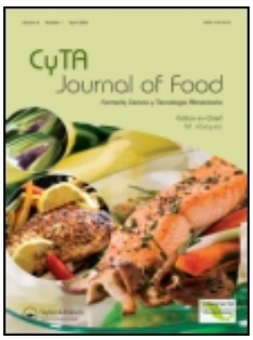

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Development of a simple method for the quantitative determination of fatty acids in milk with special emphasis on long-chain fatty acids

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Development of a simple method for the quantitative determination of fatty acids in milk with special emphasis on long-chain fatty acids

Desarrollo de un método abreviado para la determinación cuantitativa de ácidos grasos en leche con especial énfasis en los ácidos grasos de cadena larga

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A new and simple method was developed for the extraction and derivatization of fatty acid (FA) in milk. Lipid extraction of milk was carried out in H_2SO_4/methanol. Methylation was performed for 2 h at 60°C, and FA methyl esters were recovered for chromatographic analysis by the addition of hexane. The method parameters were optimized and the simple method was compared to the official reference procedure for the extraction and methylation of FAs in milk samples. For most of the 24 FAs determined, similar or significantly higher recoveries were obtained by the simple method than by the conventional method. The simple method allows processing a high number of samples, at the same time, minimizing the sample manipulation and, consequently, the sample loss and contamination. In conclusion, the proposed method is simple, rapid, low cost, and achieves good results.

Keywords: fatty acid; milk; bovine; gas chromatography

Un nuevo método abreviado se desarrolló para la extracción y derivatización de ácidos grasos en leche. La extracción de los lípidos de la leche se llevó a cabo en H_2SO_4/metanol. La metilación se realizó a 60°C durante 2 h y los ésteres metílicos de los ácidos grasos fueron recuperados para el análisis cromatográfico mediante la adición de hexano. Los parámetros del método fueron optimizados y el método abreviado fue comparado con el método de referencia para la determinación de ácidos grasos en leche. En la mayor parte de los 24 ácidos grasos analizados, el método abreviado mostró recuperaciones similares o mejores que el método convencional. El método simple permite procesar un alto número de muestras al mismo tiempo, minimizando la manipulación de la muestra y consecuentemente la pérdida de la misma y su contaminación. En conclusión, el método propuesto es simple, rápido, económico y logra buenos resultados.

Palabras clave: ácido graso; leche; bovino; cromatografía de gases

Introduction

Milk consumption is predicted to increase globally over the next 20 years, and it will cover a great proportion of human nutrient requirements as part of an everyday diet (Woods & Fearon, 2009). Due to milk's high frequency of consumption, several milk and dairy products with modified fatty acid (FA) profile have been developed in recent years to modify the human diet according to the recommendations of health agencies (WHO, 2003). These agencies have recommended to decrease the consumption of food with low content of polyunsaturated fatty acids (PUFAs), such as red meats (Luciano, 2009), and to increase the consumption of foods rich in *n*-3 PUFAs as a means of reducing the *n*-6/*n*-3 PUFAs ratio of the diet (Garg, Wood, Singh, & Moughan, 2006; WHO, 2003). This ratio is strongly correlated with the risk of developing cancer, as well as cerebrocardiovascular, inflammatory, and autoimmune diseases (Alexander, 1998). Additionally, much attention has been directed towards the presence of conjugated linoleic acid (CLA) isomers in dairy products. It is recognized that CLA isomers show anticarcinogenic properties as well as other important beneficial effects in human health (Belury, 2002).

Modification of the FAs composition of milk is mainly achieved through changes in cattle diet or by the addition of exogenous fat with high content of PUFAs (Castañeda-Gutiérrez et al., 2007; Jimenez, Garcia, & Beristain, 2008), such as fish fat (Noriega-Rodriguez et al., 2009). Rapid and precise analytical methods are necessary when verifying the final FAs profile of these modified products. The determination of FAs in foods is most often carried out by gas chromatography (GC) and usually involves lipid extraction from foods, a derivatization procedure, fatty acid methyl ester (FAME) extraction, and GC determination. This methodology was first proposed by Folch, Lees, and Stanley (1957) who used a chloroform–methanol mixture for the isolation of the total lipid content from animal tissues. However, FAs are polar, low volatility compounds that tend to self-associate or adhere to the walls of GC columns or other surfaces, and the chromatographic separation of non-modified FAs is therefore rather problematic. Thus, a derivatization process is necessary to convert the polar, nonvolatile long-chain FAs into methyl

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ester derivatives that are less polar, relatively volatile, and thermally stable (Juárez et al., 2008; Rosenfeld, 2002).

In recent years, ISO-IDF 14156 (ISO-IDF, 2001) and ISO-IDF 15884 (ISO-IDF, 2002) have been adopted as official methods for the lipid extraction and preparation of FAMEs. However, it is recognized that the conventional methods used for the analysis of FA profiles in a large number of samples are impractical since they are time consuming, require large samples, and consume large amounts of solvents (Araujo, Nguyen, Frøyland, Wang, & Kang, 2008). It has been unambiguously confirmed that the excessive handling caused by multiple steps is responsible for loss of a portion of the lipid phase and contamination (Kang & Wang, 2005). For these reasons, fast, low cost, simple, accurate, and less wasteful methods for determining FA concentrations in milk and dairy products are necessary.

Sulfuric acid has been used as a catalyst to prepare isopropyl esters in milk lipids, but the method has also resulted in extensive isomerization of conjugated dienes and artifact formation, just as for HCl/methanol. Although it has not yet been thoroughly investigated, H_2SO_4 in methanol has yielded encouraging results (Luna, Juárez, & de la Fuente, 2008). On the other hand, base methylation is the commonly used procedure to methylate milk lipids. This procedure requires strict anhydrous conditions (Carvalho & Malcata, 2005), and, for this reason, it is difficult to apply to raw milk samples directly.

The aim of this work was to develop a simple extraction–derivatization method prior to GC analysis for the quantitative determination of the FA composition in milk, with direct lipid extraction and without solvent removal. In this method, small amounts of sample and solvent are used for extraction and derivatization in a unique step. The proposed simple method was compared to the official reference procedures for extraction and methylation during analysis of the FA content in milk (ISO-IDF, 2001, 2002). The advantages, disadvantages, and applications of the proposed method are discussed.

Materials and methods

Reagents

All reagents and solvents were of analytical or high performance liquid chromatography (HPLC) grade. Ethanol, diethyl ether, ammonia solution, sodium sulfate anhydrous, sodium chloride, potassium hydroxide, sulfuric acid, methanol, *n*-pentane, and *n*-hexane were purchased from Merck (Darmstadt, Germany). Chloroform was purchased from Scharlau Chemie (Barcelona, Spain). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the study.

Standard solution and milk samples

FAME standard mixtures (C4:0 to C24:0) and individual FAMEs (*cis*-9, *trans*-11 CLA isomer, C18:1 *n*-7, C22:5 *n*-3, and methyl nonadecanoate as internal standard (IS)) were purchased from Supelco (Bellefonte, PA, USA). To prepare the stock solution, the individual FAMEs and the FAME standard mixture were mixed and diluted in *n*-hexane.

Bovine raw milk samples were obtained from a single farm owned by a Galician dairy company (Feiraco Soc. Coop. Ltda, A Coruña, Spain). The total fat content in these milk samples was determined by triplicate analyses according to Gerber's method (British Standards Institution, 1955). In all cases, the total fat content was between 32 and 34 g $kg^{-1}$of milk. Milk samples employed in the optimization of simple method were different due to the difficulty to conserve the raw milk during the time of execution of this work. Comparison of simple and conventional methods was conducted analyzing the same nine milk samples by each method.

Instrumentation and analytical conditions

Separation and quantification of the FAMEs were carried out using a 6850 GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a DB-Wax capillary column (30 m, 0.25 m id, 0.25 μm film thickness; Chrom Tech, Richmond, CA, USA).

The chromatographic conditions were as follows: the initial oven temperature was 35°C, where it was held for 2 min and then increased to 100°C at a rate of 30°C min^{-1}. The oven temperature was then increased to 225°C at 5°C min^{-1}, where it was held for 10 min. The injection port and detector temperatures were set to 250 and 300°C, respectively. Helium was used as a carrier gas at a flow rate of 1.8 mL min^{-1}. The split ratio was 10:1, and 1 μL of solution was injected. The data were recorded by integrator Software GC ChemStation version B.03.02 (Agilent Technologies).

Conventional method

Milk samples were homogenized by shaking for 10 min in a water bath at 38°C. Extractions following the conventional method were performed as outlined by ISO-IDF 14156 (ISO-IDF, 2001). An aliquot of sample (100 mL) was mixed with ethanol, NH_3 (aq), and peroxide-free diethyl ether. The funnel was shaken vigorously for 1 min and let stand to achieve phase separation. *n*-Pentane was added, mixed carefully, and left for phase separation. After discarding the aqueous layer, a Na_2SO_4 solution was added to the funnel, mixed, and left to undergo phase separation. This step was repeated twice, the remaining organic layer was transferred to a conical flask, and anhydrous sodium sulfate was added. The content of the conical flask was mixed, allowed to stand for 10 min, filtered, and evaporated in a water bath at 50°C and under reduced pressure using an R200 Buchi (Flawill, Switzerland) rotary evaporator. The separated lipids were exposed to a stream of N_2, and the FAMEs were prepared by base-catalyzed methanolysis of the glycerides using KOH in methanol as described in ISO-IDF 15884 (ISO-IDF, 2002).

Simple method

Milk samples were homogenized as described above. An aliquot of milk sample (10 μL) was placed in a tube sealed with a Teflon-lined cap, and 40 μL of a solution of nonadecanoic acid was added as internal standard. The solvent was evaporated with nitrogen (5 min, at 38°C), and 2 mL of a 25 mL L^{-1} H_2SO_4 solution in methanol was added. The tube was shaken for 30 s and stored in darkness at −20°C overnight (12–16 h) for lipid extraction. After that, methylation was carried out in a water bath for 2 h at 60°C. Then, 2 mL of a saturated NaCl solution and 1 mL of *n*-hexane were added; the mixture was shaken for 30 s and centrifuged (10 min at 2000 rpm). The aqueous layer was removed, and a small amount of anhydrous sodium sulfate was added to

eliminate any water residue. To avoid any loss of short-chain FAMEs, the hexane layer was not evaporated.

Identification, calibration, linearity, and limits of the method

FAMEs were identified by comparison of the retention times of the peaks in the sample with those of standard pure compounds. Quantitative analysis was based on the IS method. The relative mass response factor (f_i^m) was determined from the samples prepared using the stock and the IS solutions, according to the following equation:

$$f_i^m = \frac{A_{is} \times m_i}{A_i \times m_{is}}$$

where A_{is} is the peak area of the IS, m_i the mass of component i (μg), A_i the peak area of the component i, and m_{is} is the mass of IS (μg).

The methyl ester recovery during extraction and processing was determined by adding 40 μL of a 0.25 mg mL^{-1} solution of nonadecanoic acid (C19:0; Supelco) in chloroform. The study of the simple method included determination of FID response linearity; detection and quantification limits of the instrument and analytical conditions; and recovery, precision, and repeatability. The linearity of the response was determined from triplicate analyses of pure FAME solutions in *n*-hexane. For this purpose, eight 1:1 dilutions of the stock solution were used. Calibration curves were plotted using diluted solutions with a range of concentrations bracketing the concentration of FAs in the samples. The concentration of individual FAMEs in the most-concentrated working solution (Std1) was between 96 and 20 μg mL^{-1}, and, in the least-concentrated working solution (Std8), the concentrations ranged between 0.75 and 0.16 μg mL^{-1} (Table 1). The curves consisted of a plot of peak area versus concentration. Linear regression analysis of absolute areas versus injected quantities of the FAs was used.

The limit of detection (LOD) was determined as the lowest concentration that gave a signal-to-noise ratio ($S/N \geq 3$, and the limit of quantification (LOQ) was determined as the lowest concentration that gave an $S/N \geq 10$.

Simple method optimization

Before studying the simple method and comparing the results obtained by the simple method with those obtained by the conventional method, it was necessary to establish the optimal conditions in terms of sample volume, extraction time, and the time and temperature during methylation. Initially, FAs were extracted from a 50-μL milk sample by adding 2 mL of 25 mL L^{-1} H_2SO_4 in methanol. Samples were mixed and stored at -20°C for 2 h, and then derivatization was carried out in a water bath at 80°C for 1 h as described by Watts and Browse (2002).

For the determination of the ideal amount of sample, the proposed method was assayed using 5, 10, 50, and 100 μL of milk. The FAMEs extracted by the hexane layer were diluted before GC analysis in order to inject the same final concentration of FAMEs in all cases. The effect of extraction time was studied at 1, 3, 8, 12, 16, and 24 h. Samples were kept in a water bath at 40, 60, and 80°C for a period of 1 h to determine the ideal temperature during methylation. After the optimal temperature was established, this procedure was assayed in terms of time for 30 min, 1, 2, 4, 8, and 12 h in a water bath at 60°C.

Repeatability, intermediate precision, and recoveries

The repeatability [intra-day relative standard deviation (RSD), %] of the simple method was calculated based on the RSD of nine ($n = 9$) complete analyses of the same milk

Table 1. Linearity during determination of FAs studied by the simple method.

Tabla 1. Linealidad durante la determinación de los ácidos grasos estudiados mediante el método simple.

FA	t_R (min)	Standards range Std1–Std8 (μg mL^{-1})	Slope	Intercept	r	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
C4:0	2.462	64.00–0.50	0.665	−0.630	0.997	1.136	1.953
C6:0	3.918	64.00–0.50	1.570	−1.170	0.998	0.950	1.146
C8:0	5.185	64.00–0.50	2.103	−1.170	0.999	1.024	1.136
C10:0	6.992	64.00–0.50	2.447	−0.789	0.996	0.846	1.000
C12:0	8.252	64.00–0.50	2.806	−0.753	0.998	0.366	0.560
C14:0	11.338	64.00–0.50	2.960	−0.663	0.999	0.204	0.400
C14:1 *n*-5	13.037	32.00–0.25	2.945	−0.329	0.999	0.120	0.305
C15:0	13.588	32.00–0.25	2.965	−0.327	0.999	0.157	0.333
C16:0	16.475	96.00–0.75	3.079	−0.900	0.999	0.963	1.118
C16:1 *n*-7	16.826	32.00–0.25	2.980	−0.327	0.999	0.132	0.315
C17:0	18.155	32.00–0.25	2.079	−0.197	0.999	0.192	0.481
C17:1 *n*-7	18.502	32.00–0.25	2.960	−0.298	0.999	0.174	0.373
C18:0	19.797	64.00–0.50	2.994	−0.553	0.999	0.310	0.511
C18:1 *n*-9	20.057	64.00–0.50	3.158	−0.505	0.999	0.240	0.437
C18:1 *n*-7	20.174	32.00–0.25	2.853	−0.327	0.997	0.135	0.324
C18:2 *cis*-9-12	20.721	32.00–0.25	3.023	−0.316	0.999	0.138	0.347
C18:2 *trans*-9-12	20.814	32.00–0.25	2.969	−0.292	0.999	0.172	0.381
C18:3 *n*-3	21.708	32.00–0.25	2.952	−0.212	0.999	0.106	0.323
*cis*9–*trans*11 CLA	22.420	20.00–0.16	2.950	−0.280	0.999	0.138	0.349
C20:0	22.927	64.00–0.50	2.943	−0.353	0.999	0.318	0.552
C20:1 *n*-9	23.151	32.00–0.25	2.906	−0.219	0.999	0.221	0.543
C20:5 *n*-3	25.433	32.00–0.25	2.856	−0.214	0.999	0.092	0.446
C22:5 *n*-3	28.299	40.00–0.31	2.881	−0.875	0.999	0.083	0.421
C22:6 *n*-3	28.692	32.00–0.25	2.974	−0.145	0.999	0.082	0.411

Note: t_R: retention time; r: coefficient of correlation; LOD: limit of detection; LOQ: limit of quantification; CLA: conjugated linoleic acid.

Nota: t_R: tiempo de retención; r: coeficiente de correlación; LOD: límite de detección; LOQ: límite de cuantificación; CLA: ácido linoleico conjugado.

sample under the same experimental conditions by the same operator. The intermediate precision (inter-day RSD, %) was established from three complete analyses of the same milk sample on three consecutive days ($n = 9$).

The recovery of the simple method was determined from the complete analysis of nine replicate analyses ($n = 9$) of the same milk sample pre-fortified with the selected pure FA dilutions (Std3, Std4, and Std5).

Statistics

Changes in the measured FA content due to the extraction and methylation conditions were statistically analyzed by one-way analysis of variance. *Post hoc* analysis was carried out using Tukey's test. Student's *t*-test was used to determine differences in the FA content of milk analyzed by conventional and simple methods. Differences were considered to be significant when $P < 0.05$. Data analyses were conducted using the SPSS statistical package, version 13.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results and discussion

Response linearity

The response linearity for different pure FA standards (Table 1) shows an adequate linearity for all analytes, with *r* values higher than 0.99. The slope and intercept of the injected FAs were used to calculate the linear FID response of the FAs. The high slope values are indicative of the high detector sensitivity for the different FAs. The LOD and LOQ for the different pure FAs showed that standard solutions Std7 and Std8 were beyond the quantification limits for C4:0, C6:0, C8:0, *cis*9–*trans*11 CLA, and C20:1 *n*-9. For the other FAs, only standard Std8 was outside the quantification limits. The values for standard solutions outside the quantification limits were not used during further study of the method.

Method optimization

Upon examination of the importance of sample volume (Table 2), only C15:0 showed high concentrations when using 50 μL of sample. However, for most of the FAs, the highest concentrations were obtained using 5 and 10 μL of sample. For the five FAs (C16:0, C17:0, C17:1 *n*-7, C18:1 *n*-9, and C20:1 *n*-9), significantly higher concentrations were obtained using 10 μL than using 5 μL. For the other FAs (C14:0, C14:1 *n*-5, C18:3 *n*-3, and C22:5 *n*-3), the RSD using a sample volume of 5 μL was higher than 10%. Thus, a sample volume of 5 μL was considered to be inadequate, and 10 μL of milk was chosen as the optimum sample volume.

Once the optimum sample volume was established, the optimum extraction time was investigated (Table 3). The amount of FAs increased with increasing extraction time up to 12–16 h, when the highest values were obtained for all FAs. Significantly higher amounts of FAs were only found at 16 h versus 12 h for C20:0. As a result, overnight extraction (12–16 h) was selected as the optimal extraction time for the method. The extraction procedure was performed in darkness at a temperature of -20°C in order to prevent FA degradation (Araujo et al., 2008).

Table 2. Effect of sample volume on the FAME content in bovine milk (mg L^{-1} of milk) processed by the simple method.

Tabla 2. Efecto del volumen de muestra en le contenido de ácidos grasos en leche de vaca (mg L^{-1} de leche) procesada mediante el método abreviado.

	Volume of milk sample			
FA	5 μL	10 μL	50 μL	100 μL
C4:0	1,020.8 ± 39.1[a]	997.5 ± 27.7[a]	977.6 ± 23.0[a]	868.9 ± 53.1[b]
C6:0	526.1 ± 20.6[a]	503.8 ± 22.9[a]	437.5 ± 18.1[b]	383.2 ± 23.0[c]
C8:0	292.4 ± 11.9[a]	296.4 ± 08.9[a]	272.2 ± 12.2[a]	222.2 ± 11.2[b]
C10:0	663.0 ± 25.6[a]	677.4 ± 17.6[a]	576.6 ± 27.1[b]	531.5 ± 25.6[b]
C12:0	831.2 ± 54.3[a]	859.5 ± 32.6[a]	803.4 ± 44.2[b]	550.6 ± 23.0[c]
C14:0	2,690.2 ± 322.1[a]	2,893.8 ± 16.8[a]	2,899.1 ± 52.7[a]	1,880.3 ± 19.9[b]
C14:1 *n*-5	445.1 ± 55.2[a]	434.2 ± 04.1[a]	435.7 ± 3.4[a]	280.6 ± 16.4[b]
C15:0	263.8 ± 22.1[b]	240.4 ± 3.1[c]	273.4 ± 5.8[a]	170.0 ± 5.9[d]
C16:0	7,794.5 ± 582.1[b]	8,551.4 ± 404.8[a]	6,753.1 ± 56.4[c]	4,208.1 ± 311.7[d]
C16:1 *n*-7	842.3 ± 45.6[a]	830.5 ± 33.7[a]	714.4 ± 26.0[b]	388.3 ± 19.4[c]
C17:0	291.2 ± 18.2[b]	334.2 ± 3.0[a]	201.6 ± 3.1[c]	125.8 ± 9.7[d]
C17:1 *n*-7	145.2 ± 14.4[b]	164.7 ± 11.1[a]	80.9 ± 4.1[c]	43.8 ± 0.3[d]
C18:0	3,227.8 ± 232.3[a]	3,126.4 ± 245.2[a]	2,537.3 ± 213.7[b]	1,526.3 ± 106.1[c]
C18:1 *n*-9	8,122.3 ± 213.4[b]	8,771.6 ± 90.0[a]	6,811.5 ± 130.8[c]	3,313.5 ± 58.1[d]
C18:1 *n*-7	2,122.3 ± 91.2[a]	2,178.6 ± 81.0[a]	1,649.9 ± 19.1[b]	788.1 ± 19.1[c]
C18:2 *cis*-9-12	1,085.6 ± 314.5[a]	1,195.1 ± 91.8[a]	831.5 ± 60.5[b]	478.2 ± 7.4[c]
C18:2 *trans*-9-12	345.2 ± 27.6[a]	353.6 ± 7.3[a]	279.5 ± 20.7[b]	154.2 ± 1.2[c]
C18:3 *n*-3	372.3 ± 45.1[a]	358.6 ± 24.3[a]	338.9 ± 5.7[b]	211.0 ± 11.0[c]
*cis*9–*trans*11 CLA	519.1 ± 31.1[a]	551.5 ± 37.1[a]	414.4 ± 11.4[b]	187.2 ± 14.8[c]
C20:0	55.3 ± 4.1[a]	56.9 ± 1.4[a]	<LOQ	<LOQ
C20:1 *n*-9	61.7 ± 3.4[b]	67.2 ± 1.8[a]	54.9 ± 3.5[c]	<LOQ
C20:5 *n*-3	<LOQ	<LOQ	<LOQ	<LOQ
C22:5 *n*-3	55.6 ± 13.2[a]	53.4 ± 1.8[a]	<LOQ	<LOQ

Notes: Values are means ± standard deviation ($n = 9$). [a,b,c,d]Values in the same row with different letters are significantly different. LOQ: limit of quantification; CLA: conjugated linoleic acid. Only FA contents higher than LOQ are reported.

Notas: Los valores son media ± desviación estándar ($n = 9$). [a,b,c,d]Los valores en la misma fila con letras diferentes presentan diferencias estadísticamente significativas. LOQ: límite de cuantificación; CLA: ácido linoleico conjugado. Solo están recogidos aquellos ácidos grasos que presentan valores superiores al LOQ.

Table 3. Effect of the extraction time on the FAME content of bovine milk (mg L^{-1} of milk) processed by the simple method.

Tabla 3. Efecto del tiempo de extracción en el contenido de esteres metílicos de ácidos grasos de leche bovina (mg L^{-1} de leche) procesada mediante el método abreviado.

FA	Time of extraction					
	1 h	3 h	8 h	12 h	16 h	24 h
C4:0	1,275.7 ± 85.4^{c}	1,272.9 ± 63.1^{c}	1,602.4 ± 64.4^{b}	2,117.8 ± 63.2^{a}	2,168.5 ± 36.5^{a}	1,374.2 ± 82.5^{c}
C6:0	593.5 ± 12.9^{d}	667.5 ± 9.4^{c}	712.3 ± 20.5^{b}	801.9 ± 24.7^{a}	833.2 ± 17.6^{a}	648.6 ± 16.3^{c}
C8:0	366.7 ± 21.8^{d}	448.1 ± 10.4^{b,c}	467.8 ± 9.5^{b}	559.5 ± 22.5^{a}	529.9 ± 17.8^{a}	416.9 ± 19.6^{c}
C10:0	784.7 ± 29.5^{c}	959.1 ± 30.2^{b}	965.7 ± 28.6^{b}	1,163.0 ± 39.7^{a}	1,125.2 ± 26.5^{a}	901.1 ± 37.5^{b}
C12:0	986.6 ± 52.3^{b}	996.7 ± 9.7^{b}	1,312.1 ± 27.6^{a}	1,339.8 ± 73.2^{a}	1,320.8 ± 33.9^{a}	1,088.9 ± 58.1^{b}
C14:0	2,651.2 ± 152.4^{c}	2,673.6 ± 13.4^{c}	3,174.3 ± 83.4^{a}	3,212.2 ± 211.2^{a}	3,442.8 ± 92.6^{a}	2,907.8 ± 113.4^{b}
C14:1 *n*-5	275.0 ± 32.3^{b}	286.1 ± 7.9^{b}	337.8 ± 17.6^{a}	341.0 ± 27.8^{a}	358.7 ± 17.2^{a}	298.8 ± 12.4^{b}
C15:0	246.0 ± 11.2^{c}	249.3 ± 1.3^{c}	282.3 ± 6.3^{b}	315.1 ± 21.5^{a}	321.3 ± 4.1^{a}	270.8 ± 9.0^{b}
C16:0	6,580.0 ± 211.1^{c}	6,713.7 ± 56.1^{c}	7,894.5 ± 357.1^{a}	7,961.4 ± 389.5^{a}	8,739.2 ± 152.7^{a}	7,382.5 ± 261.9^{b}
C16:1 *n*-7	503.9 ± 12.3^{c}	551.1 ± 23.3^{b}	632.4 ± 23.2^{a}	643.1 ± 32.3^{a}	658.4 ± 27.8^{a}	554.4 ± 23.8^{b}
C17:0	189.2 ± 1.2^{d}	198.3 ± 1.4^{c}	228.3 ± 15.6^{a,b}	239.7 ± 28.7^{a}	261.6 ± 15.3^{a}	217.2 ± 6.9^{b}
C17:1 *n*-7	61.3 ± 1.4^{d}	66.2 ± 0.8^{c}	71.2 ± 1.1^{b}	79.7 ± 4.9^{a}	82.4 ± 3.7^{a}	69.8 ± 5.4^{b}
C18:0	2,803.0 ± 134.3^{d}	3,041.2 ± 34.4^{c}	3,597.6 ± 304.4^{a,b}	3,804.5 ± 317.6^{a}	3,836.1 ± 96.4^{a}	3,362.8 ± 121.1^{b}
C18:1 *n*-9	6,091.4 ± 327.8^{b,c}	5,884.6 ± 281.0^{c}	7,101.3 ± 272.4^{a}	7,278.2 ± 278.5^{a}	7,210.7 ± 322.0^{a}	6,209.6 ± 186.3^{b}
C18:1 *n*-7	891.0 ± 18.9^{d}	1,377.2 ± 16.0^{b}	1,694.3 ± 86.0^{a}	1,778.8 ± 92.3^{a}	1,761.9 ± 52.4^{a}	1,063.5 ± 74.5^{c}
C18:2 *cis*-9-12	693.9 ± 21.4^{c}	712.7 ± 13.6^{b,c}	812.4 ± 23.7^{a}	852.7 ± 41.0^{a}	868.9 ± 38.2^{a}	738.5 ± 26.0^{b}
C18:2 *trans*-9-12	339.9 ± 28.9^{c}	345.5 ± 18.5^{c}	423.5 ± 18.9^{a}	441.4 ± 20.3^{a}	440.9 ± 32.5^{a}	377.3 ± 19.8^{b}
C18:3 *n*-3	359.4 ± 31.1^{c}	371.3 ± 5.4^{c}	435.6 ± 35.1^{a,b}	464.8 ± 10.3^{a}	455.3 ± 20.7^{a}	412.2 ± 14.4^{b}
*cis*9–*trans*11 CLA	437.9 ± 34.2^{c}	480.3 ± 11.0^{b}	556.5 ± 13.4^{a}	547.7 ± 7.4^{a}	553.7 ± 18.4^{a}	499.0 ± 20.1^{b}
C20:0	< LOQ	63.0 ± 3.6^{c}	73.0 ± 11.3^{b}	73.8 ± 3.2^{b}	80.9 ± 4.3^{a}	74.9 ± 6.9^{b}
C20:1 *n*-9	< LOQ	< LOQ	48.1 ± 5.6^{a}	51.1 ± 5.3^{a}	50.1 ± 3.2^{a}	< LOQ
C20:5 *n*-3	< LOQ	< LOQ	< LOQ	44.9 ± 0.6^{a}	45.2 ± 1.1^{a}	< LOQ
C22:5 *n*-3	67.9 ± 4.5^{a}	52.4 ± 3.3^{b}	65.1 ± 3.1^{a}	69.8 ± 5.3^{a}	71.3 ± 10.2^{a}	49.1 ± 2.2^{c}
C22:6 *n*-3	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

Notes: Values are means ± standard deviation ($n = 9$). ^{a,b,c,d}Values in the same row with different letters are significantly different. LOQ: limit of quantification; CLA: conjugated linoleic acid. Only FA contents higher than LOQ are reported.

Notas: Los valores son media ± desviación estándar ($n = 9$). ^{a,b,c,d}Los valores en la misma fila con letras diferentes presentan diferencias estadísticamente significativas. LOQ: límite de cuantificación; CLA: ácido linoleico conjugado. Solo están recogidos aquellos ácidos grasos que presentan valores superiores al LOQ.

The derivatization process was optimized by determining the ideal temperature (Table 4) and reaction time (Table 5). The best results for most of the FAs in milk were obtained at 60°C and 80°C. Regarding individual FAs, higher amounts of C20:1 *n*-9 were obtained for transmethylation at 80°C than at 60°C, but the best results were obtained at 60°C for *cis*-9, *trans*-11 CLA and C20:0. Taking into account these results and the fact that the process at 60°C is less aggressive and more energy efficient than at 80°C, we selected 60°C as the optimal temperature.

When optimizing derivatization time, the highest FAs quantities were generally obtained at 2 and 4 h. For the shortest FAs (C4:0 and C6:0), higher values were obtained at 1 and 2 h. For C16:0, the results were better at 4 h than at 2 h, but higher concentrations were measured for the other six FAs (C14:1 *n*-5, C15:0, C18:0, C18:1 *n*-7, C20:0, and C22:5 *n*-3) when using a transmethylation time of 2 h. Thus, since a transmethylation time longer than 2 h did not improve the results and was less efficient in terms of time and energy consumption, we chose 2 h as the optimal transmethylation time.

Recovery factor

The recoveries (*R*; Table 6) were determined in spiked samples at three different concentrations, corresponding to standards Std3, Std4, and Std5 (Table 1). The highest *R* values were obtained at the intermediate level, and the lowest *R* values were found following spiking similar to the concentration of the least-concentrated standard solution (Std5). For the three different levels, the lowest *R* values were obtained for short-chain FAs. This is probably due to the loss of volatiles during concentration under the nitrogen stream. The *R* values obtained for this method were between 74.18 and 100.34.

Repeatability and intermediate precision

The simple method showed repeatability values lower than 7% (Table 6), except for C4:0. The repeatability values for the method were between 7.00% and 1.04%. The intermediate precision for the method had values under 10% except for C18:0, C18:1 *n*-9, C18:1 *n*-7, and C20:1 *n*-9, which showed the greatest RSD. The inter-day RSD varied between 10.94% and 3.09% for the same sample analyzed in triplicate on three different days.

Comparison with conventional method

Once the simple method was optimized and recovery, repeatability, and intermediate precision were conducted, the developed method was compared to the conventional method by analyzing milk samples. Figure 1 shows the chromatogram of a milk sample obtained by the simple method. The obtained results (Table 7) showed that the FA content obtained by the simple method was significantly lower than those obtained by the conventional method for the cases C4:0 and C6:0 FAs. No significant differences were obtained when milk samples were analyzed by the two methods for the case of C8:0, C10:0, C14:1 *n*-5, and C20:1 *n*-9, whereas, for the case of all the other FAs determined, differences appeared to

Table 4. Effect of the transmethylation temperature on the content of FAMEs in bovine milk (mg L^{-1} of milk) processed by the simple method.

Tabla 4. Efecto de la temperatura de transmetilación en el contenido de ésteres metílicos de ácidos grasos en leche bovina (mg L^{-1} de leche) procesada mediante el método abreviado.

	Transmethylation temperature		
FA	40°C	60°C	80°C
C4:0	1,987.2 ± 60.5[b]	2,147.3 ± 42.3[a]	2,073.4 ± 53.1[a]
C6:0	573.4 ± 24.5[b]	645.6 ± 18.9[a]	613.4 ± 20.8[a,b]
C8:0	362.1 ± 12.5[b]	404.2 ± 5.6[a]	412.2 ± 9.4[a]
C10:0	758.0 ± 17.7[b]	853.0 ± 21.4[a]	875.1 ± 8.2[a]
C12:0	871.8 ± 23.5[b]	1,088.9 ± 58.1[a]	1,094.9 ± 29.4[a]
C14:0	2,329.9 ± 96.5[b]	2,907.8 ± 113.4[a]	2,916.5 ± 96.6[a]
C14:1 *n*-5	239.4 ± 9.6[b]	298.8 ± 12.4[a]	299.5 ± 9.6[a]
C15:0	222.5 ± 13.4[b]	270.8 ± 9.0[a]	271.8 ± 8.6[a]
C16:0	6,141.7 ± 507.3[b]	7,382.5 ± 261.9[a]	7,373.1 ± 238.8[a]
C16:1 *n*-7	446.1 ± 18.7[b]	554.4 ± 23.8[a]	549.2 ± 29.8[a]
C17:0	189.2 ± 16.5[b]	217.6 ± 6.9[a]	219.2 ± 7.7[a]
C17:1 *n*-7	54.1 ± 1.4[b]	69.8 ± 5.4[a]	68.9 ± 1.7[a]
C18:0	2,646.4 ± 51.6[b]	3,362.9 ± 121.1[a]	3,336.8 ± 80.6[a]
C18:1 *n*-9	5,098.3 ± 146.8[b]	6,429.6 ± 186.3[a]	6,435.6 ± 142.4[a]
C18:1 *n*-7	1,011.6 ± 97.6[b]	1,083.5 ± 74.5[a]	1,142.3 ± 69.0[a]
C18:2 *cis*-9-12	581.4 ± 18.4[b]	738.5 ± 26.0[a]	734.7 ± 26.0[a]
C18:2 *trans*-9-12	303.2 ± 13.0[b]	377.3 ± 9.8[a]	377.6 ± 12.4[a]
C18:3 *n*-3	312.2 ± 9.5[b]	392.2 ± 14.4[a]	392.1 ± 8.8[a]
*cis*9–*trans*11 CLA	413.2 ± 12.1[b]	499.0 ± 20.2[a]	406.6 ± 13.1[b]
C20:0	58.4 ± 5.4[c]	74.8 ± 7.1[a]	65.7 ± 1.0[b]
C20:1 *n*-9	<LOQ	45.6 ± 1.4[b]	54.3 ± 4.2[a]
C20:5 *n*-3	<LOQ	46.1 ± 3.7[a]	48.4 ± 2.3[a]
C22:5 *n*-3	45.8 ± 0.8[b]	62.8 ± 4.4[a]	65.7 ± 5.8[a]
C22:6 *n*-3	<LOQ	<LOQ	<LOQ

Notes: Values are means ± standard deviation ($n = 9$). [a,b,c]Values in the same row with different letters are significantly different. LOQ: limit of quantification; CLA: conjugated linoleic acid. Only FA contents higher than LOQ are reported.

Notas: Los valores son media ± desviación estándar ($n = 9$). [a,b,c]Los valores en la misma fila con letras diferentes presentan diferencias estadísticamente significativas. LOQ: límite de cuantificación; CLA: ácido linoleico conjugado. Solo están recogidos aquellos ácidos grasos que presentan valores superiores al LOQ.

Table 5. Effect of the transmethylation time at 60°C on the content of FAMEs in bovine milk (mg L^{-1} of milk) processed by the simple method.

Tabla 5. Efecto del tiempo de transmetilación a 60°C en el contenido de ésteres metílicos de ácidos grasos en leche bovina (mg L^{-1} de leche) procesada mediante el método abreviado.

	Time of transmethylation					
FA	30 min	1 h	2 h	4 h	8 h	12 h
C4:0	1,010.6 ± 105.4[d]	1,976.4 ± 97.3[a]	2,108.2 ± 64.9[a]	1,851.9 ± 14.0[b]	1,568.6 ± 35.6[c]	852.2 ± 63.5[d]
C6:0	474.8 ± 43.4[c]	750.9 ± 51.3[a]	805.4 ± 25.4[a]	652.1 ± 20.3[b]	437.4 ± 45.3[c]	364.3 ± 74.2[c]
C8:0	365.8 ± 13.5[b]	383.9 ± 8.4[a]	396.1 ± 7.9[a]	389.9 ± 12.6[a]	362.1 ± 10.5[b]	358.7 ± 16.2[b]
C10:0	771.1 ± 28.5[b]	808.8 ± 32.5[a,b]	819.6 ± 14.6[a]	829.4 ± 19.5[a]	758.0 ± 40.2[b]	752.6 ± 26.4[b]
C12:0	986.6 ± 65.2[b]	986.2 ± 35.7[b]	1,230.3 ± 117.6[a]	1,216.8 ± 36.2[a]	977.9 ± 84.9[b]	995.3 ± 92.1[b]
C14:0	2,651.1 ± 59.9[b]	2,710.3 ± 250.8[b]	3,276.8 ± 371.0[a]	3,256.6 ± 156.1[a]	2,580.6 ± 228.0[b]	2,581.4 ± 226.4[b]
C14:1 *n*-5	286.1 ± 7.9[b,c]	308.9 ± 30.0[b]	361.7 ± 4.9[a]	323.4 ± 10.4[b]	272.5 ± 20.8[c]	285.6 ± 19.0[b,c]
C15:0	246.0 ± 11.9[c]	257.3 ± 26.0[b,c]	322.3 ± 13.2[a]	269.0 ± 14.6[b]	236.0 ± 22.2[c]	239.9 ± 22.8[c]
C16:0	6,580.1 ± 41.6[d]	6,905.5 ± 541.4[c]	7,749.3 ± 609.9[b]	8,097.5 ± 229.4[a]	6,363.0 ± 582.7[d]	6,492.7 ± 563.4[d]
C16:1 *n*-7	553.1 ± 23.3[b]	571.5 ± 23.5[b]	616.6 ± 49.1[a]	634.5 ± 56.3[a]	516.6 ± 45.1[c]	545.1 ± 31.0[b,c]
C17:0	189.2 ± 1.2[c]	200.5 ± 11.8[b]	239.4 ± 10.3[a]	241.9 ± 17.4[a]	184.0 ± 16.1[c]	192.7 ± 15.9[b,c]
C17:1 *n*-7	66.2 ± 0.8[b]	68.5 ± 4.3[b]	76.3 ± 5.9[a]	79.2 ± 04.9[a]	63.5 ± 3.8[c]	62.9 ± 5.7[c]
C18:0	2,803.0 ± 22.8[c]	2,952.6 ± 278.1[c]	3,663.3 ± 236.6[a]	3,238.7 ± 85.7[b]	2,784.2 ± 277.4[c]	2,840.6 ± 221.5[c]
C18:1 *n*-9	6,061.4 ± 79.1[b,c]	6,320.2 ± 422.0[b]	7,148.8 ± 694.2[a]	7,007.3 ± 148.6[a]	5,868.3 ± 408.3[c]	6,117.1 ± 497.8[b,c]
C18:1 *n*-7	1,377.2 ± 16.1[c]	1,477.4 ± 97.3[b]	1,629.9 ± 186.5[a]	1,438.3 ± 16.9[b]	1,347.2 ± 114.2[c]	1,385.6 ± 112.8[c]
C18:2 *cis*-9-12	712.7 ± 13.6[b,c]	755.0 ± 59.6[b]	842.1 ± 27.6[a]	833.2 ± 36.5[a]	685.7 ± 42.1[c]	716.3 ± 57.4[b,c]
C18:2 *trans*-9-12	345.5 ± 8.4[b,c]	364.8 ± 24.8[b]	393.4 ± 38.2[a]	410.2 ± 27.4[a]	329.0 ± 15.1[c]	347.5 ± 29.9[b,c]
C18:3 *n*-3	375.3 ± 5.3[b]	414.2 ± 31.6[a]	438.6 ± 29.6[a]	433.6 ± 10.2[a]	363.3 ± 22.7[b]	371.9 ± 35.1[b]
*cis*9–*trans*11 CLA	484.3 ± 15.8[a]	494.9 ± 47.7[a]	501.2 ± 35.8[a]	484.2 ± 7.6[a]	349.4 ± 28.1[b]	313.0 ± 22.4[b]
C20:0	<LOQ	56.5 ± 2.9[b]	66.9 ± 5.2[a]	60.5 ± 3.2[a]	55.2 ± 4.8[b]	<LOQ
C20:1 *n*-9	<LOQ	45.9 ± 2.4[d]	46.1 ± 4.2[d]	64.6 ± 6.6[c]	121.7 ± 12.2[b]	144.5 ± 7.2[a]
C20:5 *n*-3	<LOQ	44.9 ± 0.9[a]	46.6 ± 0.8[a]	<LOQ	<LOQ	<LOQ
C22:5 *n*-3	68.3 ± 2.8[a]	71.7 ± 4.1[a]	69.8 ± 5.2[a]	62.5 ± 2.7[b]	62.5 ± 8.5[b]	69.6 ± 0.8[a]
C22:6 *n*-3	<LOQ	<LOQ	41.9 ± 4.0[a]	41.3 ± 1.9[a]	<LOQ	<LOQ

Notes: Values are means ± standard deviation ($n = 9$). [a,b,c,d]Values in the same row with different letters are significantly different. LOQ: limit of quantification; CLA: conjugated linoleic acid. Only FA contents higher than LOQ are reported.

Notas: Los valores son media ± desviación estándar ($n = 9$). [a,b,c,d]Los valores en la misma fila con letras diferentes presentan diferencias estadísticamente significativas. LOQ: límite de cuantificación; CLA: ácido linoleico conjugado. Solo están recogidos aquellos ácidos grasos que presentan valores superiores al LOQ.

Table 6. Recovery factors at three addition levels (Std3, Std4, and Std5), repeatability, and intermediate precision (RSD, %) for the simple method.

Tabla 6. Recuperaciones a tres niveles de adición (Std3, Std4 y Std5); repetitividad y precisión intermedia (RSD, %) para el método abreviado.

FA	Recovery Std3 (%)	Recovery Std4 (%)	Recovery Std5 (%)	Repeatability (RSD, %)	Intermediate precision (RSD, %)
C4:0	79.66	87.92	74.18	7.00	8.01
C6:0	80.26	88.11	74.47	3.95	4.59
C8:0	85.87	88.22	78.67	2.80	6.52
C10:0	87.74	91.31	79.18	2.96	9.62
C12:0	88.53	91.59	81.07	3.37	10.69
C14:0	88.35	93.32	84.20	3.52	10.82
C14:1 *n*-5	89.46	92.01	84.31	3.29	9.84
C15:0	88.59	90.71	84.05	3.51	8.24
C16:0	91.59	93.05	90.09	2.88	9.85
C16:1 *n*-7	91.67	93.71	86.80	2.89	9.01
C17:0	99.23	100.34	91.71	4.13	7.91
C17:1 *n*-7	90.42	92.63	86.52	2.27	3.09
C18:0	91.20	91.49	88.26	2.47	10.94
C18:1 *n*-9	89.89	91.24	80.86	2.13	10.41
C18:1 *n*-7	95.00	90.46	80.37	1.90	10.87
C18:2 *cis*-9-12	88.31	88.77	85.36	2.26	9.65
C18:2 *trans*-9-12	86.96	91.30	83.69	3.75	9.75
C18:3 *n*-3	88.39	94.49	79.95	1.72	9.41
*cis*9–*trans*11 CLA	94.10	93.26	89.59	1.87	8.83
C20:0	89.40	91.74	82.61	1.83	4.39
C20:1 *n*-9	91.67	93.13	78.61	3.39	10.54
C20:5 *n*-3	87.37	92.71	77.43	1.04	8.57
C22:5 *n*-3	89.96	94.19	87.16	2.07	3.16
C22:6 *n*-3	87.10	93.96	87.52	3.28	4.79

Note: CLA: conjugated linoleic acid.

Nota: CLA: ácido linoleico conjugado.

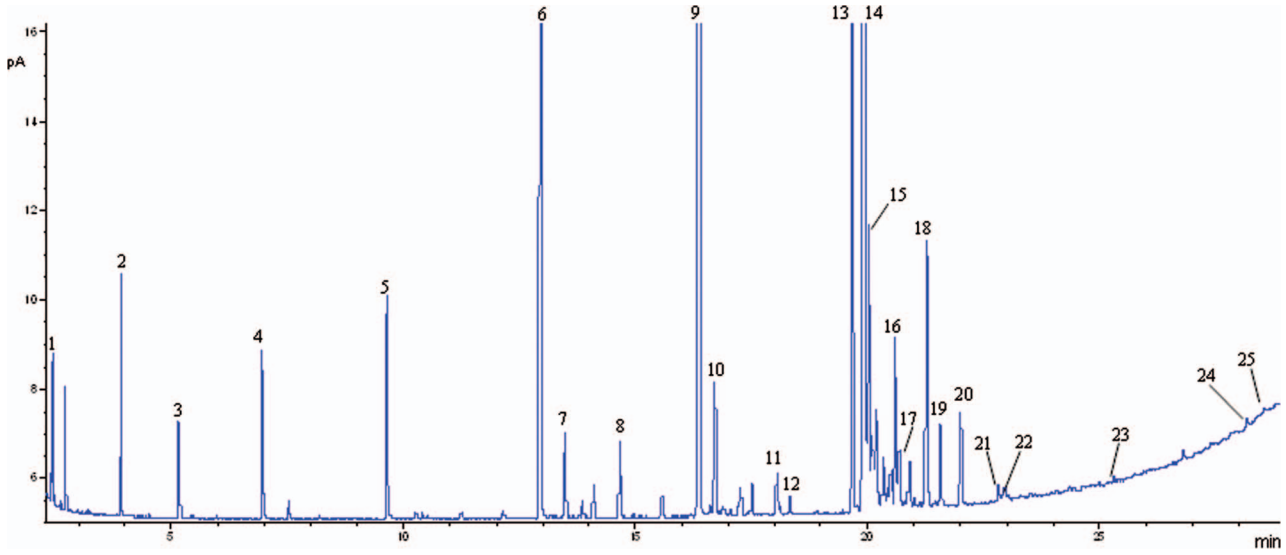

Figure 1. Chromatogram of raw bovine milk sample. Peak identification: (1) C4:0; (2) C6:0; (3) C8:0; (4) C10:0; (5) C12:0; (6) C14:0; (7) C14:1 *n*-5; (8) C15:0; (9) C16:0; (10) C16:1 *n*-7; (11) C17:0; (12) C17:1 *n*-7; (13) C18:0; (14) C18:1 *n*-9; (15) C18:1 *n*-7; (16) C18:2 *cis*-9-12; (17) C18:2 *trans*-9-12; (18) C19:0 (IS); (19) C18:3 *n*-3; (20) *cis*9–*trans*11 CLA; (21) C20:0; (22) C20:1 *n*-9; (23) C20:5 *n*-3; (24) C22:5 *n*-3; and (25) C22:6 *n*-3.

Figura 1. Cromatograma de una muestra de leche cruda de vaca. Identificación de los picos: (1) C4:0; (2) C6:0; (3) C8:0; (4) C10:0; (5) C12:0; (6) C14:0; (7) C14:1 *n*-5; (8) C15:0; (9) C16:0; (10) C16:1 *n*-7; (11) C17:0; (12) C17:1 *n*-7; (13) C18:0; (14) C18:1 *n*-9; (15) C18:1 *n*-7; (16) C18:2 *cis*-9-12; (17) C18:2 *trans*-9-12; (18) C19:0 (IS); (19) C18:3 *n*-3; (20) *cis*9–*trans*11 CLA; (21) C20:0; (22) C20:1 *n*-9; (23) C20:5 *n*-3; (24) C22:5 *n*-3; (25) C22:6 *n*-3.

be higher when using the simple method rather than using the conventional method. The differences in the FA content of milk were more evident (2-fold in some cases) for longer-chain FAs than for shorter-chain FAs (except for C20:1 *n*-9). Moreover, the concentration of the longest FAs detected using the conventional method was below the quantification limits of the GC analysis. This finding is in agreement with the fact that the alkali-based transesterification using the conventional method has two main shortcomings. Free FAs (Carvalho & Malcata, 2005; Golay, Dionisi, Hung, Giuffrida,

Table 7. FAME amounts (mg L^{-1} of milk) in bovine milk obtained by applying both conventional and simple methods.

Tabla 7. Cantidades de ésteres metílicos de ácidos grasos (mg L^{-1} de leche) en leche bovina obtenidas aplicando ambos métodos, convencional y abreviado.

FA	Simple method	Conventional method	*P*
C4:0	2,028.2 ± 149.6	2,343.0 ± 137.5	**
C6:0	723.6 ± 33.9	853.2 ± 58.5	***
C8:0	520.1 ± 15.5	494.1 ± 14.4	ns
C10:0	1,060.2 ± 22.6	1,004.4 ± 52.7	ns
C12:0	1,039.4 ± 37.7	933.2 ± 29.7	**
C14:0	3,314.1 ± 263.1	2,964.1 ± 121.9	*
C14:1 *n*-5	445.8 ± 37.5	405.6 ± 15.8	ns
C15:0	291.5 ± 22.5	249.8 ± 10.4	**
C16:0	8,252.8 ± 447.1	7,053.7 ± 296.4	***
C16:1 *n*-7	861.8 ± 31.6	706.1 ± 23.1	***
C17:0	270.9 ± 18.9	211.4 ± 19.8	***
C17:1 *n*-7	125.9 ± 10.8	65.7 ± 6.4	***
C18:0	2,512.8 ± 165.1	2,015.0 ± 40.6	***
C18:1 *n*-9	8,196.7 ± 480.2	6,738.4 ± 253.0	***
C18:1 *n*-7	3,335.3 ± 264.9	2,736.9 ± 132.9	***
C18:2 *cis*-9-12	906.2 ± 80.3	735.7 ± 24.4	***
C18:2 *trans*-9-12	528.2 ± 50.2	421.7 ± 12.6	**
C18:3 *n*-3	409.1 ± 39.8	341.2 ± 12.9	**
*cis*9–*trans*11 CLA	545.1 ± 35.5	175.4 ± 11.7	***
C20:0	65.5 ± 4.5	<LOQ	–
C20:1 *n*-9	89.6 ± 7.9	100.3 ± 13.9	ns
C20:5 *n*-3	46.3 ± 4.4	<LOQ	–
C22:5 *n*-3	54.6 ± 4.7	<LOQ	–
C22:6 *n*-3	41.4 ± 3.6	<LOQ	–

Notes: Values are means ± standard deviation ($n = 9$). ns, not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. LOQ: limit of quantification; CLA: conjugated linoleic acid. Only FA contents higher than LOQ are reported.

Notas: Los valores son media ± desviación estándar ($n = 9$). ns, no significativas ($P > 0{,}05$), *$P < 0{,}05$, **$P < 0{,}01$, ***$P < 0{,}001$. LOQ: límite de cuantificación; CLA: ácido linoleico conjugado. Solo están recogidos aquellos ácidos grasos que presentan valores superiores al LOQ.

& Destaillats, 2006) and sphingolipids may remain partially unreacted, and esters undergo saponification reactions. In the presence of methoxide, water yields free hydroxide ions. In the presence of these ions, esters and the newly generated FAMEs can be hydrolyzed (Juárez et al., 2008). For this reason, strict anhydrous conditions are required (Carvalho & Malcata, 2005). These factors help to explain the poor results found for long-chain FAs in comparison to the simple method. The higher quantities of these FAs obtained through the simple method is a very important factor since modified milks classified as functional foods most frequently contain increased concentrations of *n*-3 PUFAs and CLA isomers. These compounds are included in the long-chain FAs from milk.

Other modified methods obtained statistically indistinguishable results for most FAs when compared to the conventional method, but they displayed significant advantages in terms of time, chemical usage, and labor (Araujo et al., 2008; Feng, Lock, & Gasnsworthy, 2004; Luna, Juárez, & de la Fuente, 2005). Additionally, other simple methods carried out for other matrices obtained better results than those obtained by traditional methods. This fact is due to the prevention of FA losses by the multiple step handling and high quantities of sample and solvents (Abdulkadir & Tsuchiya, 2008; Carrapiso, Timón, Petrón, Tejeda, & García, 2000; Juárez et al., 2008; Meier, Mjøs, Joensen, & Grahl-Nielsen, 2006).

In conclusion, the method developed in this research obtained better results than the conventional method for detection of the nutritionally important long-chain FAs. In addition, this method decreases the risk of FA loss and sample contamination through reduction in sample handling, and it is suitable for the analysis of large number of samples at the same time. Moreover, the proposed simple method shows good recoveries and accuracy.

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