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Review

Total and Free Fatty Acids Analysis in Milk and Dairy Fat

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Abstract: Dairy fat is one of the most complex natural fats because of its fatty acid (FA) composition. Ruminant dairy fat contains more than 400 different FA varying in carbon chain length, and degree, position and configuration of unsaturation. The following article reviews the different methods available to analyze FA (both total and free) in milk and dairy products. The most widely used methodology for separating and analyzing dairy FA is gas chromatography, coupled to a flame ionization detector (CG-FID). Alternatively, gas chromatography coupled to a mass spectrometer (GC-MS) is also used. After lipid extraction, total FA (TFA) are commonly converted into their methyl esters (fatty acid methyl esters, FAME) prior to chromatographic analysis. In contrast, free FA (FFA) can be analyzed after conversion to FAME or directly as FFA after extraction from the product. One of the key questions when analyzing FAME from TFA is the selection of a proper column for separating them, which depends mainly on the objective of the analysis. Quantification is best achieved by the internal standard method. Recently, near-infrared spectroscopy (NIRS), Raman spectroscopy (RS) and nuclear magnetic resonance (NMR) have been reported as promising techniques to analyze FA in milk and dairy products.

Keywords: milk fat; dairy fat; total fatty acids; free fatty acids; FAME; GC-FID; GC columns

1. Introduction

Milk is an emulsion in which lipids are structured in milk fat globules (MFG). MFG contain nonpolar lipids in the interior, mainly triacylglycerols (TAG), but also cholesteryl esters and other minor lipids, covered by a membrane containing amphipathic lipids and proteins. Heat treatments and dairy product processes look to disrupt MFG structure but have little effect on lipid content and composition [1].

Lipids in milk are in a concentration between 99 g/L in ewe's milk and 33 g/L in cow's milk (Table 1). They are largely composed of TAG. Minor amounts of diacylglycerols, monoacylglycerols, free fatty acids (FFA), phospholipids (PL), glycolipids and sterols are also present in milk. Trace amounts of fat-soluble vitamins, β-carotene and fat-soluble flavoring compounds are present in the milk lipids. Because TAG account for about 98% of the total fat, they have a major and direct effect on the properties of milk fat, for example hydrophobicity, density and melting characteristics. PL account for only 0.6% of milk lipids. However, they play a major role in milk due to their amphiphilic properties. About 65% of them are found in the milk fat globule membrane (MFGM), whereas the rest remain in the aqueous phase.

Table 1. Fat content of milk from various species (wt% of the total lipids).

Lipid Class	Cow [1]	Sheep [2]	Goat [2]	Buffalo [1]
Total (wt% of total milk)	3.3–4.7	4.0–9.9	4.1–4.5	4.7
Triacylglycerol	97.5	98.1	97.3	98.6
Diacylglycerol	0.36			0.7
Monoacylglycerol	0.027	0.03	0.10	T
Cholesteryl esters	T	0.02	0.04	0.1
Cholesterol	0.31			0.3
Free fatty acids	0.027			0.5
Chol + DAG + FFA [3]		1.45	1.89	
Phospholipids	0.6	0.38	0.65	0.5

[1] From Christie [2]; [2] From Rodriguez-Alcalá et al. [3]; [3] Cholesterol + Diacylglycerols + Free fatty acids; T trace.

Thousands of scientific works describe the composition of milk fat. Many others explain how it changes according to different factors that influence its properties (animal species, breed, genotype, stage of lactation, feeding regime . . .). Most of them refer to FA composition of milk fat. A detailed description of different aspects about dairy fats can be found in various chapters of the book "Advanced Dairy Chemistry. Volume 2. Lipids" [4] or in the excellent Jensen's review [1].

Dairy fat is one of the most complex natural fats because of its FA composition. By summarizing the results of various studies, Jensen [1] reported the presence of more than 400 different FA in milk fat. Schröder and Vetter [5] separated FA fractions according to different criteria, and analyzed them by GC-MS (gas chromatography coupled to a mass spectrometer). Taking the data of all fractions together, they were able to detect 430 different FA in a butter sample. They include FA varying in carbon chain length from 4 to 26 carbons (both even and odd, in a straight or branched chain), degree of unsaturation, presenting many geometrical isomers, with double bonds in *cis* and *trans* configuration, etc. However, to the best of our knowledge, there is no a single method that allows to separate and analyze all of them. In addition, the majority of FA are present in milk fat in very small quantities (less than 0.01% of the total). Only 14 FA are above 1% [6], which makes an overall analysis more difficult.

The physicochemical properties and sensory and nutritional quality of milk and dairy fat are largely determined by its FA composition [7]. Nutritional guidelines generally encourage low consumption of saturated fats, high consumption of ω-3 polyunsaturated FA (PUFA), and avoidance of *trans* fats from partially hydrogenated fat (but not from ruminants), to promote cardiovascular health [8]. Milk fat includes almost all kinds of FA, so it is very difficult to establish its total effect. Michas et al. [9] concluded that evidence continues to accrue to support the notion that the total matrix of a food is more important than just its FA content when predicting the effect of a food on cardiovascular disease (CVD) risk.

As said before, most of the FA present in milk fat are esterified in TAG or PL. Non-esterified FA (also called FFA) are primarily formed in dairy products by the enzymatic breakdown of glycerides by lipase activities from various sources. The FFA content in milk is very low (Table 1), but can be important in some dairy products. FFA have low flavor thresholds, especially short chain FFA (SCFFA) and provide the characteristic flavor and odor of many dairy products, particularly, the flavor of fermented dairy products, and especially of cheese [10,11]. However, elevated levels of SCFFA, especially C4:0, are also responsible for rancidity in milk and other dairy products. Rancid flavor generally becomes unacceptable to the consumer [12]. Accordingly, the FFA content, together with the lipase activity control, can be considered a useful index of good quality and correct storage of food, especially for milk [13]. FFA can also contribute to texture and functionality of dairy products, as they impact on surface tension and foaming capacity of milk [14]. Furthermore, some FFA have been shown to have antimicrobial activity [12].

2. An Overview of FA Analysis

Because of the complexity of FA composition of dairy fat and its overall analysis, it is important to establish the objective of the analysis before starting. The objective may be to analyze the main FA present and degree of unsaturation; or to analyze the FA profile of lipid fractions (TAG, PL, cholesteryl esters, FFA ...); or the content of specific FA with special (favorable or unfavorable) properties (conjugated linoleic acids (CLA), *trans*-FA (*t*FA), branched chain FA (BCFA)). The method used may vary widely and can be quite simple for the first objective and almost inapproachable if the objective is to analyze accurately all FA present in a milk sample.

The most widely used methodology for separating and analyzing milk FA is gas chromatography (GC), generally coupled to a flame ionization detector (CG-FID). This methodology normally includes the following steps: (1) Lipid extraction; (2) Fractionation of lipid classes, if the objective is to analyze the FA content of one or several fractions separately; however, for the analysis of FA present in all lipid fractions (total fatty acids, TFA), this step is skipped and it is assumed that FA come largely from TAG and a small amount from PL and FFA; (3) Conversion of FA into FA methyl esters (FAME). For the analysis of FFA, in some cases, FA can be directly separated by chromatography without previous derivatization; (4) Separation and analysis by GC.

2.1. Lipid Extraction

The initial step in the analysis of any kind of lipid present in dairy fat consists in the separation of lipids from the rest of food components. It can be carried out by exhaustive extraction of all lipids present and subsequent separation of the lipid class of interest; or beginning by a selective extraction process [15]. When analyzing TFA in dairy products, the main strategy is the first one, and, since the 1960s, almost the only method used has been solvent extraction. Among solvent extraction procedures, the most widely used are the Folch method [16] and the method described by Bligh and Dyer [17], both based on a chloroform-methanol-water mixture. The mixture of solvents with a wide range of polarities allows for extracting almost all lipids present in the samples [18]. This method takes advantage of the one- to two-phase relationship of different proportions of chloroform, methanol and water. In the final step of the protocol, a purified lipid extract is obtained in an isolated chloroform layer [17]. Other solvent mixtures have also been used, such as those based in n-hexane or petroleum ether, but it should be noted that these solvents only extract neutral lipids, not PL or FFA [19,20].

As explained before, in some cases, an accurate determination of FFA in dairy products can be important for research and legislative, process development and quality control [12]. A wide range of analytical methods is available to determine the level of FFA in dairy products. Some of them allow the calculation of total acidity (total FFA), while others measure individual FFA. Most of these methods employ liquid–liquid extraction as a preliminary step using different organic solvent systems, and some have an extra chromatographic step for FFA isolation from the lipid extract prior to the analysis, as will be mentioned later. In the case of analyzing individual FFA, fat extraction techniques and quantification methods need to be able to take into account differences in solubility and volatility of the different carbon chain lengths of FFA present in milk fat. Any method for the accurate quantification of individual FFA must be efficient in extracting both water-soluble SCFFA and organic-soluble FFA, avoiding the use of evaporation steps to prevent losses of volatile SCFFA and removing any water that may be present in the sample [14]. The solvents most used in the mixtures are methanol, ethanol, n-butanol, 2-butanol, isopropanol, chloroform, diethyl ether, n-hexane, n-heptane and petroleum ether. An acid (mainly H_2SO_4 or HCl) is added most often, in order to shift the acid-base equilibrium towards the protonated form of the FFA, which is more organic-soluble.

Although solvent extraction procedures are by far the most frequently used, they need large volumes of solvent and are time consuming. Liquid–solid extraction is a good alternative when saving time is important. It is based on the adsorption of the lipid onto a solid adsorbent and its subsequent desorption using solvents. In addition, it can be used for lipid fractionation by eluting fractions using solvents of variable polarities (as explained later). As one example of these methods, Maxwell et al. [21]

described a lipid isolation procedure for milk by solvent elution from a column containing Celite 545. Total lipids were isolated by elution with a 90:10 mixture of dichloromethane: methanol.

In some cases, the analysis of TFA is carried out by direct derivatization without previous lipid extraction. These methods seem to work well for milk [22–24] whose lipid concentration is low. To the best of our knowledge, they have not been applied to other kind of dairy products, probably due to the difficulty of getting a good recovery of all lipids when they are in high concentration.

In-solution derivatization of FFA in milk has also been successfully performed. Amer et al. [25] described a simple and rapid method to derivatize FFA from milk into their ethyl esters with ethyl chloroformate without any work-up steps involving evaporation of solvent or FFA extraction before derivatization.

In some cases, only volatile SCFFA need to be determined but not the whole range of FFA. Volatile compounds in foods are generally analyzed by GC-MS with a prior step of extraction and/or preconcentration. The extraction technique formerly used was steam distillation starting from an aqueous acidified suspension of the sample. The main drawback was that, to obtain a quantitative yield of SCFFA, it was necessary to collect large amounts of distillate which had to be concentrated, and FFA extracted in an adequate solvent for GC analysis. Innocente et al. [26] proved that the aqueous acidified suspension could be used, without steam distillation, achieving valid results.

Among preconcentration methods, headspace-solid phase microextraction (HS-SPME) has proven to be quite successful in the determination of compounds responsible of cheese flavor [27–29], but it has also given good results in the case of milk [30,31]. In HS-SPME, analytes establish equilibrium among the sample matrix, the headspace above the sample and a stationary phase coated on a fused silica fiber and then are thermally desorbed from the fiber to a capillary GC column. Because no solvent is injected and the analytes are rapidly desorbed onto the column, minimum detection limits are improved and resolution is maintained [30]. Nevertheless, SPME is a non-exhaustive extraction technique. As an improvement over HS-SPME, multiple HS-SPME (MHS-SPME) has been successfully applied in the quantitation of volatile analytes from solid samples, including cheese [32]. This stepwise method implies the repeated use of HS-SPME in the same sample.

2.2. Fractionation of Lipid Classes

Total lipid extract from a milk or dairy product sample contains diverse lipid classes, as said before (Table 1). If the purpose of our analysis is to study the FA composition of different lipid classes, a lipid fractionation procedure has to be undertaken. A total lipid extract can be fractionated in its classes using solvents, based on differences of solubility in solvents of different polarities; or (more easily) by a suite of chromatographic techniques based mainly in the same principles. Thin layer chromatography (TLC) has long been used for this purpose and is still very popular. It allows performing the simultaneous analysis of various samples very rapidly and the separated lipid classes can be visualized and recovered very easily for further analysis [33]. Silica gel is the absorbent most frequently used for TLC lipid fractionation. The lipid mixture is applied on the origin and it is resolved into its components as the solvent stream passes through the absorbent by capillarity. The mobility of each lipid class will depend on its solubility in the solvent used. For instance, mobile phase containing n-hexane, diethyl ether and acetic (or formic) acid in the ratio 80:20:2 allows for separating the most common lipid classes, leaving PL at the origin [34].

Liquid–solid extraction (also called solid-phase extraction (SPE)) has also been used for lipid fractionation. In the method of Maxwell et al. [21] described above for total lipid extraction, alternatively, lipids were separated into a neutral lipid fraction by sequential elution of the SPE column with dichloromethane and a polar fraction by elution with a 90:10 mixture of dichloromethane: methanol. Nowadays, commercially available silicas with chemically bonded different functional groups make possible sophisticated lipid fractionation procedures [35,36]. In fact, this strategy is widely used for FFA isolation. Aminopropyl SPE columns are the best choice in this case. A mixture of chloroform and 2-propanol (2:1, v/v) is employed to elute the neutral lipids followed by a 2%

formic acid in diethyl ether solution to elute the FFA from the solid phase column [37]. Slight modifications are needed in samples with high levels of lactic acid [38]. By these methods, all FFA are isolated with a reportedly high degree of purity, and no further treatment is required before GC analysis. Alternative solid supports (e.g., silicic acid-KOH, anion exchange resins, deactivated alumina) and equivalent solvent mixtures are also used [39–43]. Nevertheless, some limitations have been described in all these cases. Underivatized FFA strongly interact with column phases, which can lead to irreversible adsorption. In addition, formic acid is very acidic and has an adverse effect on the chromatographic column lifespan. Such drawbacks may also affect retention times, limits of detection, limits of quantification and linearity values in the subsequent chromatographic analysis [11].

High Performance Liquid Chromatography (HPLC) represents an advance of the solid–liquid column chromatography. Although it can be directly employed for the separation of lipid classes, the technical improvements and the high degree of resolution achieved by this technique makes it more appropriate for more complex analyses of different lipid classes, and not as a simple fractionation method. For example, coupled to a light scattering detector, it has been widely used to analyze complex mixtures of TAG of milk fat, providing good resolution for more than 170 peaks corresponding to TAG with different partition numbers [1,44,45]. In the same way, Rodriguez-Alcalá and Fontecha [3] described an HPLC-Evaporative Light Scattering Detector method for the separation of major lipid classes of buttermilk.

An important advance in lipid fractionation came from the introduction of silver ion (Ag^+) chromatography along with the already existing chromatographic techniques (i.e., TLC, SPE or HPLC). The principle of the method is that silver ions interact reversibly with the π electrons of double bonds in a molecule, the stronger the complex formed the longer it is retained [46] so that lipids can be fractionated depending on the number and configuration of double bonds. Nevertheless, the main value of this methodology is to separate FA depending on the number, position and geometry of their double bonds. Momchilova and Nikolova-Damyanova [47] published a good review about silver ion chromatography.

3. FA Derivatization

The analysis of the FA composition is commonly conducted by GC. For this purpose, TFA have to be converted into less polar more volatile derivatives. The most widely employed procedure consists in converting FA into their methyl esters (FAME).

Due to the great variety of lipid classes and FA present in milk and dairy fat, it can be difficult to find a method to derivatize all of them in a quantitative manner. Quantitative recovery of short chain FA (SCFA) methyl esters, for example, can be difficult because of their high volatility. For this reason, longer chain alcohols have been used as alternatives to produce, for instance, butyl [48] and propyl [49] esters' derivatives. Nevertheless, working with FAME with proper care should be enough to avoid loss of SCFA and medium chain FA [1].

There is an overwhelming number of protocols in the scientific literature to produce FAME from milk fat. Methylation is carried out by both acid and basic catalysis. Acid-catalyzed methylation is considered quantitative as it converts FA from all lipid classes present in a sample into their correspondent FAME, including those from FFA. Boron trifluoride (BF_3) in methanol is the most commonly used catalyst, although it has been long demonstrated that acid methylation causes isomerization of conjugated dienes and produces allylic methoxy artefacts that may interfere with chromatographic analysis [6,20]. Some authors recommended the reaction with BF_3/methanol or HCl/methanol to be carried out in milder conditions for longer time, but Kramer et al. [50] showed that the methylation was not complete under these conditions and that even in mild conditions isomerization and artefacts are produced.

Based-catalyzed methods, using sodium methoxide ($NaOCH_3$) or potassium hydroxide (KOH) in methanol, have also been widely used for determining the FA profile of dairy fat. The main drawback of base-catalyzed methods is that only acyl moieties are converted to FAME. FFA, N-acyl lipids and alk-1-enyl ethers are not methylated [20]. As the proportion of these lipids classes is low in milk fat,

this fact does not cause important bias in the results. However, the amount of FFA is significant (up to 7–8% (*w*/*w*) of total fat) in very lipolytic cheeses such as Roquefort [51] or Idiazabal cheese [52] and should be taken into account when describing TFA.

Another base-catalyzed procedure suitable for milk fat derivatization was proposed in the ISO 5509 method [20] and involves the use of trimethylsulfonium hydroxide. Nevertheless, it is not recommended when cyanopropyl siloxane columns are going to be used in the subsequent GC analysis, which are, on the other hand, the most frequently used columns. Kramer et al. [50] conducted a study to compare several acid- or base-catalysts for the preparation of FAME from milk and rumen fat. They concluded that the best result for milk TFA was obtained with $NaOCH_3$ followed by HCl or BF_3, or diazomethane followed by $NaOCH_3$. Indeed, such multiple-step methods that combine both acid- and alkaline-catalyzed methylation have been widely adopted in milk TFA analysis [7].

In the case of FFA, for derivatization reactions, tetramethylammonium hydroxide (TMAH) is a commonly used derivatizing agent to convert FFA into FAME before GC-FID analysis because of its ability to simultaneously create methyl esters of glycerides and form salts of FFA (which are then converted to methyl esters in a heated injector) in separate phases. This makes it possible to analyze both components of the lipid extract without the need for prior separation [53]. However, the use of TMAH as an esterification reagent for FFA also has limitations [54,55]; the glyceride component of extracted lipids was shown to interfere with FFA determination. This led Martinez-Castro et al. [54] to modify the extraction and to include a solvent washing step of the separate layers, so as to remove interfering compounds before analysis. This issue of glyceride interference was further highlighted by Chavarri et al. [56], who reported a significant disagreement between the results obtained between FFA isolation using aminopropyl SPE columns and direct injection, and the derivatization method where FFA are converted into methyl esters using TMAH. They recommended isolating the FFA from the lipid extract before treatment with TMAH when analyzing samples with a large TAG to-FFA ratio, which is the case for most dairy samples [11]. Besides using TMAH, there are other options like isotope-labeling derivatization employing 2,4 dimethoxy-6-piperazin-1-yl pyrimidine, for example [57].

On the other hand, if the subsequent determination of FFA in milk is going to be carried out by HPLC with fluorescence detection, they must be derivatized with labeling reagents, as FFA do not contain responsive groups such as fluorophores for direct monitoring by fluorimetric detection. Among others, diazomethane-type reagents such as 9-anthryldiazomethane, and sulfonate ester reagents such as 2-(2-naphthoxy)ethyl-2-(piperidino)-ethanesulfonate, are used [58,59].

4. Separation and Analysis of FA

4.1. Separation and Analysis of FAME from TFA

GC-FID is, by far, the most widely used method to separate, analyze and quantify FAME from dairy fat. Column selectivity and separation efficiency have improved greatly in recent decades with the advent of wall-coated open tubular (WCOT) capillary columns. The International Dairy Federation (IDF) recommends this kind of column [60] for determining FA composition of milk and dairy fats.

The liquid phases of columns used for analysis of FAME are mainly polyesters with a wide range of polarities: those of low to medium polarity such as Carbowax™ (with variable proportions of polyethylene glycol) and those of high polarity, such as CP-Sil 88™, BPX70™, SP-2340™ or SP-2560™ (100% of cyanopropyl siloxane) [61]. All these phases allow separating esters having the same chain length with zero to six double bonds [6]. For example, with phases of low to medium polarity all unsaturated C18 FAME emerge from the column before any of the C20 component. In addition, the main advantage of the high polarity phases is their high capability to resolve unsaturated FAME, especially to separate *cis* and *trans* isomers.

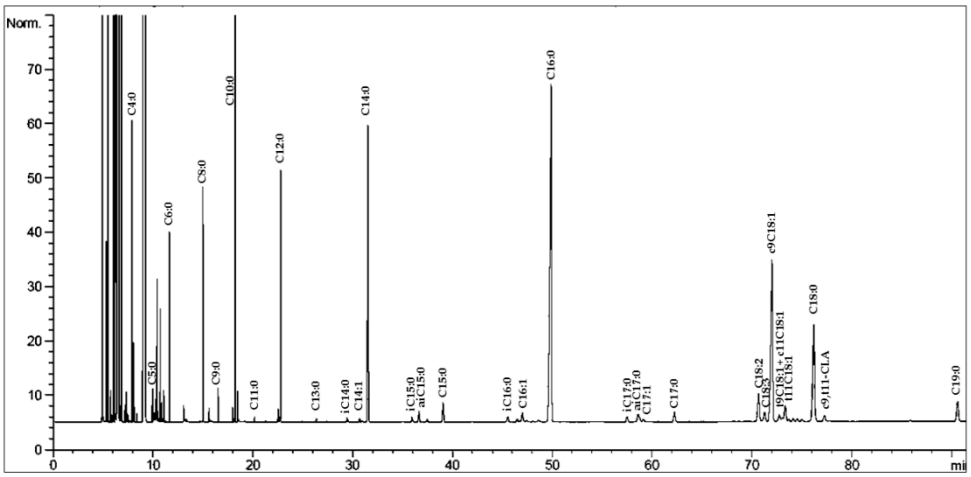
A bibliographic search in the Web of Knowledge, entering the terms “milk or dairy or cheese” in the title and “total fatty acids analysis” in the subject, and restricting the search to the last five years (in order to get an easy to handle list) and in the area of “food science and technology”, rendered a total

of 640 references. Among these, around 100 scientific articles described the protocol of FA analysis by GC and included the characteristics of the used column. One hundred meters length columns were, by far, the most frequently used, and among them CP Sil 88 was the most popular type (it was utilized in 40 works), followed by SP-2659 (Supelco Inc., Bellefonte, PA) of very similar characteristics (in 18 papers).

The type of column and its length should be chosen according to the objective of the analysis. Columns from 30 to 50 m give a good resolution, in an appreciably shorter time than longer columns, in cases where only the data about the general profile (main FA and their proportions) of FAME is required. Some representative examples of the use of this kind of columns for the analysis of FA in milk and dairy products are summarized in Table 2 [62–68]. In general, FA detected in these works included main saturated FA (SFA) from C8:0 to C20:0, monounsaturated FA (MUFA) from C14:1 to C20:1 and PUFA of 18, 20 and 22 carbon atoms. These columns do not allow resolving *cis* and *trans* isomers of any unsaturated FA. Nevertheless, some authors resolved some isomers optimizing the temperature program. For example, Ezequiel et al. [64] separated around 30 FA from cow's milk samples in 57 minutes. In addition to main SFA, MUFA and PUFA from C4 to C22, they were able to analyze *cis*9,*trans*11-C18:2, conjugated linoleic acid (*c*9,*t*11-CLA). Perna et al. [65] resolved 43 FA, from cow's milk, in 60 minutes. Their analysis included SFA of up to 24 carbon atoms (*iso* and *anteiso*) BCFA of C14:0, C15:0 and C17:0, and separated *c*7 and *c*9-C16:1 and *c*9 and *t*9-C18:1. They also analyze CLA, but they did not separate different isomers. Trigueros et al. [66] were able to resolve *c*9,*t*11- and *t*10,*c*12-CLA isomers in fermented milks using a 30 m column, in 40 minutes. Comparable results were obtained using similar columns but of 40 m or 50 m (e.g., [67,68]).

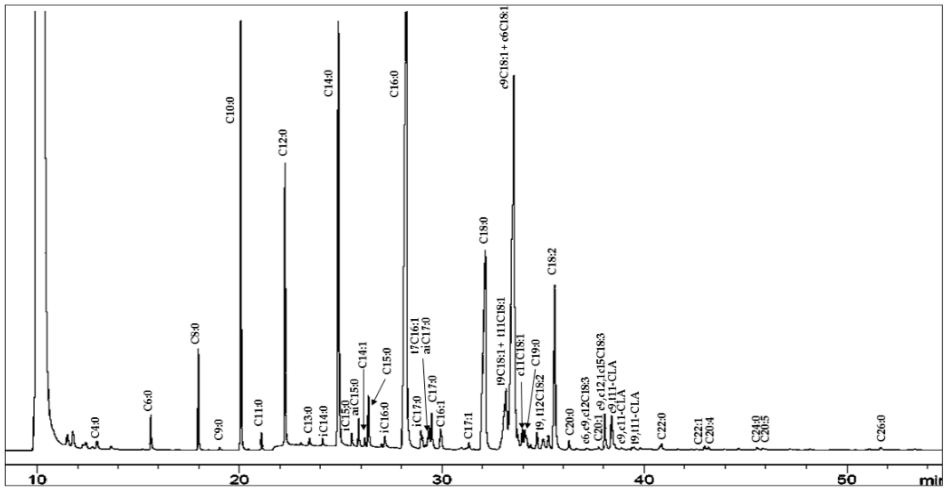
Longer columns allow separating more isomers of unsaturated FA, especially those of 16 and 18 carbons, with longer analysis times (some examples are cited in Table 2 [50,69–72]). Because of that, it is important to find a compromise between the interest and usefulness of the information obtained and the time necessary to obtain and interpret that information. Two examples of separations obtained in CP Sil columns of 60 and 100 meters are shown in Figure 1.

In any case, attempts to improve the resolution of as many as possible FA in the GC analysis have been constant during the last decades. In the case of the analysis of milk fat, efforts have been focused on identifying the *cis* and *trans* isomers of C18:1 and CLA isomers, due to their effects on human health. In particular, a special effort was made to separate *t*11-C18:1 from *t*10-C18:1 and *t*9-C18:1. Of these, only *t*11-C18:1 has been associated with human health benefits [73]. Aldai et al. [74] published an accurate review on different aspects of nutritional and health effects of *t*FA isomers and on analytical advances and challenges in resolving all of them.



(**a**)

Figure 1. *Cont.*



(**b**)

Figure 1. Gas Chromatography-Flame Ionization Detector chromatogram of fatty acids methyl esters of total fatty acids extracted from two different samples of ewes milk. (**a**) separation obtained on a CP7861 column (60 m × 0.25 mm, 0.25 µm, Agilent J&W, Santa Clara, CA, USA). Initial column temperature was held at 40 °C for 2 min, increased to 175 °C at a rate of 10 °C/min and held for 27 min, then increased to 215 °C at 0.5 °C/min. The carrier gas was helium at a flow rate of 1 mL/min, the split ratio was 1:30 and 1 µL of sample was injected. The injector and detector temperatures were 325 and 250 °C, respectively. No peaks were detected after 90 min. (**b**) separation obtained on a CP7489 column (100 m × 0.25 mm, 0.2 µm, Varian Inc., Mississauga, ON, USA). Initial column temperature was held at 60 °C for 5 min, increased to 165 °C at a rate of 14 °C/min and held for 1 min, then increased to 225 °C at 2 °C/min and held steady for 20 min. The carrier gas was helium at a flow rate of 1.2 mL/min, the split ratio was 1:5 and 1 µL of sample was injected. The injector and detector temperatures were 325 and 300 °C, respectively.

Other minor FA of interest in dairy fat are (odd and even) BCFA that contain a methyl group in (*n-1*)-position (*iso*) or (*n-2*)-position (*anteiso*). They are characteristics of ruminant fat and some bacterial species. In milk fat, they constitute about 2% of TFA but are important bioactive components due to their role in the gut and their potential activity against human breast cancer [75].

Kramer et al. [50] analyzed FAME from cow's milk using a 100 m SP-2560 column and separated 180 peaks in 90 min, using a temperature program from 70 to 215 °C. They identified SFA from 4 to 26 carbon atoms, BCFA from C13:0 to C18:0 and many PUFA of 20 and 22 carbons. However, they could not resolve important FA present in milk fat, as several *cis* and *trans* isomers of C18:1 and conjugated and not conjugated C18:2 isomers. Firl et al. [69] and Ariko et al. [70] identified more than 50 FA in bovine milk, including *t*6, *t*9, *t*10 and *t*11, and *c*9, *c*11, *c*12 and *c*13 isomers of C18:1 and three different CLA isomers (*c*9,*t*10; *c*10,*t*11 and *t*9, *t*11) using a CP7420 column, with a temperature program from 60 to 250 °C, in 90 min.

In order to get better resolution, some authors combined results from two or more separations, using different temperature programs in the same column [71] or in different columns [72,76]. Kramer et al. [71] conducted two separations, in a 100 m CP Sil 88 column, using temperature programs that plateau at 175 and 150 °C. They resolved most of the geometric and positional isomers of C16:1, C18:1, C20:1, C18:2 and C18:3 in about 200 min. Only few minor CLA isomers could not be resolved. Precht et al. [72] used two different columns (Table 2) and resolved almost all *cis/trans* C18:1 isomers present in milk fat.

Table 2. Some representative examples of the use of GC [1] columns for the analysis of FA [2] in milk and dairy products.

Column	Length (m)	Time (min)	Analyzed Sample	FA Resolved	Observations	Ref.
Supelcowax 10 (Supelco Inc.)	30	20	Human milk	23	*cis* and *trans* isomers of unsaturated FA not resolved.	[62]
Innowax (Agilent Technologies)	30	47	Cow milk	28	*cis* and *trans* isomers of unsaturated FA not resolved. Detected CLA (without specifying the isomer).	[63]
Omegawax 250 (Supelco Inc.)	30	57	Cow milk	30	*cis* and *trans* isomers of unsaturated FA not resolved. Detected *c*9,*t*11-CLA.	[64]
Omegawax (Supelco Inc.)	30	60	Cow milk	43	Included BCFA of C14:0, C15:0 and C17:0. Separated *c*7 and *c*9-C16:1 and *c*9 and *t*9-C18:1. Detected CLA (without specifying the isomer).	[65]
DB-23 (Agilent J&W)	30	40	Fermented milk from cow	21	Analyzed only main FA, but resolved *c*9,*t*11 and *t*10,*c*12-CLA isomers.	[66]
RTX-2330 (Restek Corp.)	40	60	Cow milk	37	Resolved *c*9 and *t*11-C18:1 and *c*9,*t*11 and *t*10,*c*12-CLA isomers.	[67]
CP-Sil 88 (Agilent Technologies)	50	82	Ewes milk	40	Included BCFA, resolved *c*9 and *t*11-C18:1 (and "others") and *c*9,*t*11- and *t*10,*c*12-CLA isomers.	[68]
SP-2560 (Supelco Inc.)	100	80	Cow milk	180	Among 180 resolved peaks, identified around 70 FA, but failed in resolving some *cis* and *trans* C18:1 and conjugated and not conjugated C18:2 isomers.	[50]
CP7420 (Agilent Technologies)	100	90	Cow milk	50	Column tuned for optimal *cis*/*trans* separations of FAME, especially the C18 isomers. Resolved *t*6, *t*9, *t*10 and *t*11, and *c*9, *c*11, *c*12 and *c*13-C18:1 and *c*9,*t*10; *c*10,*t*11 and *t*9, *t*11-CLA.	[69, 70]
CP-Sil 88 (Varian Inc.)	100	86 + 110.33	Cow milk	105	Two separations conducted in the same column. Only few minor CLA isomers could not be resolved.	[71]
CP-Wax 58 CB + CP-Sil 88 (Chrompack)	25 + 100	58 + 210	Cow, goat and ewes milk		Resolved almost all cis/trans C18:1 isomers present in milk fat.	[72]

[1] Gas Chromatography. [2] Fatty Acids.

With the purpose of getting a good resolution of C18:1 *trans* isomers in milk fat, some authors combine GC with some kind of Ag^+- chromatography, primarily for the fractionation of *cis* and *trans* isomers prior to GC analysis, to ensure correct separation, identification and quantification of all isomers [47]. Precht and Molketin [77] and Rodriguez-Alcalá et al. [78] fractionated FAME from cow, goat and ewes milk according to the number and geometry of double bonds by Ag^+-TLC. Then, they separated the fraction of *trans* C18:1 isomers in a CP Sil 88 100 m column. They only failed to resolve *t*6-*t*8 isomers.

Other authors use Ag^+-HPLC to complement the GC analysis. For example, Villegas et al. [79] applied a Ag^+-HPLC coupled with a photoionization mass spectrometer (Ag^+-HPLC/APPI-MS) to determine positional and geometrical isomers of C18:1 FAME from milk fat. They used a ChromSpher 5 Lipids column (250 mm × 2 mm i.d., 5 μm, Varian Inc.) and resolved nine C18:1 FAME isomers in less than 30 min. Nevertheless, the use of Ag^+-HPLC (in most cases coupled with a UV detector) for FA analysis has been used mainly for the analysis of CLA isomers. For example, Rodriguez-Alcalá et al. [80] used the same column to analyze CLA isomer composition of six commercially available CLA-fortified dairy products. They resolved eight different isomers (*t*11, *t*13; *t*10, *t*12; *c*11, *t*13; *t*10, *c*12; *c*9, *t*11; *t*8, *c*10; *c*9, *c*11; *c*10, *c*12-CLA). Sehat et al. [81] resolved and identified 12 CLA isomers in cow's milk and cheese with the same column but operating with three of them in series. In all mentioned cases, the mobile phase was 0.1% acetonitrile in n-hexane, operated isocratically at a flow rate of 1.0 mL/min.

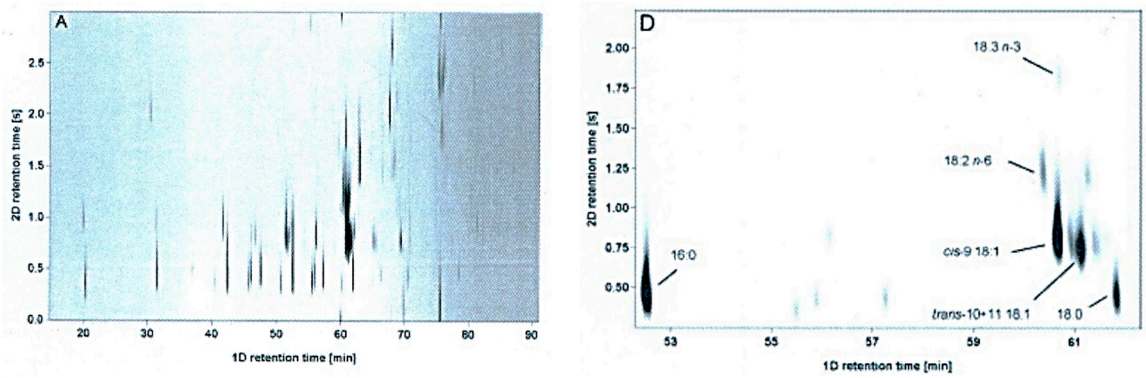
In any case, Delmonte et al. [82] established that there are some overlapping FAME that cannot be resolved under any condition using cyanopropyl siloxane phase columns (including some minor *cis* and *trans* C18:1 and CLA isomers). In recent years, several GC capillary columns containing polar ionic liquid stationary phases have been introduced on the market. Of those, SLB-IL100 and SLB-IL111 (Supelco Inc., Bellefonte, PA, USA) columns have been successfully used for the separation of selected FAME of C18:1, C18:2 and C18:3 geometric and positional isomers [83,84]. Ionic liquids are organic salts with a melting point below room temperature. They are composed of a cationic group, such as imidazolium (as in SLB-IL100) and an anionic unit (e.g., bis(trifluoromethylsulfonil)imide). These columns allow an enhanced separation of FAME of *cis* and *trans* isomers of MUFA and CLA compared to cyanopropyl siloxane columns due to their selective interaction with double bonds. The retention time for FAME increased by increase in the number of double bonds. For compounds with the same number of carbons and double bonds, the retention time increased when the double bonds were nearer to the $–CH_3$ end group and for the *cis* isomer with respect to *trans*. By applying a temperature program from 50 to 240 °C, at a rate of 1.5 °C min^{-1}, it was estimated that 464 peaks could be potentially positioned in the chromatographic space [84]. This means that all 400 FA predicted to be present in dairy fat could be resolved in only one separation Again, in all cases described in the literature, the conditions used failed to resolve some minor isomers in milk o dairy fat samples. For example, Delmonte et al. [73] coupled two 100 m SLB-IL111 columns (100 m × 0.5 mm × 0.25 μm) to a total length of 200 m for the analysis of milk fat FAME. The temperature program was from 170 °C to 185 °C and the separation time was 87.5 min. Although they were able to separate almost all FA present in milk, still some minor *cis* and *trans* C18:1 and CLA isomers were not well resolved. Gomez-Cortes et al. [85] and Bravo-Lamas et al. [76] combined the results obtained with a CP Sil 88 (100 m × 0.25 mm i.d., Supelco) and a SLB-IL111 (100 m × 0.25 mm, Supelco) in order to get a good resolution of FAME in caprine and ovine milk samples, respectively. Gomez-Cortés et al. [75] optimized the GC analysis conditions, using only the SLB-IL100 column, in order to get a good resolution of odd FA and BCFA. They assayed different programs, with different initial temperatures and concluded that, for the analysis of odd BCFA, the best initial temperature is 150 °C. Nevertheless, they also concluded that to resolve all FA it is necessary to carry out complementary temperature programs.

About two decades ago, comprehensive two-dimensional GC (GC × GC) proved to be a powerful separation method of complex samples [86]. In most GC × GC protocols, two independent types of separation are applied to a given sample. The first dimension separation typically is on a 10–30 m, non-polar column in the programmed-temperature mode. The interface between the two columns is called modulator. The modulator functions as a collection zone and as a re-injection device. Isolates from the first-column eluate into the modulator and, then, are launched in narrow pulses of about 0.01 s width into the second-dimension column. The second-dimension separation is generally on a narrow-bore (semi-)polar short (0.5–1 m) column. The separation on this column is extremely fast and results in very narrow peaks with baseline widths of, typically, 0.1–0.6 s. In addition, GC × GC

chromatograms are recorded in two dimensions (2D) plots, arising from boiling point and polarity relationships, which facilitate the compounds identification [87].

Even though GC × GC is not a novel technology, it is still perceived as such, mainly due to its limited use. Tranchida et al. [88] pointed out that there are several reasons for such a situation. Among these, they mentioned: (1) high initial instrumental plus software cost; (2) greatly increased complexity related to method optimization and to the use of GC × GC software; (3) high operational costs; and (4) the revolutionary nature of the overall technique.

Perhaps because of these difficulties, this technique has been used in a few works (to the best of our knowledge) for the analysis of milk fat FA. Vlaemick et al. [86] compared two different column sets, one nonpolar/polar and other polar/nonpolar. For the first set, they used a BPX5 column (30 m × 0.25 mm × 0.25 µm; 5% phenyl polysilphenylene–siloxane phase) connected to a BP20 column (0.85 m × 0.10 mm × 0.20 µm; polyethylene glycol phase). For the second set, a BPX80 column (30 m × 0.25 mm × 0.25 µm; 80% cyanopropyl-substituted polysilphenylene–siloxane phase) coupled to a BPX35 column (0.25 m × 0.10 mm × 0.10 µm; 35% phenyl polysilphenylene–siloxane phase). The temperature program used on both column sets was 90 to 250 °C at 2 °C/min. They obtained a better resolution of peaks with the polar/nonpolar set. However, although they improved the separation of FA compared with 1D GC methods, they suggested that further research is needed to achieve increased separation of a number of specific peak pairs such as *trans* and *cis*-C18:1 isomers, which may require a longer and/or more selective 1D columns. Indeed, they concluded that using the GC × GC technique resulted in an improved overall separation of FAME, and the well-ordered structure of the compounds in the GC × GC plot facilitated the identification and classification of known and unknown compounds. An example of 2D plots of a GC × GC chromatogram of FAME from milk fat is shown in Figure 2.



(**a**) (**b**)

Figure 2. Comprehensive two-dimensional GC (GC × GC) chromatogram of the FAME from milk fat (**a**) and close-up of the 16–18-region (**b**) separated on a nonpolar/polar column set (BPX56BP20) (Reproduced by permission from John Wiley and Sons [86]).

Bergamaschi et al. [89] and Schiavon et al. [90] used a GC × GC instrument with a FID to analyze cows' milk and cheese FA. The first column was polar (75 m × 180 µm × 0.14 µm; 23348U, Supelco). The second a nonpolar column (3.8 m × 250 µm × 0.14 µm; J&W 19091-L431, Agilent Technologies). The temperature program was from 50 °C to 240 °C, in a separation time of around 125 min. They did not explain the details of the separation, and gave data of around 65 FA. Nevertheless, in some cases, they utilized the term "sum of others" referring to some C14:1, C16:1 and C18:1 isomers that were recognized by their position in the 2D plot (not by reference standards), but it is not clear if they were well resolved in the chromatogram or not.

More complicated systems, incorporating more columns in multidimensional sets, have been also described with the objective of improving MUFA and PUFA isomers resolution in milk fat [87]. All these efforts resulted in greater number of resolved peaks. However, the counterparts are a more

sophisticated instrument, longer analysis times and more complex results, which may be difficult to interpret.

Thus, as we stated before, the analyst must value, on the one hand, the material and time investment that must be done. On the other hand, it is important to weigh the usefulness and the practical application of the results that will be obtained. It is necessary to achieve a compromise between all these factors and choose an adequate methodology according to the objectives that are pursued.

4.2. Separation and Analysis of FFA

Even though other chromatographic methods exist to separate and quantify FFA, the most popular method of analysis involves GC-FID because of its precision and reliability and relative low cost. Nowadays, the advent of WCOT capillary columns has made the original packed GC columns redundant. Over the years, many different chromatographic methods have been used, but these are relatively few in comparison to the methods employed to isolate FFA from dairy products [91]. As previously mentioned, FFA can be analyzed after derivatization or directly after extraction from the product [11,37,56]. This latter approach works without the need for derivatization because FFA are volatile and thus can be vaporized in a heated injection port. There are commercially available columns with specific FFA phases (FFAP) that achieve complete separation of FFA of chain lengths from C2:0 to C22:0. The stationary phase of the capillary column is typically any nitroterephthalic acid-modified, chemically bonded polyethylene glycol. A Split/Splitless injection can be used, but a cold on-column injection can also be employed followed by a programmed temperature ramp of the injector, as this allows for the increased separation of FFA based on their volatility within the injector. Notwithstanding, due to their nature as acids, FFA strongly interact with column phases, which can lead to irreversible adsorption, peak tailing, ghost peaks and double peak formation. The use of formic acid either in the carrier gas or as a solvent reduces the occurrence of these issues and allows for quantitative determination by GC, but the acidic nature of the extract reduces column lifetime [12,14].

Analysis of the FFA with the TMAH FAME method can overcome these issues, but it is also not without problems. An advantage of TMAH is that when pyrolysed, it degrades to TMA and methanol, which are highly volatile and thus suitable for GC analysis; however, the most volatile SCFFA can elute with the solvent peak which impacts on sensitivity, and artefact formation can periodically interfere with the quantification of other SCFFA [12,14,53]. Nevertheless, such interference is substantially reduced by using a WCOT capillary column [40,91].

Not as often as GC, but HPLC has also been widely used for FFA analysis, reverse-phase (RP) HPLC in particular. The stationary phase is typically the octadecylsilyl (ODS) type, and the mobile phase acetonitrile or methanol in water. FFA are separated on the basis of both chain length and degree of unsaturation. In fact, one of the first HPLC methods to resolve major FFA in milk fat used RP-HPLC. In this method, Reed et al. [92] separated the *p*-bromophenacyl esters of FFA. Two chromatographic separations were required due to problems of co-elution of some medium- and long-chain FFA. However, Elliott et al. [93] resolved all FFA in one separation using this method with a gradient of acetonitrile in water. Further development of the method was undertaken using a water/methanol/acetonitrile gradient to achieve faster separation of all FFA. This method is widely used for separating FFA by HPLC, but there are more [40,91,94]. For example, a useful fluorogenic derivatization method for long-chain FFA in milk was described by Lu et al. [95]. The FFA were converted to fluorescent naphthoxyethyl derivatives and separated by isocratic HPLC.

Capillary electrophoresis (CE) has also been employed for FFA separation. CE, micellar electrokinetic chromatography (MEKC) in particular, can be an attractive alternative separation technique in the case of SCFFA. This technique combines electrophoresis and chromatography. Using it, Vallejo-Cordoba et al. [96] carried out the separation of SCFFA of milk fat by a system in which they were solubilized by forming micelles with cyclodextrin. Attempts to use capillary zone electrophoresis

(CZE) for separating the whole range of FFA have been made too [97]. However, the high separation efficiency of GC is not achievable by CE.

5. Identification and Quantification of FA

FID is, by far, the most used detector in the cited works. FID does not provide structural information about the compounds, so the identification of FA is based mainly on retention time or relative retention parameters as compared with pure reference substances. Nevertheless, due to the huge number of different FA present in milk, the variability in their concentration, the limited availability of commercial standards and occurrence of overlapping peaks, in the case of TFA the identification of some minor FA is difficult and tentative at best [6].

In some cases, it is possible to identify, tentatively, the FA based on the order of elution. Ag^+-chromatographic fractionation of FA before or complementary to GC-FID analysis also helps in resolving and identifying *cis* and *trans* isomers of unsaturated FA, as commented previously. However, in some cases, it is necessary to utilize mass spectrometry (MS) to recognize some FA without any doubt.

It is beyond the scope of this review to address the fundamentals of FID and mass spectrometers. Thus, we will refer only to some representative works where MS has been used in the analysis of FA of milk and dairy fat, in order to improve the identification of FA, in comparison with all articles where FID has been used and have been already cited.

The analysis of FA by MS requires the alteration of the component from its natural state to that of an ion (positive or negative) in the gas phase, and then an ionization technique such as electron impact (EI-MS) can be applied [18]. Established databases for electron impact mass spectra of FA are readily available (e.g., The National Institute of Standards and Technology (NIST) Mass Spectral Library [98]). Specific fragmentations of FA give rise to characteristic ions, which in some cases can be used for identification. Nevertheless, electron impact ionization of FAME does not yield useful fragments to assign double bond position and configuration. Because of that, in some cases FA are acylated to produce 4,4-dimethyloxazoline (DMOX) derivatives and picolinyl and pyrrolidide esters, which produce useful fragmentation patterns for double bond determination [20].

Kairenius et al. [99] combined GC-FID of FAME and GC-MS of DMOX derivatives of cow's milk fat to detect and identify 196 FA. Destaillats et al. [100] analyzed milk fat by a combination of chromatographic techniques and utilized CG-MS of picolinyl esters and DMOX derivatives for the structural characterization of FA intermediates of ruminal biohydrogenation of *c*9,*c*12,*c*15-C18:3. Plourde et al. [101] separated a fraction containing dienoic FA as well as conjugated α-linolenic acid isomers (CLnA) from bovine milk and confirmed their double bond positions by MS of their DMOX derivatives.

In some cases, GC-MS is applied to separate and analyze FAME. Thurnhofer and Vetter [102] developed a GC-EI-MS-selected ion monitoring (SIM) analysis for verification of results obtained by GC-FID analysis of FAME from goat's milk among other food samples. By this technique, they quantified precisely selected individual FA (including BCFA and some C18:1 and PUFA isomers). Similarly, Gómez-Cortes et al. [75] and Teng et al. [103] combined GC-FID and GC-MS to identify FA for which no standards are available. Schröder et al. [5] used different methods for fractionating FAME from butter and analyzed the resulting fractions by GC-MS in SIM mode. In this way, they identified 430 FA. In all mentioned works, they used selective quadrupole MS, except one [75] in which a triple axis quadrupole is used.

Regarding FFA, during the last years, several analytical approaches have been developed for quantitation using GC-MS too [25,28,29,57,104].

Quantitative estimation of FA is carried out by measuring the areas under the chromatographic peaks. When using FID as detector, this area is proportional to the amount (by weight) of material eluting from the column, within its linearity limits. Nevertheless, response factors for each FAME or FFA need to be calculated accurately using pure standard solutions of known concentrations, prepared from commercially available individual standards or standard mixtures. Once the response factor for

individual FA has been calculated, the results of the analysis of a sample are expressed, in most cases, directly as weight (or molar) percentage of individual FA in relation to the sum of all FA detected. However, for a more accurate estimation of the amount of each FA present in a complex sample, it is necessary to apply the internal standard method, in which the recovery of all FA is based on the recovery of the internal standards (typically FA that are not present in milk fat). In the analysis of TFA from dairy fat, the most often used internal standards are C9:0 ([105–107], e.i.) and C19:0 [108]. Sometimes, the internal standard is added as FFA [105–107], others as FAME [108]. Actually, when analyzing TFA, the best practice should be to add the internal standard in the form of pure TAG (e.g., trinonanoin [69]) in order to control losses that may occur throughout the process, from the extraction to the chromatographic analysis. In addition, due to the differences in volatility between FA, including various internal standards, both for FFA and FAME analysis, covering all the range of chain length will help in getting response factors close to 1 for all FA analyzed. Finally, it should also be noted that certified reference material (CRM) is recently available in the case of some individual TFA. This is SRM 1549a Whole Milk Powder, provided by NIST [109]. However, to the best of our knowledge, it has not yet been employed on the subject we are dealing with.

As regards FFA, most of the procedures using HPLC described in the literature are based on UV or fluorescence detectors [6]. If separation has been carried out by MEKC, UV detectors are also employed, but the lack of a suitable chromophore moiety in FA excludes direct UV, and therefore indirect identification has to be used (e.g., at 270 nm, employing *p*-anisate as a chromophore) [96].

6. Complementary Methods for Analysis and Quantification of FA

Among current emerging technologies for analysis of TFA in dairy food, the optical-based methods have been reported as the most promising techniques because they have a great potential for real-time and online application [110]. Three spectroscopic techniques, near-infrared spectroscopy (NIRS), Raman spectroscopy (RS) and nuclear magnetic resonance (NMR) were used, primarily, for the analysis of milk fat. Recently, they are showing a great potential to analyze FA as well. Tao et al. [110] wrote an excellent review, summarizing the technical aspects of these techniques and their applications in dairy fat and TFA analysis.

A common feature of NIRS and RS methods is that analysis involves the development of broad-based calibration equations or models. These models, after validation, are used to predict unknown samples from the same population that was used to create the calibration set [111]. This means that, for calibration, other methods have to be applied to the samples in order to know the concentration of the compounds to be predicted.

NIRS method is based on the fact that the chemical components of a sample have absorption properties in the NIR region (780–2526 nm) of the electromagnetic spectrum. Coppa et al. [112] used NIRS to predict FA composition of cow milk. They analyzed the FA composition of 468 milk samples by GC-FID and developed predictive equations for liquid and oven-dried milk samples. The results obtained showed that NIRS can be used to satisfactorily predict FA sums and ratios (i.e., SFA, MUFA, PUFA, total *trans*-C18:1 and total *cis*-C18:1, total CLA). Good results were also obtained for individual FA present in medium-to-high concentrations, but the quality of prediction decreased when FA were present in low to very low concentrations. Andueza et al. [111] and Núñez-Sánchez et al. [113] obtained similar results for goat's milk. Lucas et al. [114] used Visible–NIRS to predict the FA composition of 445 cows' and goats' fresh and freeze-dried cheeses. Samples were scanned (400–2500 nm) and predictive equations were developed. They obtained poor predictions for C8:0, C10:0, C12:0, C18:0, *c*9-C18:1, C18:2 and C18:3. The quantification was significantly more accurate for C8:0, C10:0 and C18:3 with freeze-dried cheeses compared with fresh cheeses. This may be because water has strong absorption bands in the near infrared region, which could limit the detection of the analytes.

RS is based on the phenomenon of Raman scattering that happens when a sample is irradiated by a monochromatic light from a laser. The bands in a Raman spectrum represent vibrational characteristics of chemical bonds and functional groups of the components in the sample and offer the

basis for structural and qualitative analysis. Compared to NIR spectra, Raman spectra have significant advantage with aqueous systems, because the Raman spectra of water are weak and unobtrusive [110]. As NIRS, RS has potential to predict grouped FA, such as total SFA, MUFA, PUFA, CLA and so on. Meurens et al. [115] and Bernuy et al. [116] demonstrated that Fourier Transform-RS (FT-RS) technique has a great potential in predicting total CLA in cow's milk. Stefanov et al. [117,118] showed the capability of RS for direct semi-routine quantification of the individual or grouped *trans*-MUFA, CLA, odd and BCFA in the milk fat.

Zhao et al. [119] investigated the use of NIR, FT-mid-infrared (FT-MIR) and Raman spectroscopies and multivariate data analysis to quantify total *t*FA (TT) and to detect naturally occurring (NT) and industrially induced (IT) *t*FA in butter, Cheddar cheese and dairy spreads. They considered NT all peaks identified by CG-FID as *trans*-C18:1 and *trans*-C18:2 isomers and *t*9-C16:1. Other *t*FA were summed as IT. They concluded that models using NIR and FT-MIR spectral data performed better than those based on Raman spectra due to their lower signal-to-noise ratio. All spectroscopic methods failed to predict IT content of butter and TT contents of dairy spreads and Cheddar cheese.

NMR results from specific magnetic properties of certain atomic nuclei. Commonly, NMR spectra are 1D spectra, which provide qualitative and quantitative information of the compounds present in a sample, as the areas under the resonance peak are proportional to the concentration. In some cases, two nuclei NMR analysis are combined to obtain 2D spectra, which provide a more accurate result. Among relevant nuclei employed in NMR analysis, ^{1}H and ^{13}C have been successfully used in FA analysis of milk and dairy products. Hu et al. [120] used 1D and 2D NMR spectra to quantify FA in milk. They calculated the concentration of C4:0, total MUFA and PUFA, and observed that were in good agreement with reference values. Andreotti et al. [121,122] examined the 13 most abundant FA by ^{13}C NMR to analyze and differentiate milks from different animal species. They found that from the ^{13}C NMR spectra of TAG, the positional distribution of FA chains on the glycerol backbone could be easily evaluated. In addition, this analysis allows distinguishing goats' milk from sheep's milk, and both of these milks from cows' and buffaloes' milks. ^{1}H and ^{13}C NMR analysis has also been applied for the analysis of FA of cheeses as Asiago [123] and Pecorino Sardo [124]. In all cases, the NMR assignments allow for quantifying FA that are in high concentration. More recently, Prema et al. [125] quantified total CLA in the lipid fraction of select Canadian cheeses by ^{1}H NMR spectroscopy. In all these works, NMR analysis was applied to the lipid fraction of the dairy product. Some authors analyze FA by NMR as part of a more general metabolomics analysis, without previous lipid extraction. For example, Gómez-Gallego et al. [126] identified up to 68 metabolites in human breast milk, including C4:0. O'Callaghan et al. [127] identified and quantified 49 metabolites in cow milk samples, including six SCFA. Finally, it should be mentioned that ^{1}H NMR spectroscopy is beginning to be used for quantifying SCFFA in milk [104].

In summary, spectroscopy based methods have the potential of analyzing FA composition of dairy foods, in a rapid and non-destructive way. Once the methods have been optimized, they offer the advantage of simple sample preparation, suitability for online use and simultaneous determination of different components in a large number of samples in short time. Nevertheless, the accuracy for some FA, especially those that are in low concentration, is not good yet and further improvement is still needed [110].

7. Conclusions

As milk fat is one of the most complex natural fats, due to its FA composition, its accurate analysis is also one of the most complex and challenging tasks for any food analyst. For decades, it has been a field for constant advance and improvement. Long GC capillary columns, with more and more complex and specific phases, new chromatographic arrangements in multiple dimensions, SPE, TLC and HPLC methods for improved fractionations, mass spectrometers to identify minor FA make it possible to detect and quantify the vast majority of the FA present in milk. However, there is still

scope either to refine existing methods or to develop new methods that could facilitate and shorten the analysis.

In any case, the current options are numerous and varied and the analyst must choose the adequate methodology according to the objectives pursued.

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