Triglyceride Composition of Ewe, Cow, and Goat Milk Fat

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ABSTRACT: The separation and identification of the components in milk fat, which are mainly triglycerides, is a challenge due to its complex composition. A reverse-phase high-performance liquid chromatography (HPLC) method with gradient elution and light-scattering detection is described in this paper for the triglyceride analysis in ewes' milk fat. Triglyceride identification was carried out by combining HPLC, gas–liquid chromatography (GLC), and the calculated equivalent carbon numbers of several triglyceride standards. Quantitation of partially resolved peaks in the HPLC chromatogram was accomplished by applying a peak deconvolution program. Forty-four fatty acids were identified by GLC analysis, but only 19 were used for the following prediction of triglyceride molecular species; 181 triglycerides were identified, some of which were grouped at the same peak and needed application of the deconvolution program. Consequently, coefficients of variation were close to or lower than 5%. Moreover, the triglyceride composition of ewe, cow, and goat milk fat were compared by using these methods. These results show that ewe milk fat is richer in short- and medium-chain triglycerides, and cow milk fat is richer in long-chain and unsaturated triglycerides.
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KEY WORDS: Cow, ewe, fatty acids, GLC, goat, HPLC, milk fat, triglycerides.

Natural mixtures of triglycerides (TG) from oils and fats have usually been analyzed by reversed-phase high-performance liquid chromatography (HPLC). Milk fat, which contains a large number of different fatty acids, has presented a particular challenge to analysis in terms of the identification and separation of TG due to their complex variety of molecular species. The most complex mixtures of natural triacylglycerols require HPLC with gradient elution.

In the beginning, the refractive index detector was the most used detection system, although it has two important drawbacks: first, solvent gradients cannot be used, and second, it has low sensitivity and different responses to saturated and highly unsaturated TG (1). Moreover, use of the ultraviolet (UV) detector is difficult because the most adequate solvents also absorb in the same range and therefore cause an important baseline drift with gradient elution systems (2). In addition to these problems, different TG have nonuniform molar extinction coefficients; consequently, it is necessary to calculate their response factors (3).

Other authors have analyzed milk fat TG with a flame-ionization detector (FID) and with nonlinear gradient elution (4). They found that different TG responses were variable, although the variation was much less than with UV detection.

Introduction of the light-scattering detector has made the development of more efficient methods for the separation of natural complex mixtures of TG possible, including those of gradient elution. The evaporator temperature in the mass detector oven may influence the response of thermolabile compounds or compounds with low boiling points (5). Consequently, low temperatures would be desirable, provided that the evaporation of mobile-phase solvents is guaranteed. However, temperatures of 10 or 15°C above ambient are required for the detector to work correctly. References in the literature for the use of this detector in TG analysis are for temperatures ranging from 30 to 55°C (2,6–8), but there are no references to a study of TG response variation in relation to temperature.

Usually, mobile phases of acetonitrile and acetone have been used in the analysis of TG from milk fat, most often in isocratic elution (9–12) and in gradient elution, and they provide a resolution of 50 chromatographic peaks (4). One of the main difficulties in the analysis of TG is the identification of the chromatographic peaks because of the small number of mixed TG in a pure state. Bornaz *et al.* (11) and Dotson *et al.* (12) identified butterfat chromatographic peaks from the relationship between the retention time and the theoretical carbon number according to the model proposed by El-Hamdy and Perkins (13). An alternative method is the fractionation of total TG in milk fat by reversed-phase HPLC and analysis of the fatty acids in each fraction (10,14). Some authors have used a combination of silver-ion and reversed-phase HPLC in sequence to obtain information concerning the composition of the molecular species samples with a high amount of polyunsaturated fatty acids (15,16). Recently, HPLC linked with mass spectrometry (MS) has been demonstrated as a powerful tool in separation and identification of TG in milk fat (17).

Another problem is quantitative analysis of a multicomponent mixture, because we have to take into account the fact

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that only a small fraction of the components will appear as isolated peaks, in spite of using extremely efficient columns. For this reason, different deconvolution techniques have been elaborated (18). Meyer (19) has shown the deviation from the true area that is a result of integration as a vertical drop at the lowest point of the valley, which is usually performed by an integrator.

In this work, a reversed-phase HPLC method with a light-scattering detector is described for the analysis of TG in milk fat. The identification of TG was carried out by a combination of HPLC and gas–liquid chromatography (GLC), and was based on the equivalent carbon numbers and retention times of different standard TG. Finally, quantitation of peak areas from HPLC chromatograms was carried out after applying a deconvolution program to the parts of chromatograms with poor resolution.

EXPERIMENTAL PROCEDURES

Reagents and standards. All reagents were of analytical grade. Acetonitrile, acetone, ethanol, and hexane were purchased from Scharlau (Barcelona, Spain). Methanol, *n*-heptane, and potassium hydroxide were purchased from Merck (Darmstadt, Germany).

Fatty acid methyl esters (FAME) and TG used as standards were approximately 99% pure. Methyl esters of butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic, and arachidic acids were purchased from Poly-Science Corporation (Niles, IL). Methyl esters of *trans*-vaccenic and α-linolenic acids, homogeneous TG tributyrin, tricaproin, tricaprylin, tripelargonin, tricaprin, trilaurin, tritridecanoin, trimyristin, trimyristolein, tripentadecanoin, tripalmitin, tripalmitolein, trimargarin, tristearin, triolein, trilinolein, trilinolenin, trinonadecanoine, and mixed TG 1,2-dilauroyl-3-myristoyl glycerol, 1,2-dimyristoyl-3-lauroyl glycerol, 1,2-dilinoleoyl-3-oleoyl glycerol, 1,2-dimyristoyl-3-oleoyl glycerol, 1,2-dimyristoyl-3-palmitoyl glycerol, 1,2-dipalmitoyl-3-myrtistoyl glycerol, 1,2-dioleoyl-3-palmitoyl glycerol, 1,2-dipalmitoyl-3-oleoyl glycerol, 1,2-dioleoyl-3-stearoyl glycerol, 1-oleoyl-2-palmitoyl-3-stearoylglycerol, 1,2-distearoyl-3-myristoyl glycerol, 1,2-distearoyl-3-oleoyl-glycerol, and 1,2-distearoyl-3-palmitoyl glycerol were purchased from Sigma Chemical (St. Louis, MO).

Sample preparation. Samples of raw ewes' milk were taken from a single dairy (Complejo Agropecuario Comunidad de Madrid, Aranjuez, Spain), raw cows' milk from a single dairy (La Chirigota, Majadahonda, Spain), and raw goats' milk from another dairy (Queserías Ibéricas, Fuenlabrada, Spain). Cream was separated from milk by centrifugation, and TG were extracted with *n*-hexane as described in a previous paper (14).

TG HPLC analysis. The extracts dissolved in hexane were exposed to a stream of N_2 and evaporated to dryness under low pressure at 30°C. The residue was redissolved in HPLC-grade hexane and filtered.

The HPLC method used is based on a previously developed method (20). Two stainless-steel columns, 25 cm and 15 cm × 4.6 mm i.d. with 3 μm Spherisorb ODS-2 (Phase Separations, Queensferry, United Kingdom; Symta, Madrid, Spain) were connected in series. The mass detector (ACS 750/14; The Arsenal, Macclesfield, United Kingdom) drift tube temperature was 45°C, and the inlet gas pressure was 1.38 bar. The mobile phase consisted of a gradient elution from 0 to 70% (vol/vol) HPLC-grade acetone in HPLC-grade acetonitrile, in two stages—a linear increase of 0.7%/min in acetone for the first 50 min, an isocratic elution for 20 min, a second linear increase of 0.7%/min in acetone for another 50 min and, finally, an isocratic elution for 42 min until the end of the analysis. The flow rate was 0.9 mL/min, and the pressure was 172 bar. The gradient and data acquisition from the mass detector were controlled by the System Gold program (Beckman, San Ramón, CA).

The effect of the mass detectors' drift tube temperature on the low-molecular-mass TG was studied. Solutions of 10 mg/mL of tributyrin, tricaproin, tricaprylin, tricaprin, and trilaurin were injected twice at each of the following drift tube temperatures: 20, 25, 30, 45, and 60°C.

Solutions of 10 mg/mL of tricaprylin, tricaprin, trilaurin, trilinolein, trimyristin, tripalmitin, and tristearin (Sigma Chemical) were prepared in duplicate to test the linear behavior response. Mixtures of these were injected twice at the following amounts of each mixture: 3, 6, 9, 10.5, 12, 15, 20, and 25 μg. The regressions were obtained by using 1R analysis of BMDP Statistical Software, Inc. (Los Angeles, CA).

Five replications of the HPLC analysis were performed for one sample of ewe milk fat to determine the reproducibility of the HPLC method. Quantitation of peak areas in the TG chromatogram was carried out with a deconvolution program (Peakfit V3.11B; Jandel Scientific, GmbH, Erkrath, Germany).

Preparation of fatty acid methyl esters (FAME) for GLC. TG were placed in a 1-mL micro-reaction vial and evaporated to dryness under a stream of N_2.

Sample preparation was a modification based on a previous method (21): 20 μL *n*-heptane and 10 μL methanolic 2N KOH were added to the TG concentrate, and the mixture was stirred for 1 min; 0.1 μL of the sample was injected after 20 min. Tripelargonin was added to the sample as an internal standard (150 μg/mL into each HPLC fraction) for short-chain FAME response factor calculations, and palmitic acid methyl ester, contained in the sample, was used for unsaturated FAME response factor calculations. The response factors were assumed to be equal to 1 for the rest of FAME.

Gas chromatography (GC) analysis. The GC/FID unit used was an HRGC 5160 Mega Series (Carlo Erba, Milano, Italy), equipped with a split/splitless injector and an FID. The GC/MS was a 5890 Series II with a 5971A Mass Selective Detector both from Hewlett-Packard (Palo Alto, CA).

A fused-silica capillary column, 24 m × 0.23 mm i.d., coated in the lab with SP-1000 (Supelco, Inc., Bellefonte, PA), was used for both GC/FID and GC/MS analysis. The conditions were as follows: injector at 275°C, detector at 250°C, column initial temperature 40°C held for 4 min, then

programmed to increase at 10°C/min to 150°C, at 2°C/min to 200°C, and held at 200°C for 60 min to the end of analysis. The samples were injected in the splitless mode and held for 20 s.

Identification of fatty acids was made by using TG and FAME standards and those found in the literature (22,23), and was confirmed by GC/MS.

Calculation of the TG composition. The TG composition was estimated in accordance with the method described previously (14), based on the calculation of the equivalent carbon numbers (ECN) of the HPLC chromatographic peaks and in the molar composition in fatty acids, analyzed by GLC, collected at the HPLC chromatograph outlet. HPLC fractions (227) were collected every 40 s at the outlet of the column after 14 min; there were no peaks before that time.

The ECN was calculated according to the formula given by Herslöf *et al.* (24):

$$ECN = CN - (a' \times ND) \quad [1]$$

where *CN* is the total carbon number of the three fatty acids and *ND* the number of double bonds in the TG molecules. The value of constant a' was calculated by multiple linear regression analysis of the experimental values of the dependent variable, log k', and the independent variables *CN* and *ND* for the TG available in pure form having been injected seven times [$\log k' = q' + b'CN + c'ND$], where a' is quotient of the coefficient c' and coefficient b'.

Finally, a simple linear regression was applied to relate ECN to log k'. These linear regressions were obtained by 1R analysis of BMDP/DYNAMIC Release 7.0 (1992) (BMDP).

The percentage in which possible TG can be found in every fraction is estimated by the molar percentage of every TG. These molar percentages are calculated from the fatty acids in every fraction percentage, taking into account that the three positions in the glycerol are equivalent (25–27). This fact is justified because HPLC analysis cannot separate position isomers.

RESULTS AND DISCUSSION

Response of TG in the mass detector. The present experiments have shown that the response of TG with ECN between 18 and 36 (tributyrin, tricaproin, tricaprylin, tricaprin, and trilaurin) in the range of 20 to 60°C in the drift tube differs according to the individual TG (Fig. 1).

Tributyrin response decreased with the increase in temperature and was undetectable at 60°C. Tricaproin response remained steady up to 45°C and decreased at higher temperatures. No variation was found in the response factor for TG with ECN ≥24 within the temperature range studied. Bearing in mind all of the abovementioned items, plus the fact that TG with ECN <22 have not been described in milk (11,14,17), the temperature chosen for the detector drift tube was 45°C. However, the drift tube temperature had to be decreased to 20°C to measure retention time of pure tributyrin for the ECN calculation.

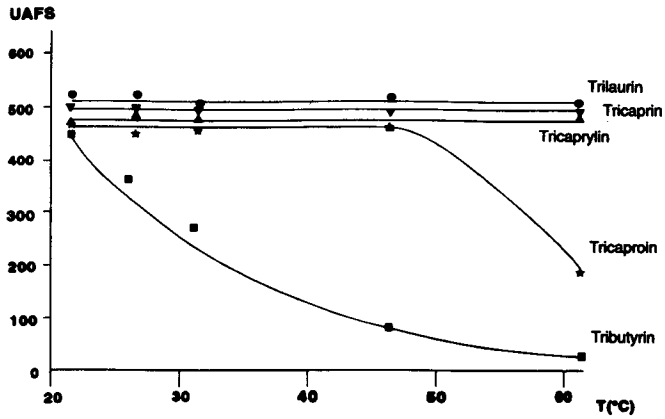

FIG. 1. Response of triglyceride standards in the mass detector.

GLC results. Forty-three fatty acids were identified by the GLC analysis, but only those in >0.5% amount in the whole fat were used to predict the TG composition of milk fat (butyric, caproic, caprylic, capric, lauric, myristic, myristoleic, *anteiso*-pentadecanoic, pentadecanoic, palmitic, palmitoleic, *iso*-margaric, *anteiso*-margaric, margaric, stearic, oleic, *trans*-vaccenic, linoleic, and α-linolenic). Figure 2 shows the GC/MS chromatogram for fatty acids of ewe whole milk fat.

Identification of TG. Figure 3 shows the HPLC chromatogram for TG of cow, ewe, and goat milk fat. The three species (ewe, cow, and goat) show chromatograms with 111 peaks (Fig. 3).

In our study, the estimate of ECN was carried out with two different groups of equations because of the complicated HPLC gradient-method used: one for the first gradient and isocratic period, for 70 min, and the other for the second gradient and isocratic period until the end of the analysis. At first, we tried to calculate the ECN with one group of equations, but the problem was that most of the TG eluted in ECN-ascending order, as expected. However, certain series of critical pairs eluted in ECN-descending order.

Standards with retention times shorter than 70 min (tributyrin, tricaproin, tricaprylin, tripelargonin, tricaprin, trilinolenin, trimyristolein, and trilaurin) were used to calculate the first a' coefficient. The resulting equations were as follows:

$$\log k' = -0.81130 + 0.06025CN - 0.1280ND \quad [2]$$

$$ECN = CN - 2.12ND \quad [3]$$

$$\log k' = -0.81143 + 0.06025ECN \text{ (SE} = 0.0289) \quad [4]$$

The second a' was calculated from the rest of the TG standards retention times (1,2-dilauroyl-3-myristoyl glycerol, tritridecanoin, 1,2-dimyristoyl-3-lauroyl glycerol, trilinolein, tripalmitolein, trimyristin, 1,2-dilinoleoyl-3-oleoyl glycerol, 1,2-dimyristoyl-3-oleoyl glycerol, 1,2-dimyristoyl-3-palmitoyl glycerol, tripentadecanoin, 1,2-dipalmitoyl-3-myristoyl glycerol, triolein, 1,2-dioleoyl-3-palmitoyl glycerol, 1,2-di-

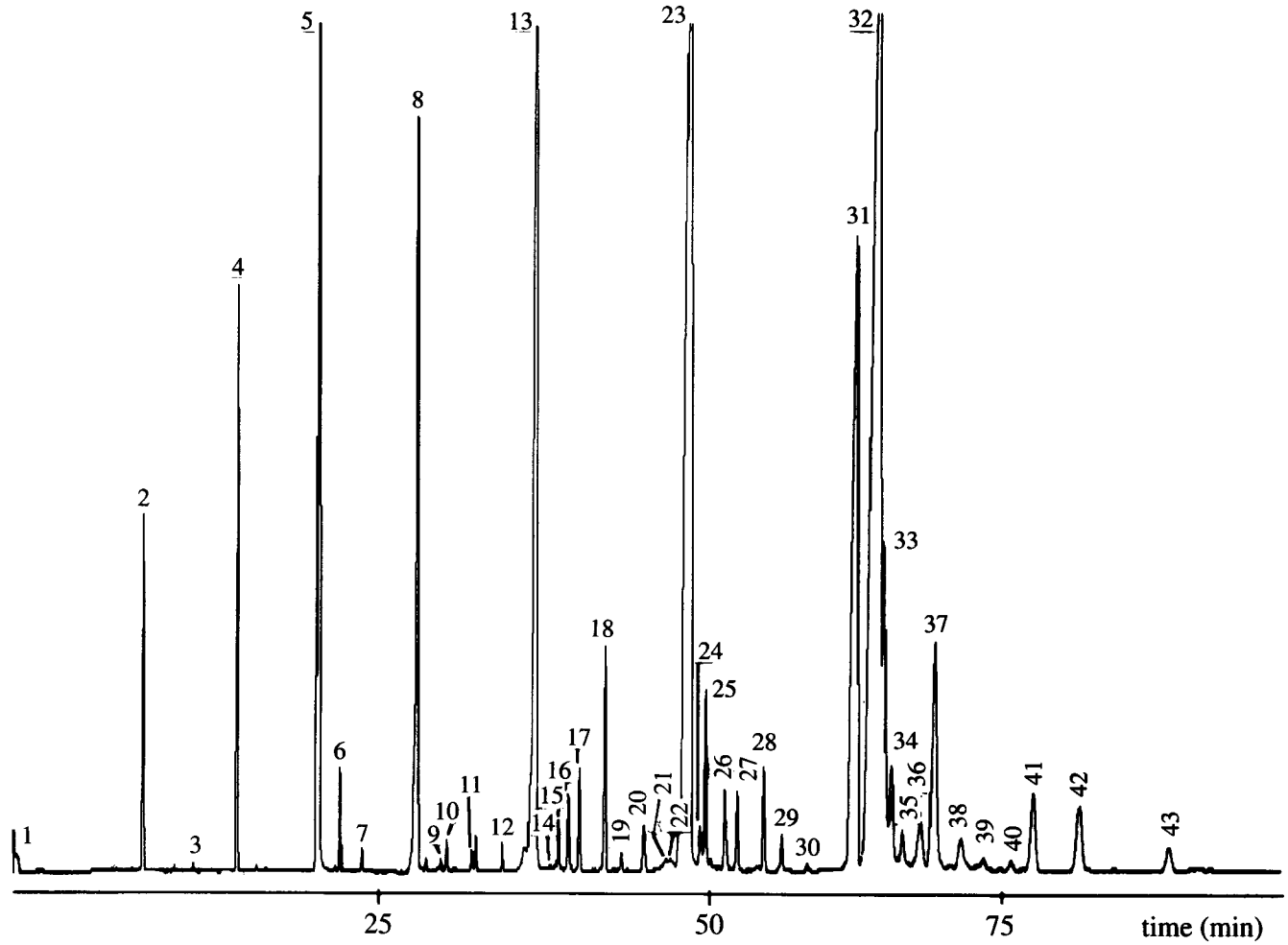

FIG. 2. Gas–liquid chromatographic analysis of ewe milk fatty acid methyl esters: **1:** 4:0; **2:** 6:0; **3:** 7:0; **4:** 8:0; **5:** 10:0; **6:** 10:1; **7:** 11:0; **8:** 12:0; **9:** 12:1; **10:** 12:1; **11:** 13:0; **12:** *i*-14:0; **13:** 14:0; **14:** 14:1; **15:** 9*c*-14:1; **16:** *ai*-15:0; **17:** *i*-15:0; **18:** 15:0; **19:** 15:1; **20:** *i*-16:0; **21:** 16:0 (branched); **22:** 16:0 (branched); **23:** 16:0; **24:** 16:1; **25:** 9*c*-16:1; **26:** *ai*-17:0; **27:** *i*-17:0; **28:** 17:0; **29:** 17:1; **30:** *i*-18:0; **31:** 18:0; **32:** 9*c*-18:1; **33:** 11*t*-18:1; **34:** 18:1; **35:** 18:1; **36:** 18:2; **37:** 9*c*,12*c*-18:2; **38:** 18:2; **39:** 19:0; **40:** 19:1; **41:** 9*c*,12*c*,15*c*-18:3; **42:** conjugated *ct*-18:2; **43:** 20:0.

palmitoyl-3-oleoyl glycerol, tripalmitin, 1,2-dioleoyl-3-stearoyl glycerol, 1-oleoyl-2-palmitoyl-3-stearoyl glycerol, 1,2-distearoyl-3-myristoyl glycerol, trimargarin, 1,2-distearoyl-3-oleoyl glycerol, 1,2-distearoyl-3-palmitoyl glycerol and tristearin). The results obtained were:

$$\log k' = 0.72097 + 0.01919CN - 0.04121ND \quad [5]$$

$$ECN = CN - 2.15ND \quad [6]$$

$$\log k' = 0.72093 + 0.01919ECN \text{ (SE} = 0.0151) \quad [7]$$

Application of these two equations reduced the number of possible TG to those containing adequate molecular parameters (*CN*, *ND*) for each peak in their retention time.

GLC analysis of fatty acids present in each of the fractions drastically reduced the number of molecular species estimated for each peak, and in some instances it limited the possibility to a single TG. The composition of the TG in each fraction was calculated from the percentage mole fraction of the main fatty acids in each fraction, taking into consideration the fact that the most probable species had values higher than 0.01% and that their ECN were included between the limit values of ECN of each fraction. When TG standards were available, retention times for their identification were considered.

The identification of some peaks clearly showed the problem that certain molecular species could correspond to more than one fraction. Taking into consideration the elution order, along with plotting the probability of finding one specific TG in one specific fraction, TG assignment was possible. Figure 4 shows the corresponding plotting of the TG with $45 < ECN \leq 46$. This figure shows that more than one maximum can exist for each TG. Nevertheless, the elution order assigned according to the ECN value in which TG must be found allows peak assignment, eluting the most unsaturated first and the saturated ones last.

One hundred and eighty-one molecular species of TG have been identified: 79 of them were saturated, 44 monounsaturated, and 58 polyunsaturated. The majority of the unsaturated

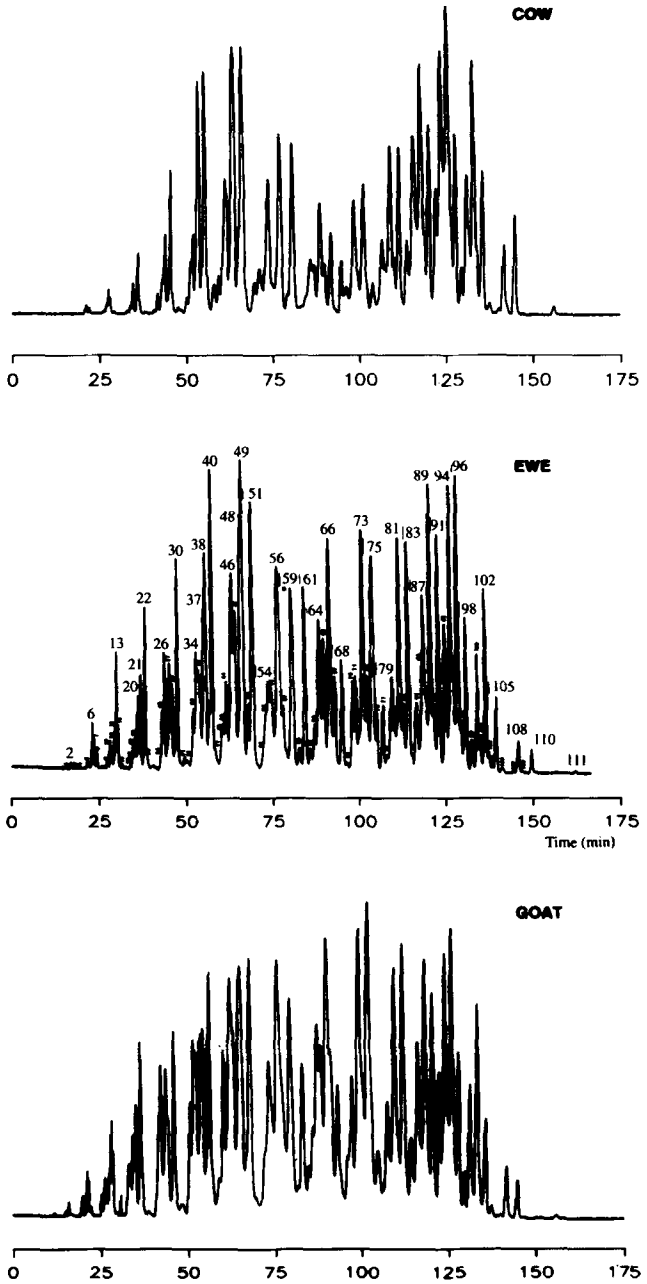

FIG. 3. High-performance liquid chromatographic analysis of cow, ewe, and goat milk triglycerides. The numbers of the peaks correspond to Table 1.

TG (61) contained only one unsaturated fatty acid, 41 contained two, and 5 had all three fatty acids unsaturated. Furthermore, ten TG that contained linear or branched odd-carbon number fatty acids have been identified. In Table 1, identified species are mentioned with retention times and peak numbers corresponding to the chromatogram in Figure 3.

This modified HPLC method has allowed us to improve the separation of peaks and, consequently, the TG assignment after the fatty acid GLC analysis of the fractions.

One hundred and fifty-one molecular species of TG have been described previously in milk fat (11,14,17); the other thirty are described in our analysis for the first time.

Due to the fact that this identification of TG is based not only on the estimation of ECN, but also on a fatty acids analysis of HPLC fractions of the TG, some of the species described previously by other authors have not been found in this study. Bornaz *et al.* (11) show 26 molecular species that contained linolenic acid. However, our study only shows 9, and they contained other fatty acids in higher amounts. The rest of them are not shown because they were found at less than 0.01%.

Our study agrees with the Myher *et al.* (17) estimation in the identification of TG with odd-carbon number fatty acids ($CN = 15$ and 17), both branched or linear. However, we have been able to differentiate between *iso* and *anteiso* isomers, not only in the GLC analysis of fatty acids but also in TG estimation. Tridecanoic and nonadecanoic acids were identified by GLC but were not included in TG estimation due to their low amounts in whole milk fat content.

In agreement with Barrón *et al.* (14), *trans*-vaccenic acid was taken into account, because it was found at 1.9% in the fatty acids analysis of the total TG fraction. The abovementioned study detected 116 TG molecular species instead of our 181, because they only considered 14 fatty acids for the calculation of the composition due to the lower sensitivity of their GLC analysis.

Quantitative analysis: response linearity. In relation to the response linearity, when the peak area for the standard TG studied was plotted vs. the amount injected, the relation was not linear. These results agree with those of other authors (5,26,27), who found that the response (A) is proportional to the injected amount (m) raised to a power ($A = am^x$). The exponent (x) is closely linked to the nebulizer shape (pressure and temperature conditions in the evaporator) and, consequently, is not dependent on the structure of the component detected (28). Christie (29) found for a wide variety of lipid classes that the detector response was approximately linear in the range of 50 to 200 µg, but tended to tail off rapidly below 10 µg. Table 2 shows the estimated equations for the log (area) relation (log A) vs. log *(amount)* injected (log c) for different standard TG, jointly with the correlation coefficients. The line slopes, which are the exponent value, are between 1.1 and 1.5.

The Student "t" analysis, applied to compare the lines, showed that there were significant differences among them because $P > 0.05$. This would indicate that the TG response is different, not because of structure but rather because of large differences in the retention times of the TG. The use of an internal standard would only have been possible if we had had all estimated TG as pure compounds to calculate their response factors. In conclusion, quantitative analysis with an internal standard was rejected and was carried out by taking the peak area percentages in the total chromatogram.

Precision of method. Table 3 shows the precision results of the quantitative HPLC analysis of a ewe milk fat sample, which was repeated five times. Peak numbers in Table 3 coincide with those in Figure 3.

It was not possible to quantitate the TG in the milk fat in

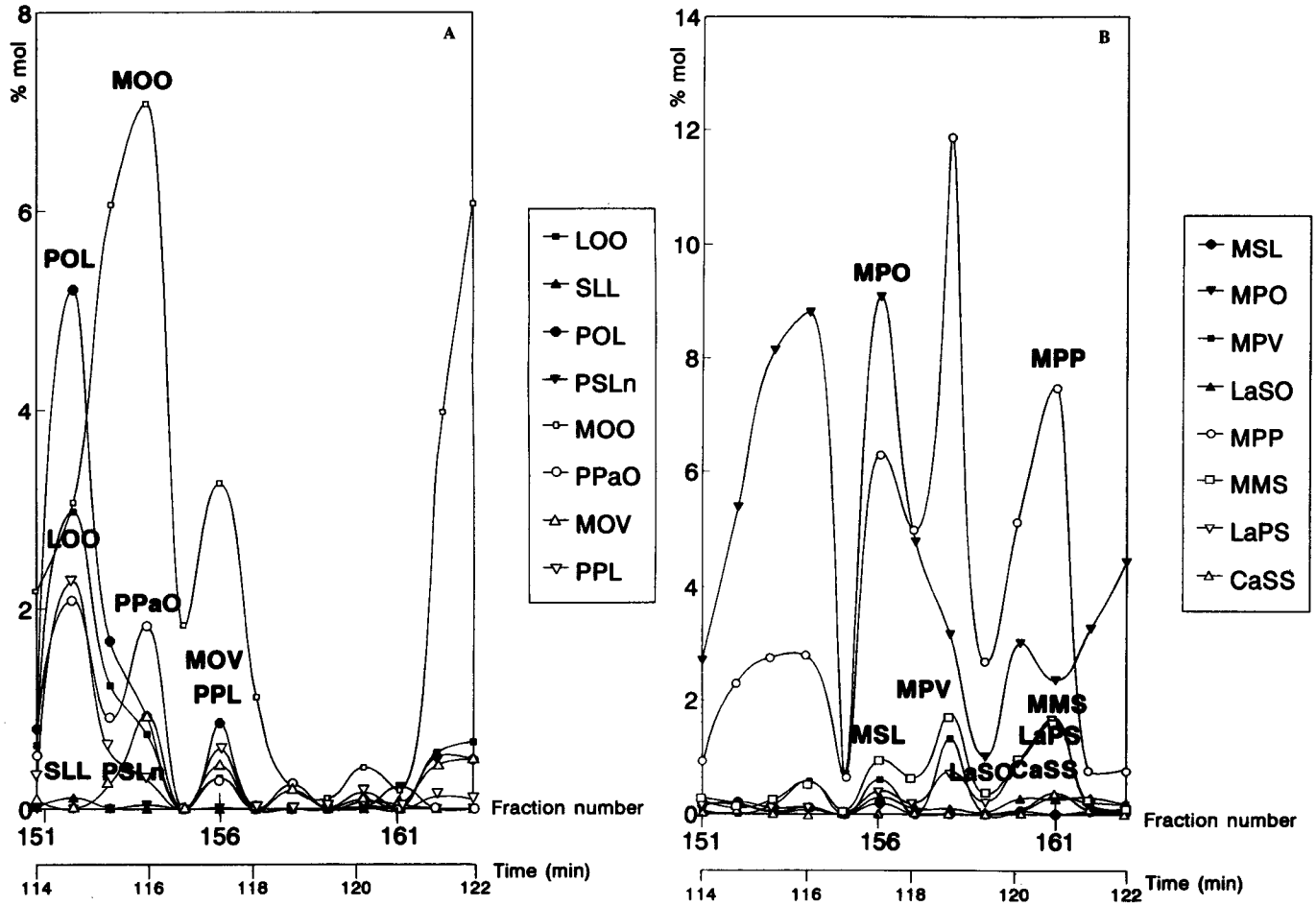

FIG. 4. Probability of finding triglycerides corresponding to 45 < equivalent carbon number ≤ 46 in ewe milk fat. **LOO:** dioleoyl-linoleoyl glycerol; **SLL:** dilinoleoyl-stearoyl glycerol; **POL:** palmitoyl-oleoyl-linoleoyl glycerol; **PSLn:** palmitoyl-stearoyl-linolenoyl glycerol; **MOO:** dioleoyl-myristoyl glycerol; **PPaO:** palmitoyl-palmitoleoyl-oleoyl glycerol; **MOV:** myristoyl-oleoyl-vaccenoyl glycerol; **PPL:** dipalmitoyl-linoleoyl glycerol; **MSL:** myristoyl-stearoyl-oleoyl glycerol; **MPO:** myristoyl-palmitoyl-oleoyl glycerol; **MPV:** myristoyl-palmitoyl-vaccenoyl glycerol; **LaSO:** lauroyl-stearoyl-oleoyl glycerol; **MPP:** dipalmitoyl-myristoyl glycerol; **MMS:** dimyristoyl-stearoyl glycerol; **LaPS:** lauroyl-palmitoyl-stearoyl glycerol; **CaSS:** distearoyl-capryl glycerol.

terms of individual molecular species because some of the HPLC peaks contained more than one molecular species. The TG were, therefore, quantitated according to peaks, although, in some cases, the poorly resolved peaks (usually those that were shoulders of other peaks) were quantitated jointly, provided that the molecular species were similar. For example, peaks 39 and 40 were quantitated together because short-chain and saturated TG with ECN = 34 had been estimated for both.

Moreover, peaks were jointly quantitated by the integrator, and then the proportions of each peak were estimated as different TG if needed. In group 72–73, saturated long-chain TG (trimyristin, lauroyl-myristoyl-palmitoyl glycerol) were quantitated with monounsaturated long-chain TG (caprylyl-stearoyl-oleoyl glycerol, myristoyl-myristoleoyl-palmitoyl glycerol).

An estimate of the peak contribution to the total area can be obtained by using a package for mathematical deconvolution of poorly resolved peaks (Peakfit; Jandel Scientific). This program adjusts the Gaussian function area for each peak to the total area from the selected part of the chromatogram, minimizing the difference between the real area and the estimated area with Gaussians. The fitting of Gaussians to the peak's group mentioned above is shown in Figure 5. The areas and their percentages in relation to the selected chromatogram for each peak are presented in Table 4. In this way, the corresponding areas to the monounsaturated TG caprylyl-stearoyl-oleoyl glycerol and myristoyl-myristoleoyl-palmitoyl glycerol and saturated trimyristin and lauroyl-myristoyl-palmitoyl glycerol were able to be measured.

Table 3 shows that the analysis of a ewe milkfat sample, repeated five times, yielded coefficients of variation (CV) below 5%, with the exception of some peaks. In general, the values of CV above 5%, which have small medium values, can be accepted as valid. For example, peaks 1–3 show small percentage values in the sample and, thus, as shows in Figure 3, the signal due to the noise detector interferes with their integration. This can justify their higher CV values.

However, there are other peaks with CV higher than 5% (which are marked with a *b* in Table 3), and for them, that ex-

TABLE 1
Identified Triglycerides (TG) in Ewe Milk Fat[a]

Peak number	Retention time (min)	TG	Peak number	Retention time (min)	Peak TG	Peak number	Retention time (min)	TG
1	15.658	BuBuP	47	63.140	BuPO	75	102.663	CyPS
2	16.602	CoCoM			BuPV			CaMS
3	17.592	BuCoP	48	64.758	CoMP	76	103.922	CoSS
4	19.017	BuBuS			CaCaP	77	105.654	OLL
5	21.282	BuCaL	49	65.084	BuPP			OOLn
		BuCyO			BuMS			SLLn
6	22.614	BuCaM	50	66.858	CoPdO	84	114.098	CySS
7	23.045	BuCyP			Bu*ai*MaO			PLL
8	23.927	BuCoS			Bu*i*MaO	85	115.205	LOO
9	26.805	CoCyO			BuMaO			SLL
10	27.632	BuCaO	58	77.800	LaLaM			POL
11	28.112	CoCyP			CaLaM	86	116.833	PSLn
12	29.017	BuLaM			CyLaP	87	117.232	MOO
13	29.329	BuCaP			CoLaS	88	118.050	PPaO
14	29.908	BuCyS	52	71.308	CaOLn			MOV
15	31.033	BuLaPd	53	71.958	CyOL	89	119.204	PPL
16	33.300	CoMyL	54	72.470	CoOO			MSL
17	34.008	BuML	55	73.279	CoSL			MPO
18	34.258	CoCaO			CaML			MPV
19	34.782	BuLaO			CyPL	90	120.220	LaSO
		BuMyP	56	75.253	CoPO	91	121.571	MPP
20	35.393	CyCaM			BuSO			MMS
		CoLaM	57	76.827	CyMO			LaPS
21	36.213	CoCaP			CyLaS			CaSS
22	37.427	BuMM			CaMPa	92	123.374	OOO
		BuLaP	65	88.462	CyPO	93	123.883	LOS
23	38.242	BuCaS			CaMM	94	125.013	POO
24	41.992	CaLnLn	59	79.345	CyMP	95	125.831	SLP
25	42.344	CoOLn			CoPP	96	127.079	PPO
		BuOL			BuPS	97	127.975	MSO
		CyMyPa			CaLaP			PPV
26	42.922	CaCaL	60	81.592	CaPaO	98	129.358	PPP
		CyLaL	61	83.021	CyOO			MPS
27	44.349	CoML	62	84.705	LaML	99	131.123	LaSS
		BuPL			CaPL	100	132.587	SOO
28	45.006	CyCaO	63	85.931	LaLaO	101	133.421	SOV
29	45.983	CoLaO	64	87.193	CaMO	102	134.924	PSO
30	46.495	BuMO			CaMV	103	135.978	PSV
		BuPPa	70	96.852	LaMO	104	137.041	N.I.
31	48.725	BuMyS			CoSO	105	138.014	PPS
32	49.650	N.I.	66	90.191	LaLaP	106	139.829	MSS
33	51.119	CaLaLa			LaMM	107	143.005	PdSS
34	51.962	CaCaM			CaMP	108	144.391	SSO
35	52.596	CyLaM			CaLaS	109	145.142	SSV
36	53.191	CyCyS			CyMS	110	148.111	PSS
37	54.027	CyCaP			CyPP	111	160.683	SSS
38	54.619	CoLaP	67	91.679	CoPS			
		CoMM			BuSS			
39	55.908	CoCaS			CaPd			
40	56.421	BuMP	68	93.723	CaOO			
		BuLaS			LaOL			
41	57.425	Bu*ai*PdP	69	95.348	LaPL			
		BuPdP			MML			
		BuPdP			CaSL			
		CaCaPd			PaOL			
42	59.550	CyOLn	71	97.712	CaPO			
		CoOL	72	98.925	CySO			
43	59.746	CaMLn			MMyP			
45	60.817	BuOV	73	99.827	MMM			
51	67.890	LaLaLa			LaMP			
46	62.248	CoPL	74	101.043	CaPP			
		CoMO			LaLaS			

[a]Bu: butyric; Co: caproic; Cy: caprylic; Ca: capric; La: lauric; M: myristic; My: myristoleic; *ai*Pd: *anteiso*-pentadecanoic; Pd: pentadecanoic; P: palmitic; Pa: palmitoleic; *ai*Ma: *anteiso*-margaric; *i*Ma: *iso*-margaric; Ma: margarie; S: stearic; O: oleic; V: *trans*-vaccenic; L: linoleic, Ln: linolenic.

TABLE 2
Estimated Equations for Standard Triglycerides (TG) in the Linearity Study of the Mass Detector

TG	Equation	R^{2a}	RSE[b]
Tricaprylin	Log A = 0.794 + 1.25 × Log c	0.977	0.055
Tricaprin	Log A = 0.628 + 1.48 × Log c	0.972	0.073
Trilaurin	Log A = 0.779 + 1.32 × Log c	0.964	0.074
Trilinolein	Log A = 0.701 + 1.42 × Log c	0.974	0.069
Trimyristin	Log A = 0.784 + 1.34 × Log c	0.975	0.063
Tripalmitin	Log A = 0.977 + 1.16 × Log c	0.970	0.062
Tristearin	Log A = 0.577 + 1.51 × Log c	0.972	0.076

[a]R^2 = coefficients of correlation.
[b]RSE = relative standard error.

planation is not valid. For instance, peaks 10, 14, and 41 show percentage values in the sample higher than 0.1%, and their peak height is so great that their integrations are not affected by the noise level. The CV values suggest that there must be an error in the integration of these peak areas, either because of the integration method or because the peaks are not Gaussian (they contained more than one TG). With the aim of solving this problem, the Peakfit deconvolution program was applied.

Table 5 shows the new values of CV percentages for the peaks with a *b* in Table 3 after application of Peakfit. In all instances, CV were lower than or close to 5%, except for peaks 41 and 69 (with 9.52 and 7.42%, respectively). However, in these cases, the percentages in the sample were small (0.0002 and 0.0005, respectively).

In addition, peak 5 was composed of two poorly resolved peaks, which coincides with the results of qualitative analysis because butyryl-capryl-linoleoyl glycerol and butyryl-caprylyl-oleoylglycerol were estimated for peak 5. The estimate of separated areas of two Gaussians for this peak gives CV values below 4%.

TABLE 3
Reproducibility Results of High-Performance Liquid Chromatographic Method

Peak number	% Peak area	CV (%)[a]	Peak number	% Peak area	CV (%)
1	0.008	20.37	59	2.71	0.35
2	0.03	19.30	60[b]	0.24	8.30
3	0.008	21.00	61	1.91	0.78
4	0.001	2.30	62	0.23	4.04
5[b]	0.04	11.50	63	0.45	5.10
6[b]	0.16	6.13	64	2.13	2.72
7	0.15	3.33	65	1.63	2.60
8[b]	0.08	11.10	66	3.25	0.90
9[b]	0.035	16.48	67	1.09	2.40
10[b]	0.12	8.14	68	1.16	1.50
11[b]	0.095	6.07	69[b]	0.08	7.80
12 + 13	0.76	2.76	70[b]	0.9	9.40
14[b]	0.21	11.53	71	0.87	4.97
15[b]	0.016	17.18	72 + 73	3.60	1.20
16 + 17 + 18	0.18	5.50	74	1.01	6.83
19	0.17	2.94	75	3.24	1.45
20	0.38	3.42	76[b]	0.49	6.70
21	0.61	0.82	77	0.69	4.75
22 + 23	1.22	1.15	78[b]	0.16	8.10
24 + 25	0.36	2.66	79	1.30	4.20
26	0.79	2.42	80	0.47	3.62
27	0.91	4.26	81	3.06	1.34
28	0.65	2.61	82	0.72	3.19
29 + 30	1.66	3.07	83 + 84	2.84	1.16
31[b]	0.05	9.80	85	0.99	4.34
32[b]	0.03	8.80	86 + 87 + 88	3.10	1.16
33	0.40	4.50	89 + 90	3.99	0.43
34	1.13	1.70	91	2.32	1.90
35	0.80	2.87	92 + 93	1.48	2.03
36	0.45	2.44	94	2.80	1.43
37	1.04	1.15	95	0.97	5.56
38	1.89	0.85	96	3.10	1.97
39 + 40	3.02	1.05	97	0.42	5.27
41[b]	0.12	11.10	98	1.60	2.74
42	0.22	5.42	99[b]	0.34	7.65
43	0.26	4.25	100	0.89	5.17
44 + 45[b]	1.01	7.90	101	0.37	4.62
46	2.75	2.80	102	1.92	1.77
47	1.53	3.46	103[b]	0.19	8.95
48 + 49	5.30	0.28	104 + 105	0.74	2.31
50	0.58	2.59	106[b]	0.085	11.57
51	3.28	1.43	107[b]	0.019	10.96
52 + 53 + 54	1.41	4.11	108 + 109	0.36	4.66
55	1.34	0.75	110	0.21	4.62
56	3.81	1.18	111	0.03	5.10
57 + 58	0.91	5.93			

[a]CV, coefficient of variation.
[b]Peaks with CV higher than 5%.

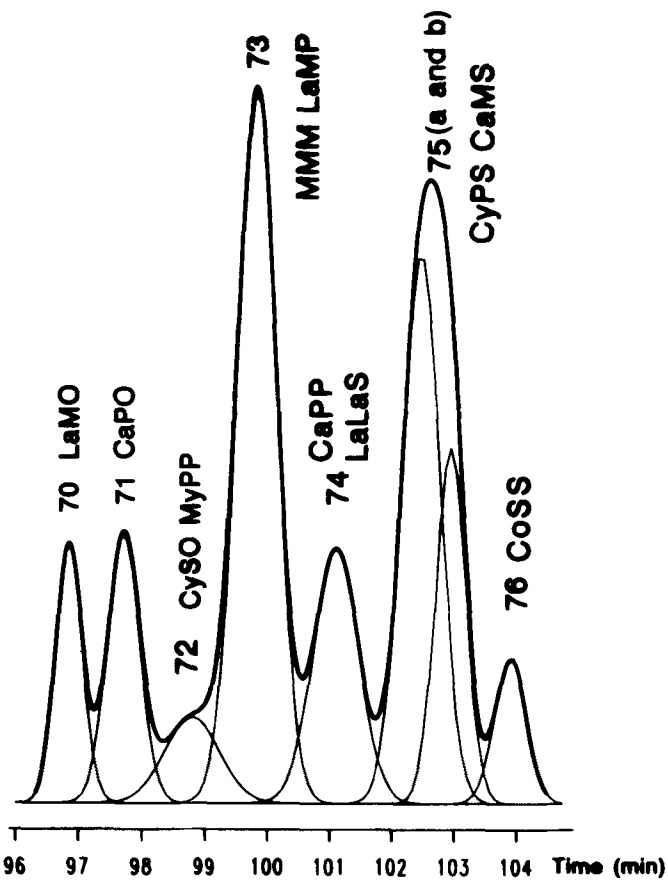

FIG. 5. Fitting of Gaussians to the 72–73 peak groups. **LaMO:** lauroyl-myristoyl-oleoyl glycerol; **CaPO:** capryl-palmitoyl-oleoyl glycerol; **CySO:** caprylyl-stearoyl-oleoyl glycerol; **MMyP:** myristoyl-myristoleoyl-palmitoyl glycerol; **MMM:** trimyristin; **LaMP:** lauroyl-myristoyl-palmitoyl glycerol; **CaPP:** dipalmitoyl-capryl glycerol; **LaLaS:** dilauroyl-stearoyl glycerol; **CyPS:** caprylyl-palmitoyl-stearoyl glycerol; **CaMS:** capryl-myristoyl-stearoyl glycerol; **CoSS:** distearoyl-caproyl glycerol.

One fact that needs mentioning is that peak 41, at first, was quantitated, by applying the simplest graphical methods of the integrator as perpendicular drops, giving an area percentage of 0.12 (Table 3). However, when Gaussian functions were adjusted, peak 41 was a small peak that elutes at the tail of peak 40, with an area percentage of 0.0002. This last value agreed with the molecular species described in the peak [butyryl-*anteiso*-margaroyl-palmitoyl glycerol, butyryl-pentadecanoyl-palmitoyl glycerol, and dicapryl-pentadecanoyl glycerol) that contain odd fatty acids, which were minority acids.

TABLE 4
Peak Areas and Percentage Calculated from the Gaussian Curve Fit to the Peak Groups 70–76

Peak number	Area	% Area
70	0.128	7.17
71	0.159	8.91
72	0.087	4.87
73	0.521	29.19
74	0.217	12.16
75 (a and b)	0.587	32.89
76	0.086	4.82
Total	1.785	100

Chromatographic separation is accomplished by ECN better than by molecular weight. The quantitative method depends on the amount injected more directly than the number of moles because the detection is not based on molecular properties but on light scattering by the solutes after spray-drying in the drift tube. In conclusion, we considered weight percentages to be more appropriate than mole fractions to show results.

Comparison between TG composition of cow, ewe, and goat milk fat. With the deconvolution program, the peaks were grouped according to the molecular features of the TG. The molecular species were grouped according to the ECN in short-chain (ECN ≤34), medium-chain (ECN = 36–40), and long-chain TG (ECN >40). In this classification, we also took into account the number of double bonds because they decrease the retention time of a TG, as we observed above in the calculation of the a' coefficient. Classification was also made based on the presence of double bonds—saturated and unsaturated. Moreover, the unsaturated species were grouped according to their number of double bonds (*ND*) in monounsaturated (1*ND*) and polyunsaturated (>1*ND*). After applying the modified HPLC method for the separation of milk fat TG, compositional differences could be seen more clearly.

TABLE 5
Coefficients of Variation (CV) Corrected After the Application of the Deconvolution Program to Peaks with a CV <5%[a]

Peak number	Average (%)	CV_I (%)	CV_F (%)
5	0.015	11.50	3.98
5′	0.017	—	2.99
6	0.161	6.13	3.62
8	0.083	11.10	3.21
9	0.022	16.48	4.30
10	0.106	8.14	2.91
11	0.096	6.07	3.22
14	0.220	11.43	3.21
15	0.018	17.18	3.30
31	0.004	9.80	6.45
32	0.009	8.80	2.82
41	0.0002	11.10	9.52
44	0.493	7.90	2.86
45	0.416	7.90	2.30
60	0.172	8.30	0.87
69	0.0005	7.80	7.42
70	0.797	9.40	0.74
74	1.126	6.83	0.72
76	0.476	6.70	0.76
78	0.116	8.10	0.82
99	0.330	7.65	3.05
103	0.187	8.95	0.56
106	0.046	11.57	2.84
107	0.018	10.96	3.15

[a]Marked with a[b] in Table 3; CV_I = coefficient of variation obtained from peak area determination with the integrator; CV_F = coefficient of variation obtained from peak area determination with the deconvolution program. Average = medium value of area peak percentage after application of the deconvolution program.

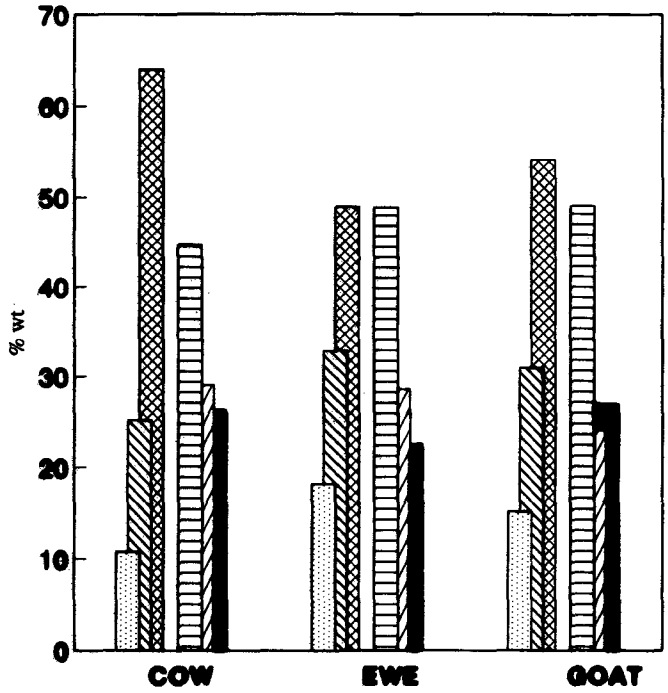

FIG. 6. Percentages of short- (dotted bars), medium- (right-slanting striped bars), long-chain (cross-hatched bars), saturated (striped bars), monounsaturated (left-slanting striped bars), and polyunsaturated (solid bars), triglycerides in cow, goat, and ewe milk.

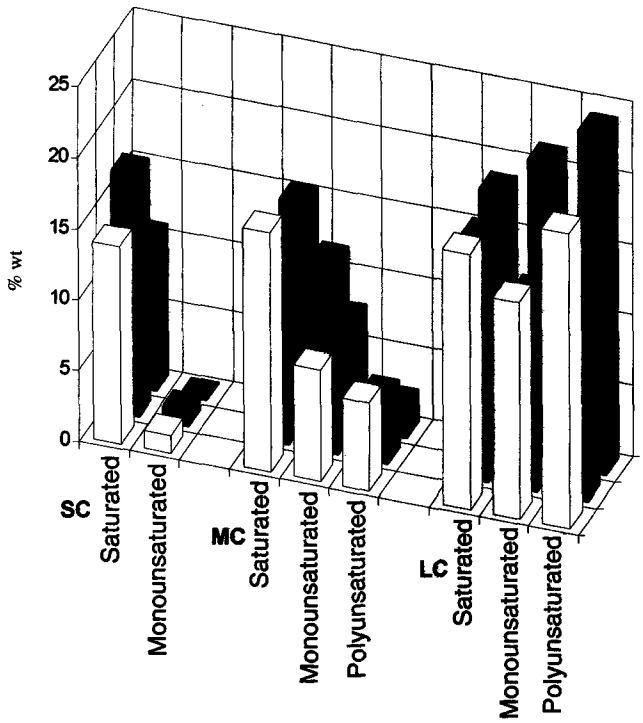

FIG. 7. Percentages of saturated, monounsaturated, and polyunsaturated triglycerides, whether they have short (SC), medium (MC) or long chains (LC), in cow (grey bars), goat (white bars), and ewe (black bars) milk.

Ewe milk had the highest short-chain TG percentage, higher then goat and cow milk (18.23, 15.21, and 10.83 %, respectively; Fig. 6). Medium-chain TG percentages were found to be in the same order (32.84, 30.83, and 25.16%, respectively). This is due to the fact that ewe and goat milk are enriched in unsaturated fatty acids, such as oleic, linoleic, and linolenic acids (30). However, cow milk had the highest long-chain TG percentage, as it is enriched in saturated long-chain fatty acids (mainly myristic and palmitic acids), followed by goat and ewe milk (64.01, 53.95, and 48.91%, respectively).

Cow milk was also confirmed to have the highest unsaturated TG quantity, while the goat unsaturated TG quantity was similar to that of ewe milk (55.31, 51.13, and 51.12%, respectively).

Figure 7 shows the short-, medium-, and long-chain TG percentages according to the degree of saturation. Cow milk fat has the highest polyunsaturated long-chain TG percentage; however, goat milk fat is the richest in polyunsaturated medium-chain TG, in accordance with the short-, medium-, and long-chain fatty acid relative quantities, and the different combinations to achieve TG in the three animal species.

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