Determination of free fatty acids in cheese: comparison of two analytical methods

By FELISA CHAVARRI*, MAILO VIRTO*, CELIA MARTIN†, ANA I. NÁJERA†, ARANTZA SANTISTEBAN*, LUIS J. R. BARRÓN†‡ and MERTXE DE RENOBALES*‡

**Bioquímica y Biología Molecular † Tecnología de Alimentos, Facultad de Farmacia, Universidad del País Vasco/Euskal Herriko Unibertsitatea, Apartado 450, E-01080 Vitoria-Gasteiz, España*

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Summary. Two methods were compared for the determination of free fatty acids (FFA) from acetic to long-chain acids in samples with a large excess of triacylglycerols (TG) (1:200, w/w), such as cheese and other dairy products. In method 1, after fat extraction, FFA were separated from TG by aminopropyl-bonded phase chromatography, injecting the fraction containing FFA directly into the gas chromatograph. In method 2, extracted fat was treated with tetramethylammonium hydroxide, the methyl ester derivatives being formed in the injector. Cheese samples and standard mixtures of FFA and TG in different proportions were analysed by both methods. The cheese sample contained 2·4 times more FFA when analysed by method 2 as compared with the result obtained with method 1. The composition of the standard mixtures analysed by method 1 closely reflected that of the original mixture and gave 90–100% recovery of FFA, regardless of their chain length and the ratio of FFA:TG (1:1 or 1:200, w/w). The composition of samples with a FFA:TG ratio of 1:200 (w/v) was severely distorted (as compared with the original composition of the sample) when analysed by method 2. Varying recoveries of FFA were also obtained, the largest differences being found for the shorter-chain components. We conclude that the FFA fraction should be separated from the TG fraction before derivatization and chromatographic analysis, particularly for samples in which the FFA represent a minor fraction of the TG.

The quantitation of free fatty acids (FFA) in biological systems has been extensively studied, and a number of excellent books have been published (Christie, 1982, 1987, 1989; Hamilton & Hamilton, 1992). In the great majority of published reports the fatty acids under study were components of neutral and polar lipid classes, with chain lengths of 16 or more carbon atoms. FFA as such are present in very small amounts in most biological systems and are often not quantified as a separate lipid class. Yet the accurate determination of the amount of individual FFA is important in certain cases, such as in milk and dairy products and vegetable oils, because of their sensory effects. Depending on their relative amounts, short-chain

‡ For correspondence.

FFA with fewer than eight carbon atoms can impart both desirable and rancid flavour notes to the end products, while long-chain FFA are primarily responsible for a soapy taste (Badings & Neeter, 1980; Jensen, 1992).

The determination of FFA in milk, cheese and other dairy products is particularly complicated owing to three main factors. (1) FFA represent $< 0{\cdot}5\,\%$ of the total fat (Jensen *et al.* 1991), which is primarily composed of triacylglycerols (TG) comprising more than a hundred different molecular species (Barrón *et al.* 1990). (2) FFA with chain lengths from 2 to 20 carbon atoms are present. (3) Short-chain FFA with fewer than eight carbon atoms are extremely volatile.

Because of these problems the methods most frequently described for the analysis of FFA in milk and dairy products involve either the separation of FFA from the bulk of the extracted lipid by some form of ion-exchange chromatography (Needs *et al.* 1983; Kaluzny *et al.* 1985; De Jong & Badings, 1990), or the preparation in a single step and in separate phases of methyl esters of both FFA and acylglycerols using tetramethylammonium hydroxide (TMAH) as a catalyst (Metcalfe & Wang, 1981; Martínez-Castro *et al.* 1986; Martín-Hernández *et al.* 1988; Nájera *et al.* 1994). In the latter method, FFA and TG are not separated prior to derivatization and analysis to facilitate the analysis of large numbers of samples. The recovery in any of these methods is obtained by analysing a given cheese sample before and after the addition of a known amount of a known mixture of FFA. The results of the analysis of the cheese sample before the addition of the FFA mixture are regarded as 'correct'. No reference is usually made to the influence of the relative amounts of different lipid classes present in the sample on the final values obtained for the total amounts of FFA. While studying lipolysis during ewes' cheese maturation, we observed very large variations in the amounts of individual and total FFA when the same cheese sample was analysed by different methods, with the TMAH method consistently giving higher results for FFA.

In this study we compared two methods for the quantitation of FFA in milk, cheese and other dairy products. In the first method (De Jong & Badings, 1990), after lipid extraction, FFA are separated from TG by aminopropyl-bonded phase chromatography, and the eluent is directly injected into the gas chromatograph. No solvent removal steps are necessary. In the second method (Martín-Hernández *et al.* 1988), after lipid extraction and solvent removal, a mixture of FFA and TG is treated with TMAH. FFA remain in the lower methanolic phase, while fatty acid methyl esters (FAME) from acylglycerols should remain in the upper phase. When a portion of the lower phase is injected into the gas chromatograph, FAME are formed in the injector. We concluded that the composition of the lipid sample, particularly the relative amounts of TG and FFA, together with the presence of TG with short-chain fatty acids, affected the amount of 'free' fatty acids determined by the TMAH method. Therefore, the composition of the sample to be analysed must be taken into consideration when selecting a method to quantify FFA.

Materials and methods

Materials

Samples from ewes' milk cheese were from Queserías Araia (Araia, Alava, Spain), taken after 90 d ripening. TMAH (200 g/kg methanol) and all FFA and TG standards were purchased from Sigma (E-28100 Madrid, Spain). FFA and TG were of the highest purity available and were not further purified. BondElut columns

(500 mg) were from Varian (Harbor City, CA 90710, USA). Solvents (Merck, D-64293 Darmstadt 1, Germany) were of the highest grade available and were not redistilled before use.

Aminopropyl-bonded phase chromatography (*method 1*)

Extraction of cheese lipids, separation of FFA and TG and gas–liquid chromatographic analysis were performed essentially as described by De Jong & Badings (1990). Briefly, cheese (1·0 g) was ground with anhydrous Na_2SO_4 (3·0 g) after which 0·3 ml 2·5 M-H_2SO_4 and 1·0 ml internal standard solution were added. Internal standard solution contained pentanoic (5:0), nonanoic (9:0) and heptadecanoic (17:0) acids. For cheese samples and standard mixtures A1:200 and B1:200, 1·0 mg/l of each fatty acid in diethyl ether–heptane (1:1, v/v) was used, while 5·0 mg/ml of each acid was used for mixtures A1:1 and B1:1. Lipids were extracted three times with 3·0 ml diethyl ether–heptane 1:1, v/v). After each extraction, the solution was clarified by centrifugation. Organic phases were combined and applied to an aminopropyl-bonded phase column, previously equilibrated with 10·0 ml heptane. TG were eluted with 10·0 ml chloroform–propanol (2:1, v/v) and FFA were eluted with 5·0 ml diethyl ether containing 20 ml formic acid/l.

The separation of TG from FFA in the eluent was checked by thin layer chromatography (Henderson & Tocher, 1992) on Silicagel plates, using trihexadecanoin, octanoic and hexadecanoic acids as standards. Plates were developed with hexane–diethyl ether–formic acid (80:20:2 by vol.), and the spots were visualized under u.v. light after spraying with Rhodamine G6 (0·5 g/l ethanol).

The FFA fraction from the aminopropyl-bonded phase column was injected directly into the gas chromatograph (model 5890, series II, equipped with a flame ionization detector; Hewlett Packard, E-28230 Las Rozas (Madrid), Spain). The underivatized, individual FFA were separated on a fused silica capillary column (25 m × 0·32 mm) coated with free fatty acid phase (cross-linked polyethylene glycol, 0·52 μm layer thickness). The carrier gas (helium) flow rate was 2 ml/min, and the temperature was raised from 65 to 240 °C at 10 deg C/min, then held at 240 °C for 20 min. The split/splitless ratio was set to 1:10. Response factors were determined for each individual fatty acid with 5:0, 9:0 and 17:0 as standards.

Trimethylammonium hydroxide derivatization (*method 2*)

Extraction of cheese lipids, derivatization and gas–liquid chromatographic analysis were performed essentially as described previously (Martínez-Castro *et al.* 1986; Martín-Hernández *et al.* 1988). In summary, ground cheese (10 g) was mixed with 10 mg each of 5:0 and 9:0 fatty acids as internal standards, 5·0 ml deionized water and 0·5 ml 5·5 M-H_2SO_4. The mixture was extracted with 15·0 ml diethyl ether and clarified by centrifugation. To the organic layer, 1·0 g anhydrous Na_2SO_4 was added. The solvent was removed under reduced pressure and 0·1 g lipid was dissolved in 3·0 ml diethyl ether in a screw-capped vial. TMAH (0·2 ml) was added and the mixture was shaken for 3 min. After 15 min, the lower layer (containing the tetramethylammonium soaps of the FFA) was washed twice with 3·0 ml diethyl ether and neutralized to pH 9 with 2 M-HCl in methanol, using thymol blue as indicator. A portion of the lower layer was injected into the gas chromatograph. FAME of the FFA were formed in the injector (Metcalfe & Wang, 1981).

Separation of individual FAME was carried out using the same gas chromatograph and capillary column described above. The carrier gas (helium) flow rate was

1 ml/min, and the split/splitless ratio was set to 1:85. The temperature was held at 40 °C for 5 min, raised to 90 °C at 2 deg C/min, immediately raised again to 180 °C at 10 deg C/min, held at 180 °C for 15 min, raised to 220 °C at 10 deg C/min and finally held at 220 °C for 20 min.

Mixtures of free fatty acids and triacylglycerols of known composition

A mixtures. The FFA and the fatty acids of the TG were of different chain length. Two different mixtures were prepared, one in which the total FFA: total TG ratio was 1:1 (w/w, mixture A1:1) and one with the total FFA:total TG ratio 1:200 (w/w, mixture A1:200). The amounts of each component are given in Table legends.

B mixtures. The FFA and the fatty acids of the TG were of the same chain length. Two different mixtures were prepared in the same way as for the A mixtures, one designated B1:1 and the other B1:200.

All results are presented as means ± SD for four different experiments. All mixtures of FFA and TG were prepared fresh for each experiment.

RESULTS AND DISCUSSION

Free fatty acids of a cheese sample

As shown in Table 1 (columns 1 and 2), the amounts of individual and total FFA from the same cheese sample varied considerably depending on the method used for their quantitation. With the exception of oleic acid (18:1*n*–9) the amounts of all fatty acids determined by method 2 were significantly higher (up to one order of magnitude for 6:0) than those obtained by method 1. In addition, no 2:0 was detected with the former method, most likely owing to the presence of a large peak at the solvent front (results not shown). The total amount of FFA was 2·4 times that when method 2 was employed. Because the two extraction procedures and the total amounts of sample extracted were different, it was reasonable to think that the extraction carried out with method 2 was somehow more efficient, thus yielding higher amounts of FFA. To check this possibility, cheese samples were extracted as described for each method, using the follow-up procedure of the other method for the final analysis (Table 1, columns 3 and 4). Comparison of columns 1 with 3 and 2 with 4 indicates that whenever the TMAH derivatization method (method 2) was used (regardless of extraction procedure), significantly higher amounts of FFA were found. The much lower amounts of 2:0 obtained in this second set of experiments by using aminopropyl-bonded phase chromatography (column 3 *v.* column 1) can be explained by the solvent evaporation step that the TMAH extraction method uses.

In this particular cheese sample undecanoic acid (11:0) was not detected. Thus, we used triundecanoin as internal standard (1·0 mg/g cheese) to quantitate fatty acids from TG. We observed the appearance of 'free' 11:0 when method 2 was used, but not when the FFA fraction was previously separated from the TG by aminopropyl-bonded phase chromatography, as described in method 1. This observation indicated to us that with method 2 TG in the upper phase were partly hydrolysed under the alkaline conditions of the reaction, and appeared in the lower phase. The largest differences in the amounts of individual FFA obtained by the two methods were observed for the shorter-chain fatty acids (Table 1, columns 1–2 and 3–4). This is consistent with the higher solubility of FAME of shorter-chain length fatty acids in methanolic solvents. Thus, after the TMAH reaction, the composition of the 'free fatty acid' lower phase was altered by the presence of other fatty acids from the partly hydrolysed TG.

Table 1. *Free fatty acids in a ewes' milk cheese measured by different methods*

(Values are mg/kg cheese, means ± SD for $n = 4$)

Free fatty acid†	Method 1‡	Method 2§	Extraction by method 2; separation of free fatty acids and triacylglycerols by method 1‖	Extraction by method 1; derivatization by method 2¶
2:0	264 ± 94	ND	83·5 ± 18·9	ND
4:0	58·6 ± 7·2	917 ± 136	40·8 ± 8·2	1114 ± 175
6:0	33·0 ± 4·4	340 ± 47	32·1 ± 2·4	431 ± 66
8:0	38·6 ± 1·0	238 ± 19	32·9 ± 1·3	271 ± 31
10:0	102 ± 10	363 ± 46	82·3 ± 51·3	420 ± 47
12:0	73·5 ± 9·9	227 ± 10	64·2 ± 5·1	243 ± 29
14:0	156 ± 6	502 ± 25	135 ± 7	529 ± 25
16:0	345 ± 47	662 ± 65	291 ± 19	645 ± 125
18:0	142 ± 26	193 ± 32	114 ± 6	173 ± 46
18:1*n*–9	412 ± 39	311 ± 40	358 ± 14	296 ± 69
18:2*n*–6	58 ± 13	254 ± 61	53·5 ± 8·0	208 ± 220
Total	1683	4007	1284	4330

ND, not detected.

† 2:0, ethanoic; 4:0, butanoic; 6:0, hexanoic; 8:0, octanoic; 10:0, decanoic; 12:0, dodecanoic; 14:0, tetradecanoic; 16:0, hexadecanoic; 18:0, octadecanoic; 18:1*n*–9, octadec-9-enoic (oleic); 18:2*n*–6, octadeca-9,12-dienoic (linoleic).

‡ Extraction and analysis were performed as described in Materials and Methods, as aminopropyl-bonded phase chromatography (method 1).

§ Extraction, derivatization and analysis were performed as described in Materials and Methods as trimethylammonium hydroxide derivatization (method 2).

‖ Extraction was performed as described in Materials and Methods under trimethylammonium hydroxide derivatization (method 2) and free fatty acids were separated by aminopropyl-bonded phase chromatography (method 1).

¶ Extraction was performed as described in Materials and Methods under aminopropyl-bonded phase chromatography (method 1) and derivatized with trimethylammonium hydroxide derivatization (method 2).

Standard lipid mixtures

To investigate the possible transfer of methyl esters of the acylglycerol fatty acids from the upper to the lower phase during the TMAH derivatization reaction, and to determine the effect of the nature of the TG and the relative proportions of TG and FFA on the amounts of 'free' fatty acids quantitated by the two methods, four different mixtures of TG and FFA were prepared. The FFA and the fatty acids of the TG were of different (A mixtures) or the same (B mixtures) chain length. For both A and B mixtures, the ratios of FFA to TG were 1:1 (w/w) and 1:200 (w/w). The latter mixtures resembled the lipid composition of milk, cheese and other dairy products (Jensen *et al.* 1991).

Results of the analysis of mixture A1:1 are summarized in Table 2. Separation of the FFA from TG by aminopropyl-bonded phase chromatography prior to analysis by gas–liquid chromatography (method 1) yielded a clean FFA fraction, with only traces of fatty acids coming from the TG. These trace amounts were most likely present in the commercial TG used (see Materials and Methods). The recovery of the FFA present in the original standard mixture (4:0, 8:0, 12:0 and 18:1*n*–9) was very good and the determined composition of the sample closely reflected its original composition. With method 2, higher amounts of fatty acids from the TG fraction appeared as 'free' fatty acids in the FFA fraction, and the determined composition of the sample was somewhat different from the original. Method 2 gave a substantially lower recovery of 4:0 due, most likely, to the solvent removal step.

Table 2. *Composition of standard mixture A 1:1 analysed by two different methods*†

Fatty acids	Method 1‡ Free fatty acid, mg	Method 1‡ Composition, g/kg total fatty acids	Method 2§ Free fatty acid, mg	Method 2§ Composition, g/kg total fatty acids
4:0	5·3±0·04	278	27·5±5·0	177
6:0‖	0·01±0·01	0	1·7±0·3	11
8:0	4·9±0·06	257	51·0±4·1	329
10:0‖	0·04±0·00	2	1·3±0·1	8
12:0	4·3±0·06	226	45·7±1·5	294
14:0‖	0·03±0·01	2	1·0±0·2	6
18:0‖	0·06±0·01	3	0·28±0·04	2
18:1*n*–9	4·4±0·03	231	26·7±2·1	172

† Methods 1 and 2 and the standard mixture are described in the text.
‡ The initial mixture contained 5·0±0·1 mg of each component (free fatty acids, 4:0, 8:0, 12:0 and 18:1*n*–9; triacylglycerols, tricaproin, tricaprin, trimyristin and tristearin).
§ The initial mixture contained 51·7±1·5 mg of each component (free fatty acids, 4:0, 8:0, 12:0 and 18:1*n*–9; triacylglycerols, tricaproin, tricaprin, trimyristin and tristearin).
‖ Fatty acids from the triacylglycerols present in the standard mixture.

Table 3. *Composition of standard mixture A 1:200*† *analysed by two different methods*

Fatty acids	Method 1‡ Free fatty acid, mg	Method 1‡ Composition, g/kg total fatty acids	Method 2‡ Free fatty acid, mg	Method 2‡ Composition, g/kg total fatty acids
4:0	0·98±0·01	224	0·51±0·06	37
6:0§	0·40±0·01	91	7·53±0·95	542
8:0	0·93±0·01	212	0·95±0·02	68
10:0§	0·01±0·00	2	2·32±0·21	167
12:0	0·98±0·02	224	0·79±0·07	57
14:0§	0·04±0·00	9	1·54±0·34	111
18:0§	0·17±0·01	39	ND	0
18:1*n*–9	0·87±0·01	199	0·24±0·09	17

ND, not detected.
† The initial mixture contained 1·02±0·02 mg of each free fatty acid (4:0, 8:0, 12:0 and 18:1*n*–9) and 201·5±4·1 mg of each triacylglycerol (tricaproin, tricaprin, trimyristin and tristearin).
‡ Methods 1 and 2 are described in the text.
§ Fatty acids from the triacylglycerols present in the standard mixture.

When the ratio of FFA:TG in the sample was 1:200 (mixture A1:200, Table 3), the appearance of acylglycerol fatty acids (particularly shorter-chain fatty acids) in the FFA fraction was much more significant for method 2 than for method 1. This observation is consistent with the higher solubility of short-chain FAME in a methanolic phase and of longer-chain FAME in the ether phase. The increase in the proportion of TG in the sample affected the recovery of 18:1*n*–9, which was considerably lower when method 2 was employed. Method 2 gave a distorted composition of the original sample, with the fatty acids from acylglycerols predominating over the actual, original FFA.

When the fatty acids of the TG and of the FFA were of the same chain length (B mixtures), the contamination of the FFA fraction with short-chain acylglycerol FAME (method 2) was not readily apparent if the ratio of FFA:TG in the sample

Table 4. *Composition of standard mixture B 1:1 analysed by two different methods*†

	Method 1‡		Method 2§	
Fatty acids	Free fatty acid, mg	Composition, g/kg total fatty acids	Free fatty acid, mg	Composition, g/kg total fatty acids
4:0	5·6±0·3	290	31·6±10·3	212
8:0	4·9±0·1	254	49·0±0·2	328
12:0	4·4±0·1	228	43·0±0·3	288
18:1*n*–9	4·4±0·4	228	25·6±0·4	172

† Methods 1 and 2 and the standard mixture are described in the text.

‡ The initial mixture contained 5·1±0·1 mg of each component (free fatty acids, 4:0, 8:0, 12:0 and 18:1*n*–9; triacylglycerols, tributyrin, tricaprylin, trilaurin and triolein).

§ The initial mixture contained 48·7±1·3 mg of each component (free fatty acids, 4:0, 8:0, 12:0 and 18:1*n*–9; triacylglycerols, tributyrin, tricaprylin, trilaurin and triolein).

Table 5. *Composition of standard mixture B 1:200† analysed by two different methods*

	Method 1‡		Method 2‡	
Fatty acids	Free fatty acid, mg	Composition, g/kg total fatty acids	Free fatty acid, mg	Composition, g/kg total fatty acids
4:0	0·97±0·00	241	13·6±2·9	551
8:0	0·98±0·00	243	6·5±1·3	263
12:0	1·00±0·01	248	3·8±1·1	154
18:1*n*–9	1·08±0·04	268	0·8±0·5	32

† The initial mixture contained 1·02±0·02 mg of each free fatty acid (4:0, 8:0, 12:0 and 18:1*n*–9) and 204·3±9·4 mg of each triacylglycerol (tributyrin, tricaprylin, trilaurin and triolein).

‡ Methods 1 and 2 are described in the text.

was 1:1 (mixture B1:1, Table 4). The recovered amounts of the different fatty acids obtained with mixture B1:1 were similar to those obtained with mixture A1:1 (Table 2, method 2) except perhaps for 4:0, which gave better recovery. Published reports (Martínez-Castro *et al.* 1986; Martín-Hernández *et al.* 1988) refer to this type of mixture, implying the general validity of the method.

However, when a B mixture with an FFA:TG ratio of 1:200 (as could be expected in milk, cheese and other dairy products) was analysed by method 2 (mixture B1:200, Table 5), unexpected problems became apparent. The amount of each fatty acid recovered increased as its chain length decreased, yielding a composition for the sample entirely different from the original. Moreover, the total amount of FFA recovered was several-fold higher than that present in the original mixture. These results can be explained by considering the results shown in Table 3 for method 2: the shorter the chain of the acylglycerol fatty acids, the more readily their FAME will transfer to the lower layer, thus appearing as 'free' fatty acids. As a result, the original sample appeared to contain a larger amount of FFA when analysed by method 2 than it actually did. By contrast, method 1 provided a clean separation of FFA and TG, even in the presence of large excess of TG, and the composition of FFA obtained closely reflected that of the original sample.

In conclusion, the results presented here clearly indicated that to determine the amounts (or relative composition) of FFA in samples from milk, cheese and other dairy products where FFA are present in very low amounts as compared with TG, separation of the FFA fraction from the rest of the lipid is necessary before

derivatization and analysis by gas–liquid chromatography. In addition, and although method 1 could be more laborious when large numbers of samples need to be analysed, it is important to realize that samples can be prepared ahead of time and injected into the gas chromatograph with an automatic injector. With method 2, samples have to be injected immediately after the TMAH reaction.

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