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REVIEW

Free fatty acids quantification in dairy products

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Free fatty acids (FFA) in dairy products have experienced a resurgence of interest in recent years due to their associated health benefits, flavour potential and antimicrobial activity, as a direct cause of hydrolytic rancidity, and also because of the importance of accurate determination for quality, research and legislative purposes. Gas chromatographic analysis remains the technique of choice for FFA analysis; however, little advancements in method development have occurred over the last twenty years. The purpose of this review is to provide an overview of the methodology used to quantify FFA in dairy products by gas chromatography since its inception, to highlight some of the challenges experienced, to suggest aspects that could be improved to increase accuracy and throughput and reduce labour costs and solvent usage and to widen applications.

Keywords Free fatty acids, Dairy, Gas chromatography, Esters.

INTRODUCTION

The accurate determination of free fatty acids (FFA) in dairy products is important for research, legislative, process development and quality control. Free fatty acids have strong sensory properties and are important compounds in the flavour and aroma of many dairy products, especially cheese and fermented dairy products (Fox and Wallace 1997; Collins *et al.* 2003). However, elevated levels of short chain FFA are also responsible for rancidity in milk and other dairy products (Deeth 2006). Free fatty acids also contribute to texture and functionality, as they impact on surface tension and foaming capacity of milk (Kuzdzal-Savoie 1980; Kamath *et al.* 2008). FFA particularly, butyric acid and conjugated linoleic acid, have also been shown to have beneficial health and nutritional effects, such as anticarcinogenic properties (Parodi 1999), and can act as bioactive compounds *in vivo* (Kritchevsky *et al.* 2000; Nielsen *et al.* 2015). There is also some evidence to suggest that they may help to reduce body fat (Wahle *et al.* 2004).

A major difference between animal and vegetable fats is the presence of water-soluble short chain fatty acids (SCFA) (Jensen *et al.* 1967; Jensen 2001). These SCFA require special consideration in terms of extraction and quantification due to their solubility and volatility (IDF 1991; Evers 2003). The current International Dairy Federation (IDF) and the International Standard Organisation (ISO) (ISO/IDF, 2001), which details fat extraction of milk and milk products, describes a six-hour reflux for fat extraction for cheese and does not incorporate a strategy for the extraction of FFAs for many other dairy products such as milk, condensed milk, dried milk products, cream or fermented milk. The current IDF standard method (ISO/IDF, 2001) is also very laborious and limited in its potential applications. Widely used fat extraction methods (Hickey *et al.* 2006, 2007; Kilcawley *et al.* 2012; Martini *et al.* 2013; Firl *et al.* 2014; Sert *et al.* 2014; Juan *et al.* 2015) are based on older methods such as those described by De Jong and Badings (1990) or Folch *et al.* (1957). Thus, there is considerable scope to either refine existing methods, or to develop new methods that are more reliable and accurate, use less solvents and are applicable for a wide range of sample types.

FAT IN DAIRY PRODUCTS

The primary component found in milk lipids are triacylglycerides (TAG) (Figure 1), which

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account for >95% of the total lipid content (Jensen 2001). These compounds consist of a glycerol molecule linked to three fatty acid chains to yield a tri-ester. The length of these individual FA chains varies and can be saturated or unsaturated. As well as TAG, which contain three FA chains, there are diacylglycerides (which contain two fatty acid chains) and monoacylglycerides (which contain one fatty acid chain). When a fatty acid is not bound to glycerol, it is termed a FFA. Free fatty acids are primarily formed in dairy products by the enzymatic breakdown of glycerides (Deeth and Fitz-Gerald 1995).

Milk fat differs from other lipids in that it contains fatty acid carbon chain lengths ranging from $C_{4:0}$ to $C_{18:3}$, either in their free state or bound as glycerides (Bills *et al.* 1963; Jensen *et al.* 1991). As opposed to other lipids, such as vegetable oils, which contain chain lengths of carbon number greater than $C_{14:0}$. The SCFA ($C_{4:0}$-$C_{8:0}$) also have very strong sensory characteristics with low flavour thresholds and impart distinctive flavours and odours in dairy products (Fox and Wallace 1997; Collins *et al.* 2003). Rancidity in milk is associated with increases in FFA levels due to enzymatic activity and is generally unacceptable to the consumer when levels exceed 1.5 mmol/L (Deeth 2006). The fact that these SCFA are water-soluble and volatile impacts on the approach required to quantify FFA in dairy samples. Therefore, an appropriate method must be capable of extracting both water-soluble SCFA and organic-soluble FA, avoid the use of an evaporation steps to prevent losses of volatile SCFA and also remove or negate any water that may be present in the sample.

FREE FATTY ACIDS IN DAIRY PRODUCTS

The most immediate application of the determination of FFA in dairy products is in measuring milk quality. Milk with elevated FFA levels develops a rancid flavour due to the liberation of SCFFA, especially butyric acid, and generally becomes unacceptable. Glycerides in pasteurised milk are susceptible to breakdown due to bacterial lipases, or in raw milk this breakdown can also occur due to the indigenous lipolytic enzymes (lipoprotein lipase) present in milk (Antonelli *et al.* 2002). The majority of lipids are encased in a lipoprotein complex termed the milk fat globule membrane (MFGM), which prevents lipoprotein lipase coming into contact with these lipids. However, this MFGM can rupture due to external stresses (agitation, pumping, homogenisation), allowing lipolysis to proceed (Deeth and Fitz-Gerald 1978; Deeth 2006). This can be an issue with automatic milking systems (AMS). The development of larger dairies, resulting in more pumping, increased farm to dairy transport distances and storage times can all impact on the integrity of the MFGM (Klungel *et al.* 2000). The increased milking frequency with AMS when compared to parlour systems is another factor implicated in increasing FFA levels (Abeni *et al.* 2005). Higher temperatures of pumping (Wiking *et al.* 2005) and air intake (Rasmussen *et al.* 2006) have been shown to have a significant impact on FFA levels.

The monitoring of FFA levels during milk production is important from a health and safety point of view, as bad hygiene practises can lead to the growth of psychotropic bacteria, which in turn increases lipolysis (Antonelli *et al.* 2002). Unlike lipoprotein lipase, some bacterial lipases are not affected by the MFGM and can readily access lipids, as many are also heat resistant (Fitzgerald and Deeth 1983), they can therefore potentially survive pasteurisation or ultra-high temperature (UHT) treatments. Today, hygiene is strictly controlled, so microbial contamination is less likely; however, milk production is increasing in many parts of the world where industrial practises are not as well developed.

The FFA content of dairy products is an important indicator of lipolysis in ripened and fermented products and is a useful marker when establishing and defining process conditions. While high FFA levels are generally considered undesirable, they are important flavour compounds in many dairy products, such as cheese (Fox and Wallace 1997), and

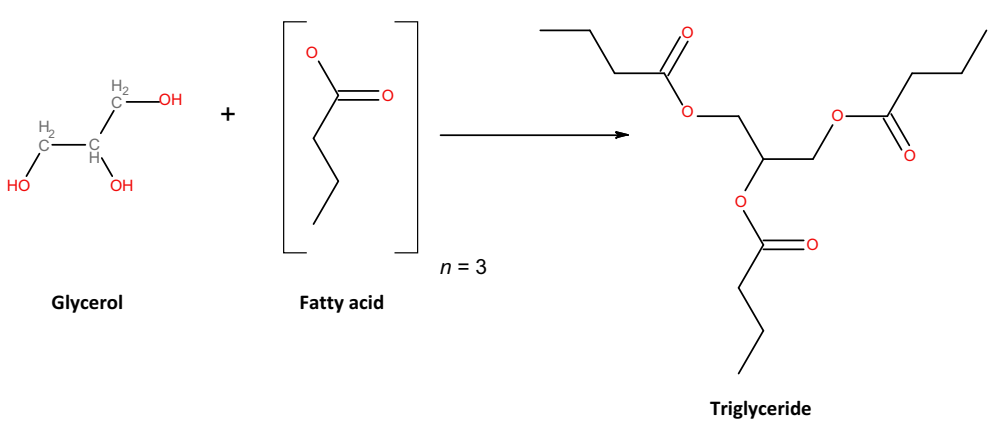

Figure 1 Formation of triglycerides.

act as precursors to other flavour compounds (Collins *et al.* 2003); and also have functional properties (Deeth and Smith 1983). Free fatty acids have also been shown to have beneficial health, nutritional and bioactive properties (Parodi 1999; Kritchevsky *et al.* 2000).

The determination of FFA has been an important component of many studies involving bovine, ovine and caprine milks and their derived products (Table 1). Lipolysis in milk and milk fat composition, particularly bovine milk, has also been extensively studied (Kintner and Day 1965; Cartier and Chilliard 1990; Kondyli and Katsiari 2002; Chilliard *et al.* 2003) and reviewed (Jensen 1964, 2001; Jensen *et al.* 1991; Deeth 2006). Free fatty acids determination is necessary for understanding the overall flavour profile and has been performed on a range of dairy products, for example milk powder (Deeth *et al.* 1983), Cheddar cheese (De Jong and Badings 1990), ewes milk cheeses (Fernández-García *et al.* 2006), hard cheese (Malacarne *et al.* 2009), soft cheese (Calzada *et al.* 2014), goats milk cheese (Juan *et al.* 2015) and yoghurt (De Jong *et al.* 1994).

Dairy processes are constantly evolving with the development of new technologies, and FFA determination is an important tool in gaining more knowledge into process development and for research purposes. Lipids in milk can be characterised further into glycerides, FFA, phospholipids and unsaponifiable lipids (Jensen *et al.* 1991). The presence of this wide range of compounds leads to complicated reaction mechanisms and the formation of diverse chemical products some of which influence flavour, texture and composition of the final product. Thus, accurately determining FFA leads to a better understanding of these processes as well as identifying some of the key compounds involved in flavour, texture, health and food safety.

FATTY ACID METHYL ESTERS (FAMEs)

Gas chromatographic (GC) analysis is one of the most popular techniques for the analysis of lipids (Christie 1993; Delmonte *et al.* 2009). The technique involves the analysis of a gaseous mixture, usually a volatised liquid sample, which is introduced to a chromatographic column where the components are separated by temperature based on their affinity with the column phase. These separated components are then analysed in a detector. The flame ionisation detector (FID) is most widely used because of its relative low cost, simplicity and wide availability. In order for an analyte to be analysed by GC, it must be volatile. For the majority of lipids, this is not the case and a technique known as chemical derivitisation is often employed to volatilise the sample. The most well-established technique for the analysis of FA by GC is to convert them into fatty acid methyl esters (FAMEs) (Christie 1993). This same approach is used in the current international standard for the analysis of fatty acids in milk fat (ISO/IDF, 2002). Gas chromatographic analysis of fat in dairy products can be categorised into two types: total fatty acid (TFA) analysis, or free fatty acid (FFA) analysis. For TFA, the triglyceride ester-bound fatty acids (FA) are quantified, but for FFA analysis, only the FFA isolated from the fat are subsequently quantified. The reaction for the formation of FAMEs from FFA is termed an esterification reaction.

FAMES: ACID-CATALYSED ESTERIFICATION OF FREE FATTY ACIDS

Free fatty acids can be converted into FAMEs using methanol in the presence of a suitable acid catalyst, such as sulphuric acid (H_2SO_4) or boron trifluoride (BF_3) (Figure 2). The mechanism is initiated with the protonation of the acid with methanol forming an intermediate. This intermediate loses a proton to yield the FAME. All the steps are reversible and require a large excess of alcohol to drive the reaction to completion. Also water must be excluded from the reaction as it is a stronger electron donor than aliphatic alcohols and the formation of the intermediate product is not favoured (Christie 1993).

IMPACT OF DIFFERENCES IN SOLUBILITY OF DAIRY FATTY ACIDS FOR FREE FATTY ACID DETERMINATION

As mentioned, milk fat differs from other natural lipids in that it contains a large proportion of SCFA, either in their free state or bound as glycerides. The challenge in dealing with methyl esters of SCFA resides in their high volatility and water solubility. A quantitative recovery can be difficult to achieve without special precautions. Many fat extraction techniques employ the use of solvent extraction of the fat or oil followed by evaporation, where substantial losses of SCFA can occur. Also as SCFA are water soluble, they may be poorly recovered during extractions involving phase separation. Extraction procedures that employ the use of high temperatures, such as refluxing or distillation, are also prone to increased risk of losing these volatile SCFA.

Several methods have been developed that are considered quite successful for FFA analysis of dairy products using derivitisation techniques. These involve using reagents that derivitise FFA at room temperature, and do not employ aqueous solvents or evaporation steps. Christopherson and Glass (1969) described the determination of glycerides in milk, but also described a simple subsequent step for FFA determination in milk using 10% hydrochloric acid in methanol and leaving to stand at room temperature for one hour prior to GC analysis. Luddy *et al.* (1968) used boron trifluoride (BF_3) in methanol to esterify all FFA in butter oil. These methods have been reviewed and recommended (Badings and De Jong 1983; Christie 1993).

While the above methods are useful for FFA analysis, this was only achieved postderivitisation of glycerides in the

Table 1 List of recent publications for determination of FFA in dairy products, and the referenced FFA extraction method

Application	*Reference*
Study of lipolysis of cheeses made from goat milk[a]	(Juan *et al.* 2015)
Study of lipolysis in Tulum cheese made from raw and pasteurised milk[b]	(Sert *et al.* 2014)
Study of the effect of high pressure processing on lipolysis and volatile compounds in Brie[a]	(Calzada *et al.* 2014)
Lipolysis and volatile compounds in Galotyri-type cheese[a]	(Kondyli *et al.* 2013)
Study of effect cold storage on raw milk quality[c]	(Gargouri *et al.* 2013)
Study of refrigerated raw goat milk effect on whole milk powder[d]	(Fonseca *et al.* 2013)
Effect of goat genotypes on milk yield and composition[e]	(Chilliard *et al.* 2003)
Lipolysis in probiotic and synbiotic cheeses[f]	(Rodrigues *et al.* 2012)
Evaluation of commercial enzymes to accelerate cheese ripening[a]	(Kilcawley *et al.* 2012)
Study on effect of probiotic bacteria on conjugated linoleic acid in curdled milk[f]	(Silva *et al.*2011)
Comparison of FFA composition between low fat and full fat goat milk cheeses[g]	(Nouira *et al.*2011)
Evaluation of volatile compounds and FFAs in yoghurts made of ewe's and goat's milk[g]	(Güler and Gürsoy-Balcı 2011)
Effect of cooling on the distribution of lipoprotein lipase in milk[c]	(Dickow *et al.* 2011)
Effect of frozen storage and packaging on Turkish Motal cheese[a]	(Andic *et al.* 2011)
Probiotic in lamb rennet enhances rennet lipolytic activity[a]	(Santillo *et al.* 2009)
FFA profile of Parmigiano–Reggiano cheese[a]	(Malacarne *et al.* 2009)
Effect of lactic acid bacteria esterases to the release of fatty acids in ewe's milk cheese[h]	(Abeijón Mukdsi *et al.* 2009)
Relationship between surface tension, FFA content and foaming properties of milk[d]	(Kamath *et al.* 2008)
Effect of high pressure treatment on FFA during ripening of ewe's milk cheese[a]	(Juan *et al.* 2015)
Starter strain related effects on properties of Cheddar cheese[a]	(Hickey *et al.* 2007)
Influence of air intake on FFAs in milk during automatic milking[c]	(Rasmussen *et al.* 2006)
Influence of seasonal milk supply on properties of Cheddar cheese[a]	(Hickey *et al.* 2006)
Seasonal variation of FFA content in Spanish ovine ewe's milk cheeses[a]	(Fernández-García *et al.* 2006)
Evaluation of cooling strategies during pumping of milk[c]	(Wiking *et al.* 2005)
Milk quality and automatic milking[i]	(Abeni *et al.* 2005)
FFAs and volatile compounds in low fat Kefalograviera cheese[a]	(Kondyli *et al.* 2003)
Fatty acid composition of raw goat milk[a]	(Kondyli and Katsiari 2002)

FFA extraction applied as described by:
[a]De Jong and Badings (1990). [b]Folch *et al.* (1957). [c]IDF (1991). [d]Deeth *et al.* (1975). [e]Koops and Klomp (1977). [f]Alonso *et al.* (2003). [g]Deeth *et al.* (1983). [h]Kuzdzal-Savoie (1980). [i]Shipe *et al.* (1980).
FFA, free fatty acids

sample. Thus, it involved two separate derivitisation steps resulting in increased labour and reagents used. In applications where individual FFA determination is not necessary, titration of the fat extract with a suitable base like KOH can be used to measure the total acidity. This is a common technique used to measure milk quality and is described in the reference method of the Bureau of Dairy Industries (IDF 1991) for the determination of total free fatty acid level in milk, where the FFA content is expressed in millimoles of FFA/100 g fat. For applications that describe solvent extraction of FFA, the type of solvent employed is dependent on the targeted FFA. For SCFA determination, it is possible to use water as a solvent. The authors Kosikowsky and Dahlberg (1946) and Horwood and Lloyd (1980) used steam distillation to collect the SCFA in cheese. In the latter method, the determination of acetic acid ($C_{2:0}$) to caprylic acid ($C_{8:0}$) was achieved by GC-FID analysis of the distilled alkali salts of the acids dissolved in formic acid. However, recoveries of FFA by steam distillation decreased as the carbon chain length increased (Kosikowsky and Dahlberg 1946). To extract the full range of FFA from dairy products, solvents such as chloroform (Gray 1975), acidified diethyl ether (Salih *et al.* 1977), hexane/diethyl ether (Deeth *et al.* 1983) and heptane/diethyl ether (De Jong and Badings 1990) have been used. These organic solvents have been reported to achieve high recoveries (>92%) of FFA in samples such as cheese. However, these were shown to be less reliable when applied to milk, due to the natural oil in water emulsion of milk (IDF 1991). In many procedures, anhydrous sodium sulphate is often added to the sample to absorb moisture that may interfere with analysis prior to solvent extraction. When solvent mixtures are employed, recoveries of SCFA can drop when the nonpolar component of the solvent is

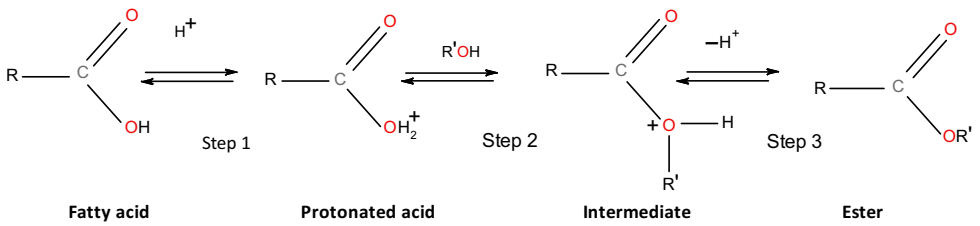

Figure 2 Acid-catalysed esterification. Adapted from (Christie 1993)

increased. This was demonstrated by Salih *et al.* (1977) where the addition of hexane to diethyl ether was used to make a 30% v/v solution in diethyl ether in the initial extraction step, resulting in only 82% recovery of $C_{4:0}$, where 100% was achieved using diethyl ether alone.

Solvents capable of extracting the complete range of FFA from samples will also extract the rest of the lipid mixture, and depending on the application, it might be necessary to isolate the FFA component prior to analysis. There have been a number of chromatographic techniques employed, which involved silicic acid/KOH columns (Harper 1953; Gray 1975; Woo and Lindsay 1982), ion exchange resins (Needs *et al.* 1983; McNeill *et al.* 1986; McNeill and Connolly 1989), deactivated alumina columns (Deeth *et al.* 1983; De Jong and Badings 1990) and aminopropyl columns (Kaluzny *et al.* 1985; De Jong and Badings 1990) to isolate FFA from fat extracts. Due to their strongly alkaline nature, hydrolysis of glycerides is known to occur if silicic acid columns/KOH (Stark *et al.* 1976; Woo and Lindsay 1980), and ion exchange resins (Stark *et al.* 1976) are used and can result in an overestimation of FFA levels. Improvements of these methods have been described. For the isolation of FFA from Cheddar cheese, where Woo and Lindsay (1982) implemented the use of two separate columns, a pre-column to remove lactic acid from the sample extract, and a silicic acid-KOH arrestant column to isolate FFA, which were eluted using 2% v/v formic acid in ethyl ether. Needs *et al.* (1983) described another method to isolate FFA in milk, which used a pretreated amberlite resin that contained no water or fine particles. The lipid extract was mixed with the resin for one hour followed by solvent removal and washing, and the isolated FFA were subsequently converted to FAMEs for GC analysis. However, these methods are very labour intensive and time-consuming. The use of deactivated alumina columns was first reported by Deeth *et al.* (1983) and was considered a novel alternative to the solid supports using silicic acid/KOH or ion exchange resins, and the authors reported high recoveries of $C_{4:0}$-$C_{18:1}$ FFAs from milk, cheese and butter. However, the nature of the final extract (6% v/v formic acid in diisopropyl ether) was reported to have detrimental impact on column lifetime and repeatability (De Jong and Badings 1990) leading to a modification of the procedure using a lower concentration of formic acid (3% v/v) in diethyl ether. De Jong and Badings (1990) compared the performance of aminopropyl columns in isolating FFA using a reference mix where they reported 96–101% recovery of all FFA, against the deactivated alumina, where only 82–89% recovery was achieved. They concluded that aminopropyl isolation provided a better alternative for FFA determination than deactivated alumina. The procedure employed was a modification of work done by Kaluzny *et al.* (1985) who detailed the separation of various lipid classes using aminopropyl columns. This method was later used for FFA determination in yoghurt (De Jong *et al.* 1994). Kaluzny *et al.* (1985) had demonstrated that the components of a lipid mixture can be separated and collected with excellent recovery and purity (101.4% and 98% for FFA, respectively) using aminopropyl columns; however, it is important to note that the fat extraction procedure employed prior to FFA isolation must be sufficient to extract all of the FFA from the sample.

ISSUES RELATING TO THE ACIDIC NATURE OF FATTY ACID EXTRACTS IN RELATION TO COLUMN PERFORMANCE

Another consideration for the determination of FFA by GC is the acidic nature of the final extract. By their nature as acids, FFA have strong interactions with column phases, which can lead to irreversible adsorption, peak tailing, ghost peaks and double peak formation (Emery and Koerner 1962; Ackman and Burgher 1963) particularly when dealing with high moisture conditions (Woo *et al.* 1984). The use of formic acid either in the carrier gas (Gray 1975) or as a solvent (Horwood and Lloyd 1980) reduced the occurrence of these issues and allowed for quantitative determination by GC, but the acidic nature of the extract reduced column lifetime. Today, selective capillary columns with specific free fatty acid phases (FFAP) are well established to achieve complete separation of FFA of chain length $C_{2:0}$-$C_{22:0}$ without derivitisation. However, these columns still suffer the effects of FFA adsorption and reduced column lifetime when used with acidic FFA extracts (De Jong and Badings 1990). Derivitising the FFA to esters can overcome these issues and was employed in other studies (McNeill *et al.* 1986; McNeill and Connolly 1989; Kim Ha and Lindsay 1990).

THE USE OF TETRAMETHYLAMMONIUM HYDROXIDE AS AN ESTERIFICATION REAGENT

Tetramethylammonium hydroxide (TMAH) was first reported as an esterification reagent for carboxylic acids by Prelog and Picentanida (1936), who discovered that the ammonium salts of these acids decomposed when heated to yield trimethylamine (TMA) and methyl esters. TMAH was applied in the methylation of benzoic acid by Fuson *et al.* (1939) with yields of 60–90% of the methyl esters at temperatures of 200–250 °C. The utilisation of TMAH as a means to form methyl esters of organic acids followed by GC analysis was described by Robb and Westbrook (1963), who found that the TMAH reaction proceeds rapidly at room temperature and that the salts readily decompose in the heated injection port (365 °C) of the GC to yield methyl esters, which are immediately introduced onto the column. They prepared the tetramethylammonium salts by two procedures: (i) titration of the dissolved acid with TMAH in methanol; and (ii) ion exchange using an Amberlite resin. On the range of acids tested, which included mono carboxylic, dicarboxylic, keto, hydroxy and aromatic acids, they found that oxalic, malonic, citric and malic acids were not converted to methyl esters by this method. This was attributed to the fact that these compounds decomposed to lower molecular weight compounds than their methyl ester counterparts. The authors also reported that the yield of methyl ester tended to drop if the acid was present in small quantities and that maximum yields were observed if the total quantity of acid present was greater than 20 μg/μl. The yields obtained ranged from 85% to 95%, and it was concluded that these variable yields made the method unsuitable for quantitative analysis, but useful for qualitative purposes. A limitation of many methylation procedures is the necessity to extract the acids from an aqueous solution before the esterification can be performed. Downing (1967) investigated the preparation of tetramethyl ammonium salts in an aqueous solution followed by GC analysis. The method used a capillary probe, an apparatus developed to collect the sample aliquot followed by drying in an oven before directly injecting into the heated injection port of the GC. They found this analysis was both quantitative and reproducible. The quantity of acid was also investigated and they reported that as little as 0.3 μg of acid could be analysed with no significant loss of yield. Downing and Greene (1968) investigated the use of TMAH to esterify polyunsaturated fatty acids (PUFA). The authors found that the strong alkaline nature of the TMAH solution interfered with the esterification of these acids when compared to analysis performed with BF_3/methanol as the methylating agent. This problem was overcome by neutralising the salt solution to pH 7.5–8.0 using 5% v/v acetic acid in distilled water. The use of TMAH to form ammonium salts of carboxylic acids, which could pyrolyse to pure esters within the injection port of a GC, was considered to be a significant advantage over other reagents such as BF_3/methanol, as GC methods employed at the time described the need to saponify the acids with a suitable base before the addition of esterifying agents. Due to the strong alkali nature of TMAH and the high temperatures needed to pyrolyse its product salts, other quaternary ammonium salts were investigated for their use in methylating carboxylic acids. Brochmann-Hanssen and Olawuyi Oke (1969) employed a modification of the TMAH method as described by Stevenson (1966) for the methylation of barbiturates. In this method, trimethylanilinium hydroxide was used as the esterification catalyst, which yielded the methyl ester and dimethylaniline. Incomplete methylation of the barbiturate compounds was experienced when they employed TMAH, and they also noticed a number of small secondary peaks in their analysis. They showed that dimethylaniline was a better leaving group than TMA, allowing for milder temperature conditions (200 °C) in the GC injector to achieve complete methylation for analysis. MacGee and Allen (1974) described a GC method using trimethyl(α,α,α-trifluoro-m-tolyl)ammonium hydroxide (TMTFTH) (Figure 3), which again was considered a milder base providing a better leaving group than TMAH or trimethylanilinium hydroxide, for the preparation of FAMES in serum samples. Along with the reduced temperature conditions for complete methylation, the group also reported that the lower alkaline nature of TMTFTH yielded increased recoveries of PUFA when compared with TMAH. This group also reported a technique termed as 'ester neutralisation' where the addition of methyl propionate in excess was hydrolysed by remaining TMTFTH, thus neutralising the solution. This was considered to be a much more convenient method of neutralisation than adding an exact amount of acid as any remaining methyl propionate would not interfere with analysis.

Common analysis of FA mixtures involved saponification techniques with a strong base such as sodium hydroxide

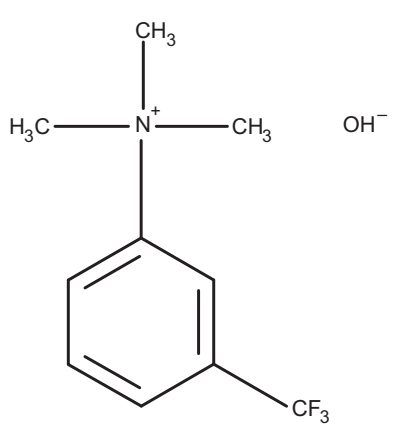

Figure 3 Structure of trimethyl (α,α,α-trifluoro-m-tolyl) ammonium hydroxide.

(NaOH) or KOH followed by esterification with a methanolic acid, such as HCl, sulphuric acid (H_2SO_4) or BF_3 to determine FFA content. In applications where the glyceride-bound FA needed to be determined, a methanolic base, such as sodium methoxide (CH_3NaO), was used to form the methyl esters. As quaternary ammonium hydroxides are inherently strongly basic, a methanolic solution can form methyl esters of the glyceride-bound FA, and given that the ammonium salts of FFA degrade to form methyl esters, their use in analysing common lipid mixtures was investigated. TMTFTH in methanol was employed by McCreary *et al.* (1978) in the characterisation of the fatty acid content in common edible oils (Corn, Peanut and Olive). They reported that this was a more suitable reagent than TMAH or trimethylanilinium hydroxide due to the PUFA content. They prepared the oils in benzene and compared this catalyst against the more common CH_3NaO and achieved comparable results. They also described the added benefit of being able to both simultaneously trans-esterify glycerides while forming ammonium salts of the FFA, which upon injection were esterified, in a single preparation step. This was a significant advantage over existing procedures, which required FFA and glycerides to be derivitised and analysed separately. Metcalffe and Wang (1981) used TMAH in methanol on various fat mixtures dissolved in diethyl ether. This single step process resulted in separate phases: the trans-esterified methyl esters of the glycerides (organic phase), and the ammonium salts of the FFA (aqueous phase). Conveniently each phase was suitable for direct injection to a GC instrument for FFA or glyceride characterisation: furthermore with the addition of enough methanol, a single phase was formed, which could be injected for total fat composition determination.

THE APPLICATION OF TETRAMETHYLAMMONIUM HYDROXIDE DERIVATISATION FOR THE ANALYSIS OF FREE FATTY ACIDS IN DAIRY PRODUCTS

Although butter fat was analysed by Metcalffe and Wang (1981) using TMAH in methanol, the results obtained were only qualitative. Martínez-Castro *et al.* (1986) applied this approach to the analysis of milk fat for quantitative determination, which was later adapted for cheese extracts (Martin-Hernández *et al.* 1988; Juárez *et al.* 1992; Chavarri *et al.* 1997). One of the factors investigated by Martínez-Castro *et al.* (1986) was the effect of making the reaction mixture pH neutral, prior to analysis (which is usually recommended for GC analysis to protect the column and any PUFA that may be present in a sample). The authors discovered that neutralising the basic TMAH solution to pH 7.5–8.0 resulted in losses of SCFA and increased standard deviations in the analytical data, which adversely affected the robustness of the method. They attributed this to the dissociation of the ammonium salts at the selected pH values. As PUFA content in milk fat is generally quite low, and the development of SCFA in milk is a good indicator of the level of lipolysis (Jensen *et al.* 1991), avoiding neutralising the sample could be considered a necessary step in obtaining reliable quantifiable results of FA content in dairy products. An advantage of TMAH is that when pyrolysed, it degrades to TMA and methanol (McCreary *et al.* 1978), which are highly volatile and thus suitable for GC analysis; however, TMA was subsequently reported to interfere with peak determination (Martin-Hernández *et al.* 1988). The application described for the analysis in cheese highlights obvious benefits in being able to prepare and analyse both the FFA and the triglyceride components from a sample in a single extraction method. One of the limitations of the TMAH reaction highlighted by Martínez-Castro *et al.* (1986) was the apparent development of secondary reactions, which can lead to the glyceride and FFA fractions dissociating into the opposite layers during extraction. This phenomenon was evident when the fatty acids of the glyceride layer were detected in the FFA layer. This led to a modification of the method by the authors where the layers were separated and washed with the appropriate solvent before analysis. Chavarri *et al.* (1997) noted that if there was a large triglyceride to FFA ratio, which is the case in most dairy products, the issue with FFA dissociation was even more pronounced. These authors also reported a comparison of the TMAH extraction esterification procedure versus an on-column chromatographic procedure (De Jong and Badings 1990) where FFA were isolated using aminopropyl columns before direct injection to GC-FID. Substantial discrepancies were evident between both methods in the analysis of FFA in Cheddar cheese, where they obtained total FFA measurements of 1683 mg/kg and 4007 mg/kg using the aminopropyl and TMAH methods, respectively. The higher FFA levels for the TMAH method were subsequently shown to be due to the dissociation of glycerides into the FFA layer, and they concluded that it was necessary to isolate FFA from the lipid mixture before employing the TMAH extraction/esterification method.

POTENTIAL ADVANTAGES OF BUTYL ESTER OVER METHYL ESTER DERIVATISATION

The use of higher molecular weight alcohols may be considered an alternative to overcome many issues experienced with methyl esters. Studies by Parodi (1967) demonstrated increased recoveries of SCFA when butyl esterification was carried out instead of methyl esterification. Parodi (1967) evaluated several different methylation methods; using BF_3 in methanol (Metcalfe *et al.* 1966), sodium methoxide (DeMan 1964) and butylation methods using H_2SO_4 in butanol (Gander *et al.* 1962), di-n-butyl carbonate (Sampugna *et al.* 1966), and BF_3 in butanol (Parodi 1967) to determine the fatty acid composition of butter fat. Parodi

Table 2 Brief summary of some of the validation data reported by Firl *et al.* (Firl *et al.* 2014). Recovery is expressed as % recovery of added TG standards from milk samples. Accuracy is expressed as % fat content of sample; results obtained from method of Firl *et al.* (2014) were compared against a gravimetric determination. Precision is expressed as coefficient of variance between sample replicates. Intraday precision determined from six replicates analysed in 1 day, interday precision determined by analysing two samples in six replicates over 4 weeks. Limit of detection (LOD) and limit of quantification (LOQ) determined based on the analysis of C24:1 TG spiked into milk samples and reported as mg/g fat.

Recovery[a]	102.5–115.0%
Accuracy[b]	
Gravimetric determination[c]	4.18%[d],5.2%[e]
Method of Firl *et al.* (2014)	4.25%[d],5.15%[e]
Precision	
Intraday	0.30–7.59%
Interday	5.3–20.76%
LOD	0.1 mg/g fat
LOQ	0.2 mg/g fat

[a]Determinations based on the addition of TG standards to milk samples before sample treatment and analysis. [b]Determined based on the analysis of certified milk standards. [c]Applied as described by Iverson *et al.* (2001). [d]Analysis of certified milk standard with defined fat content 4.24%. [e]Analysis of certified milk standard with defined fat content 5.335%.

(1967) expressed the data as ratio amounts of each fatty acid ester relative to the corresponding myristate ester, and for $C_{4:0}$ reported 0.042 and 0.25 for the BF_3 in methanol and sodium methoxide methylation methods, respectively. When butyl esters were employed, results of >0.33 for $C_{4:0}$ were obtained. Greater responses were observed for other short chain acids ($C_{6:0}$, $C_{8:0}$). Butyl esters were also employed by Parodi (1970) utilising KOH in butanol, a modification of the method that was described by Christopherson and Glass (1969), and by Kim Ha and Lindsay (1990) where they utilised BF_3 in butanol to determine the FFA content in milk and cheese.

The direct on-column approach of De Jong and Badings (1990), while capable of isolating FFA with high recoveries, suffers several drawbacks, mainly relating to column longevity and phase binding resulting in carry-over. TMAH as a derivitising agent can be quite effective for individual FFA determination as it overcomes the volatility issues by forming salts of the FFAs, but suffers limitations when dealing with high triglyceride levels in dairy products (Martínez-Castro *et al.* 1986; Chavarri *et al.* 1997). Employing butyl esters may be a more suitable alternative to these methods. To date, very little information exists comparing the effectiveness of different extraction (amino propyl solid-phase extraction) and esterification (TMAH or butyl esters) techniques in the determination of FFA in a wide range of dairy products.

ADVANCEMENTS IN METHODOLOGY

Many publications reference long-established FFA extraction methods (Table 1), but little or no further development or validation has taken place since these techniques were first published. There have been some efforts to improve and validate some of the existing fat extraction methods. Firl *et al.* (2014) modified the method of Folch *et al.* (1957) to extract lipids from various milk samples, which were converted to methyl esters using trimethylsulfonium hydroxide (TMSH) and subsequently analysed by GC-FID. They performed a very useful validation approach based on the spiking milk samples with triglycerides and reported limits of detection (LODs), limits of quantification (LOQs), accuracy and precision (a brief summary of which is

Table 3 Brief summary of the validation data reported by Amer *et al.* (2013)

Fatty Acid	*Linear range (ug/mL)*	R^2	*LOD (ug/mL)*	*LOQ (ug/mL)*	*Method Repeatability RSD (%)*[a]	*Method stability RSD (%)*[b]	*Recovery (%)*
C4:0	0.5–50	0.9994	0.02	0.5	1.57	1.35	99.46
C6:0	0.5–50	0.9996	0.02	0.5	0.15	0.29	100.28
C8:0	0.5–50	0.9989	0.02	0.5	0.15	0.3	99.98
C10:0	0.5–50	0.9993	0.02	0.5	0.31	0.13	100.00
C12:0	0.5–50	0.9993	0.0005	0.5	0.08	1.27	100.09
C14:0	0.5–50	0.9995	0.0005	0.5	0.65	0.94	100.40
C16:0	0.5–50	0.9994	0.0005	0.5	1.69	0.42	100.37
C18:0	0.5–50	0.9996	0.0005	0.5	0.32	0.38	100.00
C18:1	0.5–50	0.9987	0.02	0.5	0.83	1.04	100.80
C18:2	0.5–50	0.9998	0.02	0.5	0.9	2.32	100.18
C18:3	0.5–50	0.9998	0.02	0.5	1.34	2.54	101.17

[a]Mean relative standard deviation (%) of FFA concentrations of five raw milk samples. [b]Mean relative standard deviation (%) of FFA concentrations of the same five raw milk samples analysed after 3 days.

shown in Table 2). Free fatty acids quantification of dairy samples was not included in this study. Reis *et al.* (2011) proposed a new method utilising thermal desorption (TD) followed by GC–mass spectrometry (MS) to determine the fatty acid profile of milk. They described the use of TMSH to convert the triglycerides into methyl esters. The reagent and milk sample were simply added together within the autosampler vial and heated; a process they referred to as thermochemolysis (THM) took place when the instrument analysis started. They compared this to the Rose–Gotlieb extraction method (Walstra and Mulder 1962) where conventional trans-esterification using KOH in methanol had achieved comparable results in relation to recoveries and repeatability with the exception of $C_{4:0}$, where significant differences in the relative peak area abundance were reported. Using the conventional method, they reported a relative peak area abundance of $C_{4:0}$ ranging from 2.222 to 2.347% across various milk samples in comparison with their reported method, where 1.11–1.432% was achieved with the same samples. They had also reported limitations when dealing with raw nonhomogenised milk with poor repeatability between analyses. This was attributed to the small volume of milk that was employed for the analysis not being representative of the entire milk sample. When dealing with homogenised milk, where the milk fat is distributed in smaller droplets, better repeatability was obtained (0.24–10.83% RSD). The evaluation of the THM technique was based on linearity, repeatability and as a comparison against a conventional extraction method. Free fatty acids determination was not incorporated. However, this could potentially be a very useful method in high-throughput laboratories as only 0.125 mL of milk sample is required for the analysis, and the sample preparation is minimal with only a mild heating step of 37 °C to disperse any cream in the sample, while needing no extraction or clean-up.

A new approach for the quantification of individual FFAs in milk was described by Amer *et al.* (2013). They reported the use of ethyl chloroformate to form ethyl esters of milk FFAs in-solution where pyridine was added as a catalyst. The resulting ethyl esters were extracted in chloroform and analysed by GC-MS. Quantitation of each FFA was determined against deuterated fatty acid internal standards. Method validation was performed and repeatability, linearity, recoveries, LOD and LOQ were reported, a brief summary of which is shown in Table 3. The method was applied to different bovine milk samples, which consisted of raw, full fat (3.55% w/w), semiskimmed (1.5% w/w) and skimmed milk (0.1% w/w). The stability of the method appears excellent with RSD <4% being achieved across all FAs, and recoveries >99% reported. The use of ethyl esters instead of the more common methyl esters overcomes some of the issues experienced with volatility and water solubility of the SCFAs. There is also the benefit of eliminating the requirement of prior extraction before the addition of the derivitising agent as the reaction occurs in solution, where the esters are collected from the aqueous medium using chloroform. However, the future application of this method appears limited to aqueous samples only, but it appears to be a much more suitable alternative to methods such as the BDI reference standard.

CONCLUSIONS

Free fatty acids determination in dairy products is important for research, legislative, process development and quality control. However, due to the lack of development and validation of existing methodologies, information in relation to analytical robustness, linearity, LOD and LOQ is lacking. The application of most methods is usually limited to specific dairy samples, and very little information exists in relation to their suitability for a wide range of dairy products with different fat levels and FFA content. In addition, the use of the most common FAME techniques for analysis of milk appears to be less suitable for the recovery of SCFA. Recent developments to improve existing extraction methods, where validation was carried out and reported, and the procedures adapted to employ more modern instrumentation are a positive step forward (Reis *et al.* 2011; Amer *et al.* 2013; Firl *et al.* 2014). However, more development is required to create methods that can accurately and precisely quantify FFA in a wide variety of dairy products in an efficient and robust manner.

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