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Direct quantification of fatty acids in dairy powders with special emphasis on *trans* fatty acid content

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Abstract

In this study a validated procedure for accurate determination of fatty acids in dairy products, with special emphasis on total *trans* fatty acids (TFA) content is presented. Dairy fat naturally contains 4–6% of *trans* fatty acids, mainly *trans*-octadecenoic acids (i.e. vaccenic acid), and 0.3–1.5% of conjugated linoleic acids (CLA). The proposed procedure does not require lipid extraction, and transesterification of lipids could be carried out directly on dairy products. Optimal analytical conditions have been developed to allow accurate determination of TFA content without prior fractionation of *cis*/*trans* FAME isomers by thin-layer chromatography. The methodology requires the use of a highly polar open tubular capillary column having at least 100 m length. CLA and other fatty acids from C4:0 (butyric) acid to long-chain polyunsaturated fatty acids (LC-PUFAs) could also be analyzed. Therefore, the methodology presented is versatile and could be used for both targeted analysis (e.g. determination of TFA in dairy products) and determination of the broad fatty acid profile in dairy products.
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1. Introduction

Dairy fat is one the most complex dietary fat and has unique nutritional and physical properties. It was reported that dairy fat contain about 30–40 major fatty acids and a myriad of minor fatty acids such as branched-chain, oxo-, keto-, hydroxy- fatty acids for a total of 400 fatty acids (Jensen, 2002). Acyl chains are organized in phospholipids and acylglycerol clusters and acyl chains length ranging from C4 to C24 with ethylenic double bond (*cis* or *trans*) number from 0 to 6 (Jensen, 2002). A unique feature of dairy fat is the occurrence of a specific *trans* fatty acid, i.e. *trans*-11 (vaccenic) 18:1 acid and *cis*-9,*trans*-11 (rumenic) 18:2 acid, a conjugated isomers of linoleic acid (Bauman & Griinari, 2003; Kramer et al., 1998).

Accurate separation of almost all *trans* 18:1 fatty acid (TFA) isomers could be achieved using highly polar capillary column having at least 100 m length (Precht & Molkentin, 1996; Wolff & Bayard, 1995). A pre-separation by Ag-TLC is required to avoid overlap between some *cis*- and *trans*-18:1 acid isomers. Recently, an high-performance liquid-chromatographic methodology suitable for separation of *cis*- and *trans*-18:1 acid isomers using reversed-phase stationary phase have been published (Juanéda, 2002). To date, these methodologies remain the most powerful analytical tools for research purposes. However, for quality control laboratories, where more straightforward techniques are required, these procedures are time and cost consuming.

All the methodologies currently used for the analysis of *trans* fatty acids (TFA) and CLA in dairy powders require lipid extraction. Nevertheless, direct transesterification of food matrices and biological samples could be achieved using acid or alkaline reagents (Destaillats, Golay,

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Giuffrida, Hug, & Dionisi, 2004). The first type of direct method was developed for analysis of lipids in microorganisms (Abel, Deschmertzing, & Peterson, 1963). This method, which consists of mixing the sample with a methanolic hydrogen chloride (HCl) solution, was further modified and applied to a wide range of food products (Ulberth & Henninger, 1992). A variation of this method, involving mixing the samples with a 5% (v/v) methanolic acetyl chloride solution, was developed for the fatty acid analysis of human milk and adipose tissues (Lepage & Roy, 1984). For analysis of CLA containing products, base-catalyzed procedure is preferred (Christie, Sébédio, & Juanéda, 2001). This catalytic condition avoids degradation or geometrical isomerization of CLA isomers. Free fatty acids (FFA) are not derivatized by alkoxyde catalyst but FFAs are not present in significant amounts in common dairy products. This methodology had been applied with several experimental conditions to a wide range of products. A two step procedure which required the use of a methanolic sodium hydroxide solution at 90 °C for 10 min, followed by reaction of the mixture with boron trifluoride (BF3) in order to methylate FFA, have been developed (Park & Goins, 1994). Another procedure, that does not require heating or the addition of BF3, was developed by Grob and co-workers (Grob, Suter, & Pacciarelli, 1997a, 1997b). The procedure requires the use of a 5% (v/v) methanolic sodium methoxide solution, which reacts with esterified fatty acids at room temperature for about 1 min. A pre-treatment step may be required, depending on the nature of the food matrix. This methodology has been adapted for the quantification of fatty acids in infant formula (Cantellops, Reid, Eitenmiller, & Long, 1999).

The work presented here is a validated direct procedure for accurate determination of fatty acids in dairy products, with special emphasis on TFA and conjugated linoleic acids content.

2. Materials and methods

2.1. Direct preparation of fatty acid methyl ester (FAMEs) samples

The mixture of internal standards was prepared in a volumetric flask (50 ml) by dissolving equal amounts of tritridecanoin (the triacylglycerol synthesized from tridecanoic acid) and methyl undecanoate (150 ± 0.1 mg) in *n*-hexane. The mixture of internal standards was prepared in a volumetric flask (100 ml) by dissolving equal amounts of tritridecanoin and methyl undecanoate (200 ± 0.1 mg) in methyl *tert*-butyl ether (MTBE). A sample was weighed (to the nearest 0.1 mg) into a centrifuge tube (25 ml) with a screw cap in order to have approximately 50 mg of fat. For powdered infant formulas and milk powder, the sample should be dissolved in water (2 ml, 15 min). The derivatization was conducted at room temperature for 3.5–4.0 min after adding internal standard solution (5 ml), methanolic sodium methoxide (5% solution, 5 ml) to the sample and shaking continuously for 10 s. The reaction was stopped by adding *n*-hexane (2 ml), and by adding an aqueous solution (10 ml) of disodium hydrogen citrate (0.1 g/ml) and sodium chloride (0.15 g/ml). To prevent hydrolysis of FAMEs, the transesterification time should not exceed 240 s. After phase separation, the supernatant was diluted before GLC analysis.

2.2. Preparation of fatty acid methyl esters (FAMEs) from total lipid extract

Non-water-soluble dairy matrices such as cheese should be first extracted using Röse-Gottlieb method. Preparation of FAMEs could be carried out as presented previously (see above) on 50 mg of lipid extract.

2.3. Fractionation of FAME by argentation-thin layer chromatography (Ag-TLC)

FAMEs were fractionated by TLC on silica gel plates impregnated with silver nitrate. The plates were immerged in a 5% silver nitrate solution in acetonitrile for 15 min in the dark, and activated at 100 °C for 1 h (Wolff, 1995). Fractionation was performed according to number and configuration of double bonds, using a mixture of hexane and diethyl ether (90:10, vol/vol) as developing solvent. At the end of the chromatographic runs, the plates were sprayed with a solution of 2′,7′-dichlorofluorescein, and viewed under UV light. Bands corresponding to *trans* and *cis* monounsaturated FAs, respectively, were scraped off and transferred into a test tube, and methanol (1.5 mL), hexane (2 mL) and an aqueous solution of sodium chloride (5% wt/vol, 1.5 mL) were successively added with thorough mixing after each addition. After standing for ca. 1 min, the hexane phase was withdrawn, and the sample was concentrated prior to GLC analysis.

2.4. Gas-liquid chromatography analysis of FAMEs

Analysis of total FAMEs and Ag-TLC fractions were performed on a gas chromatograph (Carlo-Erba, Milano, It), equipped with a fused-silica CP-Sill 88 capillary column (100% cyanopropyl-polysiloxane; 100 m, 0.25 mm i.d., 0.25 μm film thickness; Varian, Palo Alto, CA) cold on-column injector and flame-ionization detector (300 °C). Oven temperature programming was 60 °C isothermal for 5 min, increased to 165 °C at 15 °C/min, isothermal for 1 min at this temperature then increased to 225 °C at 2 °C/min and held isothermal for 17 min at 225 °C. The inlet pressure of the carrier gas (H_2) was 200 kPa.

2.5. Identification and quantification of fatty acids

For optimal GLC separation, we recommend the use of long (at least 100 m), polar, open-tubular capillary columns, which permit accurate separation of FAMEs including the *cis* and *trans* isomers. Response factors for each

FAMEs could be calculated using commercially available FAME standard mixtures. In the present study a mixture containing the following methyl esters of butyric acid (4:0), caproic acid (6:0), caprylic acid (8:0), capric acid (10:0), undecanoic acid (11:0), lauric acid (12:0), tridecanoic acid (13:0), myristic acid (14:0), myristoleic acid (14:1$n-5$), pentadecanoic acid (15:0), pentadecenoic acid (15:1$n-5$), palmitic acid (16:0), palmitoleic acid (16:1$n-7$), heptadecanoic acid (17:0), heptadecenoic acid (17:1$n-7$), stearic acid (18:0), elaidic acid (*trans*-18:1$n-9$), oleic acid (18:1$n-9$), linolelaidic acid (all *trans*-18:2$n-6$), linoleic acid (18:2$n-6$), arachidic acid (20:0), γ-linoleic acid (18:3$n-6$), eicosenoic acid (20:1$n-9$), linolenic acid (18:3$n-3$), heneicosanoic acid (21:0), eicosadienoic acid (20:2$n-6$), behenic acid (22:0), eicosatrienoic acid (20:3$n-6$), erucic acid (22:1$n-9$), eicosatrienoic acid (20:3$n-3$), arachidonic acid (20:4$n-6$), docosadienoic acid (22:2$n-6$), lignoceric acid (24:0), eicosapentanoic acid (20:5$n-3$), nervonic acid (24:1$n-9$), docosahexaenoic acid (22:6$n-3$) was obtained from Nu-Check-Prep (Elysian, MN).

The response factor R_i, mean of three injections of the calibration standard solution for each $FAME_i$ present in the calibration standard solution, is calculated relative to the 11:0 internal standard as follows:

$$R_i = \frac{m'_i \cdot A'_0}{m'_0 \cdot A'_i}$$

where m'_i is the % mass of $FAME_i$ in the calibration standard solution; A'_0 the peak area of 11:0 in the calibration standard solution chromatogram; m'_0 the % mass of 11:0 in the calibration standard solution; A'_i the peak area of $FAME_i$ in the calibration standard solution.

The content of each FA, expressed in g FA_i/100 g product, is calculated as follows:

$$\frac{m_0 \cdot A_i \cdot R_i \cdot S_i(\mathrm{FA}) \cdot 100}{A_0 \cdot m}$$

and in g FA_i/100 g total fat, is calculated as follows:

$$\frac{\text{g } FA_i/100 \text{ g product} \cdot 100}{\%\mathrm{Fat}}$$

where m_0 is the mass of 11:0 internal standard added to the sample solution, in mg; A_0 the peak area of 11:0 internal standard and m the mass of test portion in mg. S_i(FA), the stoichiometric factor, is used to express correctly results as fatty acids (g fatty acid/100 g product or oil). S_i(FA) is the ratio between the methyl esters and fatty acids molar masses.

2.6. Transesterification procedure performance test

The performance of the transesterification methodology could by monitored by recording the areas of the two internal standard peaks (methyl undecanoate and tritridecanoin) in the analyzed samples, in the blank and in the reference sample. The performance of transesterification (PT) expressed in %, is calculated on the recovery of the tritridecanoin as second internal standard as follows:

$$\frac{m_{11:0} \cdot A_{13:0} \cdot R_{13:0} \cdot S_{13:0}(\mathrm{TAG}) \cdot 100}{A_{11:0} \cdot m_{13:0}}$$

where $m_{11:0}$ is the mass of 11:0 internal standard added to the solution, in mg; $A_{13:0}$ the peak area of 13:0 internal standard; $R_{13:0}$ the response factor of 13:0 relative to 11:0; $S_{13:0}$ the stoichiometric factor to convert 13:0 FAME into 13:0 TAG; $A_{11:0}$ the peak area of 11:0 internal standard and $m_{13:0}$, the mass of 13:0 internal standard added to the solution, in mg.

The difference in recoveries between the blank and sample (or the reference sample), should not exceed 1.0% of the mean of the duplicate determinations. The performance of the transesterification method should be always 100.0% ± 2.0%. If the performance of the transesterification is >102.0% or <98.0% the origin of the problem could be incomplete transesterification, partial degradation of one internal standard, or matrix effect. In this case, analysis should be repeated using both fresh reagents and internal standard solution.

3. Results and discussion

3.1. Sample preparation

The direct methods were optimized to extract lipids efficiently from the samples tested. Preliminary results showed that the acid catalyzed methodology (HCl/methanol) was not suitable for dairy products because short-chain fatty acids could be lost during derivatization (results not shown). Moreover, these tests confirmed that the acid catalyst gave rise to degradation of CLA (Christie et al., 2001). However, the modified Grob method (see details in Section 2) proved to be suitable for dairy samples.

The direct method was compared to indirect method, which involve Röse-Gottlieb extraction, subsequent derivatization using methanolic solution of sodium methoxide ($NaOCH_3$), followed by GLC analysis, under the same chromatographic conditions (Table 1). As illustrated, the modified Grob method proved to be as good as the Röse-Gottlieb method for fatty acid quantification. The direct method prevents several operations that can lead to degradation, loss of material, which increase measurement uncertainty.

3.2. trans Fatty acid content determination in dairy product by gas-liquid chromatography (GLC)

The use of unsuitable chromatographic tools (e.g. capillary columns) could underestimate the TFA content of samples. Indeed, commercially available GLC capillary columns having length lower than 100 m are not suitable for quantification of TFA in dairy products (Table 2). A pre-fractionation step before GLC analysis remains the unique methodology available for accurate measurement

Table 1
Fatty acid (FA) analysis of whole milk powders

Methodology	Expression of the results					
	gFA/100 g product		gFA/100 g fat		gFA/100 g total FA	
Analyzed matrices ($n = 6$)	DM powder	IM fat	DM powder	IM fat	DM powder	IM fat
Total fat (Röse-Gottlieb method)			28.17	28.17		
Fatty acids						
4:0	0.984	0.984	3.49	3.49	3.90	3.87
6:0	0.578	0.573	2.05	2.03	2.29	2.25
8:0	0.341	0.342	1.21	1.21	1.35	1.35
10:0	0.770	0.770	2.73	2.73	3.05	3.03
12:0	1.196	1.201	4.25	4.27	4.74	4.73
14:0	3.048	3.070	10.82	10.90	12.08	12.08
14:1	0.280	0.282	0.99	1.00	1.11	1.11
15:0	0.293	0.296	1.04	1.05	1.16	1.17
15:1	0.000	0.000	0.00	0.00	0.00	0.00
16:0	7.944	8.026	28.20	28.49	31.50	31.57
16:1	0.450	0.460	1.60	1.63	1.78	1.81
17:0	0.083	0.080	0.29	0.28	0.33	0.31
17:1	0.061	0.063	0.22	0.23	0.24	0.25
18:0	2.345	2.374	8.32	8.43	9.30	9.34
18:1 TFA	0.619	0.621	2.20	2.20	2.46	2.44
18:1	4.462	4.506	15.84	16.00	17.69	17.72
18:2 TFA	0.099	0.102	0.35	0.36	0.39	0.40
18:2	0.410	0.396	1.45	1.41	1.62	1.56
18:2 CLA	0.106	0.108	0.38	0.38	0.42	0.43
18:3	0.134	0.132	0.47	0.47	0.53	0.52
20:0	0.037	0.037	0.13	0.13	0.15	0.15
20:3	0.020	0.019	0.07	0.07	0.08	0.07
20:4	0.025	0.025	0.09	0.09	0.10	0.10
20:5	0.021	0.019	0.08	0.07	0.08	0.08
22:0	0.022	0.023	0.08	0.08	0.09	0.09
Other FA	0.896	0.914	3.18	3.25	3.55	3.60
Total fatty acids	25.223	25.425	89.54	90.26	100.00	100.00

Comparison of results obtained using the direct methodology (DM) and the indirect methodology (IM; lipid extraction followed by transesterification using KOH/methanol procedure).

of all 18:1 TFA isomers. However, an estimation of up to 95% of total 18:1 TFA could be directly achieved using a 100 m CP-Sil 88 capillary column. As illustrated in Fig. 1, baseline resolution of both oleic (*cis*-9 18:1) and *trans*-15 18:1 acid isomers remain the critical chromatographic parameters to be optimized. Chromatographic conditions allowing this separation without prior fractionation of total FAMEs have been published (Precht & Molkentin, 1996) but this resolution could become difficult to maintain after several GLC runs. By adding the area corresponding to the *trans*-16 18:1 FAME to the area corresponding to the *trans*-4 to *trans*-13/14 18:1 FAMEs, up to 95% of TFA could be estimated. The underestimation due to the co-elution of *trans*-15 18:1 and *cis*-9 18:1 FAMEs is minimized if we consider that positional *cis*-6/8 and *cis*-14 18:1 FAMEs co-elute with *trans*-13/14 18:1 and *trans*-16 18:1 FAMEs, respectively. Results on 1765 milk samples, have shown that the relative content of these minor *cis*-18:1 acid isomers is constant (Precht & Molkentin, 1996). Therefore, the error associated with the calculation proposed could be considered as negligible.

Table 2
Accuracy associated with length of CP-Sil 88 capillary column (Varian) used for *trans* 18:1 acid quantification

CP-Sil 88 (Varian) capillary column	18:1 TFA measured (% of 18:1 TFA)
50 m	63
100 m	83
100 m, conditions presented in this study	>95
Tandem Ag-TLC and GLC analysis on 100 m	100

18:2 TFA could easily quantified by summing all the minor peaks which elute before the linoleic acid methyl ester peak (see Fig. 2). These peaks correspond to an important number of methylene and non-methylene interrupted octadecadienoic acid isomers (Dionisi, Golay, & Fay, 2002; Precht & Molkentin, 1997, 1999; Ulberth & Henninger, 1994). CLA FAMEs are well separated from other octadecadienoate peaks (see Fig. 2). The peak labeled as CLA correspond to the co-elution of the two main CLA isomers present in dairy fat, *cis*-9,*trans*-11 and *trans*-7,*cis*-9 18:1 acid isomers. These compounds are quantitatively formed in the mammary gland by Δ9-desaturation of their TFA precursors: *trans*-11 and *trans*-7 18:1 acid isomers (Corl et al., 2002). Baseline separation of these CLA could be achieved by silver ion HPLC but appears not possible

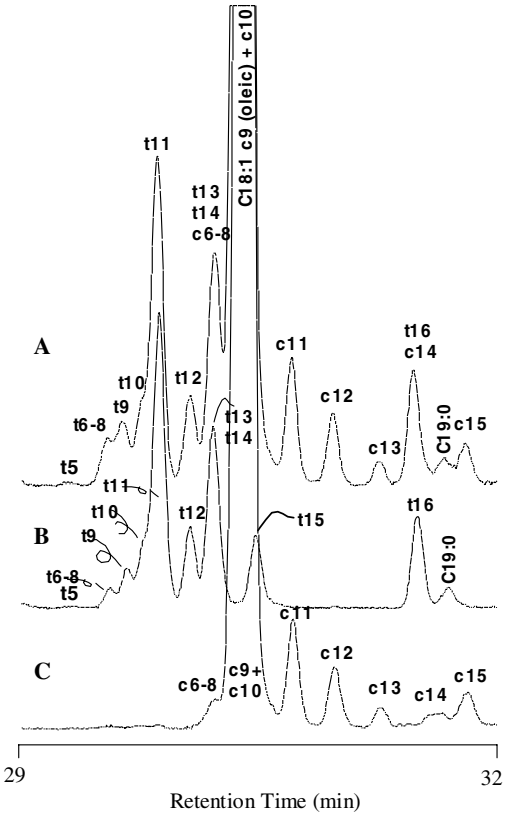
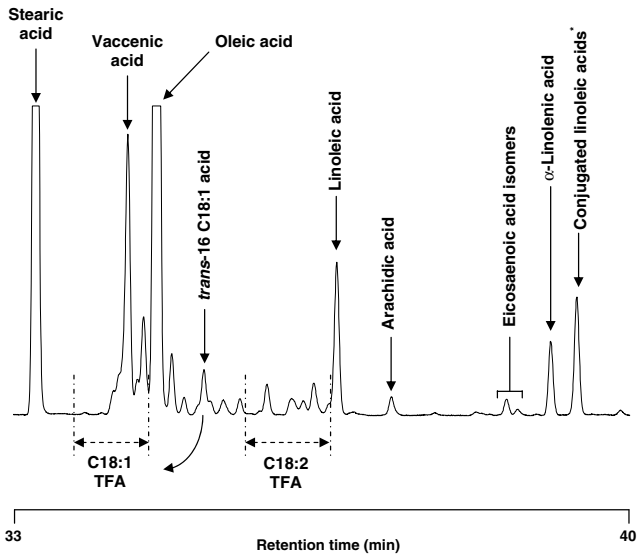


Fig. 1. Resolution of total 18:1 FAME (A); *trans*-18:1 FAME (B) and *cis*-18:1 FAME (C) derived from milk fat with gas-liquid chromatography on a 100-m column (CP-Sil 88). Fractions have been obtained by argentation thin-layer chromatography (Ag-TLC).



* *cis*-9,*trans*-11 and *trans*-7,*cis*-9 conjugated linoleic acids (CLA) isomers

Fig. 2. Resolution of 18 fatty acid methyl esters (FAMEs) derived from milk fat with gas-liquid chromatography on a 100-m column (CP-Sil 88) allow accurate estimation of both *trans*-18:1 FAME isomers, and *trans*-18:2 FAME isomers.

with a commercially available GLC column. The CLA peak is principally composed by *cis*-9,*trans*-11 18:2 acid isomer, which trivial name is rumenic acid (Kramer et al., 1998).

3.3. Validation of the method

The method was validated according to standard procedures (ISO-17025) and results for TFA content ranging from 0.4 to 4 g/100 g of fatty acids are presented in Table 3. Analytical conditions that were optimized and used here, allowed fatty acid quantification between 0.10 and 80.0 g per 100 g total fatty acids. The estimated detection limit expressed as three times the standard deviation of the background signal (noise) lies around 0.03 g/100 g total FA. The limit of quantification, for each fatty acid, is about 0.10 g/100 g total FA.

4. Conclusion

Direct preparation of FAME sample from liquid or powered dairy product without prior lipid extraction is less time and cost consuming than the two step experimental protocol. Base catalysis methodology used for the preparation FAMEs and consequently performed at room temperature is suitable for CLA quantification. As expected, degradation of CLA was observed during acidic catalyzed

Table 3
Uncertainty measurement of the method developed for direct quantification of TFA and CLA in dairy products

Uncertainty parameter	Value	Unit	Standard uncertainty	
			Absolute $u(x)$	Relative $u(x)/x$
Repeatability of the method	0.2	g(FA)/100 g FA	0.0144	0.0720
	4.0		0.1440	0.0360
Recovery of the method (extractability)	0.991	ratio	0.0100	0.0101
Linearity	0.02	ratio	0.0006	0.0320
(Peak area FA/peak area IS)	0.4		0.0288	0.0720
Mass of C11 added	10	mg	0.0300	0.0030
Mass of sample	200	mg	0.0250	0.0001
Response factor	1	ratio	0.0100	0.0100
Total uncertainty	0.2	g(FA)/100 g FA	0.0254	0.1272
	4.0		0.5249	0.1312

The final uncertainty is expressed as an interval, ex: FA = 0.2 ± 0.05 g FA/100 g FA and FA = 4.0 ± 1.05 g FA/100 g FA.

FAMEs preparation. The proposed methodology allows accurate quantification of total TFA in dairy product and this methodology could be implemented in a quality control laboratory. However, for research purpose, when detailed TFA isomers profile is required, it is preferred to perform a pre-separation of *cis*- and *trans*-18:1 FAMEs by Ag-TLC or reversed-phase HPLC.

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