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Validation of the sensitive and accurate quantitation of the fatty acid distribution in bovine milk

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Abstract

A method for the precise analysis of the complex mixture of fatty acids in milk has been developed and validated. The triacylglycerol of nonanoic acid was applied as the internal standard (ISTD) for absolute quantification of fatty acids. Milk lipids were extracted by miniaturized ultrasonication and methylated with trimethylsulfonium hydroxide (TMSH), which has been proven to be rapid and effective. Resulting fatty acid methyl esters (FAME) were determined by GC/FID with excellent resolution, including separation of several 18:1 isomers. The low quantitation limit (0.01 mg mL^{-1} milk) indicates that the sensitivity of the method is sufficient to quantify up to 50 fatty acids, from 4:0 to 23:0. Measurements of precision provided excellent results for different bovine milk samples of different fat content (COV of 1.9% and 9.8% for intra- and interday precision, respectively). Recovery was on average 108 ± 3.5%. Evaluation of methods for determining the total fat content showed that gravimetry is no longer needed when using the ISTD. In conclusion, the present method is completely validated and readily applicable to the quantification of fatty acids in milk.

1. Introduction

Milk fat is characterized by high contents of saturated fatty acids (average 70%) and only less than 5% polyunsaturated fatty acids (PUFA). In addition, bovine milk fat has a very complex composition of fatty acids, as they are derived from both microbial metabolism in the rumen, body storage, dietary fatty acids and *de novo* synthesis of short-chain fatty acids. Fatty acid composition is furthermore affected by many factors such as intrinsic (e.g., stage of lactation, breed or genotype) and extrinsic ones (environmental factors like season and farming system or feeding factors). Therefore, many approaches have been reported in the literature to modify the fatty acid composition of milk fat with different types of forages or different feed-supplements (Altenhofer, Spornkraft, Kienberger, Rychlik, Meyer & Viturro, 2013; Ferlay, Glasser, Martin, Andueza & Chilliard, 2011; Kennelly, 1996; Kalac & Samkova, 2010).

To evaluate these alterations and enhancements of the fatty acid composition, methods to analyze the precise fatty acid composition are necessary. In this regard, the challenge is the huge variety of fatty acid isomers and the varying amounts of fatty acids, from major fatty acids like palmitic and oleic acid to minor fatty acids like *trans* isomers, highly unsaturated fatty acids, conjugated linoleic acids (CLA) and branched-chain fatty acids.

The most important analytical tool for fatty acid determination is gas chromatography, which allows precise and reproducible determination of fatty acids and separation of various individual isomers (Delmonte, Kia, Hu & Rader, 2009). There are standard methods for fatty acid determination. One of the most frequently used official methods, AOAC Official method 996.06, allows the determination of total, saturated and unsaturated fat in different foods with the use of TG-11:0 as internal standard (ISTD). However, samples which may contain *trans* fatty acids are explicitly excluded

from the measuring due to incomplete separation and identification and furthermore, validation data is lacking (AOAC Official method 996.06, 1997).

Generally, either on-column or split/splitless injection is used. On the one hand, the on-column technique is superior for high boiling compounds, which can be discriminated in a split/splitless system, and for thermal unstable or volatile analytes, because it avoids thermal degradation and discrimination of volatile substances. On the other hand, there is a serious risk of column contamination. Therefore, split/splitless injection with a liner that minimizes column contamination is the current method of choice. New techniques like the temperature-programmed evaporation (PTV) balance the discrimination between analytes with a wide range of boiling points and those with high volatility (Hübschmann, 2009).

Gas chromatography is most conveniently coupled with flame ionization detection (FID). As the detector response is proportional to the number of carbons in the fatty acid chain, FID requires the use of response correction factors (Ackman, 1972). These can be either theoretically calculated from the carbon number or experimentally determined by measuring a fatty acid methyl ester (FAME) standard mixture. In the method presented here, response factors were determined by analyzing methylated mixtures of triacylglycerol (TG) standards to incorporate both differences in the detector response and discriminations during the derivatization procedure (Firl, Kienberger, Hauser & Rychlik, 2012). Derivatization is a critical procedure for PUFA and especially for linoleic acid and CLA, as they are subject to isomerization reactions. In this regard, differences between methylation methods are observed, whereby base-catalyzed procedures are supposed to be milder, causing less isomerization. Moreover, time and temperature can be influential (Collomb, Schmid, Sieber, Wechsler & Ryhänen, 2006; Park et al., 2002; Yamasaki, Kishihara, Ikeda, Sugano & Yamada, 1999). Trimethylsulfonium hydroxide (TMSH) is a base-

88 catalyzed methylation reagent, which was reported to generate no isomerization
89 products (Ishida, Wakamatsu, Yokoi, Ohtani & Tsuge, 1999). In the approach
90 presented here, the derivatization procedure for CLA in milk, which originated from
91 linoleic acid, was tested.
92 Generally, the focus of research has been the resolution of the complex fatty acid
93 spectrum and identification of numerous fatty acids in milk fat (Delmonte *et al.*, 2009,
94 Destaillats & Cruz-Hernandez, 2007; Kramer, Blackadar & Zhou, 2002; Ledoux,
95 Laloux & Wolff, 2000). In contrast to this, the goal of the present study was the exact
96 quantification of specific fatty acids rather than the fatty acid pattern. There are few
97 publications that report on targeted analysis of fatty acids in dairy products. These
98 either focused on the quantitation of trans 18:1 isomers in milk powder and other
99 food matrices (Golay, Dionisi, Hug, Giuffrida & Destaillats, 2006; Golay, Giuffrida,
100 Dionisi & Destaillats, 2009), or validated a method for milk samples (Simionato *et al.*,
101 2010). However, these methods are not as accurately validated as the approach
102 presented. The whole extraction and derivatization process was subjected to a
103 thorough validation by using an appropriate ISTD, which was the TG of 9:0. This is
104 not done routinely, jet, and offers the benefit that the ISTD undergoes the same
105 clean-up and type of derivatization as the analytes. This standard was also used to
106 determine response factors for absolute quantitation of individual fatty acids. The
107 response factors consider the clean-up process as well and, therefore, losses of
108 individual fatty acids during clean-up process and GC measurement are included and
109 accounted for easily in the quantitation, which improves the accuracy of the method.
110 Furthermore a very detailed validation procedure, based on valid methods for
111 determiningrecovery and detection and quantification limits (DL and QL) was
112 performed. Recovery experiments have been done by spiking with several different
113 fatty acids (applied in form of the TG) and DL have been determined based on a

114 statistically valid method (Vogelgesang and Hädrich, 1998). In addition, different milk
115 types were included in the validation and the different behaviors during the clean-up
116 process were analyzed. This is also a new feature showing that the procedure is
117 valid for a high variety of milk samples.

2. Materials and methods

2.1. Chemicals and samples

For validation, different bovine milk types were used, depending on the particular objective: Type A (UHT milk, 3.5% fat, commercial product from Germany); two certified milk standards with defined fat content, 4.24% (type B) and 5.335% fat (type C), respectively (QSE, Wolzach, Germany), and two milk samples from local producers, type D (0.52% fat) and type E (4.46% fat). The following chemicals were obtained commercially from the sources given in parentheses: methyl-*tert*-butyl-ether (MTBE), chloroform, methanol and sodium chloride (Merck, Darmstadt, Germany); TMSH (Machery and Nagel, Duren, Germany); butyric acid, trinonanoylglycerol, tritridecanoylglycerol, trimyristoylglycerol, tripentadecanoylglycerol, tripalmitoylglycerol, tripalmitoleoylglycerol, trioleoylglycerol, trilinoleoylglycerol tritetracosenoylglycerol (trinervonylglycerol) (Sigma, Taufkirchen, Germany) and sn 1,2-distearoyl-3-butyroylglycerol, tristearoylglycerol, trilinolenoylglycerol, trieicosatetraenoylglycerol (triarachidonylglycerol) and tridocosahexaenoylglycerol (LGC Standards, Wesel, Germany). The following standards and standard mixtures were used for identification of FAME: Supelco 37 Component FAME Mix, vaccenic acid, cis-9-decenoic acid methyl ester, palmitelaidic acid methyl ester, cis-12-octadecenoic acid methyl ester, cis-13-octadecenoic acid methyl ester (Sigma, Taufkirchen, Germany); cis/trans FAME Mix (Restek, Bellefonte, PA), branched-chain FAME Mixtures BR 1 - 4 (Larodan, Malmö, Sweden), CLA (9c,11t) methyl ester, CLA (10t,12c) methyl ester, and CLA (9c,11c) methyl ester (Biotrend, Cologne, Germany); methyl docosapentaenoate (LGC Standrads, Wesel, Germany).

2.2. Extraction of milk lipids

Milk samples were frozen immediately after delivery and stored at -20°C until use. For clean-up, samples were thawed in a water bath at 42°C for at least 40 min and

144 stirred cautiously to avoid frothing. Trinonanoylglycerol (1 mg in chloroform) was
145 added as ISTD to 1 mL homogenized milk sample. Lipid extraction was performed by
146 a modification of the methods of Folch *et al.* (Folch, Ascoli, Lees, Meath & Le Baron,
147 1951, Folch, Lees & Sloane Stanley, 1957), which was simplified by Hallermayer
148 (1976). Ten mL chloroform/methanol (1:2, v/v) were added and processed using an
149 ultrasound 'Sonotrode' (type UW 2070, Bandelin, Berlin, Germany) for 1 min at 40 Hz
150 at room temperature. After centrifugation (4000 g for 5 min at 4°C), the organic phase
151 was collected in a separating funnel. The residue was processed again, as detailed
152 before, for its complete extraction. Both organic supernatants were combined and
153 extracted with aqueous sodium chloride solution (14 mL, 0.1 mol L^{-1}). After phase
154 separation, the chloroform layer was drained and evaporated at 37°C under vacuum.
155 The lipid extract was dissolved in 1 mL MTBE. Of this solution, 100 µL were
156 methylated with 50 µL TMSH, vortexed and directly injected into the gas
157 chromatograph (GC).

158 *2.3. Gas chromatographic analysis of FAME*

159 FAME were resolved on a Hewlett Packard 6890 GC equipped with an Agilent 7683
160 autosampler and FID. A CP 7420 column (coating select FAME, 100% bonded
161 cyano-propyl-phase, 100 m × 0.25 mm) with 0.25 µm film thickness was applied
162 (Agilent Technologies, Boeblingen, Germany). The split/splitless injector was used
163 with split 50, and samples were injected at 60°C. Then, the oven temperature was
164 raised by 8°C min^{-1} to 120°C and by 1.5°C min^{-1} to 242°C. Finally, the temperature
165 was raised by 1°C min^{-1} to 250°C as the final temperature. Total run time was
166 101 min, and injector and detector temperatures were 260°C and 270°C,
167 respectively. Hydrogen was used as carrier gas with a constant pressure of 1 bar and
168 Nitrogen was used as make up gas with a flow of 23 ml min^{-1} (Westfalen, Muenster,
169 Germany). Peaks were identified by comparison of retention times with known FAME

standards. RF for the quantitation of individual fatty acids were determined by using corresponding TG standards in appropriate mixtures, with TG-9:0 as ISTD, after applying the derivatization procedure described above.

2.4. Method validation

Response factors for individual fatty acids were calculated by the formula given in equation 1 from mixtures of TG [13:0, 14:0, 15:0, 16:0, 16:1 (9), 18:0, 18:1 (9), 18:2 (9,12), 18:3 (9,12,15), 20:4 (5,8,11,14) and 22:6 (4,7,10,13,16,19)] in chloroform, which were prepared in quadruplicate in the amounts expected in 1 mL milk sample. TG-9:0 was added as ISTD (1 mg) and the mixture processed and esterified as described in Section 2.2. In addition, butyric acid (0.4 mg in chloroform) and TG-9:0 (0.1 mg in chloroform) were combined, evaporated, dissolved in 100 µL MTBE and esterified as described in Section 2.2.

$$RF = \left(A_{Analyte} \cdot c_{ISTD}\right) / \left(c_{Analyte} \cdot A_{ISTD}\right) \quad (1)$$

DL and QL were determined according to the method of Vogelgesang and Hädrich (1998). Milk type C was spiked with TG-24:1 as the latter endogenously appears only in negligible traces in milk. The spiking was carried out at four different concentration levels (each in triplicate) starting slightly above the estimated DL and covering one order of concentration magnitude. TG-9:0 was added as ISTD and samples prepared as described above. Samples were run with split 10 and 30 in addition to split 50 to check for optimization options. DL and QL were derived statistically from the resulting regression line and the confidence interval according to the method of Vogelgesang and Hädrich (1998).

Recovery was determined by analyzing milk samples (type A) in triplicate, which were spiked with TG [13:0, 14:0, 15:0, 16:1 (9), 18:1 (9), 18:2 (9,12), 18:3 (9,12,15), 20:4 (5,8,11,14) and 22:6 (4,7,10,13,16,19)] and sn 1,2-distearoyl-3-butyroylglycerol to approximately double the amount that is present in milk (except for minor fatty

196 acids, which were used in higher amounts). The recoveries were calculated from the
197 difference of spiked and unspiked milk as the mean of the addition experiments.
198 Recovery was also determined by analyzing different milk types (B, C, D and E) that
199 were spiked with TG-15:0, and TG-18:1. For all analyses, TG-9:0 was used as ISTD.
200 Determination of the total fat content was evaluated by comparison of gravimetry with
201 the determination by using TG-9:0 as ISTD. For this purpose, two milk samples with
202 defined fat content (type B and C) were analyzed in 6 replicates with both methods
203 as described above.
204 Intra-day precision was determined by analyzing one sample in 6 replicates within
205 one day. Inter-day precision was determined by analyzing two samples in 6
206 replicates during 4 weeks. Precision was determined for milk types B and C.

3. Results and discussion

A typical chromatogram of FAME from bovine milk using a 100 m highly polar capillary column (CP 7420, Agilent Technologies) is shown in Fig. 1. Separation and identification of over 50 different fatty acids was achieved, ranging from short-chain (C4) to very long-chain (23:0) and highly unsaturated fatty acids (22:5n-3), particularly including various branched-chain fatty acids, 18:1 isomers and CLA.

3.1. Screening for isomerization products

The derivatization of linoleic acid and CLA was screened for isomerization products by comparing the CLA of a milk sample spiked with linoleic acid to those of an unspiked milk.. As displayed in Fig. 2, no increase in CLA content could be detected. By derivatizing a plain linoleic acid standard sample, the level of isomerization products was less than 1%, which is within the range of the COV. Ishida et al. (1999) previously tested TMSH for isomerization products of linoleic and linolenic acid and achieved similar results, with only negligible formation of 18:2 and 18:3 isomers. Time and temperature are crucial factors for isomerization. Accordingly, tetramethylammonium hydroxide, which needs higher temperatures for the formation of methyl esters than TMSH, leads to almost 35% isomerization products (Ishida et al., 1999). Other derivatization methods, like use of BF_3 or acidic methanol, equally promote isomerization and convert PUFA into isomerization products amounting to 36% of their precursors (Yamasaki et al., 1999, Chen, Cao, Gao, Yang & Chen, 2007). Methanolic potassium hydroxide, which is recommended by ISO (EN ISO 5509:2000), and sodium methoxide are other commonly used reagents for methylation. Their advantage is that no losses of PUFA are observed. However, both are not able to derivatize free fatty acids as they are only transmethylation reagents and, like BF_3 and acidic methanol, the procedure is far more time consuming and laborious then the TMSH procedure (Firl *et al.*, 2012, Chen *et al.*, 2007).

3.2. Determination of response factors

Results of response factors are shown in Table 1.; obviously, the response factors are dependent on the number of double bonds and chain length, which is due to the derivatization method. Losses of PUFA were previously reported during the derivatization procedure with TMSH (Firl *et al.*, 2012, Ishida, Katagiri & Ohtani, 2009). The latter authors recovered about 70% of PUFA, which is consistent with the results presented here. Butyric acid also has a very low response factor, which is due to discrimination of this very short chain fatty acid in the GC injector as already described in the DGF methods (Deutsche Gesellschaft für Fettwissenschaft e.V., 2010). Valeric acid methyl ester, which is also very short-chained, has been recommended as an ISTD, and should behave similar to butyric acid methyl ester in the GC injector. However, as small coefficients of variation (COV) indicate that the behavior of butyric acid during derivatization and GC measurement is constant, it does not seem to be necessary to use an additional ISTD. Nevertheless, the clean-up process for the determination of response factors was necessary to be considered as the relatively short-chained TG-9:0 is subject to losses during work-up compared to the endogenousTG being composed of mixed long-chained fatty acids. The calculation of response factors would lead to too small response factors and, therefore, result in too high results, if the clean-up would not be included..

3.3. Validation of the method

For validation of the method, DL and QL were determined following the procedure detailed by Vogelgesang and Hädrich (1998). Accordingly, a DL of 0.007 mg mL^{-1} and a QL of 0.01 mg mL^{-1} were determined for a split ratio of 50. These values could not be optimized by decreasing the split ratio of the GC injection system. To the best of our knowledge, DL and QL have never been determined for the whole extraction procedure for FA determination in milk by spiking the matrix with a TG and

subsequent statistical calculations. Simionato *et al.* (2010) and Golay *et al.* (2006) determined the signal to noise ratio (SNR) and estimated DL and QL from SNR values of 3 and 10, respectively. These two groups obtained DL of 0.2 and 0.3 and QL of 0.5 and 1 mg g^{-1} fat, respectively, which is higher than the results presented here (0.1 and 0.2 mg g^{-1} fat, respectively).

Recovery was determined for various fatty acids, which were applied in the form of the respective TG. Results are given in Table 2 and averaged at 108.5 ± 3.5%. All recovery values were slightly above 100%. This could be due to the structure of the ISTD, which is relatively short-chained. Even though the response factors went through the same clean-up process, matrix effects might cause further discrimination. As different milk types can behave quite differently during the clean-up process, recovery experiments were performed with different milk types (Table 3). Recovery ranged between 87 and 110%, and differences between the different milk types become apparent in the varying COV from 1 to 15%. In particular, milk with higher fat content and milk from local producers, which is not thoroughly homogenized, showed higher variations. This underlines the assumption that matrix effects can cause variations of recovery. However, all recovery values were in the range of 87 - 115%, which shows the suitability of the method.

The total fat content was determined either by gravimetry, as applied by Iverson, Lang, & Cooper (2001) e.g., or by using the ISTD TG-9:0 (Table 4). The mean values were comparable for both milk samples. The differences in accuracy become apparent when considering the variances and deviances. The absolute deviances of the individual measurements from the declared fat content were significantly smaller for the ISTD method (p=0.02). The gravimetrical method shows a COV about 5 times higher. Accordingly, even if the results appear to be similar in this case, the alternative using an ISTD is considerably more precise than the gravimetric method,

285 which is in agreement with results published previously (Aued-Pimentel, Kus,
286 Kumagai, Ruvieri, & Zenebon, 2010).
287 Precision was determined for two milk types with different fat content (milk type B
288 and C); no differences in precision were observed between the samples.
289 Reproducibility within one day was very good for all fatty acids determined (Table 5)
290 with COV ranging between 0.3 and 10.8%. The mean was 1.6 and 2.1% for milk
291 types B and C, respectively. Intermediate precision showed slightly higher results,
292 with a mean COV of 9.8%, which is still excellent. The values ranged up to 20.8% for
293 lignoceric acid, which is a minor fatty acid.
294 Finally, milk type B and C were analyzed six times and the levels of fatty acids were
295 calculated (Table 5). Depending on the total fat content, a few fatty acids were below
296 DL. CLA (9t,11t) and (10t,12c) could not be detected in either milk sample. In milk
297 type B additionally, heneicosanoic acid was not detectable and eicosadienoic acid
298 was below QL. In milk type C, which had a higher fat content, only heneicosanoic
299 acid was detectable but below QL. However, around 50 fatty acids could be
300 separated and quantified in both milk samples. This included various 18:1 isomers,
301 many branched-chain fatty acids, and PUFA.

4. Conclusion

The thorough and successful validation of the GC methodology for quantitation of fatty acids in cow milk underlines the complexity of the fatty acid distribution and the value of this sensitive tool with high separation efficiency. To date, no other methodology is able to resolve this high number of components from TG. The determination of FAME in cow milk samples of different origin and consistency is important in numerous studies in animal physiology (Cruz-Hernandez et al., 2007; Ferley *et al.*, 2011; Kalac & Samkova, 2010; Khiaosa-ard, Klevenhusen, Soliva, Kreuzer & Leiber, 2010; Kennelly, 1996), which demonstrates the necessity of this kind of effective methodology. Therefore, GC of FAME will remain an important technique in lipidomics.

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Table 1.

Response factors for fatty acids relative to nonanoic acid used as internal standard in form of its TG.

Fatty acid	4:0	13:0	14:0	15:0	16:0	16:1 n7	18:0	18:1 n9	18:2 n6	18:3 n3	20:4 n6	22:6 n3
RF	0.59	1.02	1.01	1.05	1.07	1.02	1.04	1.05	0.86	0.96	0.87	0.79
COV % [a]	1.31	1.07	1.24	1.80	1.93	0.96	1.18	1.82	1.16	2.15	2.33	2.78

RF, response factor; TG, triacylglycerol; [a] COV of triplicate determination as described in Section 2.4.

418 **Table 2.**

419 Recoveries (%) of fatty acids of lipid standards in spiked milk samples (n=4) [a]

Fatty Acid	Recovery	COV %
4:0	112.1	3.3
13:0	105.2	0.5
14:0	111.2	1.0
15:0	105.7	0.9
16:1 (9)	106.2	1.5
18:1 (9)	102.5	1.9
18:2 (9,12)	110.1	1.7
18:3 (9,12,15)	109.8	1.2
20:4 (5,8,11,14)	110.6	0.6
22:6 (4,7,10,13,16,19)	115.0	0.3

420 [a] Determined in spiking experiments at milk concentration of the respective fatty acids as described in Section
421 2.4.

422 **Table 3.**

423 Recoveries (%) of fatty acids of TG standards in spiked milk samples (n=3) with different fat contents [a]

Milk type (% fat)	15:0	COV (%)	18:1 (9)	COV (%)
A (3.5)	105.7	0.9	102.5	1.9
B (4.24)	103.6	3.2	102.9	4.4
C (5.334)	101.7	3.6	93.6	9.9
D (0.52)	104.0	2.5	110.3	1.5
E (4.46)	99.1	4.0	87.4	15.3

424 [a] Determined in spiking experiments at milk concentration of the respective fatty acids as described in Section
425 2.4. Milk types are defined in Section 2.1. TG, triacylglycerol.

426 **Table 4.**

427 Comparison of measured fat contents of milk samples by the gravimetric and the ISTD-methods [a]

	Milk type B			Milk type C		
	fat content %	COV [b] %	absolute deviance [c]	fat content %	COV [b] %	absolute deviance [c]
Gravimetric method	4.18	13.55	0.5	5.20	8.82	0.4
ISTD method	4.25	2.54	0.1	5.15	2.17	0.2

428 [a] determined as described in Section 2.4; [b] of six replicate determinations; [c] mean of absolute deviances of all six
429 determinations; milk types are defined in Section 2.1.

Table 5.

Fatty acid contents in different milks as well as reproducibility and intermediate precision (%) of their measurement [a]

		QSE milk A			QSE milk B			Inter-
Fatty acid	Non-systematic name	mg mL^{-1}	COV %	g 100g^{-1} fat	mg mL^{-1}	COV %	g 100g^{-1} fat	mediate precision[b]
4:0	butric acid	2.03	1.25	4.33	2.36	1.64	4.22	9.22
6:0	caproic acid	0.83	0.55	1.76	1.02	1.12	1.82	7.85
8:0	caprylic acid	0.53	0.88	1.13	0.67	0.48	1.20	6.95
10:0	capric acid	1.26	0.32	2.69	1.64	0.65	2.93	6.32
10:1 (9)		0.12	1.39	0.25	0.15	1.38	0.26	7.30
11:0		0.02	1.49	0.04	0.03	6.00	0.06	10.58
12:0	lauric acid	1.54	0.70	3.28	2.01	0.54	3.59	5.99
12:1 (5)	lauroleic acid	0.03	4.32	0.06	0.04	1.99	0.07	10.84
13:0 (anteiso)		0.02	3.10	0.04	0.03	0.70	0.05	8.40
13:0		0.04	2.15	0.09	0.06	1.26	0.10	9.13
14:0 (iso)		0.05	2.99	0.11	0.06	2.58	0.11	10.71
14:0	myristic acid	5.53	0.31	11.82	6.86	0.86	12.23	5.31
15:0 (iso)		0.09	1.38	0.19	0.10	2.13	0.18	9.42
14:1 (9)	myristoleic acid	0.42	0.93	0.91	0.51	1.63	0.90	5.61
15:0 (anteiso)		0.20	1.20	0.42	0.21	2.23	0.38	7.32
15:0		0.51	0.75	1.08	0.61	0.70	1.08	5.69
16:0 (iso)		0.14	0.80	0.30	0.16	0.70	0.28	10.72
16:0	palmitic acid	14.90	0.30	31.85	18.09	0.91	32.27	5.64
16:1 (trans 9)	palmitelaidic acid	0.02	3.61	0.05	0.02	3.00	0.04	10.97
16:1 (9)	palmitoleic acid	0.80	0.33	1.72	0.90	0.91	1.61	5.98
17:0 (iso)		0.14	2.02	0.30	0.17	1.11	0.30	9.14
17:0 (anteiso)		0.20	0.87	0.43	0.23	0.38	0.40	10.38
17:0		0.27	1.66	0.58	0.30	0.84	0.54	7.99
17:1 (9)		0.13	2.47	0.29	0.14	1.56	0.24	7.39
18:0 (iso)		0.03	0.93	0.06	0.03	3.64	0.05	11.32
18:0	stearic acid	4.36	0.52	9.32	5.38	0.91	9.60	6.40
18:1 (trans 6)		0.08	0.55	0.18	0.10	2.19	0.18	
18:1 (trans 9)	elaidic acid	0.09	1.59	0.19	0.08	1.88	0.15	12.71
18:1 (trans 10)		0.09	1.87	0.19	0.12	2.71	0.21	12.51
18:1 (trans 11)	vaccenic acid	0.45	1.02	0.96	0.46	1.56	0.82	7.21
18:1 (9)	oleic acid	9.89	0.36	21.13	11.00	0.95	19.62	6.64
18:1 (11)	cis-vaccenic acid	0.27	0.71	0.57	0.29	1.06	0.52	8.90
18:1 (12)		0.08	2.32	0.17	0.11	2.08	0.20	10.24
18:1 (13)		0.04	0.40	0.09	0.05	1.53	0.09	12.82
18:2 (9,12)	linoleic acid	0.78	0.83	1.66	1.10	0.88	1.96	6.82
18:3 (6,9,12)	y-linolenic acid	0.01	3.97	0.03	0.02	6.26	0.04	15.14
18:3 (9,12,15)	linolenic acid	0.26	0.85	0.55	0.31	0.74	0.55	7.75
20:0	arachidic acid	0.06	1.31	0.13	0.07	1.00	0.13	11.05
CLA (9c,11t)		0.26	1.18	0.55	0.26	1.39	0.47	7.55
CLA (10t,12c)		n.d.			n.d.			
CLA (9t,11t)		n.d.			n.d.			
20:1 (11)		0.02	2.70	0.03	0.02	0.83	0.04	11.46
21:0		n.d.			n.q.			
20:2 (11,14)		n.q.			0.01	10.80	0.02	
20:3 (8,11,14)		0.03	1.91	0.06	0.04	2.56	0.08	9.17
20:4 (5,8,11,14)	arachidonic acid	0.05	1.32	0.11	0.07	1.17	0.12	9.87
22:0	behenic acid	0.02	1.95	0.03	0.02	6.27	0.04	14.07
22:1 (13)	erucic acid	0.02	1.92	0.04	0.02	4.51	0.04	17.61
20:5 (5,8,11,14,17)		0.03	1.24	0.06	0.03	3.24	0.06	13.33
22:2 (13,16)		0.01	7.59	0.02	0.01	6.28	0.02	15.29
24:0	lignoceric acid	0.02	1.80	0.04	0.02	1.07	0.04	20.76
22:5 (7,10,13,16,19)		0.05	2.50	0.11	0.05	1.20	0.10	11.68

433 [a] determined as described in Section 2.2 and Section 2.3; [b] determined for milk type B; n.q., below QL, n.d. below
434 DL

Figurelegends

Fig. 1 Typical chromatogram of milk lipid FAME (milk type A).

(1) butyric acid, (2) caproic acid, (3) caprylic acid, (4) nonanoic acid (ISTD), (5) capric acid, (6) decenoic acid, (7) undecanoic acid, (8) TMSH, (9) lauric acid, (10) lauroleic acid, (11) anteiso-tridecanoic acid, (12) tridecanoic acid, (13) iso-myristic acid, (14) myristic acid, (15) iso-pentadecanoic acid, (16) myristoleic acid, (17) anteiso-pentadecanoic acid, (18) pentadecanoic acid, (19) iso-palmitic acid, (20) palmitic acid, (21) palmitelaidic acid, (22) palmitoleic acid, (23) iso-heptadecanoic acid, (24) anteiso-heptadecanoic acid, (25) heptadecanoic acid, (26) heptadecenoic acid, (27) iso-stearic acid, (28) stearic acid, (29) 9t-octadecenoic acid, (30) 10t-octadecenoic acid, (31) 11t-octadecenoic acid, (32) 9c-octadecenoic acid , (33) 11c-octadecenoic acid, (34) 12c-octadecenoic acid, (35) 13c-octadecenoic acid, (36) linoleic acid, (37) γ-linoleinic acid, (38) linolenic acid, (39) arachidic acid, (40) 9c11t-CLA, (41) 10c12t-CLA, (42) eicosenoic acid, (43) 9t11t-CLA, (44) heneicosanoic acid, (45) eicosadienoic acid, (46) eicosatrienoic acid, (47) arachidonic acid, (48) behenic acid, (49) erucic acid, (50) eicosapentaenoic acid, (51) docosadienoic acid, (52) lignoceric acid, (53) docosapentaenoic acid. See also Table 5 for specifications.

Fig. 2 Formation of CLA from linoleic acid during the derivatization process.

(36) linoleic acid, (38) linolenic acid, (40, 41 and 43) CLA; black line – unspiked milk sample; grey line - milk sample spiked with linoleic and linolenic acid as described in section 2.4 (milk type A).