono-, di- and triacylglycerides (Olivecrona, Vilaro, & Bengtsson-Olivecrona, 1992). Therefore, short- and medium-chain fatty acids are preferentially released by LPL. LPL is of more significance in raw-milk cheeses than in cheeses made from pasteurized milk, since its activity is not reduced by pasteurization. According to Deeth and Fitz-Gerald (1983), it is generally accepted that high-temperature short-time (HTST) treatment (72°C for 15 s) inactivates the enzyme very extensively. However, it is still thought to contribute to lipolysis in pasteurized-milk cheese, as 78°C × 10 s is required for its complete inactivation (Driessen, 1989). More recently, Chavarri, Santisteban, Virto, and de Renobles (1998) studied the enzymology of industrial raw and pasteurized ewes' milk and Idiazabal cheese made from these milks. LPL activity was assayed on the following substrates: trioctanoic, tridecanoic, tridodecanoic, trihexanoic acids, olive oil and ewe milk fat. Highest rates of hydrolysis were noted for trioctanoic acid, with lower and comparable levels noted for tridecanoic, tridodecanoic and olive oil; hydrolysis of ewe milk fat yielded octanoic, decanoic, and hexadecanoic acids. Overall, these workers found that LPL activity in milks decreased significantly as lactation progressed, with pasteurization of milk causing an average 73–95% inactivation of LPL. Activity of LPL determined in aqueous cheese extracts during ripening was quite low and not very reproducible. However, this activity appeared to be associated with the extracted fat layer rather than the aqueous phase.

2.2. Rennet paste

Commercial rennets are normally free from lipolytic activity. However rennet paste, used in the manufacture of some hard Italian varieties (e.g., Provolone, Romano), contains the lipase, pregastric esterase (PGE) (Nelson, Jensen, & Pitas, 1977). PGE is highly specific for short chain acids esterified at the *sn*-3 position (Nelson et al., 1977; Fox & Stepaniak, 1993). Suckling stimulates the secretion of PGE at the base of the tongue, and it is washed into the abomasa with the milk.

Rennet paste is prepared from the abomasa of calves, kids or lambs slaughtered after suckling. The abomasum is partially dried and ground into a paste, which is slurried in milk before being added to cheese milk (Fox & Stepaniak, 1993). Some interspecies differences in specificity have been reported for calf, kid and lamb PGEs which result in slight differences in flavour characteristics of the cheese, depending on the source of PGE (Nelson et al., 1977; Fox & Stepaniak, 1993). Most other lipases investigated are unsuitable for use in the manufacture of Italian varieties due to incorrect specificity; certain fungal lipases (e.g., a lipase secreted by *Rhizomucor miehei*) may be acceptable alternatives (Fox, 1988). Chaudhari and Richardson (1971) identified a second lipase, termed gastric lipase, in an extract of cleaned gastric tissue and reported that a combination of calf gastric lipase and goat PGE resulted in Cheddar and Provolone of superior quality to cheese made with PGE alone. However, Nelson et al. (1977), claimed it was doubtful whether the stomach wall secretes an intrinsic lipase and attributed gastric lipase activity to oral secretions or the regurgitation of intestinal contents containing pancreatic lipase.

2.3. Microbial lipolytic enzymes

Lipases and esterases of lactic acid bacteria (LAB) appear to be the principal lipolytic agents in Cheddar and Dutch-type cheeses made from pasteurized milk (Fox et al., 2000). Evidence for this comes from the very low levels of FFA in aseptic starter-free cheeses made using glucono acid-δ-lactone, rather than in cheese made using starter culture (Reiter et al., 1967). Early studies on the role of LAB and lipolysis by Stadhouders and Veringa (1973) concluded that partially hydrolysed milk fat was a better substrate for lipolysis by starter bacteria than unhydrolysed milk fat. To hydrolyse milk fat in milk and cheese, LAB possess esterolytic/lipolytic enzymes capable of hydrolyzing a range of esters of FFA, tri-, di, and monoacylglyceride substrates (Holland & Coolbear, 1996; Chich et al., 1997; Fox & Wallace, 1997; Liu, Holland, & Crow, 2001). Despite the presence of these enzymes, LAB, especially *Lactococcus* and *Lactobacillus* spp. are generally considered to be weakly lipolytic in comparison to species such as *Pseudomonas*, *Acinetobacter* and *Flavobacterium* (Stadhouders & Veringa, 1973; Fox et al., 1993; Chich et al., 1997). However, because of their presence in cheese at high numbers over an extended ripening period, LAB are considered likely to be responsible for the liberation of significant levels of FFA.

To date, lipases/esterases of LAB appear to be exclusively intracellular and a number have been identified and characterized (Chich et al., 1997; Castillo, Requena, Fernandez de Palencia, Fontecha, & Gobbetti, 1999; Liu et al., 2001). El-Soda, El-Wahab, Ezzat, Desmazeaud, and Ismail (1986) found intracellular esterolytic activities in four strains of lactobacilli: *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. acidophilus*. All lactobacilli showed activity against substrates up to $C_{5:0}$; *Lb. delbrueckii* subsp. *lactis* and *Lb. acidophilus* displayed the highest esterolytic activities. None of the strains tested hydrolysed *o*- and *p*-nitrophenyl (*p*-NP) substrates containing fatty acids of the even numbered carbon atoms from 6 to 14. The presence of lipases and esterases has been demonstrated in nine strains of *Lc. lactis* subsp. *lactis*, citrate positive lactococci and *Lc. lactis* subsp. *cremoris* (Piatkiewicz, 1987). β-Naphthyl dodecanoic acid ($C_{12:0}$) and β-naphthyl ethanoic acids ($C_{2:0}$) were the substrates used for determination of lipase activity and esterase activity, respectively. Esterase activity was higher than lipase activity in all strains. Kamaly, Takayama, and Marth (1990) reported the presence of lipases in the cell-free extracts of a number of strains of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*; these lipases were, in general, optimally active at 37°C and pH 7 to 8.5. *Lc. lactis* subsp. *cremoris* showed the highest lipolytic activity of the strains studied on tributanoic acid and milk fat emulsions. Activity of all lipases was stimulated by reduced glutathione and low (ca. 2 g 100 mL^{-1}) concentrations of NaCl but inhibited by high concentrations of NaCl (ca. 20 g 100 mL^{-1}). Khalid and Marth (1990) reported the quantitative estimation of the lipolytic activity of *Lb. casei* L-7, *Lb. casei* L-14, *Lb. plantarum* L-34 and *Lb. helveticus* L-53. Milk fat, olive oil and tributanoic acid emulsions were used as substrates; the three emulsions were hydrolysed by the four strains of lactobacilli with the exception of *Lb. casei* L-7, which failed to hydrolyse olive oil. According to Lee and Lee (1990), esterolytic and lipolytic enzymes were produced by cell lysis of *Lb. casei* subsp. *casei* LLG. Maximum lipolytic activity was observed at pH 7.2 and 37°C; enzyme activity was inhibited by Ag^{+} and Hg^{2+} ions and stimulated by Mg^{2+} and Ca^{2+} (Lee & Lee, 1990). Chich et al. (1997) reported the presence of esterolytic activities in an intracellular extract of *Lactococcus lactis* subsp. *lactis* NCDO 763. Activity was detected using β-naphthyl butanoic acid ($C_{4:0}$) as substrate and the purified enzyme was active on *p*-NP from C_2 to C_{12} with pH and temperature optima of 7.0–8.0 and 55°C, respectively. *Lb. fermentum*, a species found in the starter used in the manufacture of Parmesan cheese (Battistotti & Bosi, 1987), contains a cell surface-associated esterase specific for $C_{4:0}$ which can hydrolyse β-naphthyl esters of fatty acids from $C_{2:0}$ to $C_{10:0}$ (Gobbetti, Smacchi, & Corsetti, 1997).

Gobbetti, Fox, Smacchi, Stepaniak, and Damiani (1996) reported the purification of an intracellular lipase from a *Lb. plantarum* strain isolated from Cheddar cheese. This enzyme had a molecular mass of 65 kDa

with pH and temperature optima of 7.5 and 35°C, respectively. This enzyme was relatively heat stable to a temperature of 65°C but was irreversibly inactivated on heating to 75°C for 2 min. Substrate specificity studies were carried out on triacylglycerides, β-monoacylglycerides (β-MAG) and β-naphtyl esters of $C_{2:0}$ to $C_{12:0}$ FA. Hydrolysis of triaclyglycerides indicated that the enzyme had highest activity on tributanoic acid, less activity on tridodecanoic and trihexadecanoic acids and no activity on tri-*cis*-9-octadeceneoic acid. In common with other studies, highest activity on β-naphtyl esterified fatty acids was found against butanoic acid, with high activity also noted against, ethanoic acid, hexanoic acid, octanoic acid, decanoic acid, and dodecanoic acid substrates. Lowest activity was found against tetradecanoic acid, hexadecanoic acid, octadecanoic acid and *cis*-9-octadeceneoic substrates. Profiles of β-MAG after hydrolysis by the purified lipase indicated that β-MAGs were generated with fatty acids $C_{14:0}$ to $C_{18:1}$ but those fatty acids of chain length shorter than $C_{14:0}$ were not produced.

Liu et al. (2001) identified three intracellular esterases in *Streptococcus thermophilus*, two of which were purified to homogeneity and designated esterase I and II with molecular masses of ~34 and 60 kDa, respectively. Differences in substrate specificities between esterase I and II were noted, with esterase I hydrolyzing *p*-nitrophenyl esters of short chain FFA C_2 to C_8 while esterase II hydrolysed C_2–C_6 *p*-nitrophenyl esters. Both enzymes had maximum activity on *p*-NP butanoic acid. Esterase I, which was tested against a range of glyceride substrates, hydrolysed di- and monoacylglycerides up to $C_{14:0}$. The impact of cheese compositional parameters on esterase I activity on *p*-NP butanoic acid substrate indicated that enzyme activity was reduced by decreasing pH in the range 5.5–8.0, decreasing temperature in the range 25–37°C, and decreasing water activity (a_w) in the range 0.99–0.80. Interestingly, esterase activity was increased on elevation of NaCl concentration from 3.7 to 7.5 g 100 mL^{-1}, an effect of which has not been previously reported.

As the location of most LAB esterase/lipase activities appears to be intracellular. It is evident that they may require release into the cheese matrix through cell autolysis for maximum efficiency. However to date, few studies have been undertaken to establish whether a relationship exists between the extent of autolysis of LAB and lipolysis in various cheese varieties. Early work by Walker and Keen (1974) found that Cheddar cheeses made with *Lc. lactis* subsp. *cremoris* AM2 developed higher total levels of odd-numbered C_3–C_{15} methyl ketones compared with cheese made with *Lc. lactis* subsp. *cremoris* HP. This finding indicated that starter strain properties may influence the levels of certain lipolytic end products in cheese. However, these workers did not monitor cell viability and autolysis of these strains in cheese during ripening. Later work by Wilkinson, Guinee, O'Callaghan, and Fox (1994) demonstrated that strain AM2 was highly autolytic in comparison to strain HP and that secondary proteolysis was higher in Cheddar cheese made using strain AM2. The higher levels of secondary proteolysis in cheese made using this strain were ascribed to an early and more extensive release of intracellular peptidases on autolysis. Recently, Collins, McSweeney, and Wilkinson (2003) examined the influence of starter autolysis on lipolysis during a 238 d ripening period, as measured by the concentrations of FFA from $C_{4:0}$ to $C_{18:3}$ in Cheddar cheese made using either *Lc. lactis* subsp. *cremoris* AM2 or *Lc. lactis* subsp. *cremoris* HP as starters. These workers found that Cheddar cheese made using the highly autolytic *Lc. lactis* subsp. *cremoris* AM2 developed significantly higher levels of a number of FFA including octanoic acid ($C_{8:0}$), tetradecanoic acid ($C_{14:0}$), hexadecanoic acid ($C_{16:0}$), and octadecanoic acid ($C_{18:0}$) during ripening compared with cheese made with the less autolytic strain *Lc. lactis* subsp. *cremoris* HP. Cell free extracts prepared from both strains had generally similar levels of activity on lipase (tri *cis*-9-octadeceneoic acid emulsion) or esterase (*p*-NP butanoic acid) substrates and these workers concluded that there was preliminary evidence for a relationship between autolysis of starter bacteria and lipolysis in cheese. However, Meyers, Cuppett, and Hutkins (1996) found that release of FFA from either butter oil or a range of triacylglyceride substrates by incubation with whole cells of various LAB strains was higher than that found when the substrates were incubated with intracellular extracts prepared by sonication of the cells. These authors suggested that the microenvironment within whole cells may be more conducive to lipase activity. The genetic characterization of lipolytic enzymes of LAB by Fernandez et al. (2000) confirmed the intracellular nature of a tributanoic acid esterase from *Lc. lactis* subsp. *cremoris* B1014. These workers showed that the 744 base pair *EstA* gene encoded for a 258 amino acid protein of molecular mass ~29,000 Da; however, this gene did not encode for a signal sequence at the N-terminal required for extracellular secretion. Cloning and up to 170-fold overproduction of this enzyme was possible using a nisin-controlled expression system which allow detailed characterization of enzyme specificity and kinetics. The tributanoic acid esterase displayed highest activity on short chain *p*-NP esters of fatty acids with highest activity against *p*-NP hexanoic acid ($C_{6:0}$). This enzyme was not active on *p*-nitrophenyl esters of fatty acids of chain length $> C_{12:0}$. The triacylglycerol substrate, tributanoic acid, was readily hydrolysed while activity was also detected against phospholipid substrates with medium chain fatty acid residues. However, increasing concentrations of the phospholipid substrate to levels favouring micelle

formation did not lead to an increase in enzyme activity through interfacial activation, further confirming the esterolytic nature of this enzyme. The work of Drouault, Corthier, Ehrlich, and Renault (2000) and Drouault et al. (2002) has provided a good insight into the complexity of the molecular processing and transmembrane secretion mechanisms required for the secretion of an active extracellular lipase of *Staphylococcus hyicus* which was cloned and overexpressed in *Lc. lactis*. The lipase of *S. hyicus* consists of a signal peptide of 38 amino acids, a pro-peptide of 207 residues and a mature lipase of 396 amino acid residues. Secretion of a pro-protein of 86 kDa occurs followed by further cleavage by a metalloproteinase to generate the mature 46 kDa lipase. Expression of this lipase in *Lc. lactis* was possible up to 30% of total cellular proteins; however, levels beyond this were toxic to *Lc. lactis*. In addition to the production of pre-prolipase and prolipase, several other truncated lipase forms were produced intracellularly and also secreted extracellularly. The authors suggest that various unidentified cell wall-associated proteinases may be responsible for this cleavage, these strains did not possess the PrtP enzyme. Despite the presence of the correct signal sequence from *S. hyicus* and its replacement with a lactococcal leader peptide, most of the lipase activity (80%) in *Lc. lactis* was concentrated intracellularly. Half of the intracellular activity was in the precursor pro-enzyme form anchored to the cell membrane while the remainder was trapped by the cell wall and cleaved at the N-terminal by cell wall associated proteinases. Interestingly, the kinetics of lipase degradation and cleavage specificities of cell wall-associated proteinases differed between *Lc. lactis* and *Lc. cremoris* strains indicating the involvement of different extracellular proteinases in the processing of the lipase. The significance of this work for cheese ripening is not clear as the authors did not report whether the various truncated forms of the enzyme possess lipase activity. Drouault et al. (2002) subsequently identified a chromosomal gene *pmpA* coding for a PrsA-like lipoprotein (PLP) which has been shown to be involved in protein secretion in *Bacillus subtilis*. PmpA and its gene product PLP also appears to be involved in protein folding and secretion in *Lc. lactis*, as overproduction of PrsA reduced the production of intracellular truncated lipase fragments and increased the production of active correctly secreted prolipase of the extracellular lipase of *S. hyicus* when cloned and overexpressed in *Lc. lactis*. These authors suggest that *PmpA* allows correct folding of the lipase which prevents its degradation by the surface protease HtrA. The fact that deletion of *PmpA* did not exert an adverse effect on growth of *Lc. lactis* in a rich medium suggests a limited role for this gene. However, under salt-induced stress conditions with 0.25 M NaCl in the medium, growth rate was much reduced, indicating that *PmpA* may become a limiting factor in salt stress resistance factors in *Lc. lactis*.

It is therefore important that research is carried out to establish the contribution to lipolysis of intact or autolysed LAB cells and whether any trans-membrane glyceride or fatty acid active transport system is involved in the formation of FFA and other lipolytic end products by LAB.

Picante cheese is a traditional cheese manufactured in Portugal from a mixture of ovine and caprine milks and is manufactured without deliberate addition of starter cultures. The major species present in Picante cheese throughout ripening are adventitious LAB and yeasts. Welch Baillargeon, Bistline, and Sonnet (1989) reported lipase activity in three strains of *Geotrichum candidum*. Emulsified oleic and palmitic acid esters were used as substrates; optimum pH and temperature for lipolytic activity were 7 and 37°C, respectively. Freitas, Pintado, Pintado, and Malcata, (1999) isolated four species of bacteria (*Enterococcus faecium*, *E. faecalis*, *Lactobacillus plantarum* and *Lb. paracasei*) and three species of yeasts (*Debaryomyces hansenii*, *Yarrowia lipolytica* and *Cryptococcus laurentii*) from Picante cheese and assayed each for proteolytic and lipolytic activities. High lipolytic activity was reported for *Y. lipolytica*, using tributyrin as a substrate; the other species studied released lower concentrations of FFA.

Brevibacterium linens is a constituent of the flora of surface-ripened cheeses (e.g., Limburger) which are characterized by a significant level of lipolysis during ripening. Lipolytic activity has been demonstrated in *Br. linens* using emulsified olive oil as a substrate (San Clemente & Vadehra, 1967). Sørhaug and Ordal (1974) reported esterolytic and lipolytic activities in five strains of *Br. linens* using tributanoic acid, emulsified tributanoic acid and emulsified olive oil as substrates.

In mould-ripened cheeses such as Brie, Camembert and Roquefort, *Penicillium* spp. are essential lipolytic agents (Gripon, 1993; McSweeney & Sousa, 2000). *P. roqueforti* possesses two lipases, one with a pH optimum of 7.5–8.0, the other with a more alkaline pH optimum (9–9.5) (Morris & Jezeski, 1953; Kman, Chandan, & Shahani, 1966; Niki, Yoshioka, & Ahiko, 1966). The neutral lipase is more active on trihexanoic acid and the alkaline lipase is more active on tributanoic acid (Menassa & Lamberet, 1982). *P. camemberti* produces an extracellular lipase optimally active on tributanoic acid at pH 9 and 35°C (Lamberet & Lenoir, 1976).

It is well recognized that propionic acid bacteria (PAB) are between 10 and 100 times more lipolytic compared to LAB (Kraut & Mazurek, 1974; Dupuis, 1994). Using emulsified tributanoic acid as a substrate, Oterholm, Ordal, and Witter (1970) showed that *Propionibacterium freudenreichii* subsp. *shermanii*, present in the microflora of Swiss-type cheese, possesses an

intracellular lipase with pH and temperature optima of 7.2 and 47°C, respectively. The maximum rate of hydrolysis on triacylglycerides was observed on tripropanoic acid ($C_{3:0}$), followed in order by tributanoic acid ($C_{4:0}$), trihexanoic acid ($C_{6:0}$) and trioctanoic acid ($C_{8:0}$). Hydrolysis of soluble substrates was small compared to hydrolysis of emulsified substrates and these workers therefore suggested that the enzyme be considered a lipase. Dupuis, Corre, and Boyaval (1993) screened a number of strains of propionibacteria for both esterolytic activity against ethanoic acid, propanoic acid and butanoic acid esters and lipolytic activity against tributanoic acid substrates. Intracellular fractions derived from strains grown in liquid media showed both esterase and lipase activities. However, in this study evidence was provided, for the first time, for the secretion of extracellular esterase and lipase activity during growth; however the extent to which this activity may have arisen as a result of cell lysis was not determined. More recently, Kakariari, Georgalaki, Kalantzopoulos, and Tsakalidou (2000) purified and characterized an intracellular esterase from *Propionibacterium freudenreichii* subsp. *freudenreichii*. Esterase activity was assayed for in the cell-free growth medium, cell wall fractions and a sonicated intracellular extract. In contrast to Dupuis et al. (1993) esterase activity was not found in the cell-free medium, or associated with the cell wall fractions. The enzyme purified by Kakariari et al. (2000) was either cytoplasmic or cell membrane associated.

3. Catabolism of fatty acids

In cheese, FFA released as a result of lipolysis, especially short- and medium- chain fatty acids directly contribute to cheese flavour. FFA also act as precursor molecules for a series of catabolic reactions leading to the production of flavour and aroma compounds, such as methyl ketones, lactones, esters, alkanes and secondary alcohols (Gripon, Monnet, Lamberet, & Desmazeaud, 1991; Fox & Wallace, 1997; McSweeney & Sousa, 2000). Pathways of fatty acid catabolism are outlined in Fig. 2 and catabolism will be dealt with under the following headings: (1) methyl ketones, (2) esters, (3) secondary alcohols, (4) lactones, and (5) aldehydes.

Methyl ketones (alkan-2-ones) are important fatty acid catabolites, particularly in Blue cheese. Dartley and Kinsella (1971) found the total concentration of methyl ketones in blue-veined cheese increased steadily up to 70d of ripening and subsequently decreased. Concentrations of methyl ketones have also been shown to increase throughout the ripening period of Emmental cheese (Thierry, Maillard, & Le Quere, 1999). Methyl ketones are formed in cheese due to the action of mould lipases, e.g., *Penicillium roqueforti* (Urbach, 1997), *Penicillium camemberti* and *Geotrichum candidum* (Lawrence, 1966; Lamberet, Auberger, Canteri, & Lenoir, 1982; Cerning, Gripon, Lamberet, & Lenoir, 1987; Molimard & Spinnler, 1996). Spores, as well as vegetative mycelia, have been shown to produce methyl



Fig. 2. Catabolism of free fatty acids (Molimard & Spinnler, 1996).

ketones (Chalier & Crouzet, 1998) and spores have been used in the bioconversion of medium chain (C_6 to C_{12}) fatty acids (Dartey, Kinsella, & Kinsella, 1973; Larroche, Besson, & Gros, 1994). Metabolism of fatty acids by *Penicillium* spp. involves four main steps and the pathway by which methyl ketones are formed is referred to as β-oxidation, the pathways involved are illustrated in Fig. 3. The steps include: release of FFA by lipases, oxidation of the released FFA to α-ketoacids, decarboxylation of keto acids to alkan-2-ones, of less carbon atom, followed by the reduction of alkan-2-ones to the corresponding alkan-2-ol. The final step is reversible under aerobic conditions (Kinsella & Hwang, 1976a). *Penicillium roqueforti* spores have been shown to produce methyl ketones when long chain fatty acids, e.g., hexadecanoic acid and *cis*,*cis*-9,12-octadecadienoic acid, are added to a culture medium (Chalier & Crouzet, 1993), while the presence of glucose and amino acids have been known to stimulate methyl ketone formation by spores of *Penicillium roqueforti* or *Aspergillus niger* (Lawrence, 1966; Demyttenaere, Koninckx, & Meersman, 1996). Chalier and Crouzet (1998) studied the bioconversion of copra oil (rich in direct precursors of methyl ketones; octanoic, decanoic, dodecanoic and tetradecanoic acids) by two strains of *Penicillium roqueforti* spores, in the presence or absence of exogenous lipase. Without exogenous lipase action, methyl ketone production was low, 3.3–6.1 mmol 100 g^{-1} of oil, respectively, for both strains. Strain dependant formation of methyl ketones resulted from the bioconversion of FFA present in the oil. Lipolysis of copra oil by *Candida cylindracea* lipase, resulted in a large increase in methyl ketone concentration (91.2 and 193.5 mmol 100 g^{-1} of oil, respectively).

It has been suggested that fatty acids are not the only methyl ketone precursors (Dartey et al., 1973; Kinsella & Hwang, 1976a). The high concentrations of heptan-2-one and nonan-2-one found in Blue and Camembert cheeses were not in proportion to the quantities of octanoic and decanoic acids present in milk fat (Kinsella & Hwang, 1976a); the main fatty acid in milk fat is hexadecanoic acid ($C_{16:0}$). It has been shown that methyl ketones can be formed also by mould cultures from the ketoacids naturally present at low concentrations in milk fat or by oxidation of monounsaturated fatty acids (Kinsella & Hwang, 1976b). The rate of production of methyl ketones in cheese is affected by temperature, pH, physiological state of the mould and the concentration of fatty acids. Both resting spores and fungal mycelia are capable of producing alkan-2-ones at a rate that does not directly depend on the concentrations of FFA precursors. In fact, high concentrations of FFA are toxic to *P. roqueforti* spores (Fan, Hwang, & Kinsella, 1976). Growth of the mycelium of *P. camemberti* is more

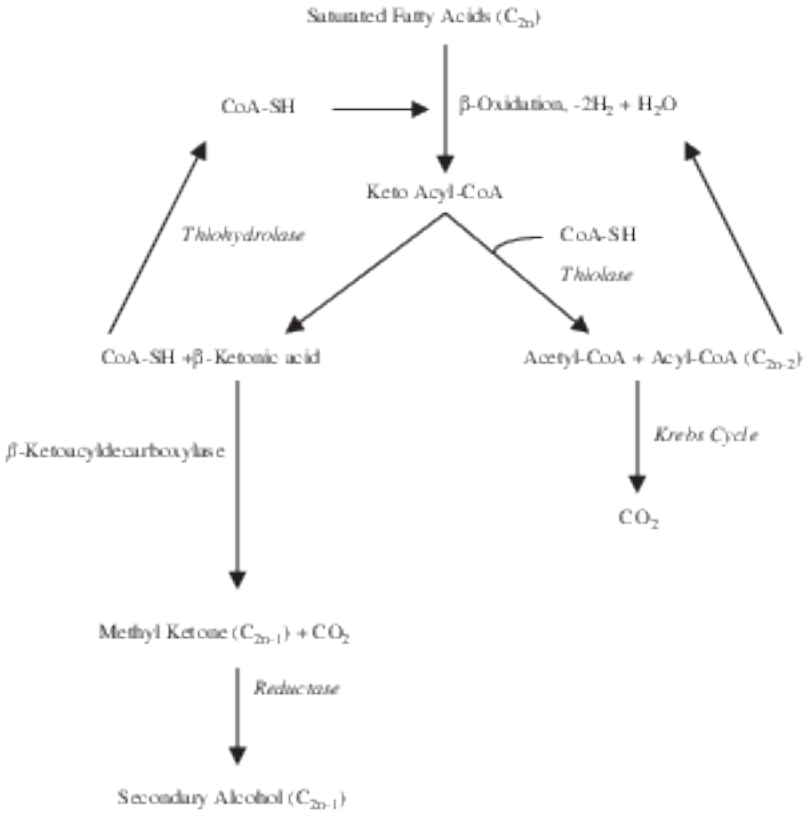


Fig. 3. Catabolism of fatty acids by *Penicillium* spp. (McSweeney & Sousa, 2000).

sensitive to inhibition by fatty acids than that of *P. roqueforti*, even though *P. camemberti* catabolizes the individual fatty acid more rapidly (Fan et al., 1976). In contrast, Kinsella and Hwang (1976a) reported a positive correlation between free fatty acid level and the concentration of methyl ketones produced. Spores of *P. roqueforti* have been found to oxidize fatty acids containing 2–12 carbon atoms with octanoic acid being the substrate which is most rapidly converted. Mycelia oxidize fatty acids over a wide pH range, with an optimum between pH 5 and 7, similar to that of mature Blue cheese. Hence, mycelia are provided with optimum pH conditions for methyl ketone production in mature cheese (Dwivedi & Kinsella, 1974; Gripon, 1993). A β-oxidative pathway has been indicated in mycelial metabolism of FFAs to methyl ketones (Lawrence & Hawke, 1968). Addition of fatty acids to a slurry system, in which Blue cheese flavour was produced, was found to inhibit lipolysis but to increase concentrations of methyl ketones (King & Clegg, 1979). When FFA are present at low concentrations in cheese, they are completely oxidized to CO_2 and low amounts of methyl ketones are formed (Margalith, 1981).

Methyl ketone formation in Camembert cheese has been studied by Dumont, Roger, and Adda (1974a) and Dumont, Roger, Cerf, and Adda (1974b, c). Eleven methyl ketones were identified; levels of nonan-2-one, heptan-2-one and undecan-2-one were found to increase steadily during ripening. Methyl ketones with even-numbered chains appeared late in ripening and were never present in large amounts, except in very mature cheeses. Heptan-2-one has been found in significant concentrations in Parmigiano-Reggiano cheese (Meinhart & Schreier, 1986). In a study of artisanal Blue cheese, heptan-2-one and nonan-2-one were found to be the predominant methyl ketones; their concentrations increased during the first part of the ripening process to a maximum at 60 d, after which time levels began to decrease (de Llano, Ramos, Rodriguez, Montilla, & Juarez, 1992). Methyl ketones are the most important flavour components present in Blue cheese and methyl ketones are present at the highest concentrations. In full-fat Cheddar cheese, levels of heptan-2-one, nonan-2-one and undecan-2-one increased for approximately 14 weeks and then decreased. Concentrations of methyl ketones in low fat cheeses were found to be ca. 25% of the levels observed in the full-fat cheese (Dimos, 1992). Inhibition of lipolysis in low fat cheese was suggested as an explanation for this difference (Dimos, Urbach, & Miller, 1996) as FFA are the principal methyl ketone precursors. Engels, Dekker, de Jong, Neeter, and Visser (1997) compared the volatile compounds in the water-soluble fraction of 7 cheese varieties (Gouda, Proosdij, Gruyere, Maasdam, Edam, Parmesan and Cheddar). Nine ketones, mostly methyl ketones, were identified. Diriack and De Winne (1999) reported levels of 2-heptanone and 2-nonanone ranging from 290.3 to 321.0 μg $100\,g^{-1}$ and 184.1 to 196.0 μg $100\,g^{-1}$, respectively, in Gouda cheese; levels varied between 333.2 and 359.8 μg $100\,g^{-1}$ and 176.8 and 198.6 μg $100\,g^{-1}$, respectively, in Emmental. In another study, pentan-2-one and heptan-2-one were found to be the most abundant methyl ketones in aged ewes milk cheese (14 samples were analysed, 9 of which were Manchego cheese), with mean levels of 73.7 and 36.8 μg $100\,g^{-1}$, respectively (Villaseñor, Valero, Sanz, & Martinez Castro, 2000).

Esters and thioesters are other products of fatty acid catabolism, and are common components of cheese volatiles (Urbach, 1997). A great diversity of esters is present in cheese (Molimard & Spinnler, 1996). Esters are highly flavoured and are formed when FFA react with alcohols. Esterification reactions resulting in the production of esters occur between short- to medium-chain fatty acids and the alcohols derived from lactose fermentation or from amino acid catabolism. Ethyl esters arise from esterification of ethanol with acetyl-coenzyme A (Yoshioka & Hashimoto, 1983). *Geotrichum candidum* is also able to produce esters, some of which have a very pronounced melon odour (Jollivet, Chateaud, Vayssier, Bensoussan, & Belin, 1994). *Pseudomonas fragi* hydrolyses milk fat and esterifies certain of the lower fatty acids with ethanol, producing fruity flavours. Similar esters have been identified in some lactic cultures used in the manufacture of Cheddar cheese (Molimard & Spinnler, 1996). While ethyl, methyl, propyl and butyl esters of even $C_{2:0}$ to $C_{10:0}$ fatty acids have been reported in various cheese varieties (Meinhart & Schreier, 1986), a more recent study found that all of the fatty acid esters in Cheddar were ethyl derivatives (Arora, Cormier, & Lee, 1995).

Thioesters are formed when FFA react with free sulphydryl groups (Molimard & Spinnler, 1996). Lamberet, Auberger, and Bergere (1997) compared the ability of various strains of coryneform bacteria, *Micrococcaceae* and commercial starters of *Lactococcus lactis* and *Leuconostoc* spp. to form *S*-methyl thioesters. All strains synthesized *S*-methyl thioacetate. Strains of *Brevibacterium linens* and *Micrococcaceae* were able to form branched and straight-chain thioesters. Coryneform bacteria (other than *B. linens*) and strains of *L. lactis* synthesized thioesters up to *S*-methyl thiobutyrate; however, branched-chain thioesters were not produced. Cavaille-Lefebvre and Combes (1997) demonstrated the ability of an immobilized lipase from *Rhizomucor miehei* to catalyse the synthesis of short-chain flavour thioesters such as thioethyl, thiobutyl and thioexyl propanoic acid, butanoic acid and pentanoic acid. In a later study, the synthesis of thioethyl-2-methylpropanoate, butanoate, 3-methylbutanoate, hexanoate and of thiobutyl propanoate, butanoate and pentanoate was achieved via esterification of ethanethiol or butanethiol with

short-chain fatty acids using a commercial immobilized *Rhizomucor miehei* lipase (Cavaille-Lefebvre, Combes, Rehbock, & Berger, 1998).

Thirty eight esters were identified in Parmigiano-Reggiano cheese by Meinhart and Schreier (1986), with ethyl ethanoate, ethyl octanoate, ethyl decanoate and methyl hexanoate being the most abundant. Roger, Degas, and Gripon (1988) reported that 2-phenylethyl acetate and 2-phenylethyl propanoate are qualitatively important in Camembert cheeses; however, precursors (alcohols) of these esters may be produced from amino acids, fatty acids or via glycolysis. Methyl and ethyl esters have been found in high proportions in artisanal Blue cheese (de Llano et al., 1992). Fourteen different esters have been identified in Emmental cheese (Imhof & Bosset, 1994; Rychlik, Warmke, & Grosch, 1997). When the aqueous phase of Emmental cheese was studied by dynamic head space analysis, it was found that esters increased during the warm room stage of ripening and most esters showed a 4–20-fold increase between days 3 and 62 (Thierry et al., 1999). Fatty acid ethyl esters and smaller quantities of methyl esters have been identified in Manchego cheese (Villaseñor et al., 2000).

Secondary alcohols can be formed in cheeses by enzymatic reduction of methyl ketones (Engels et al., 1997). *Penicillium* spp. are responsible for the production of secondary alcohols (e.g., 2-pentanol, 2-heptanol and 2-nonanol) in blue-veined cheese due to reduction of methyl ketones (Martelli, 1989). de Llano et al. (1992) found 2-heptanol and 2-nonanol to be the main alcohols in artisanal Blue cheese. Production of 2-propanol from acetone and 2-butanol from butanone has been reported in Cheddar cheese (Urbach, 1993), while Thierry et al. (1999) reported a 10–50-fold increase in the levels of secondary alcohols in the aqueous phase of Emmental during ripening.

Lactones are cyclic compounds (Fox et al., 1993) formed by the intramolecular esterification of hydroxy fatty acids (Christie, 1983), through loss of water, and the resultant formation of a ring structure (Molimard & Spinnler, 1996). Basic studies of lactone formation in milk fat have illustrated that lactones are produced by heat, in the presence of water, from their precursor hydroxyacids (Eriksen, 1976). α- and β-lactones are highly reactive and unstable in cheese (Fox & Wallace, 1997). In contrast, however, γ- and δ-lactones are stable and have been identified in cheese; they have 5- and 6-sided rings, respectively (Eriksen, 1976).

The precursors of lactones, hydroxyacids, in freshly drawn milk are formed in the mammary gland by oxidation of fatty acids (Eriksen, 1976). It has been reported that the mammary glands of ruminants have an δ-oxidation system for fatty acid catabolism (Fox et al., 2000). It has been reported that lactones may be formed from keto acids after reduction to hydroxyacids (Wong, Ellis, & LaCroix, 1975). γ- and δ-Lactones can also be formed spontaneously from the corresponding γ- and δ-hydroxyacids following their release from triacylglycerides by lipolysis (Eriksen, 1976); the concentration of these lactones in cheese should, therefore, correlate with the extent of lipolysis. The presence of disproportionate amounts of high molecular mass lactones has been reported in rancid Cheddar cheese, which has led to the suggestion of other pathways for the formation of lactones (Wong et al., 1975). $C_{12:0}$ lactones may be formed by *P. roqueforti* spores and vegetative mycelium from long-chain saturated fatty acids ($C_{18:1}$ and $C_{18:2}$) (Chalier & Crouzet, 1992). Hydroxylation of fatty acids can also result from normal catabolism of fatty acids. Lactones may also be generated from unsaturated fatty acids by the action of lipoxygenases or hydratases (Dufossé, Latrasse, & Spinnler, 1994). The potential for lactone production depends on such factors as feed, season, stage of lactation and breed (Fox et al., 2000). The sweet flavoured γ-dodecanolactone and γ-dodec-*Z*-6-enolactone occur at much higher levels in milk from grain-fed cows than in milk from pasture fed cows (Urbach, 1997).

Jolly and Kosikowski (1975b) found that the concentration of lactones in Blue cheese was higher than the levels reported by Wong et al. (1975) in Cheddar, and concluded that the extensive lipolysis in Blue cheese influences the formation of lactones. δ-Dodecalactone and δ-tetradecalactone were found to be the principal lactones in Blue cheese at 75 d of ripening (Jolly & Kosikowski, 1975b). In Cheddar cheese, lactone levels increased most rapidly to a concentration well above their flavour threshold early in the ripening period (Jolly & Kosikowski, 1975b). Wong et al. (1975) reported levels of δC_{10}, γC_{12}, δC_{12} and δC_{14} of 150, 80, 490 and 890 μg 100 g^{-1}, respectively, in Cheddar cheese at 14 months. Several lactones have been identified in Parmigiano-Reggiano cheese; quantitatively, the most significant lactone found was δ-octalactone (Meinhart & Schreier, 1986). The Lactones found in Camembert cheese include γ-decalactone, δ-decalactone, γ-dodecalactone and δ-dodecalactone (Gallois & Langlois, 1990).

Aldehydes are formed from amino acids by transamination, resulting in the formation of an imide that can be decarboxylated. It is also proposed that aldehydes are formed by Strecker degradation of amino acids (Keeney & Day, 1957). Aldehydes may also be formed microbially. It has been reported that *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* possess the enzyme, threonine aldolase, which can catalyse the direct conversion of threonine and glycine to acetaldehyde (Marshall & Cole, 1983; Wilkins, Schmidt, Shireman, Smith, & Jezeski, 1986). However some straight-chain aldehydes, e.g., butanal, heptanal and nonanal, may be formed as a result of the β-oxidation of unsaturated fatty acids. Gruyere and Parmesan have high levels of lipolysis and contain high

concentrations of linear aldehydes. Straight-chain aldehydes are characterized by "green grass-like" aromas (Moio, Dekimpe, Etievant, & Addeo, 1993). Aldehydes were also detected in the water-soluble-fractions of all of the cheese varieties analysed by Engels et al. (1997).

4. Contribution of lipolysis and metabolism of FFA to cheese flavour

The flavour of mature cheese is the result of a series of biochemical changes that occur in the curd during ripening, caused by the interaction of starter bacteria, enzymes from the milk, enzymes from the rennet and accompanying lipases and secondary flora (Urbach, 1997). The numerous compounds involved in cheese aroma and flavour are derived from three major metabolic pathways: catabolism of lactate, protein and lipid (Molimard & Spinnler, 1996). Lipid hydrolysis results in the formation of FFA, which may, directly, contribute to cheese flavour and also serve as substrates for further reactions producing highly flavoured catabolic end products.

Cheese flavour is very complex and differs from one cheese variety to another (Tomasini, Bustillo, & Lebeault, 1993). Early research on Cheddar cheese flavour sought to identify a single compound or class of compounds responsible for characteristic flavours (see Aston & Dulley, 1982). When no such compound or class was found, the Component Balance Theory was proposed by Mulder (1952). The theory suggests that cheese flavour is made up of a balance of flavours contributed by a number of compounds, which must be present at certain levels and in the correct balance to produce a flavour typical of a given variety. However, for some cheese varieties, a specific class of compound is recognized as being the major contributor to flavour. In the case of hard Italian cheeses, FFA are significant contributors to the flavour (Woo & Lindsay, 1984; Brennand, Ha, & Lindsay, 1989). For mould ripened cheeses, methyl ketones are important flavour contributors (Molimard & Spinnler, 1996). However, in the case of Cheddar cheese and similar varieties, little is known about the exact contribution of individual compounds to flavour (Wijesundera & Drury, 1999).

As well as being a source of flavour compounds, it has been proposed that the fat in cheese provides a fat–water–protein interface for flavour forming reactions to occur. Fat also acts as a solvent for fat-soluble flavour compounds, allowing their retention in cheese and release during consumption (Manning, 1974; Olson & Johnson, 1990; Lawrence, Giles, & Creamer, 1993; Wijesundera & Drury, 1999). In this respect, the physical presence of fat in cheese is important for flavour development.

Long-chain FFA (>12 carbon atoms) are considered to play a minor role in cheese flavour due to their high perception thresholds (Molimard & Spinnler, 1996). Short and intermediate-chain, even-numbered fatty acids ($C_{4:0}$–$C_{12:0}$) have considerably lower perception thresholds and each gives a characteristic flavour note. Butanoic acid contributes "rancid" and "cheesy" flavours. Hexanoic acid has a "pungent", "blue cheese" flavour note, octanoic acid has a "wax", "soap", "goat", "musty", "rancid" and "fruity" note. Depending on their concentration and perception threshold, volatile fatty acids can either contribute positively to the aroma of the cheese or to a rancidity defect. The flavour effect of FFA in cheese is regulated by pH. In cheeses with a high pH, e.g., surface bacterially ripened cheese, the flavour effect of fatty acids may be negated due to neutralization (Molimard & Spinnler, 1996).

Woo, Kollodge, and Lindsay (1984) found high levels of butanoic and hexanoic acids in Limburger cheese which were related to the development of the strong, characteristic aroma of the cheese. FFA are important flavour contributors in hard Italian varieties such as Romano, Parmesan and Provolone (Aston & Dulley, 1982). Of these three Italian varieties, Romano has the highest concentration of FFA (and the strongest FFA-generated flavours), Parmesan the lowest, while Provolone contains intermediate levels. The latter variety contains relatively high levels of butanoic and hexanoic acids (Woo & Lindsay, 1984). Woo and Lindsay (1984) found butanoic acid, hexadecanoic acid and C_{18} congeners at levels of 175.6, 78.5 and 122.4 mg kg^{-1} cheese, respectively, in Romano cheese. Butanoic and hexadecanoic acids and C_{18} congeners were reported at levels of 14.0, 175.0 and 189.0 mg 100 mL^{-1} cheese, respectively, in Parmesan cheese. de la Feunte, Fontecha, and Juárez (1993) found levels of butanoic, hexadecanoic and *cis*-9-octadecenoic acids of 100.5, 389.6 and 347.1 mg kg^{-1} cheese in Parmesan cheese. Mozzarella cheese has a low FFA concentration, which is associated with its mild flavour (Woo & Lindsay, 1984). The importance of butanoic acid in Camembert flavour was indicated by the generation of a Camembert-like flavour in a cheese base containing a mixture of butanoic acid, methyl ketones, oct-1-en-3-ol and other compounds (Woo et al., 1984). Free fatty acids, in particular butanoic, propanoic and ethanoic acids, have been correlated with Swiss cheese flavour notes (Zerfiridis, Vafopoulou-Mastrogiannaki, & Litopoulou-Tzanetaki, 1984; Vangtal & Hammond, 1986). Ha and Lindsay (1991, 1993) reported that 4-ethyloctanoic acid contributed the characteristic goat- and mutton-like flavours of cheeses manufactured from goat (fresh, semi-soft cheeses) and sheep milk (Pyrenees, Roquefort cheeses), respectively. FFA have been reported to play an important role in the flavour of Serra da Estrela cheese (Partidario, Barbosa, & Boas, 1998).

Literature on the contribution of FFA to the flavour of cheese is vast and conflicting, the role of FFA in the flavour of Cheddar cheese and similar varieties is much less apparent than with the varieties already mentioned. Woo et al. (1984) reported low concentrations of FFA in Edam and Colby cheeses which had low-intensity, smooth flavours. Butanoic acid appeared to be present at high enough concentrations to contribute to the flavours of these cheeses, but a significant role for the other FFA was less apparent.

Research concentrating on relating Cheddar flavour to levels of FFA is based on three approaches: (1) determination of FFA levels individually, collectively or as ratios in order to correlate with flavour development, (2) addition of fatty acids to bland bases to determine if Cheddar flavour can be produced or improved, or selective removal of FFA from Cheddar extracts to detect any alteration in flavour, and (3) manufacture of reduced fat Cheddar cheese or cheeses with vegetable fat as a substitute for milkfat (Aston & Dulley, 1982).

FFA were indicated as important precursors of other flavour compounds in New Zealand Cheddar cheese (Lawrence, 1967). Singh and Kristoffersen (1970) reported a relationship between the formation of active sulphydryl groups, FFA and flavour development. In contrast to these findings, Law and Sharpe (1977) reported that the ratio of FFA to H_2S was found to be unrelated to Cheddar flavour (Law & Sharpe, 1977). Deeth and Fitz-Gerald (1975) reported that 3 month old Cheddar cheeses with an acid degree value >3 were described as unclean or butyric. In another study (Law, Sharpe, & Chapman, 1976) rancidity was characterized by soapy off-flavour in cheeses manufactured from milk with added lipases from *Pseudomonas fluorescens* and *Pseudomonas fragi*. These cheeses contained FFA concentrations 3–10 times higher than control cheeses Patton (1963) found that removing fatty acids from Cheddar cheese distillates had a significant effect on aroma. In a later study, Cheddar samples were presented to a taste panel at 1, 3, 6 and 12 months of ripening and relationships between Cheddar flavour and chemical analyses were presented. It was found that Cheddar flavour rose and then declined as the concentrations of butanoic and hexanoic acids increased (Barlow et al., 1989). Best Cheddar flavour was associated with 4.5–5.0 mg kg^{-1} butanoic acid and 2.0–2.5 mg kg^{-1} hexanoic acid. However, when these fatty acids were removed by neutralization, the flavour was unchanged.

Many studies have shown that reduced-fat Cheddar cheese lacks typical flavour and contains lower concentrations of FFA. This supports the theory that FFA are important to Cheddar flavour (Tanaka & Obata, 1969; Foda et al., 1974; Manning & Price, 1977; Olson & Johnson, 1990; Reddy & Marth, 1993; Dimos et al., 1996; Wijesundera et al., 1998). Cheddar cheese manufactured with vegetable or mineral lipids has also been reported to develop atypical flavours (Foda et al., 1974; Wijesundera & Watkins, 2000). To illustrate the importance of milk fat to Cheddar flavour, Foda et al. (1974) examined flavour development in cheeses made from skim milk homogenized with milk fat, mineral oil, or two commercial vegetable fats. Cheese containing vegetable fats had very little Cheddar flavour; cheese containing mineral oil had a slight Cheddar flavour. Cheese containing milk fat gave the best results, however, flavour was still inferior to the whole-milk cheese, this suggests that the milk fat globule membrane, removed on homogenization of the milk fat, may have important enzymes or other factors which play a role in the development of Cheddar flavour. It is also plausible that the interface between the lipid and aqueous phase in the cheese is important to flavour development. However, Wijesundera and Drury (1999) reported no significant difference in Cheddar cheese flavour intensity between whole-milk cheese and cheeses made from skim-milk homogenized with cream or anhydrous milk fat.

The release of secondary metabolites is of great importance to cheese flavour. Given suitable conditions of maturation, these compounds will enhance the flavour complexity (Nicol & Robinson, 1999). The secondary metabolites resulting from lipolysis include: methyl ketones, lactones, esters and secondary alcohols.

Methyl ketones are responsible for the unique flavour of Blue cheese, especially, heptan-2-one and nonan-2-one (Jolly & Kosikowski, 1975a). Fatty acids and secondary alcohols are also major flavour components (Arnold, Shahani, & Dwivedi, 1975; King & Clegg, 1979). While methyl ketones are more important in relation to the flavour of Blue cheeses, they are also present in Camembert cheese at 25–60 μmol 100 g^{-1} of fat (Molimard & Spinnler, 1996). The two major methyl ketones in Blue and Camembert cheeses are nonan-2-one and heptan-2-one (Anderson & Day, 1966; Gripon, 1993). The homologous series of odd-chain methyl ketones, from C_{3-0} to C_{15-0}, constitute some of the most important components in the aroma of surface-mould ripened cheese, e.g., St. Paulin, Tilsiter and Limburger (Dartley & Kinsella, 1971). The significance of methyl ketones to Cheddar flavour has not been established. Significant levels of pentan-2-one and heptan-2-one in the headspace of Cheddar has been attributed to mould contamination (Urbach, 1997). Wijesundera and Watkins (2000) provided evidence of the significance of methyl ketones to Cheddar cheese flavour, as cheese made with milk containing vegetable fat was low in characteristic Cheddar flavour and in methyl ketones.

In general, the flavour thresholds of methyl ketones are quite low ranging from 0.09 μg 100 g^{-1} for heptan-2-one in water to 4.09 to 50.0 mg 100 g^{-1} for propan-2-one in water (Moio, Semon, & Le Quéré, 1994; Molimard &

Spinnler, 1996). Octan-2-one, nonan-2-one, decan-2-one, undecan-2-one and tridecan-2-one are described as having "fruity", "floral" and "musty" notes, heptan-2-one has a blue cheese note (Rothe, Engst, & Erhardt, 1982). The mushroom and musty notes of methyl ketones are important contributors to the flavour of Camembert cheese (Molimard & Spinnler, 1996).

According to Eriksen (1976) lactones possess a strong flavour. Although the aromas of lactones are not cheese-like, they may contribute to overall cheese flavour (Fox et al., 1993; Fox & Wallace, 1997; Fox et al., 2000) and have been reported to contribute to a buttery character in cheese (Dirinck & De Winne, 1999). δ-Lactones have low flavour thresholds compared to other volatile flavour compounds (O'Keefe, Libbey, & Lindsay, 1969) and are generally characterized by very pronounced, fruity notes ("peach", "apricot" and "coconut") (Dufossé et al., 1994). δ-Lactones have generally higher detection thresholds than those of γ-lactones. Thresholds are relatively low for γ-octalactone, γ-decalactone and γ-dodecalactone (0.7–1.1 μg $100\,g^{-1}$ in water) and even lower for shorter chain lactones (Dufossé et al., 1994). γ-C_{12}, γ-C_{14}, γ-C_{16}, δ-C_{10}, δ-C_{12}, δ-C_{14}, δ-C_{15}, δ-C_{16}, and δ-C_{18} lactones have been identified in Cheddar cheese (O'Keefe et al., 1969), and their concentrations correlate with age and flavour intensity (Wong et al., 1975). This would suggest that certain lactones are important in relation to Cheddar cheese flavour (Fox et al., 2000). In a study of lactone levels in Cheddar cheese, Wong et al. (1975) reported that greater quantities of higher molecular weight lactones, particularly δ-C_{14} (1.41 mg $100\,g^{-1}$ cheese) and δ-C_{16} (~1.8 mg $100\,g^{-1}$ cheese), were produced in rancid cheeses; normal cheeses contained ~0.7 mg $100\,g^{-1}$ and ~0.3 mg $100\,g^{-1}$ cheese, respectively. According to Dimos (1992), δ-decalactone increased in full-fat Cheddar cheeses to a maximum at about 14 weeks and decreased to half the maximum value by 26 weeks. In low fat cheeses, the level of δ-decalactone remained fairly constant throughout maturation. In a later study, decreases in the level of δ-decalactone were associated with an improvement in Cheddar cheese flavour (Dimos, 1996). However, cheeses manufactured with vegetable fat which have a low Cheddar flavour have been shown to be deficient in γ- and δ-lactones, suggesting their importance in relation to the flavour of this variety (Wijesundera & Drury, 1999). Dirinck and De Winne (1999), characterized the flavour of Gouda and Emmental cheeses. δ-Decalactone and δ-dodecalactone were more abundant in Gouda cheeses than in Emmental cheeses and the higher buttery notes, in Gouda cheeses, were attributed to high concentrations of lactones.

It has been reported that esters are important contributors to the flavour of Parmigiano-Reggiano cheese (Meinhart & Schreier, 1986). In a survey of various cheese varieties, Engels et al. (1997) found high concentrations of ethyl butanoate in cheeses with a "fruity" note such as Gruyere, Parmesan and Proosdij. This fruity flavour, which arises due to esters, is considered undesirable in Cheddar cheese (Urbach, 1997; McSweeney & Sousa, 2000).

Thioesters often have characteristic aromas in many foods, e.g., in onions, garlic and some fruits (Cavaille-Lefebvre et al., 1998) and Law (1984) reported that thioesters have a "cheesy" aroma. Arora et al. (1995) analysed the odour-active volatiles in Cheddar cheese headspace and found that most of the esters separated had a "buttery" to "fruity" aroma. However, thioesters formed by the reaction of esters of short-chain fatty acids with methional imparted the characteristic "cheesy" aroma to Cheddar cheese. According to Lamberet et al. (1997), *S*-methyl thioesters contribute a characteristic strong flavour to various smear-ripened soft cheeses (e.g., Tilsit, Limburger and Havarti).

Secondary alcohols, also resulting from lipolysis, may contribute to cheese flavour (Arora et al., 1995). Propan-2-ol, butan-2-ol, octan-2-ol and nonan-2-ol are encountered in most soft cheeses and are typical components of the flavour of Blue cheeses (Engels et al., 1997). Moinas, Groux, and Horman (1975) found that heptan-2-ol and nonan-2-ol represented 10–20 and 5–10 g $100\,g^{-1}$, respectively, of all aromatic compounds in Camembert cheese. Oct-1-en-3-ol has a raw mushroom odour with a perception threshold of 0.001 mg $100\,g^{-1}$, and has been suggested as one of the key compounds in the global aromatic note of Camembert cheese (Molimard & Spinnler, 1996).

5. Patterns of lipolysis in various cheese varieties

Levels of lipolysis measured as release of FFA vary considerably between cheese varieties from moderate (e.g., Cheddar, Cheshire, Caerphilly) to extensive (e.g., mould-ripened, hard Italian and surface bacterially ripened (smear) varieties (McSweeney & Fox, 1993; Fox & Wallace, 1997; Fox et al., 2000; McSweeney & Sousa, 2000). The level of lipolysis should not exceed 2% of triacylglycerides in Gouda, Gruyere or Cheddar cheeses (Gripon, 1993). Excessive lipolysis is considered undesirable and cheeses of the latter varieties containing a moderate level of FFA may be considered as rancid by some consumers (IDF, 1980; Fox et al., 1993). Limited lipolysis is thought to be desirable in Dutch-type cheeses. In Emmental cheese, moderate levels of FFA, of the order of 2–7 g kg^{-1}, are liberated during ripening and make an important contribution to characteristic flavour and aroma in both raw and pasteurized milk cheese (Steffen, Eberhard, Bosset, & Ruegg, 1993; Chamba & Perreard, 2002). Recent data for lipolysis in Emmental cheese manufactured using raw, and microfiltered milk with and without various strains of propionibacteria

indicated that FFA released during ripening originate from lipolytic activity of the added propionibacteria and that the extent of FFA developed in the cheese is strain specific (Chamba & Perreard, 2002).

The highest levels of lipolysis have been observed in traditional mould-ripened cheeses; 5–10% of total triacylglycerides are hydrolysed in Camembert and up to 20% are hydrolysed in other blue-vein cheeses (Gripon et al., 1991; Gripon, 1993). Levels of 18–25% of total fatty acids as FFA have been reported for Danish Blue cheese (Anderson & Day, 1966).

Extensive lipolysis can be attained in mould-ripened cheeses without rancidity, this may be due to neutralization of fatty acids on elevation of pH (Gripon, 1993). The essential lipolytic agents in mould-ripened cheeses are the enzymes of *Penicillium* spp. and the high proportion of free oleic acid in Camembert has been attributed to the lipase of *Geotrichum candidum*. Cream, used for the manufacture of the Danish blue cheese, Danablu, is homogenized and held before pasteurization thereby allowing lipolysis to proceed at a high rate (Nielsen, 1993). Homogenization damages the MFGM, reduces fat globule size and increases the total fat globule surface area, thereby homogenization provides a larger lipid-serum interface for lipase activity. Extensive lipolysis is also characteristic of certain Italian varieties (e.g., Grana Padano, Parmigiano-Reggiano, Romano and Provolone) (Bosset & Gauch, 1993; Woo & Lindsay, 1984), surface bacterially ripened (smear) cheeses (e.g., Limburger) (Woo et al., 1984). Arnold et al. (1975) found a direct relationship between the flavour intensity of ripened Romano type cheese (an Italian variety) and its butyric acid content. Many Italian varieties are manufactured from raw milk (e.g., Parmigiano-Reggiano, Grana Padano, Provolone) which leads to higher levels of lipolysis in the ripened cheese due to the action of LPL. However, the high curd cooking temperatures used in the manufacture of Parmigiano-Reggiano, Grana Padano reduces the activity of LPL during ripening. Kid or lamb rennet paste is also used for coagulation of Provolone and Romano cheeses, and contains pregastric esterase (Battistotti & Corradini, 1993). PGE is responsible for extensive lipolysis, resulting in the characteristic "piccante" flavour of these varieties (Fox et al., 2000; McSweeney & Sousa, 2000). This "piccante" flavour is due primarily to release of short chain FFAs, i.e., $C_{4:0}$ to $C_{10:0}$ (Nelson et al., 1977). *Brevibacterium linens* is a major constituent of the microflora of the smear of surface bacterially ripened cheeses and produces lipolytic enzymes (Reps, 1993).

6. Measurement of lipolysis

The FFA compositions of some selected cheese varieties are shown in Table 1. Various methods are used to quantify FFA. Gas chromatography (GC) has been the method most commonly used to quantify levels of individual FFAs in cheese and is the dominant technique for the routine analysis of FFA; the flame ionization detector is robust with a wide and dynamic range enabling accurate FFA quantification. In a review of the determination of FFA in milk and milk products (IDF, 1991) methods for analysis of FFA by GC were grouped under two headings. The first group permits direct analysis of FFA and includes the method of Nieuwenhof and Hup (1971). FFA are isolated on an alkaline silica gel column, the eluate is concentrated and FFA are directly quantified by GC. However, the silicic acid column used by Nieuwenhof and Hup (1971) was later shown to induce hydrolysis of the fat. The method of Woo and Lindsay (1982) is also included in this group; this method involves removal of lactic acid by a partition pre-column followed by isolation of FFA on a modified silicic acid–potassium hydroxide arrestant column. FFA are then separated in formic acid-mobilized elutes on a glass column packed with diethylene glycol succinate (DEGS-PS) by GC. This GC procedure enables rapid separation and quantification of FFA. The method has since been used in other studies to quantify individual FFA in various cheeses (Woo & Lindsay, 1984; Woo et al., 1984). The GC methods of Iyer, Richardson, Amundson, and Boudreau (1967), McNeill and Connolly (1989), Fontecha et al. (1990) belong to the second group of GC methods which involves the preliminary preparation of methyl esters. In general, the third group of GC methods quoted in Table 1 involve solvent extraction of the lipid fraction in the cheese, followed by separation of FFAs by e.g., saponification (Martinez-Castro, Alanso, & Juarez (1986); Martin-Hernandez, Juarez, Ramos, & Martin-Alvarez, 1990; Martin-Hernandez & Juarez, 1992; de la Feunte et al., 1993; Poveda, Pérez-Coello, & Cabezas, 1999), chromatographic columns (Deeth, Fitz-Gerald, & Snow, 1983; Lesage, Voilley, Lorient, & Bézard, 1993), or methylation to fatty acid methyl esters (FAME) (Ha & Lindsay, 1990; Kinderlerer, Matthias, & Finner, 1996; Partidario et al., 1998; Partidário, 1999). The method of Dulley and Grieve (1974) utilizes steam-distillation followed by GC to quantify volatile fatty acids.

Few studies have been performed to compare different methods of FFA quantification of the same sample. According to IDF (1991), isolation of FFA from the sample material is a vital step in any method. FFA must be isolated from both the aqueous phase and the fat phase. Organic solvents are used in the extraction procedures of the majority of the methods referenced. According to IDF (1991), it is difficult to combine a good extraction of short chain FFA from the aqueous phase together with a good extraction from the fat phase. While these extraction methods commonly involve the use of internal standards of fatty acids

Table 1
Free fatty acids (FFA) composition (mg kg^{-1} cheese) of some selected cheese varieties

Cheese class	FFA												Total FFA	Method of FFA determination	Reference
	$C_{2:0}$	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$			
I. Rennet coagulated															
RC. 1B-R. EH.[a]															
Parmesan		1055	451	243	440	439	1540	3896	1171	3471	123		13697	GC	de la Fuente et al. (1993)
		140	106	84	158	181	684	1750	1890[b]					GC, Woo and Lindsay (1982)	Woo and Lindsay (1984)
Romano		1756	843	328	942	428	448	785	1234[b]						
RC. 1B-R. H.[c]															
Cheddar	476	952	143	175	159	571	952	1556	794	2841	635	238	9492	HPLC	Kilcawley et al. (2001)
	1587	952	191	159	175	619	746	1253	508	1476	413	175	8254		
	1270	794	111	111	48	238	397	619	270	667	206	111	4842		
		15	2	6	25	37	103	285	534[b]				997	GC Woo and Lindsay (1982)	Woo et al. (1984)
		111	33	38	67	68	183	397	131[b]				1028		
		308[d]	144[d]	185[d]	387[d]	225[d]	423[d]	1148[d]	3299[b,d]				6079[d]		
		10	24	28		45	117	309	131	503	41	23		GC	McNeill and Connolly (1989)
		5	16	20		46	110	270	123	524	40	27			
		0	25	27		46	120	299	139	496	45	26			
		0	6	20		37	86	231	81	200	21	0			
			~8	~12	~25	~45	~70	~275	~130	~370			947	GC	Reddy and Marth (1993)
		37	34	22	16	18	38	117	168[b]				450	GC	Woo and Lindsay (1982)
		43	26	16	29	25	61	202	299[b]				701		
		45	35	18	16	13	27	110	169[b]				433		
		47	33	25	55	30	59	196	387[b]				832		
		56	45	29	22	35	76	216	365[b]				844		
		60	44	29	22	35	74	229	385[b]				878		
		142	68	73	134	94	175	433	1377[b]				2306		
	865	115	38	41	49	81	218	503	172	467	69	40		Titration	Bills and Day (1964)
		69	105	13	12	15	48	76	61	209	36			GC, Deeth et al. (1983)	Marsili (1985)
	10	43	12	6	11									GC	Dulley and Grieve (1974)
			~128	~490	~69	~90	~171	~295	~299					GC, Martínez-Castro et al. (1986)	McSweeney and Fox (1993)
Cheshire		8	12	32		59	162	394	132	418	36	12		GC	McNeill and Connolly (1989)
		0	10	27		49	112	275	94	276	29	8			
Roncal		905	302	377	372	392	1007	2243	620	1438	100		8178	GC	de la Fuente et al. (1993)
Idiazabal		285	118	65	578	358	667	1553	372	1299	40		5577		
Manchego		2001	1030	768	1642	1169	2359	6443	1807	12643	1069		32404		
		255	133	110	318	202	443	994	348	816				GC, Juárez et al. (1992)	Poveda et al. (1999)

		876	704	426	398	340	608	1925	508	1384	106		7285	GC, Martin-Hernandez et al. (1988)	Fernandez-Garcia, Lopez Fandiño, Alonso, and Ramos (1994)
RC. 1B-R. SH.[a]															
Caerphilly		18	15	31		57	150	387	127	419	36	13		GC	McNeill and Connolly (1989)
Mahon		383	377	274	394	307	810	2107	902	2235	82		8743	GC	de la Fuente et al. (1993)
Colby		81	9	33	49	22	67	196	93[b]				356	GC, Woo and Lindsay (1982)	Woo et al. (1984)
Monterey Jack		93	3	10	37	20	110	252	211[b]				736		
Majorero		431	400	513	1964	1378	2327	5877	1327	4921	886		20794	GC	de la Fuente et al. (1993)
		216	290	225	723	364	663	1745	528	1400			6144	Martin-Hernandez et al. (1990)	Martin-Hernandez and Juarez (1992)
RC. 1B-R. CWE. ST.[f]															
Swiss		170	90	45	122	208	311	1904	1427[b]				4277	GC, Woo and Lindsay (1982)	Woo et al. (1984)
		345	21	25	53	88	267	930	1197[b]				2926		
		392[d]	135[d]	190[d]	381[d]	522[d]	1337[d]	4262[d]	2664[b,d]				9843[d]		
Emmental		112	45	71		68	207	515	256	842	99	31		GC	McNeill and Connolly (1989)
Gruyere		1037	83	6	16	13	58	193	75[b]				1481	GC, Woo and Lindsay (1982)	Woo et al. (1984)
	288	120	26	21	44	51	160	475	tr	14	50[g]			GC, Dulley and Grieve (1974), Neuwenhof and Hup (1971), Iyer et al. (1967)	Zerfiridis et al. (1984)
RC. 1B-R. CWE. DT.[h]															
Edam		60	8	9	14	47	39	122	57[b]				356	GC, Woo and Lindsay (1982)	Woo et al. (1984)
RC. 1B-R. P-FC.[i]															
Mozzarella		54	7	1	120	12	27	76	66[b]					GC, Woo and Lindsay (1982)	Woo and Lindsay (1984)
Provolone		782	308	81	172	122	120	199	334[b]						
		127	63	65	141	104	210	537	1424[b]				2671		
RC. M-R. SM-R.[j]															
Camembert		35	5	14	35	43	69	270	210[b]				681	GC, Woo and Lindsay (1982)	Woo and Lindsay (1984)
	208	101	58				448	1028		1421				GC	Lesage et al. (1993)
		361	287	160	225	298	622	1442	303	1043			5066	GC	de la Fuente et al. (1993)
Brie		124	9	19	44	74	255	839	1314[b]				2678	GC, Woo and Lindsay (1982)	Woo et al. (1984)

Table 1 (*Continued*)

Cheese class	FFA											Total FFA	Method of FFA determination	Reference
	$C_{2:0}$	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$			
R.C. M-R. 1M-R.[k]														
Roquefort		992	751	715	2104	1403	2632	6452	17404[h]				32453	GC, Woo and Lindsay (1982)	Woo et al. (1984)
		961	626	707	2280	1295	3185	6230	2241	6282	896		25969	GC	de la Fuente et al. (1993)
Blue		1146	777	546	1275	1835	4147	11416	14088[h]				35230	GC, Woo and Lindsay (1982)	Woo et al. (1984)
Blue d' Auvergne															
Blue (with conidia)		105	700	815	2135	4460								GC[d]	Kindstedt et al. (1996)
White (no conidia)			620	160	235	185									
Gamonedo blue		937	804	1040	3366	2329	7850	19768	9157	30434			75685	GC, Fontecha et al. (1990)	de Llano et al. (1992)
R.C. S-R.[m]															
Port Salut		41	4	8	54	33	86	275	199[h]				700	GC, Woo and Lindsay (1982)	Woo et al. (1984)
Limburger		1475	688	24	90	92	602	565	709[h]						
Brick		72	2	8	35	22	63	161	39[h]				402		
		62	27	68	193	153	357	952	538[h]				2350		
		995[d]	232[d]	446[d]	1067[d]	613[d]	1805[d]	2835[d]	5977[h,d]				13970[d]		
Serra de Esterla		37	34	33	89	46	98	198	106	220	12	7		GC	Partidario et al. (1998)
		357	34	35	97	52	102	201	103	208	11	3		FAME, Partidario (1998)	Partidario (1999)
Munster		163	102	66	154	206	704	2057	833	1412	99	504		GC, Ha and Lindsay (1990)	de Leon-Gonzalez, Wendorff, Ingham, Jaeggi, and Houck (2000)
2. *Whey cheese*															
Gjetost		106	35	31	180	49	170	456	631[h]				4558	GC, Woo and Lindsay (1982)	Woo et al. (1984)

[a] Rennet coagulated, internal bacterially ripened, extra hard.
[b] C18:0 congeners.
[c] Rennet coagulated, internal bacterially ripened, hard.
[d] commercial samples exhibiting distinct flavour defects.
[e] Rennet coagulated, internal bacterially ripened, semi-hard.
[f] Rennet coagulated, internal bacterially ripened, cheeses with eyes, Swiss-type.
[g] C18:2 + C18:3.
[h] Rennet coagulated, internal bacterially ripened, cheeses with eyes, Dutch-type.
[i] Rennet coagulated, internal bacterially ripened, Pasta-Filata cheeses.
[j] Rennet coagulated, mould-ripened, surface mould-ripened.
[k] Rennet coagulated, mould-ripened, internal mould-ripened.
[l] Limit of detection for method = 10 mg/kg^{-1} cheese.
[m] Rennet coagulated, surface-ripened.

dissolved in an organic solvent, this does not guarantee the completeness of the extraction procedure.

In the work of Chavarri et al. (1997), two methods of sample preparation were compared for the determination of FFA in ewes' milk cheese by GC. In method 1, after fat extraction, FFA were separated from triacylglycerides by aminopropyl-bonded phase chromatography. The fraction containing FFA was then injected directly onto the gas chromatograph. In method 2, extracted fat was treated with tetramethylammonium hydroxide and the methyl ester derivatives were then formed in the injector. It was concluded that the FFA fraction should be separated from the triacylglyceride fraction before derivatization and chromatographic analysis, particularly for samples in which a minor fraction of the triacylglycerides have been hydrolysed to FFA.

The method described by de Jong and Badings (1990) is now a commonly used procedure for the quantification of FFA in cheese. Accurate and rapid determination of FFA from $C_{2:0}$ to $C_{20:0}$ is achieved by direct separation of underivatized FFA using capillary GC. In brief, the method involves FFA extraction using ether/heptane from a cheese paste containing anhydrous sodium sulphate and sulphuric acid. Extracted FFA are then isolated using alumina or an anion-exchange method (aminopropyl columns) and separation of FFA ($C_{2:0}$ to $C_{20:0}$) is achieved by capillary GC.

Three gas chromatographic methods were compared by Ardö and Polychroniadou (1999) for the analysis of free fatty acids in cheese. The accuracy and recovery rates are reported for the first method only. Method 1 included the preparation and methylation procedures described by Martinez-Castro et al. (1986). This involves diethyl ether extraction of fat followed by methylation with tetramethylammonium hydroxide, which results in an upper layer containing methyl esters from glycerides and a lower layer which holds the tetramethylammonium soaps of FFA. The upper layer may be used to analyse the fatty acid composition of glycerides. For FFA analysis the bottom layer is separated, washed with ethyl ether and adjusted to pH 9. The methyl esters are then analysed by programmed GC as described by Juarez, de la Feunte, and Fontecha (1992). N_2 or He were used as carrier gases; the column was a silica capillary column with silicone, 50% phenyl, 50% cyanopropyl as the stationary phase. Injecting samples at 300°C gave the best quantitative results. The derivatization technique tested resulted in no loss of the volatile components, because the FFA were injected in the form of soaps.

Ardö and Polychroniadou (1999) reported the accuracy of the above method using two types of cheese; one was a fresh cheese with a low FFA content and the other a cheese that had undergone moderate lipolysis. Reproducibility values for the fresh cheese were comparable to those reported by Woo and Lindsay (1982) (GC method) for Cheddar cheese; however, they were lower than those reported by McNeill and Connolly (1989) and Deeth et al. (1983) (GC methods) also for Cheddar cheese. Accuracy was reported to have increased substantially for the cheese with the higher FFA content and there was an improvement in the overall coefficient of variation reported by Martin-Hernandez, Alonso, Juarez, and Fontecha (1988). Recovery rates were examined by adding standard mixtures of FFA to a sample from a cheese of known FFA content. Percentage recoveries ranged from 91% for butanoic acid ($C_{4:0}$) to 103% for octadecanoic acid ($C_{18:0}$). It was concluded that this technique provides a fast and reliable method of FFA analysis in cheeses with low FFA contents and particularly in those with high FFA contents.

HPLC should not be ignored as a method for FFA analysis (Marsili, 1985). HPLC can operate at ambient temperature ensuring relatively little risk to sensitive functional groups (Christie, 1997). In the method of Kilcawley, Wilkinson, and Fox (2001), $C_{2:0}$, $C_{3:0}$ and $C_{4:0}$ were recovered by steam-distillation and quantified by ligand-exchange, ion-exclusion HPLC. $C_{6:0}$–$C_{18:3}$ are derivatized to bromophenacyl esters following solvent extraction and overall separation is achieved by reversed-phase HPLC.

Bills and Day (1964) used an ion-exchange silicic acid column to separate FFA, which were then quantified by titration with potassium hydroxide, using phenolphthalein as an indicator. Removal of CO_2 from the air stream when titrating is vital to maintain the stability of the end point. In this method the air stream was freed from CO_2 by bubbling through 20% KOH, and IDF (1991) recommend titration under nitrogen. It was later shown that the ion-exchange resin used induced fat hydrolysis (McNeill & Connolly, 1989). When using a titration method to quantify FFA, the eluate of each FFA must be titrated separately and the method is not as accurate as more sophisticated GC or HPLC methods.

The "copper soaps" method is a classical colorimetric method which enables the determination of total levels of FFA (IDF, 1991). Copper salts of FFA are selectively transferred to an organic phase; a coloured complex is then formed between the copper salts and sodium diethyl dithiocarbamate, the intensity of which is related to the concentration of copper salts. While the method is sensitive and rapid (IDF, 1991), only a global estimation of FFA levels may be obtained.

Acid degree value (ADV) is defined as the number of milliequivalents of alkali required to neutralize the FFA in 100 g of fat. Cheddar with an ADV >3.0 meq $100\,g^{-1}$ fat is usually classed as rancid (Dairy Research and Development Corporation, 2000). Classification of

cheese by ADV has recently been used by Park (2001), in a study of proteolysis and lipolysis of Goats' milk cheese.

At present, the most suitable methods for determination of lipolysis in cheese are GC and HPLC methods; levels of FFA and FFA profiles may be determined. There is potential for increased use of established technologies, such as gas chromotography/mass spectroscopy (GC/MS) and emerging technologies, e.g., liquid chromotography/mass spectroscopy (LC/MS), for the measurement of FFA. GC/MS provides very accurate qualitative and quantitative measurement of FFA as well as the other volatile components of dairy products (Esteban, Martínez-Castro, & Sanz, 1992; Esteban et al., 1997; Valero, Miranda, Sanz, & Martínez-Castro, 1997).

Barbieri et al. (1994) used dynamic headspace and simultaneous distillation–extraction techniques to analyse the aroma fraction of Parmesan cheese by means of GC and GC/MS. A total of 167 compounds were identified, with the prevalent classes of compounds being: hydrocarbons, aldehydes, ketones, alcohols, esters and acids. In a later study, the flavour of Gouda and Emmental-type cheeses was characterized and the cheeses were then classified using GC/MS profiling, (Dirinck & De Winne, 1999). The aim of the study was to measure cheese aroma characteristics objectively using GC/MS in order to obtain the necessary reference information for steering the biotechnological production of enzyme-modified cheese towards typical cheese flavour characters, such as Gouda and Emmental. Extraction was by means of simultaneous steam distillation, and semi-quantitative composition was determined by GC/MS profiling; principle component analysis was used to interpret the data matrix. The authors claim that an effective approach for instrumental characterization of cheese aroma and flavour should be a combination of a suitable aroma isolation procedure, i.e., GC or GC/MS profiling, and processing of the semi-quantitative data by multivariate (pattern recognition) statistics. GC/MS has also been used to analyse the volatile components of ewes' milk cheese (including Manchego); 57 volatile compounds were quantified, the most abundant were: FFA, acetoin, butanone, methyl ketones as well as primary and secondary alcohols (Villaseñor et al., 2000). LC/MS involves the separation of components on a HPLC column followed by detection using a mass spectrometer. Careri, Mangia, and Musci (1998) reviewed the application of different LC/MS techniques for the analysis of natural compounds in foods. The use of this technique in analysis of food extracts provides important advantages because of the combination of the separation capabilities of LC and the power of MS as an identification and conformation method (Careri et al., 1998).

7. Conclusion

While the existing knowledge of how lipolysis impacts on the flavour of certain cheese varieties, e.g., Blue and hard Italian varieties, is extensive, additional research would undoubtedly be of benefit for varieties such as Cheddar, which is of major economic importance in many countries. The outcomes of this research should allow a better understanding of the catabolic reactions resulting from the release of FFA during cheese ripening as well as more detailed knowledge of how the catabolic products impact on final cheese flavour.

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