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High Performance Liquid Chromatographic and Gas Chromatographic Methods for Lipid Analysis

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19.1. Introduction

Milk fat contains a number of different lipids, but is predominately made up of triacylglycerols (TAG) (98%). The remaining lipids are diacylglycerols (DAG), monoacylglycerols (MAG), phospholipids, free fatty acids (FFA) and sterols. Milk fat contains over 250 different fatty acids, but 15 of these make up approximately 95% of the total (Banks, 1991); the most important are shown in Table 19.1. The unique aspect of bovine, ovine and caprine milk fat, in comparison to vegetable oils, is the presence of high levels of short-chain volatile FFAs (SCFFA), which have a major impact on the flavor/aroma of dairy products. Most cheeses are produced from either bovine, ovine or caprine milk and the differences of their FFA profile are responsible for the characteristic flavor of cheeses produced from such milks (Ha and Lindsay, 1991).

Milk fat composition and esterase/lipase activity are also very important in the dairy industry in terms of flavor, functionality and nutrition. Therefore, it is necessary to have accurate methods to enable quantification of the key milk fat components and lipolytic activity. Methods specifically relating to the quantification of esterase and lipase activity are described in detail in Chapter 11. However, methods incorporating chromatography for the determination of lipolytic activity are described briefly in this chapter

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Table 19.1. Principal free fatty acids in milk fat

Fatty Acid	Notation	% (w/w)
Butyric (Butanoic acid)	$C_{2:0}$	4.2
Caproic (Hexanoic acid)	$C_{4:0}$	2.9
Caprylic (Octanoic acid)	$C_{6:0}$	1.5
Capric (Decanoic acid)	$C_{8:0}$	3.7
Lauric (Dodecanoic acid)	$C_{12:0}$	4.4
Mytristic (Tetradecanoic acid)	$C_{14:0}$	12.3
Palmitic (Hexadecanoic acid)	$C_{16:0}$	31.3
Stearic (Octadecanoic acid)	$C_{18:0}$	3.5
Oleic (*cis*-9-Octadecenoic acid)	$C_{18:1}$	19.5
Linoleic (*cis*-9, *cis*-12-Octadecadienoic acid)	$C_{18:2}$	1.8
Linolenic (*cis*-9, *cis*-12, *cis*-15-Octadecatrienoic acid)	$C_{18:3}$	1.2

together with a detailed account of chromatographic methods used to quantify individual FFAs, MAGs, DAGs and TAGs.

19.2. Quantification of FFAs

19.2.1. Gas Chromatography

The most widely used method for quantifying FFAs is gas chromatography (GC), which has attained widespread favor due to its versatility, high sensitivity and relatively low cost. GC complexed with a flame ionization detector is used routinely to quantify FFAs, either directly or derivatized as fatty acid methyl esters (FAME). GC with mass spectroscopic detection has become the favored technique for quantification of volatile compounds derived from lipids (esters, lactones, ketones, alcohols and acids).

Over the last 20 years, the selectivity and separation efficiency of columns has increased markedly with the advent of capillary wall coated open tubular (WCOT) columns, which have in many cases made the original packed GC columns redundant. Capillary columns are very narrow (0.1–0.3 mm internal diameter) and typically 25–50 m long. They consist of fused silica on a flexible polymeric coat with a thin internal bonded liquid phase. The choice of internal phase depends on the nature of material to be resolved and the narrow diameter and length give optimum partitioning of the sample constituents (Christie, 2003a). The thickness of the film (liquid phase) has a marked impact on retention time, the thicker the film, the longer the retention time, but the greater the capacity of the column. Film thickness can vary from 0.1 to 5.0 μm; the general rule is that low boiling point compounds

require a thin film, while volatile compounds require a thick film. As the fatty acids are in the gaseous phase (usually helium or hydrogen), they travel down the column with the carrier gas and diffuse into the liquid phase to varying degrees according to their equilibrium constant and are thus separated.

Modern GCs have an automatic injector which greatly reduces premature evaporation of the sample, which occurred with manual injection. There are a number of different types of injection systems, such as split injection, splitless injection, on-column injection and programmed-temperature injection. Split injectors are used widely to extend the life of WCOT columns, which are prone to overloading. In this system, after injection and vaporization, the sample vapors are mixed with the carrier gas. The gas line is spilt between the column and the split-line; only a small volume of gas (sample) enters the column, as most is vented off to the atmosphere. The amount of sample loaded onto the column and the volume passing through the split-line is controlled by the spilt ratio (Rood, 1999a). This system is designed to introduce the sample to the column rapidly as the injector has a very high gas flow rate. A major disadvantage of this technique is that it may discriminate between FFAs due to their wide range of boiling points.

In splitless injection, the sample is not split as most of it enters the column and the remainder is vented to the atmosphere. It is used mainly for the analysis of trace compounds. In this system, the sample is injected and vaporized with a solvent and mixed with the carrier gas in the injection chamber. The solvent typically has a high boiling point relative to the column temperature and will condense when it enters the column, forming a narrow band containing the sample. After a pre-set time, the spilt line is opened which maintains the narrow band of sample at the top of the column (Rood, 1999a; Christie, 2003a). Most injectors operate in either split or splitless mode. With on-column injection, a liquid sample is deposited directly onto the column without vaporization. Discrimination problems are avoided since the amount of each compound entering the column depends on its concentration, not volatility. The primary purpose of an on-column injector is to guide the syringe needle into the column (Rood, 1999a). In this procedure, care must be taken to not overload the column; therefore, sample dilution must be gauged correctly prior to injection (Christie, 2003a). Temperature-programmable injectors are widely available and offer the analyst an enhanced opportunity to resolve, and thus quantify, components with similar properties. In this system, the injector can be heated very rapidly during a GC run. This is useful for samples containing compounds with a wide range of volatilities. The system is typically used in conjunction with on-column injection where components move from the injector to the column once they reach their boiling point as the injection temperature is increased (Rood, 1999a; Christie, 2003a).

The most commonly used detector for FFA analysis is the flame ionization detector (FID) due to its versatility, sensitivity and stability. Organic compounds exiting the column enter a hydrogen-oxygen flame. Combustion of the compounds in the flame creates ionic species, which are attracted to a charged collector, creating a current. This current is measured as a signal and differences are registered as peaks (Rood, 1999b). An example of a modern GC-FID system is shown in Figure 19.1.

19.2.2. High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is the most widely used chromatographic technique. Its popularity is due to high separation power, excellent selectivity and the high diversity of analytes that can be quantified (Cserháti and Forgács, 1999). The main difference between HPLC and GC is the use of a liquid mobile phase and different methods of detection. Quantification of components depends on the type of technique used, column, mobile phase, temperature, flow rate, and most importantly, type of detector. Spectrometric detectors in the ultra violet (UV)/visible range are the most widely used. The most useful types for lipid analysis are those that offer a range of variable wavelengths, the best being photodiode array detectors. Spectrophotometric detectors can analyze only a few lipid components directly, but are widely used in conjunction with suitable derivatization methods (Christie, 2003a). Refractive index and evaporative

Figure 19.1. A Varian 3800 gas chromatograph coupled to a flame ionization detector and a Varian CP-8400 automatic injector operated by Varian Star Workstation Software Version 5.

light-scattering detectors are also used, but are not as common due to their high cost.

Reverse-phase (RP) HPLC has been used widely for FFA analysis. The stationary phase is almost always the octadecylsilyl (ODS) type. The mobile phase is typically acetonitrile or methanol in water and detection is by UV between 205 and 210 nm. FFAs are separated on the basis of both chain length and degree of unsaturation (Christie, 1997). An example of a modern HPLC system is shown in Figure 19.2.

19.2.3. Isolation of FFA

Quantification of FFAs in dairy products, especially in cheese, is particularly important due to the impact of some FFAs on flavor. However, FFAs act as precursors of a wide range of flavor compounds (e.g., methyl ketones, lactones, esters and aldehydes), (Singh *et al*., 2003). The extent of lipolysis in cheese varies widely between varieties (Table 19.2). Typically, those cheeses with more than ~3000 mg/kg have a characteristic lipolytic aroma/flavor and lipolysis plays an important role in their ripening. A major difficulty in quantifying FFAs in cheese is the distribution of FFAs of different chain length within the cheese matrix. SCFFA ($C_{4:0}-C_{8:0}$) partition mainly into the aqueous phase, whereas medium ($C_{10:0}-C_{14:0}$) and longer

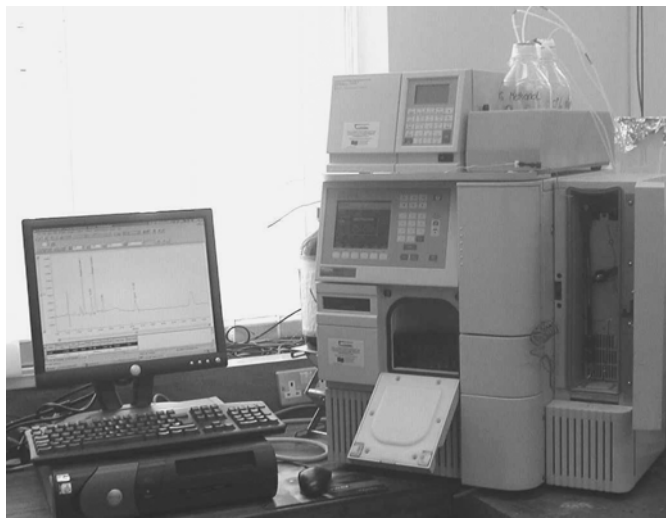

Figure 19.2. A Waters 2695 high performance liquid chromatograph with a Waters 2487 dual absorbance detector operated by Waters Empower Software.

Table 19.2. Average free fatty acid content (mg/kg) of a number of important cheese varieties

Cheese Variety	FFA (mg/kg)	Reference
Blue (US)	32,230	Woo *et al*., 1984b
Brick	2,150	Woo *et al*., 1984b
Brie	2,678	Woo *et al*., 1984b
Cabrales	57,266	de la Feunte *et al*., 1993
Caciocavallo Silano	15,171	Corsetti et al., 2001
Caerphilly	1,253	McNeill and Connolly, 1989
Camembert	681	Woo *et al*., 1984b
Cheddar	1,028	Woo *et al*., 1984b
Cheshire	1,265	McNeill and Connolly, 1989
Colby	550	Woo *et al*., 1984b
Edam	356	Woo *et al*., 1984b
Emmental	2,206	McNeill and Connolly, 1989
Gorgonzola	31,600	Contarini and Toppino, 1995
Gruyere	1,481	Woo *et al*., 1984b
Idiazabal	5,577	de la Feunte *et al*., 1993
Jarlsberg	3,538	Kilcawley (unpublished)
Limburger	4,187	Woo *et al*., 1984b
Mahon	8,743	de la Feunte *et al*., 1993
Majorero	20,794	de la Feunte *et al*., 1993
Manchego	32,404	Poveda *et al*., 1999
Monterey Jack	736	Woo *et al*., 1984b
Mozzarella	583	Kilcawley (unpublished)
Munster	6,260	de Leon-Gonzalez *et al*., 2000
Parmesan	4,993	de la Feunte *et al*., 1993
Pecorino Romano	6,311	Bütikofer, 1996
Picante	17,161	Freitas and Malcata, 1998
Port Salut	700	Woo *et al*., 1984b
Provolone	2,118	Woo and Lindsay, 1984
Roncal	8,178	de la Feunte *et al*., 1993
Roqueforti	32,453	Woo *et al*., 1984b
White Pickled Cheese	603	Akin *et al*., 2003

($C_{16:0}$–$C_{18:3}$) chain FFAs partition with the fat (IDF, 1991). Therefore, the choice of method to isolate or separate FFAs from the cheese matrix is very important if all FFAs are to be determined accurately. Typically, separated FFAs are then quantified by either GC or HPLC. Over the years, many different chromatographic methods have been used to quantify FFAs but these are relatively few in comparison to the methods employed to isolate FFAs from cheese.

19.2.3.1. Steam Distillation

Kosikowski (1977) described a distillation method for extracting volatile FFAs from cheese. However, individual FFAs were not quantified as the extract was titrated to a specific end point, with the amount of alkali used relating to the level of volatile acids present. Steam distillation was used successfully by Horwood and Lloyd (1980) to isolate FFAs from cheese. Formic acid was used to form FFAs from the salts obtained after distillation of the acids from cheese into alkali. This method was also used by Parliament *et al.* (1982) who extracted SCFFAs from an acidified aqueous suspension of cheese. Contarini *et al.* (1988) evaluated steam distillation for the extraction of volatile FFAs from Grana cheese and obtained very good recoveries. Kilcawley *et al.* (2001) also used steam distillation to isolate $C_{2:0}$, $C_{3:0}$ and $C_{4:0}$ from enzyme-modified cheese.

19.2.3.2. Solvent Extraction and Solid-Liquid Partitioning

The methods for isolation of FFAs fall generally into two categories; solvent extraction or solid–liquid partitioning, but in many cases combinations of both have been used. Due to differences in the solubility of fatty acids in solvents, care must be taken to select those which offer the best opportunity for extracting the whole range of FFAs. Solubility increases as the chain length of the fatty acids in lipids decreases, or as the chain length of the solvent alcohol increases. The most commonly used solvents for extraction are diethyl ether and hexane. Their wide use is due predominantly to their low toxicity and the high solubility of SCFFAs therein. Spangelo *et al.* (1986) devised a method for isolating FFAs involving their extraction from milk using acetonitrile in the presence of H_2SO_4 (reduced pH aids extraction of the FFAs) and anhydrous Na_2SO_4 which binds moisture also aids the extraction of FFA. The mixture was filtered and dried. FFAs were methylated using a strong anion-exchange resin prior to analysis by GC. Contarini *et al.* (1988) extracted FFAs from Taleggio cheese using ethyl ether–petroleum ether (40–60%, v/v) after the addition of anhydrous Na_2SO_4 and H_2SO_4. The salts of the FFAs were obtained by titration with ethanolic NaOH, dried and converted to FFAs prior to GC analysis.

19.2.3.3. Supercritical fluid extraction (SFE)

In this technique, highly compressed CO_2 above its critical pressure is used to extract FFAs, followed by chromatography. The density of a supercritical fluid resembles that of a liquid, but its viscosity is similar to that of a gas (Christie, 2003a). Its main advantage is the ability to adjust solvent power by regulating pressure and temperature. CO_2 is used widely due to

its low toxicity. Tuomala and Kallio (1996) used this technique to separate volatile compounds from Swiss cheese using on-line GC-FID and mass spectrometry. SCFFAs were extracted, but some minor volatile components co-eluted with some of the FFAs, interfering with resolution.

19.2.3.4. Solid supports

A number of different solid supports have been used to isolate FFAs from dairy products. Early methods used alkaline or acid silicic-based columns (Nieuwenhof and Hup, 1971; Gray, 1975). However, Stark *et al.* (1976) reported that such columns may hydrolyse TAGs. Woo and Lindsay (1982) used a similar approach to isolate FFAs from a range of dairy products using a modified silicic acid-KOH column, which included a preliminary step to remove lactic acid that interfered with the subsequent chromatography of SCFFAs. FFAs were eluted from the column using a solution of 2% formic acid in diethyl ether and concentrated by rotary evaporation prior to GC analysis. This method was used to quantify FFAs in several cheese varieties (Woo and Lindsay, 1984; Woo *et al.,* 1984a). The method was modified by Woo *et al.* (1984b) who used an ethylene glycol pre-column to remove lactic acid. However, this pre-column was reported to be sensitive to water and therefore $MgSO_4$ instead of Na_2SO_4 should be used with samples with a high moisture content to increase the life of the column.

Other investigators have used an anion exchange resin as solid support (Bills *et al.*, 1963; Deeth *et al.*, 1983; Needs *et al.*, 1983; McNeill *et al.*, 1986; McNeill and Connolly, 1989; de Jong and Badings, 1990; Ha and Lindsay, 1990).

Bills *et al.* (1963) used pre-treated Amberlite resin dispersed in hexane to isolate FFAs from milk. Fat was removed from the resin using hexane, absolute ethanol and methanol and the FFAs were esterified prior to analysis by GC. Needs *et al.* (1983) extracted lipids from milk by using ether and the FFAs were isolated using a strong basic anion exchange resin (Amberlyst 26, BDH Ltd, Poole Dorset, UK). The FFAs were methylated and resolved by GC. McNeill *et al.* (1986) also used Amberlyst resin to isolate FFAs in conjunction with silicic acid to remove phospholipids. Extracted FFAs were then analyzed by GC. This method was used by McNeill and Connolly (1989) to quantify FFAs in a number of semi-hard cheeses.

Deeth *et al.* (1983) isolated FFAs from milk and dairy products by first extracting the FFAs using diethyl ether, or mixtures of hexane-diethyl ether under acidic conditions in the presence of Na_2SO_4. These extracts were then passed through a glass column containing deactivated alumina to remove neutral TAGs. The alumina with the adsorbed FFAs was dried. Di-isopropyl ether containing 6% formic acid was added, mixed and centrifuged. The

supernatant, containing the FFAs, was analysed by GC. de Jong and Badings (1990) also used alumina as adsorbent, but modified the procedure of Deeth *et al.* (1983) by using 3% formic acid in diethyl ether. They reported a higher recovery and better repeatability than Deeth *et al.* (1983). FFAs ($< C_{12:0}$) and total branched chain FFAs in cheese and milk fat were analyzed by Ha and Lindsay (1990), who also used neutral alumina to isolate FFAs after the fat was extracted in diethyl ether:hexane (1:1, v/v). FFAs were eluted from the alumina using formic acid in di-isopropyl ether. SCFFAs were separated from long-chain FFAs by simultaneous distillation–extraction. Butyl esters of the FFAs were prepared and quantified by GC.

de Jong and Badings (1990) also evaluated a solid phase aminopropyl column conditioned in heptane to isolate FFAs from milk prior to quantification by GC. Milk fat, extracted with ether:heptane (1:1, v/v) was added to the aminopropyl column and the neutral lipids removed by chloroform: 2-propanol (2:1, v/v). The FFAs were eluted from the column using diethyl ether containing 2% formic acid. The authors compared this method to the alumina method and found considerably higher recovery of all FFAs. FFAs were quantified directly by capillary GC. Further work by de Jong *et al.* (1994) highlighted the need to use an excess of adsorbent aminopropyl, as some dairy samples contain a high level of lactic acid, which has an affinity for aminopropyl columns, reducing binding capacity for FFAs. They also demonstrated that interference in the resolution of FFAs by GC caused by lactic acid is reduced as only a very low level of lactic acid elutes with the FFAs. A collaborative study using numerous extraction procedures for FFAs highlighted the robustness of aminopropyl columns (Bütikofer, 1996). This study recommended the use of hexane:2-propanol (3:2, v/v) instead of chloroform:2-propanol (2:1, v/v) due to the toxicity of chloroform. It was also found that most commercial aminopropyl cartridges are washed with acetic acid ($C_{2:0}$) prior to packaging and therefore the level of $C_{2:0}$ varies between batches, making it difficult to quantify $C_{2:0}$ by this procedure.

A novel method for the determination of SCFFAs in cheese was reported by Innocente *et al.* (2000). Grated cheese was mixed with water and crotonic acid. The mixture was centrifuged and the aqueous phase acidified to pH 3 or 4 to aid the extraction of FFA salts. The FFAs were then extracted in diethyl ether and determined by GC.

19.2.4. GC Analysis

Gas chromatographic analysis of FFAs isolated from dairy products has been preformed by a number of different techniques. Examples of

methods used to quantify FFAs by GC involve injecting the FFAs directly (Nieuwenhof and Hup, 1971; Contarini *et al.,* 1988; de Jong and Badings, 1990; Innocente *et al*., 2000), or indirectly in the form of potassium salts dissolved in formic acid (Horwood and Lloyd, 1980; Gray, 1975), or as volatile butyl esters (Parodi, 1970; Ha and Lindsay, 1990) or as methyl esters (Martín-Hernández *et al*., 1988; McNeill and Connolly, 1989).

Innocente *et al*. (2000) quantified volatile FFAs directly in a diethyl ether extract, with crotonic acid as an internal standard, on a Nukol fused-silica wide-bore GC column. This method is rapid and reproducible for the quantification of acids from $C_{2:0}$ to $C_{7:0}$. The method of Gray (1975) involved titration of isolated FFAs with methanolic KOH and concentrating by drying. The FFAs were extracted in a mixture of acetone and water, then analyzed using a packed GC column containing diethylene glycol succinate. Parodi (1970) prepared butyl esters of FFAs from milk fat. Fat was extracted by hexane, mixed with butanolic KOH and saturated with NaCl. This solution was centrifuged and the top hexane layer, containing the butyl esters, then separated on a packed GC column. Ha and Lindsay (1990) prepared butyl esters by drying FFAs previously isolated from cheese in diethyl ether under nitrogen. BF_3/butanol was added to the dried mixture which was then boiled for 10 min, after which 10 ml of methanol:water (15:100, v/v) and 10 ml of pentane were added and the solution mixed and centrifuged. The resulting butyl esters of the FFAs were concentrated prior to analysis on a capillary GC column. Bills *et al*. (1963) prepared methyl esters from FFAs previously extracted from milk fat by adding ethylene chloride, then saturating with NaCl. The ethylene chloride layer, which contained the methyl esters, was concentrated prior to GC analysis in a packed column.

However, the methods most widely used for quantification of FFAs in dairy products are adaptations of the methods of Martín-Hernández *et al*. (1988) and de Jong and Badings (1990). Martín-Hernández *et al*. (1988) developed a rapid method to quantify FFAs in cheese. Lipids were extracted from an acidified cheese paste with diethyl ether and tetramethylammonium hydroxide (TMAH). An upper phase contains transesterified TAGs, while the lower phase contains TMA soaps of FFAs, which were subsequently converted to methyl esters in the GC injector. This procedure was developed originally with a packed column, but excess TMAH can pyrolyze to trimethylamine and interfere with chromatographic resolution. Interference due to trimethylamine was eliminated by using a capillary WCOT column (typically chemically bonded polyethylene glycol) and a temperature programmable injector with an initial temperature of 300°C (Juárez *et al*., 1992). Chavarri *et al*. (1997) found that the FFA fraction should be separated from the TAG fraction prior to derivatization with TMAH as transesterified

TAGs can transfer to the lower phase and be converted to methyl esters, giving false high results.

In the method described by de Jong and Badings (1990), FFAs are isolated using an aminopropyl column; the isolated extract is injected directly onto a capillary GC column using cold on-column injection. The stationary phase of the capillary column is typically any nitroterephthalic acid-modified, chemically bonded polyethylene glycol capillary column; however, it is necessary to use a column with good film thickness ($>0.5\,\mu m$) to prolong performance of the column. Direct on-column injection without cooling is possible but reduces the longevity of the column considerably. Chavarri *et al.* (1997) found that this method gave 90–100% recovery of FFAs regardless of their chain length or the ratio of FFAs to TAGs. A major advantage of this method is that it is readily amenable to automation and that the use of on-column injection eliminates discrimination between FFAs of different volatility.

The methods described above have been used principally to quantify FFAs in cheese, but can be used for other milk products with some slight modifications. All the above methods use internal standards (typically FFAs which are not present in milk fat), and the recovery of all FFAs is based on the recovery of these internal standards. It is best to use both volatile and non-volatile FFAs as internal standards. Currently, the International Standard for the extraction of lipids and lipo-soluble compounds from milk and milk products is ISO 14156 (ISO, 2001) and involves solvent extraction. Determination of the fatty acid composition of milk fat involves the preparation of fatty acid methyl esters (FAME) by transesterification (ISO, 2002a), followed by quantification by GC (ISO, 2002b).

19.2.5. HPLC Analysis

One of the first HPLC methods to resolve the major FFAs in milk fat used RP-HPLC (Reed *et al.*, 1984). In this method, FFAs in milk are converted to *p*-bromophenacyl esters *via* a crown ether-catalyzed reaction. Two chromatographic runs were required to quantify all FFAs due to problems of co-elution of some medium- and long-chain FFAs. However, Elliott *et al.* (1989) accomplished complete separation of all FFAs in one run using this method with a gradient of acetonitrile in water at a column temperature of 10°C. Further development of the method was undertaken by Garcia *et al.* (1990), who used a water/methanol/acetonitrile gradient to achieve faster separation of all FFAs. This method is one of the most widely used for quantifying FFAs by HPLC. FFAs, without esterification, can also be separated by HPLC using an ODS stationary phase column and acetic or phosphoric acid in the mobile phase to suppress ions (Christie, 2003a). This

method appears to have been applied mainly to vegetable fats to detect adulteration (Hein and Isengard, 1997).

Sanches-Silva *et al*. (2004) compared RP-HPLC and GC-FID methods to quantify FFAs in potato crisps. Sample preparation for the RP-HPLC method involved saponification of the FFAs with 0.5M NaOH in ethanol-water (94:4, v/v). The ethanol dissolves the sodium salts of FAs, which are then separated from other salts and water-soluble impurities using 0.6 M HCl. The sodium salts of the FAs are converted to free acids and then analyzed on an ODS2 column using a gradient of acetonitrile and water. The GC-FID technique involved the production of FAME followed by separation on a polyethyleneglycol capillary GC column. The authors found very little difference between the methods for the quantification of the long-chain FFAs, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$. The RP-HPLC method may be applicable to the wide range of FFAs in dairy products.

A useful fluorogenic derivatization method for long-chain FFAs in milk was described by Lu *et al*. (2002). The FFAs were converted to fluorescent naphthoxyethyl derivatives and separated by isocratic HPLC and monitored using a fluorometric detector at an excitation wavelength of 235 nm and an emission wavelength of 350 nm. Other fluorometric detection methods appear to be used mainly to quantify long-chain FFAs in vegetable oils. Kotani *et al*. (2002) described an HPLC method for quantifying FFAs using electrochemical detection. The basis of the method involves the voltametric reduction of quinone, after it has been associated with FFA. The method does not require derivatization and separation is achieved using an ODS column. The method has been only applied to the isolation of long-chain FFAs, but appears to be reproducible and sensitive. Kilcawley *et al*. (2001) quantified $C_{2:0}$, $C_{3:0}$ and $C_{4:0}$ acids in cheese and in enzyme-modified cheese using a monosaccharide hydrogen ionic column (ion-partioning) with an isocratic mobile phase of 0.1 N H_2SO_4 with detection at 220 nm, after the SCFFAs had been isolated by steam distillation.

19.2.6. Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) has attracted much interest recently, due its potential anti-cancer, anti-atherogenic and anti-inflammatory therapeutic properties (Weiss *et al*., 2004; see Chapter 3). CLA occurs as several positional and geometric isomers of $C_{18:2}$ with a conjugated double bond. The *cis*-9, *trans*-11 CLA isomer occurs most often naturally. Commercial sources of CLA also contain the *trans*-10 and *cis*-12 isomer, which is also biologically active. Since various isomers of CLA differ in biological activity, methods of analysis for the various CLA isomers have become very

important. It is necessary to be able to separate and quantify each geometrical and positional isomer and to avoid isomerization during any dervitization steps. CLA is commercially available as a free acid in large amounts and is therefore relatively easy to quantify. However, naturally-occuring CLA is present in its esterified form at very low concentrations which makes it much more difficult to quantify (Christie, 2003b).

Capillary GC is widely used to quantify CLA. Best results are attained using base-catalyzed rather than acid-catatylzed transesterification due to geometrical isomerization, which increases the relative proportions of all *trans* isomers. Depending upon the nature of the sample, capillary polyethylene glycol columns will achieve sufficient separation of the main CLA isomers, but minor isomers may co-elute. Best separation of CLA isomers is achieved using high polarity polyethylene glycol capillary columns with a stabilized cyanopropyl or cyanosilicone phase (Christie, 2003b). Silver-ion HPLC is also very useful for separating and quantifying positional and geometrical isomers of CLA. UV quantification of methyl esters separated by silver-ion HPLC is used routinely. Silver-ion thin-layer chromatography (TLC) is often required for the enrichment of CLA isomers prior to GC analysis. GC-MS is used widely to identify specific isomers (Christie, 2003b).

19.3. Lipid-Derived Volatile Aroma and Flavor Compounds

Lipid-derived volatile compounds play an important role in the flavor of foods. These compounds contribute to the characteristic notes of many dairy flavors, but are also responsible for many off-flavors. Parliament and McGorrin (2000) reviewed those volatile compounds important in milk, cream, butter, cultured creams and cheese. The pathways involved in the degradation of milk fat have also been reviewed by McSweeney and Sousa (2000) and compounds include FFAs, methyl ketones, lactones, esters, aldehydes, primary and secondary alcohols, hydroxyacids, hydroperoxides and ketoacids.

19.3.1. Gas Chromatography Mass Spectrometry

Virtually all volatile aromatic and flavorsome lipid-derived compounds are analyzed using gas chromatography-mass spectrometry (GC-MS). The components of interest are isolated initially, concentrated, then injected onto a suitable capillary column and detected using a mass spectrometer.

The principle of GC-MS involves the bombardment of organic molecules of interest in the vapor phase with electrons to form positively charged ions, which fragment in a number of different ways to give smaller ionized entities. These ions are propelled through magnetic or electrostatic fields and

Figure 19.3. A Varian 3400 gas chromatograph with a Tekmar 3000 purge-and-trap concentrator and a Varian Saturn 2000 mass spectrometer detector operated by Varian Saturn GC/MS Workstation Software Version 5.41 k.

separated according to their mass to charge ratio (Christie, 2003a). Mass spectrometry is used widely as a mass-selective GC detector and is unequalled as an identification tool (Reineccius, 2002). An example of a modern GC-MS system is shown in Figure 19.3.

19.3.2. Isolation and Concentration of Volatile Lipid-Derived Components

Numerous techniques have been developed to isolate and concentrate aroma compounds derived from lipids or other components, from other constituents than dairy foods. The most widely used methods are based on volatility and/or solubility.

Methods based on volatility include static and dynamic headspace analysis, high vacuum and steam distillation and direct injection (Reineccius, 2002). Static headspace involves extracting the vapor of a food under vacuum and directly injecting it onto a GC capillary column, followed by mass spectrometry; however, the method lacks sensitivity. Dynamic headspace methods usually involve purge-and-trap. These methods use an inert gas (helium or nitrogen) to purge a sample, releasing volatile constituents, which are then trapped on sorbants (Tenax, charcoal, activated carbon) or cryogenically (liquid nitrogen). The approach can be problematic as the choice of trapping material can discriminate between compounds and the use of cryogenics

causes sample dilution due to the entrapment of water (Reineccius, 2002). Problems can also occur due to the complexity of the equipment and contamination of traps.

Distillation techniques, such as steam distillation, are typically carried out in a rotary evaporator after the sample has been solubilized in an organic solvent. The distillate is injected directly onto a suitable GC column. This method is used widely due to its simplicity and because components with high boiling points are recovered easily. High-vacuum distillation is used widely to isolate volatile components from solvent extracts. This procedure often requires an extraction step to remove water.

Direct solvent extraction techniques are widely used and range from very simple procedures where solvent and sample are mixed and dried to the use of supercritical CO_2 and pressure chambers. The efficiency of the technique is influenced greatly by the choice of solvent.

Solid phase micro-extraction (SPME) allows isolation and concentration of volatile components rapidly and easily without the use of a solvent. These techniques are independent of the form of the matrix; liquids, solids and gases can be sampled quite readily. SPME is an equilibrium technique and accurate quantification requires that the extraction conditions be controlled carefully. Each chemical component will behave differently depending on its polarity, volatility, organic/water partition coefficient, volume of the sample and headspace, speed of agitation, pH of the solution and temperature of the sample (Harmon, 2002). The techniques involve the use of an inert fiber coated with an absorbant, which govern its properties. Volatile components are adsorbed onto a suitable SPME fiber (which are usually discriminative for a range of volatile components), desorbed in the injection chamber and separated by a suitable GC column. To use this method effectively, it is important to be familiar with the factors that influence recovery of the volatiles (Reineccius, 2002).

Odor-active components in cheese flavor, many of which are derived from milk lipids, can be detected using GC-olfactometry (GC-O). GC-O is defined as a collection of techniques that combine olfactometry, or the use of the human nose, as a detector to assess odor activity in a defined air stream post-separation using a GC (Friedich and Acree, 1988). The data generated by GC-O are evaluated primarily by aroma extract dilution analysis or Charm analysis. Both involve evaluating the odor activity of individual compounds by sniffing the GC outlet of a series of dilutions of the original aroma extract and therefore both methods are based on the odor detection threshold of compounds. The key odourants in dairy products and in various types of cheese have been reviewed by Friedich and Acree (1988) and Curioni and Bosset (2002).

19.4. Tri-, Di- and Mono-Acylglycerols

TAGs consist of a glycerol backbone, with the three hydroxyl groups esterified to a fatty acid. They are by far the most abundant lipids in animal and vegetable fats. Most lipolytic enzymes cleave TAGs at the *sn*-1 and *sn*-3 positions, with relatively few enzymes showing a preference for the *sn*-2 position. Removal of one fatty acid produces a DAG, removal of two produces a MAG and removal of three FAs produces a glycerol molecule. 2,3-and 1,2-DAGs and 2-MAGs are inherently unstable and undergo acyl migration to 1,3-DAGs and 1-MAGs, respectively (Kilara, 1985) (see Figure 19.4).

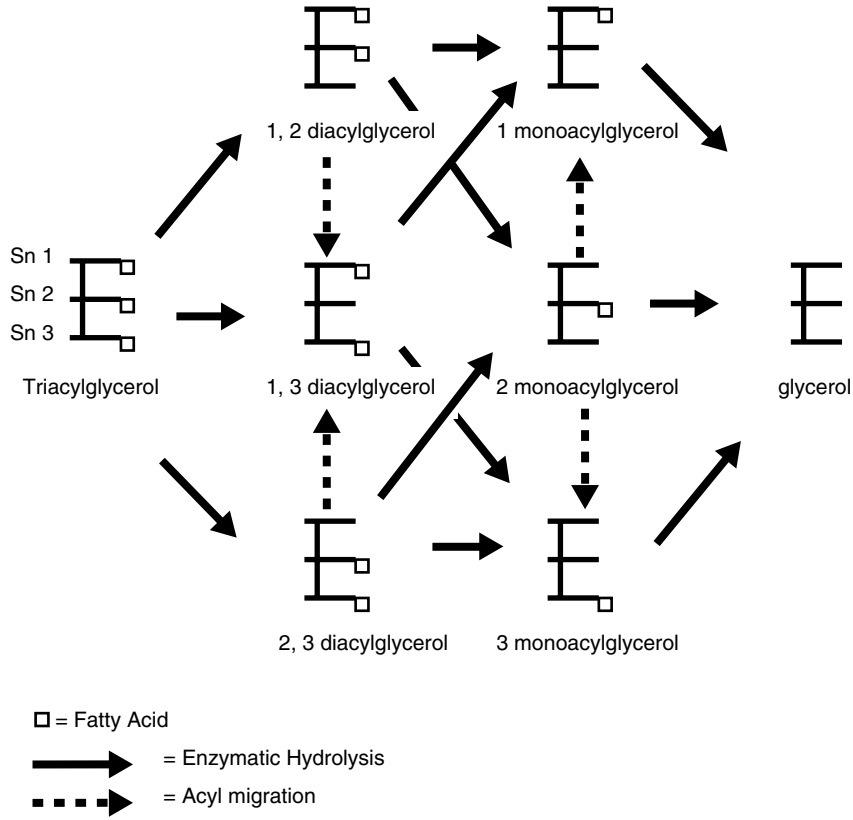

Figure 19.4. Enzymatic degradation of triacylglycerol to diacylglycerols monoacylglycerols and glycerol.

Due to the great diversity of their molecular species, it is difficult to separate all species by one method alone. The unstable nature of some DAGs and MAGs increases this difficulty. TAGs, DAGs and MAGs can be separated by TLC on silica gel using various mixtures of hexane, diethyl ether, acetic or formic acid as the solvent (Christie, 2003a). TAGs, DAGs and MAGs are separated based on their overall polarity and TLC is widely used to isolate and purify TAG fractions. Another useful method to separate TAGs, DAGs and MAGs is silver ion TLC where separation is achieved according to the number of double bonds in the acyl residues. RP-TLC has been used widely to separate TAGs according to their polarity. A combination of silver ion-TLC and RP-TLC has been used to elucidate the structure of TAGs (Nikolova-Damyanova, 1999). High temperature gas chromatography has also been used widely to separate TAGs according to the sum of the molecular weights of their constituent fatty acids. This type of analysis requires columns with coating materials stable up to 380°C; various nonpolar and polar columns have been used successfully to analyze TAGs of various vegetable and butter oils. Fused silica open tubular columns appear to give the best separation of TAG according to the distribution of the double bonds on the fatty acyl moieties and by the molecular mass of each TAG.

Chromatographic and spectrophotometric methods used for the analysis of TAGs have been reviewed by Andrikopoulos (2002). Details are given of HPLC (normal, RP, silver-ion, size exclusion/gel permeation), GC, supercritical fluid chromatography, mass spectrometry and TLC methods. Laakso (2002) has also reviewed mass spectrometric methods used to characterize TAGs. Most of the above methods have been used to characterize TAGs, DAGs and MAGs in vegetable oils and therefore are concerned mainly with TAGs containing predominantly long-chain FFAs. Some methods dealing specifically with TAGs, DAGs and MAGs in milk fat have been reported. Contarini and Toppino (1995) studied TAGs, DAGs and MAGs in Gorganzola cheese. Fat was extracted in hexane and diethyl ether, followed by TLC. Chromatograms were developed using 2,7-dichlorofluorescin in ethanol. The bands corresponding to TAGs, DAGs or MAGs were recovered and extracted with chloroform and diethyl ether. MAGs and DAGs were separated as trimethyl silane derivatives using GC and TAGs were chromatographed directly. García-Ayuso and Luque De Castro (1999) compared chromatographic and non-chromatographic techniques for the quantification of TAGs and FFAs in dairy products. Nájera *et al.* (1999) achieved good resolution for many TAGs in cheese by a RP-HPLC method with a light scattering detector.

19.5. Chromatographic Methods for the Analysis of Lipolytic Activity

Fatty acids released by lipases can be determined quantitatively by TLC, GC and HPLC. TLC methods are used in conjunction with densitometric methods or autoradiographic methods using radiolabelled TAG. Many GC and HPLC methods that have been outlined earlier are widely used to isolate and quantify FFAs in lipolytic assays. Additionally a method using *p*-nitrophenyllaurate as a substrate was described by Maurich *et al*. (1991) who quantified activity by the release of *p*-nitrophenol. Veeraragavan (1990) used a RP-HPLC method with triolein as the substrate. Triolein was emulsified in buffer with the aid of a surface active agent and the lipase added under controlled conditions. Lipolytic activity was measured by the release of oleic acid and quantified by absorbance at 208 nm.

Patel *et al*. (1996) produced trimethylsilyl derivatives of FFAs, MAGs, DAGs and TAGs in butterfat hydrolyzed by different lipases. GC was used to quantify these compounds; the method enabled FFAs of acyl carbon numbers from 4 to 18, MAGs with acyl carbon numbers of 12–18, DAGs with acyl carbon numbers of 16–36 and TAGs with acyl carbon numbers of 30–46 to be identified.

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