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A MODIFICATION OF GAS CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF FATTY ACID COMPOSITION OF MILK FAT

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Abstract

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Gas chromatography method, described in ISO 15885, was modified using a certified reference material (CRM), different from that specified in the method, capillary column with selective polar phase and preparation of methyl esters was carried out with other esterifying reagents. The applicability of the modified method for determination of fatty acid composition was studied via evaluation of some important characteristics: selectivity, linearity and linear range, accuracy, precision and measurement uncertainty of the method. The selectivity and linearity of the method were investigated with CRM 47885 – Supelco 37 Component FAME Mix. Due to the column efficiency, the results for selectivity of four fold greater concentration were better than required in ISO 15885. The linearity range for 37 fatty acids was (22.03 ÷ 399.7) $\mu g.ml^{-1}$. To determine the accuracy of the modified method we analyzed milk fat of an interlaboratory test. The results met the range 90% ÷ 110% and there was no need for recovery. To evaluate the precision of the method five dairy products were analyzed. The obtained results were significantly better than required in ISO 15885, as the method for esterification of fatty acids was simplified and easy to perform. The uncertainties for the quantities of saturated and unsaturated fatty acids of five dairy products were low, as a result of relatively small deviations in measured precision.

Key words: determination of fatty acid composition of milk fat, FAMEs, gas chromatography
Abbreviations: FAs – fatty acids, FAMEs – fatty acid methyl esters

Introduction

Regulation (EU) 1169/2011 requires by December 2016 mandatory nutrition declaration on the label of the product to ensure complete information on the content and composition of foodstuffs with the aim to protect health and consumer interests. The amount of saturated fatty acids and in particular the amount of monounsaturated and polyunsaturated fatty acids is very important component of the nutrition declaration. To commit to such legislation, laboratories must be able to verify, by means of analysis, the contents declared on labels.

The determination of the amount of saturated, monounsaturated and polyunsaturated fatty acids in milk and milk products is carried out by gas chromatography method described in ISO 15885/2002. As indispensable for the application of ISO 15885/2002 are recognized ISO 14156/2001 (Extraction methods for lipids and liposoluble components) and ISO 15884/2002 (Preparation of fatty acid methyl esters).

Some researchers, in an effort to reduce the analysis time, chemical usage and labor were modified conventional methods. Feng S. et al. (2004) have presented rapid method for milk lipids separation – double centrifugation of milk at high speed centrifuge at 4°C, instead to use solvents method ISO 14156/2001. Luna, P. et al. (2005) has improved method for separating lipids from milk, using two centrifugations at room temperature (20°C). For identification of fatty acid

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composition they used Certified Reference Material 164, recommended in the ISO 15885/2002.

A new and simple method was developed by Martınez B. et al. (2012) for the extraction of milk lipids and next derivatization of fatty acids without solvent removal. Lipid extraction of milk was carried out in H_2SO_4/methanol and stored in darkness at -20°C overnight (12–16 h). After that, esterification has continued for 2 h at 60°C.

Butte W., (1983) has evaluated the fatty acid profile of almond oil, sesame oil, olive oil and butter after transesterification with trimethylsulphonium hydroxide.

W.W. Christie (1993) has summarized all methods for preparation of ester derivatives of fatty acids for chromatographic analysis. His conclusion was that the best esterification procedures for short chain fatty acids, which occurrence in milk fats, are those in which heating of the reagents is avoided. Short-chain fatty acid esters (methyl especially) are volatile and may be lost selectively on refluxing the esterification medium.

This paper presents the applicability of a modified ISO 15885/2002 for determination of the fatty acid composition of milk fat: certified reference material different from that specified in the method, capillary column with selective polar phase and preparation of fatty acid methyl esters using other esterifying reagents.

The following characteristics of the modified method were studied: selectivity, linearity and linear range, accuracy, precision (repeatability and reproducibility) and measurement uncertainty.

Materials and Methods

Equipment

Gas chromatographic analyses were performed using a gas chromatograph "Agilent Technologies" 6890 N, equipped with a flame – ionization detector, capillary column: Supelco SP™ – 2560 Fused Silica with parameters – 100.0 m x 0.2 μm x 0.25 mm, highly polar 100% biscyanopropyl phase; Software – G 2070 AA.

Chromatographic conditions

Temperature of the injector – 250°C, split mode 1:100 carrier gas – nitrogen, temperature regime: initial 60°C – 2 min.; 20° C/min to 160°C – 1min., 2° C/min to 240° C – 12 min.

Sample preparation

The used milk products were commercially available and were supplied on official control. We analysed 55 samples white pickled cheese, 30 samples kashkaval, 20 samples buttermilk, 20 samples milk (raw and pasteurized) and 25 samples yogurt. All dairy products were produced from cow milk. The isolation of the fat was performed according to ISO 14156. Methyl esters of fatty acids were obtained by transesterification method described in Bulgarian State Standard EN ISO 12966 – 3/2010. This method was preferred because the derivatization was performed in a single, fast reaction step. To (10 ± 2) mg separated milk fat, weighted in test tube, were added 500 μl tert – butyl methyl ether (TBME) and 250 μl thrimethylsulfonium hydroxide (TMSH). 1 μl of the solution was injected into gas chromatograph for quantification of FAMEs. As the methyl esters were formed completely during injection, the inlet temperature was 250°C. This procedure prevented losses by the multiple step esterification of the traditional method, described in ISO 15884/2001.

Reagents

Tert – butyl methyl ether (TBME), quality ≥ 99.0% (GC) and three – methyl sulfonic hydroxide (TMSH) intended for derivatization were purchased from Fluka Analytical. All reagents were of analytical grade.

Standards

The certified reference material CRM 47885, Supelco 37 Component FAME Mix (Traceable Certified Reference Materials) was purchased from Supelco SIGMA – ALDRICH. CRM 47885 contains methyl esters of 37 fatty acids with concentration range of 199.90÷399.7 $\mu g.ml^{-1}$.

Selectivity of the method

Selectivity of the method was determined by using the software of apparatus, after six injections of CRM 47885 with concentration of (0.2÷0.4 $mg.ml^{-1}$). ISO 15885 required the largest possible separation of methyl butyrate from the solvent peak and resolution of 1.5 (baseline resolution) between methyl stearate and buthyl oleate at a concentration of each of three esters equal to 0.1 $mg.ml^{-1}$.

Linearity and linear range of the method

Linearity was evaluated by linear regression analysis included in the software of the gas chromatograph. The linear range was determined by building calibration graphs of 37 FAMEs, contained in the CRM, at four concentration levels – 199.90 ÷ 399.7 $\mu g.ml^{-1}$, 99.95 ÷ 199.95 $\mu g.ml^{-1}$, 44.06 ÷ 99.92 $\mu g.ml^{-1}$and 22.03 ÷ 49.96 $\mu g.ml^{-1}$. The last three concentration levels were prepared by dilution with dichloromethane of CRM. We performed 6 injections for each concentration point. The criterion for linearity of the method was: the obtained correlation coefficients of calibration graphs of 37- FAMEs to be ≥ 0.990.

We calculated the uncertainty of the established from the analysis amounts of saturated and unsaturated FAMEs contained in CRM in the lowest, average and highest concentration.

Accuracy

The conformity of the value obtained from measurements of the concentration of the analyte with the true value.

To determine accuracy of the method we analyzed milk sample of an interlaboratory test RVQS 548, organized in 2014 by the company MUVA – Kempten. The evaluation of the accuracy of the method was done by comparing the results obtained from our measurements with those from the report RVQS 548. After six analyses of the sample from RVQS 548 was determined percentage of recovery (R,%) of methyl esters of fatty acids with concentration higher than 1g.100 g^{-1} fat. The recovery was calculated by the formula:

$$R\ \% = b/a\ x100\ ,$$

where: b – the changes in concentration of the analyte in the sample; a – the true amount of the sample referred to in the report of MUVA – Kempten.

The results for the accuracy of the method were acceptable if they meet the range 90% ÷ 110% compared to figures for RVQS 548.

Precision of the method

The precision of the analytical method was evaluated in terms of repeatability and reproducibility.

The repeatability

The repeatability of the method was assessed by investigation of fatty acid composition of milk, yogurt, white pickled cheese, kashkaval and butter.

We carried out 6 independent determinations of the same sample by the same analyst, under the same conditions, using the same equipment within a short period of time, according to analytical procedure. Statistically average value of fatty acids (, expressed as g.100 g^{-1} fatty acids and standard deviation (Sr) were calculated.

The reproducibility

The reproducibility of the method was evaluated as it was determined fatty acid composition of the same 5 dairy product from two analysts under the same conditions, the same apparatus at different days for a short period of time, according to the analytical procedure. Statistically average value of fatty acids (, expressed as g.100 g^{-1} fatty acids and standard deviation (Sr) were calculated.

Measurement uncertainty

The presentation of the result for the fatty acid composition of dairy products required calculation of uncertainty. The uncertainty was reported as expanded uncertainty (**U),** with coverage factor (k) of 2, which corresponded to 95% confidence interval:

$\mathbf{U = 2.Uc}$, where

$\mathbf{U_c}$ was the combined uncertainty and summarizes all measurable contributors of uncertainty

$\mathbf{U_c = \sqrt{U_a^2 + U_b^2}}$

$\mathbf{U_a}$ – uncertainty based on the measurements from analyses;

$\mathbf{U_b}$ – reference concentration uncertainty (CRM and of technical devices)

The uncertainties of five dairy products were calculated, based on the results of precision of the samples.

Results and Discussion

Selectivity of the method

The results for selectivity are presented in Table 1. By combining selectivity of the phase, column efficiency (long column length) and temperature gradient, the chosen capillary GC column provided high resolution separation of FAMEs and complied requirements of ISO 15885 at four fold greater concentration of the three esters (Figure 1- chromatogram).

Table 1
Selectivity and resolution of the modified method

FAMEs	Retention time – min	Concentration in mg.ml^{-1}		Selectivity from the previous peak		Resolution – baseline	
		according to ISO 15885	CRM 47885	a requirment of ISO 15885	Results	a requirment of ISO 15885	Results
Butiric Acid Methyl Ester (C4:0)	16.946	0.1	0.4	largest possible separation	1.067		4.962
Stearic Acid Methyl Ester (C18:0)	35.424	0.1	0.4		1.014	≥ 1.5	3.098
Oleic Acid Methyl Ester (C18:1n9c)	37.019	0.1	0.4		1.014	≥ 1.5	3.308

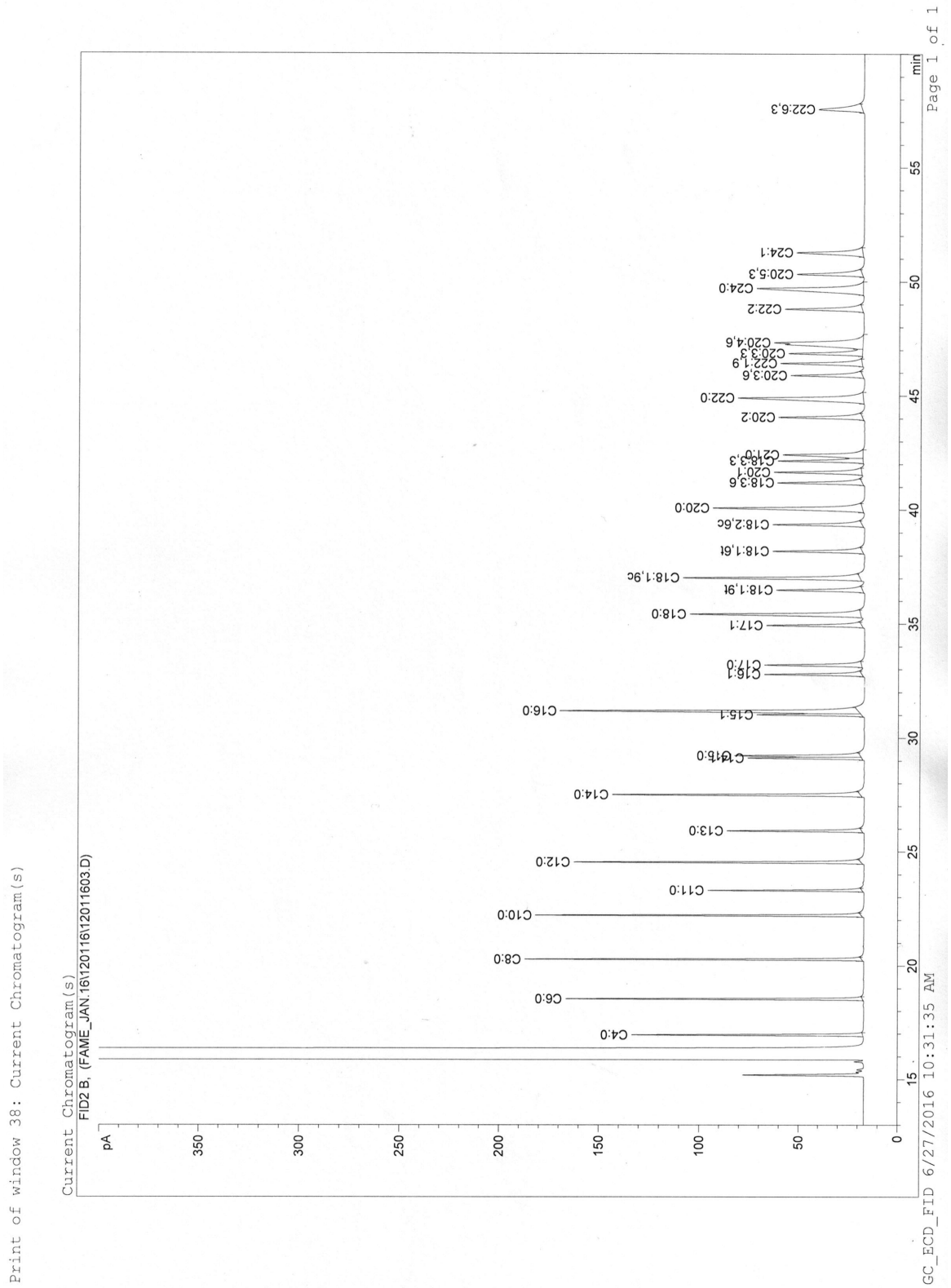

Fig. 1. Chromatogram of CRM 47885 – the concentrations of FAMEs were from 199.90 ÷ 399.70 µg.ml^{-1}. The concentration of palmitic acid methyl ester was 598.60 µg.ml^{-1}

Linearity and linear range of the method

Calibration graphs of the 37 components of CRM had correlation coefficients from 0.995 to 0.999. They were ≥ 0.990, which complied the criterion for linearity of the method in the studied range 22.0 ÷ 399.7 μg.ml^{-1}.

The linearity range was confirmed by the data in Table 2. There was proportional relationship between the total quantities of saturated and unsaturated FAMEs in the studied concentration levels.

Table 2
Quantities of saturated and unsaturated FAMEs (mean value ± SD), contained in CRM 47885 at three concentration levels, μg.ml^{-1}

Concentration levels, μg.ml^{-1}	Saturated FAMEs	Unsaturated FAMEs
22.03 ÷ 49.96	698.3 ± 16.7	521.3 ± 8.3
99.95 ÷ 199.95	2992.0 ± 59.8	2343.2 ± 28.1
199.90 ÷ 399.70	5792.0 ± 92.6	4173.6 ± 41.7

Accuracy

The results for accuracy of the modified method (Table 3) were between 91.5% and 110%. The received recoveries were very good and there was no need for correction for recovery. It was due to suitable chosen CRM for the quantification of fatty acids in milk fat.

The preferred esterification method contributed also to the good recoveries, because the methylation process in the inlet of GC, didn't allow loss of the volatile short-chain fatty acids. Feng et al. (2004) calculated around 20% loss of volatile fatty acids during methylation process, described in ISO 15884 (2002). Martınez B. et al. (2012) had the same finding even when sealed tubes were used.

Table 3
Results for accuracy of the modified method

FAMEs	The true quantity (report of RVQS 548) – g.100g^{-1} fat	Determined quantity, mean value ± SD, g.100g^{-1} fat	Accuracy – (R, %)
Butiric Acid Methyl Ester (C4:0)	3.41	3.44 ± 0.09	100.88 ± 2.64
Caproic Acid Methyl Ester (C6:0)	1.98	2.14 ± 0.04	108.08 ± 2.02
Caprilic Acid Methyl Ester (C8:0)	1.18	1.23 ± 0.03	104.23 ± 2.54
Capric Acid Methyl Ester (C10:0)	2.8	2.94 ± 0.08	105 ± 2.85
Lauric Acid Methyl Ester (C12:0)	3.2	3.38 ± 0.09	105.62 ± 2.81
Myristic Acid Methyl Ester (C14:0)	11.54	11.34 ± 0.34	98.26 ± 2.94
Palmitic Acid Methyl Ester (C16:0)	31.74	29.75 ± 1.14	93.73 ± 3.59
Stearic Acid Methyl Ester (C18:0)	8.67	8.33 ± 0.24	96.08 ± 2.76
Oleic Acid Methyl Ester (C18:1n9c)	19.42	19.03 ± 0.55	98 ± 2.83
Linoleic Acid Methyl Ester (C18:2n6c)	1.53	1.44 ± 0.05	94.12 ± 3.27

Table 4
Results for repeatability of the modified method, obtained from the analyses of five dairy products – g.100g^{-1} fatty acids[a]

FAMEs	milk	yogurt	white pickled cheese	kashkaval	butter
Butiric Acid Methyl Ester (C4:0)	4.00 ± 0	4.23 ± 0.05	4.47 ± 0.08	4.55 ± 0.08	4.20 ± 0
Caproic Acid Methyl Ester (C6:0)	2.58 ± 0.04	2.58 ± 0.04	2.82 ± 0.04	2.80 ± 0	2.62 ± 0.04
Caprilic Acid Methyl Ester (C8:0)	1.52 ± 0.08	1.40 ± 0	1.48 ± 0.04	1.50 ± 0	1.50 ± 0
Capric Acid Methyl Ester (C10:0)	3.60 ± 0.15	3.25 ± 0.05	3.53 ± 0.05	3.27 ± 0.05	3.38 ± 0.04
Lauric Acid Methyl Ester (C12:0)	3.87 ± 0.10	3.52 ± 0.04	3.72 ± 0.04	3.50 ± 0	4.03 ± 0.05
Myristic Acid Methyl Ester (C14:0)	11.87 ± 0.10	11.25 ± 0.05	11.72 ± 0.04	11.80 ± 0	12.45 ± 0.08
Palmitic Acid Methyl Ester (C16:0)	32.00 ± 0	31.17 ± 0.08	30.82 ± 0.07	30.48 ± 0.09	32.75 ± 0.08
Stearic Acid Methyl Ester (C18:0)	10.00 ± 0	11.50 ± 0.06	10.80 ± 0.06	11.05 ± 0.12	10.28 ± 0.04
Oleic Acid Methyl Ester (C18:1n9c)	23.00 ± 0	23.22 ± 0.04	22.58 ± 0.04	22.77 ± 0.15	21.0 ± 0
Linoleic Acid Methyl Ester (C18:2n6c)	2.18 ± 0.04	2.52 ± 0.04	2.30 ± 0	1.52 ± 0.04	1.87 ± 0.05

[a] According to ISO 15885:
The requirement of ISO 15885 for repeatability was: the relative difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will not more than 5% of cases be greater than:
- for fatty acid components present in excess of 5 g.100 g^{-1} of total fatty acids: 5%, with an absolute maximum of 1 g.100 g^{-1};
- for fatty acid components present in amounts of 1 g to 5 g.100^{-1} g total fatty acids: 12%, with an absolute maximum of 0.5 g.100 g^{-1} total fatty acid.

Precision of the method

Repeatability

The results for repeatability of the method from the analyses of five dairy products are shown in Table 4. The results obtained complied with the requirements for repeatability of ISO 15885.

Reproducibility

The method was reproducible because the results of this study (Table 5) met the criterion set by ISO 15885. The study for the applicability of the modified method presented significantly better results for the precision than required in ISO 15885. The coefficients of variation (RSD, %) of reproducibility for FAMEs of five dairy products were less than 4.0 %. The main reason for the reduced variations in repeatability and reproducibility was the chosen method for esterification of fatty acids. Bulgarian State Standard EN ISO 12966 – 3/2010 provided a method suitable for the esterification of milk fat, simpler and very easy for performance.

Luna, P. et al. (2005) have determined profile of fatty acid methyl esters of ewes'milk fat, which were prepared as described in ISO 15884/IDF 182:2002. They observed least precision in determining the short-chain fatty acids (C4-C6-C8), where the coefficients of variation for these compounds were between 3 and 7%.

Martınez et al. (2012) have used combined method for extraction and esterification of fatty acids of bovine milk. Their results showed inter-day RSD between 10.94% and 3.09% and could be attributed to the greater volatility of the short-chain fatty acids during the methylation process.

Measurement uncertainty

The quantities of saturated and unsaturated fatty acids with expanded uncertainty, determined for five dairy products are presented in Table 6 – quantity of saturated and unsaturated fatty acids, determined in five dairy products.

Table 6

Quantity of saturated and unsaturated fatty acids, determined in five dairy products – g.100g^{-1} total fatty acids

Type fatty acids	milk	yogurt	white pickled cheese	kashka-val	butter
Saturated fatty acids	71,8 ± 2.3	71,1 ± 2.1	71,6 ± 2,2	71,9 ± 2,2	73,5 ± 2,2
Unsaturated fatty acids	28,2 ± 1.1	28,9 ± 0,8	28,4 ± 1,0	28,1 ± 0,8	26,5 ± 0,8

The values of uncertainty of saturated and unsaturated fatty acids for five dairy products were low due to the relatively small deviations in measured precision.

Conclusions

The modified ISO 15885 is suitable for determination of fatty acid composition of milk fat. The used modifications improved the parameters of ISO 15885.

Table 5

Results for reproducibility of the modified method, obtained from the analyses of five dairy products – g.100g^{-1} fatty acids[a]

FAMEs	milk	yogurt	white pickled cheese	kashkaval	butter
Butiric Acid Methyl Ester (C4:0)	3.98 ± 0.04	4.24± 0.05	4.45 ± 0.08	4.55 ± 0.07	4.17 ± 0.06
Caproic Acid Methyl Ester (C6:0)	2.57 ± 0.04	2.57 ± 0.04	2.81 ± 0.05	2.80 ± 0.04	2.62 ± 0.04
Caprilic Acid Methyl Ester (C8:0)	1.52 ± 0.06	1.42 ± 0.04	1.48 ± 0.04	1.51 ± 0.05	1.48 ± 0.06
Capric Acid Methyl Ester (C10:0)	3.55 ± 0.14	3.24 ± 0.05	3.50 ± 0.06	3.26 ± 0.06	3.38 ± 0.04
Lauric Acid Methyl Ester (C12:0)	3.90 ± 0.10	3.50 ± 0.04	3.70 ± 0.06	3.52 ± 0.04	4.05 ± 0.07
Myristic Acid Methyl Ester (C14:0)	11.85 ± 0.08	11.24 ± 0.05	11.68 ± 0.06	11.78 ± 0.04	12.45 ± 0.07
Palmitic Acid Methyl Ester (C16:0)	32.01 ± 0.09	31.19 ± 0.10	30.82 ± 0.07	30.47 ± 0.07	32.75 ± 0.08
Stearic Acid Methyl Ester (C18:0)	9.96 ± 0.09	11.45 ± 0.09	10.78 ± 0.06	11.04 ± 0.11	10.29 ± 0.05
Oleic Acid Methyl Ester (C18:1n9c)	22.98 ± 0.07	23.17 ± 0.06	22.56 ± 0.05	22.74 ± 0.15	21.04 ± 0.06
Linoleic Acid Methyl Ester (C18:2n6c)	2.15 ± 0.08	2.51 ± 0.05	2.27 ± 0.06	1.52 ± 0.04	1.85 ± 0.05

[a] According to ISO 15885:
The relative difference between two independent single test results, obtained using the same method on identical test material in different laboratory with different operator using different equipment within a short interval of time, will not more than 5% of cases be greater than:
- for fatty acid components present in excess of 5 g.100 g^{-1} of total fatty acids: 15%, with an absolute maximum of 4.0 g.100 g^{-1};
- For fatty acid components present in amounts of 1 g to 5 g.100^{-1} g total fatty acids: 20%, with an absolute maximum of 1.0 g.100 g^{-1} total fatty acids.

Due to the column efficiency, the selectivity of the modified method was better than the required in ISO 15885.

The chosen CRM is suitable for quantitative analysis of fatty acid composition.

The linearity was very good for the entire concentration range, R^2 values of 37 calibration curves were ≥ 0.995.

The recovery was between 90 and 110%. There was no need for correction for recovery.

As the method for esterification of fatty acids was simpler and very easy to perform, the precision was significantly better than required in ISO 15885.

The uncertainties for the quantities of saturated and unsaturated fatty acids of five dairy products were low, due to the relatively small deviations in measured precision.

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