A Reverse-Phase High-Performance Liquid Chromatographic Method for Analyzing Complex Mixtures of Triglycerides. Application to the Fat Fraction of an Aged Cheese

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ABSTRACT: Analysis of complex mixtures of triglycerides that span a broad range of partition numbers can entail a number of difficulties. The present study was therefore intended to develop a method of reverse-phase high-performance liquid chromatography using a light scattering detector for analyzing such mixtures. Stationary phase type, mobile phase gradient, column temperature, and injection solvent were the factors used to develop the method. The method improved the selectivity of critical pairs of milk fat triglycerides, providing good resolution for 115 peaks with partition numbers of between 22 and 53. The use of tristearin as internal standard and the relative response factors for different partition number ranges made it possible to more accurately estimate the weight of the sample triglycerides.

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Reverse-phase high-performance liquid chromatography (RP-HPLC) is one of the most efficient means of separating triglycerides according to the combined chainlengths of the fatty acyl residues and the total number of double bonds. Still, simultaneous analysis of mixtures such as milk fat, which contains a large number of short-, medium-, and long-chain triglycerides with differing degrees of unsaturation spanning a broad range of partition numbers (PN), remains a very complicated task. The main difficulty lies in achieving good resolution of the triglycerides with shortest retention time, particularly those with the same PN value, while at the same time achieving narrow elution bands within reasonable analysis times for the triglycerides with the longest retention times (1–4). Under such conditions of analysis, identification and quantitation of molecular species are extremely difficult, both because of the paucity of pure samples of many natural mixed triglycerides and because many critical pairs or groups of triglycerides with the same PN value have not yet been resolved (5–7). The above mentioned difficulties are minimized when the triglycerides are analyzed by silver ion HPLC, because the separation is based only on the degree of unsaturation of the fatty acyl residues. However, fewer triglyceride fractions are obtained (8). Thus, several authors have proposed to combine these two techniques sequentially to obtain more useful information on the molecular makeup of natural triglyceride mixtures (9).

Different chromatographic factors have been studied with a view to improving the efficacy of RP-HPLC triglyceride analysis. Such factors include the type of stationary phase, mobile phase composition, injection solvent, and analysis temperature. The best separations have been achieved using octadecylsilane (ODS) stationary phases, especially at particle diameters of 3–4 μ (10–13). Two or three columns connected in series have been used to analyze simultaneously mixtures of triglycerides with PN values between 12 and 54 (14–18).

Acetonitrile is the organic solvent most frequently used in the mobile phase, and acetone has proved to be the most efficacious organic modifier (3,7,19–22). Nonlinear elution gradients and specific step-wise gradients have also been suggested as means of improving the separation of critical pairs in complex mixtures (17,23,24).

Several workers have reported that the injection solvent may exert a major influence on the resolution of a chromatographic system, suggesting that the mobile phase itself is the most appropriate injection solvent (25). However, when the mobile phase does not provide sufficient solubility for the triglycerides, other solvents capable of dissolving all of the sample triglycerides can be used as sample diluents, whenever they are compatible with the mobile phase (25,26).

Column temperature also exerts a sizable influence on the efficacy of triglyceride separations, with shorter retention times and lower selectivity as the temperature is increased (26,27). Temperature gradients of between 15 and 60°C have been proposed to enhance the solubility of the most highly saturated triglycerides (4,7).

By adjusting the aforementioned chromatographic factors in combination with UV and refractive index detectors, some workers obtained between 46 and 62 peaks from milk fat at analysis times of between 40 and 80 min (4,6,28). Subsequently,

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use of light-scattering detectors has made it possible to develop more effective methods of separating complex mixtures of triglycerides, achieving separations of milk fat yielding up to 111 peaks with PN values ranging from 24 to 54 in 160 min (18).

Despite the improvements in the RP-HPLC analysis of triglycerides, there is still no single satisfactory method for overcoming all the difficulties posed by the analysis of complex mixtures. Accordingly, the object of this study was to develop an RP-HPLC method using gradient elution and a light-scattering detector for analyzing and quantifying complex mixtures of triglycerides spanning a broad range of PN values, and then to apply that method in analyzing the fat fraction of an aged cheese. The composition of cheese fat exerts a strong influence on cheese quality. The cheese firmness is affected by the melting interval of the fat, depending upon the temperature and the triglyceride composition (29). The cheese triglycerides contribute to adhesion and help to impart a smooth texture to the cheese (30). In addition, the fat exerts an influence on cheese aroma formation because the hydrolysis of the triglycerides increases the concentration of free fatty acids during ripening (31).

EXPERIMENTAL PROCEDURES

Sample preparation. Samples of Idiazabal cheeses ripened for 90 d were analyzed. The cheeses were manufactured from raw milk collected in March from ewes of the Lacha breed using natural lamb rennet as a coagulant, in accordance with the procedures approved by the Regulatory Board of Idiazabal Cheese Appellation of Origin (32). The fat fraction of 5 g of ground cheese was extracted for analysis of the triglycerides, according to the official analysis procedure of the International Dairy Federation (IDF) (33) using *n*-pentane (Panreac, Barcelona, Spain) as the extraction solvent. Tristearin (45 mg) (purity 99%, Sigma Chemical Co., St. Louis, MO) was added to ground cheese as an internal standard. The solvent was removed in a model RV05-ST rotary vacuum evaporator (Janke & Kunkel, Staufen, Germany) at a temperature of less than 35°C. A 30-mg sample of fat was then dissolved in 1 mL of *n*-hexane (Merck, Darmstadt, Germany).

HPLC analysis. The HPLC equipment comprised one model 422M and one model 422S pump (Kontron, Milan, Italy), a model 7161 injector with a 5 µL loop (Rheodyne, Cotati, CA), and a model Sedex 45 light-scattering detector (Sedere, Alfortville, France) equipped with a model Compact 106 automatic air compressor (Cedime, Arrancudiaga, Spain). Detector conditions were an air pressure of 2.2 bars and a temperature of 40°C. The system was operated by a model AT 486 work station (Kontron) running MT-450 software (Kontron) connected to a model LQ-570 daisy wheel printer (Seiko Epson, Nagano, Japan). Column temperature was controlled by means of a model Precisterm thermostated water bath (Selecta, Barcelona, Spain).

Before injection, 200 µg of triglyceride-containing fat extract was filtered through a Durapore filter with a pore diameter of 0.22 µm (Millipore, Milford, MA) and warmed to 30°C in a hot-water bath.

Acetone, *n*-hexane, dichloromethane, chloroform, and mixtures (vol/vol) of acetonitrile/acetone (50:50), acetonitrile/dichloromethane (50:50), chloroform/acetone (50:50), and hexane/acetone (50:50) (Merck) were used as injection solvents for the HPLC analyses.

Two particle shapes were tested, a spherical particle shape (Spherisorb ODS-2, Phase Separations, Queensferry, United Kingdom) and an uneven particle shape (Nucleosil 120 C-18, Machery Nagel, Düren, Germany), using two columns (20 cm × 4.6 mm, Symta, Madrid, Spain) connected in series. Particle size was 3 µm in both cases.

The mobile phase consisted of the solvents acetonitrile and acetone (HPLC grade, Scharlau, Barcelona, Spain). Five nonlinear gradients were tested, varying the initial proportion of acetone between 0 and 10% and the final proportion between 70 and 85% (gradients 1–5). The flow rate was 1.0 mL/min in all cases.

Three isothermal analysis temperatures (30, 35, and 40°C) and two temperature gradients (T_1 and T_2) were tested. The gradients involved increasing the analysis temperature from an initial level of 30°C to either 45 or 60°C at a rate of 2°C/min, starting at an analysis time of 65 min. Once the maximum temperature had been reached, isothermal temperature conditions were employed until completion of the analysis.

The triglycerides were quantified based on the chromatographic peak areas, using tristearin as internal standard. Response factors were calculated from five replications using solutions of 1 mg/mL of the pure triglycerides tricaproin, tricaprylin, trinonanoin, tricaprin, trilinolenin, trimyristolein, trilaurin, 1,2-dilauroyl-myristin, tritridecanoin, 1,2-dimyristoyl-laurin, trilinolein, trimyristin, 1,2-dilinoleoyl-olein, 1,2-dimyristoyl-olein, 1,2-dimyristoyl-palmitin, tripentadecanoin, 1-myristoyl-2-oleoyl-palmitin, 1,2-dipalmitoyl-myristin, triolein, 1,2- dioleoyl-palmitin, 1,2-dipalmitoyl-olein, tripalmitin, 1,3-dioleoyl-stearin, 1-palmitoyl-2-oleoyl-stearin, 1,2-distearoyl-myristin, triheptadecanoin, 1,3-stearoyl-olein, 1,2-distearoyl-palmitin, and tristearin (purity 99%, Sigma) in *n*-hexane (Merck).

The relative response factor for each chromatographic peak was calculated based on the PN value for that peak. The PN value for each peak was calculated using the equation obtained by linear regression of the independent chromatographic variable, log k', found experimentally from the analyses of known pure triglycerides, on the PN values for those same known triglycerides ($PN = c_1 + c_2 \cdot \log k'$). The regression was performed using the 1R analysis program from BMDP Statistical Software, Inc. (Los Angeles, CA).

The analytical method was validated by calculating the linearity of response, the detection and quantification limits, and the reproducibility of the quantitative method. The linearity of response was determined by analyzing solutions of pure tricaprylin, trimyristin, triolein, and tripalmitin in *n*-hexane, yielding injection quantities of 0.5, 1, 2, 5, 10, and 20 µg. Three replications were carried out, and the regressions were performed using the 1R analysis program from BMDP Statistical Software, Inc.

The detection limit (L_D) and quantification limit (L_Q) values for the analytical method were the means of the limit values calculated from the calibration curves for the pure triglycerides tricaprylin, trimyristin, triolein, and tripalmitin, and from the mean area of the noise for five replicate analyses of a blank (*n*-hexane) (34–38). Repeat analysis of five samples from a single cheese was used to establish the reproducibility of the method.

RESULTS AND DISCUSSION

Of the different injection solvents or solvent mixtures tested, only chloroform, dichloromethane, and *n*-hexane completely dissolved the cheese triglycerides. However, the effect of these three solvents on the resolution of the early eluting peaks was different.

While *n*-hexane and dichloromethane provided clearly defined peak shapes without doublets and narrows, chloroform showed doubled peaks for the triglycerides up to PN 28. These results could be explained by invoking the solvophobic theory of Horvath *et al.* (39), who put foward a model in which, for the mobile phase to carry a solute molecule, that molecule must create an appropriate hole in the mobile phase followed by a reduction in a free volume caused by van der Waals forces and electrostatic interactions. This allows the solute, in this case a triglyceride, to interact with the mobile phase and the hydrocarbon ligand groups on the stationary phase. *n*-Hexane and dichloromethane were the solvents that yielded good interaction among the cheese triglycerides, the acetonitrile/acetone mobile phase, and the silica gel particle-bound ODS. Because the large differences in the theoretical polarity index between *n*-hexane and the mobile phase could possibly induce solute precipitation at the head of the column, dichloromethane was chosen as the injection solvent. To ensure complete solubility of the triglycerides in the injection solvent, the sample was warmed to 30°C in a hot water bath.

Table 1 shows the effect of gradient type on the retention times (rt) and selectivity (α) for five pairs of peaks for cheese triglycerides from different regions of the chromatogram (number of peaks, as in Fig. 1). The results show that the gradient that started with an initial proportion of 10% acetone (gradient 1) shortened the retention times substantially for analysis times of up to 70 min. Furthermore, the retention times for peaks 97 and 98 were very similar for those gradients, with a final proportion of between 70 and 85% acetone for analysis times of between 135 and 150 min (gradients 1–4), whereas for gradient 5 the retention times for those same peaks were lower, a result of the more rapid increase up to the final proportion of acetone (70% in 105 min).

On evaluating the effect of gradient type on the α values for the peak pairs considered, none of the gradients proved capable of separating critical pairs 51–52 and 58–59 on the stationary phase employed (Spherisorb ODS-2). For the remaining critical pairs considered, the α values were quite similar for all the gradients tested, except gradient 5, which yielded selectivity values closer to 1 after analysis times of more than 70 min, probably because of an excessively fast increase in the proportion of acetone in the mobile phase.

Regardless of gradient type, the number of theoretical plates (*N*) was much higher for the peaks with the longest retention times (between 320,000 and 420,000 plates) than for those with the shortest retention times (between 15,000 and 25,000 plates). However, there was no clear pattern in the value of *N* for the different gradient types tested. All these results were indicative of the major effect of acetone on triglyceride resolution in RP-HPLC, as reported by other workers (2,19,20). It should also be noted that the gradients in which the final proportion of acetone did not exceed 70% (gradients 2 and 5) experienced difficulties from precipitation of the

TABLE 1
Retention Time (rt) and Selectivity (α) Values in the High-Performance Liquid Chromatographic (HPLC) Analyses of Certain Pairs of Idiazabal Cheese Triglycerides Using Different Nonlinear Gradients of Acetone in Acetonitrile

Peak number[a]	PN[b]	rt (min), Gradient type					α, Gradient type				
		1[c]	2[d]	3[e]	4[f]	5[g]	1[c]	2[d]	3[e]	4[f]	5[g]
42	34	43.97	49.49	49.86	50.88	51.86	0.993	0.991	0.986	0.990	0.995
43	34	44.26	49.92	50.51	51.36	52.11					
51	36	54.35	59.05	60.13	60.78	61.46	1.000	1.000	1.000	1.000	1.000
52	36	54.35	59.05	60.13	60.78	61.46					
58	38	63.30	67.43	69.11	69.77	70.16	1.000	1.000	1.000	1.000	1.000
59	38	63.30	67.43	69.11	69.77	70.16					
74	42	90.22	92.10	93.96	93.94	89.95	0.964	0.966	0.967	0.967	0.974
75	42	93.48	95.26	97.09	97.07	92.24					
97	48	117.84	119.25	119.37	119.11	103.02	0.983	0.982	0.984	0.983	0.986
98	48	119.79	121.35	121.35	121.06	104.40					

[a] Number of peak as in Figure 1.
[b] PN = partition number.
[c] Gradient 1: 10 to 35% (50 min), isocratic (20 min), 35 to 80% (75 min), and final isocratic (10 min).
[d] Gradient 2: 0 to 35% (50 min), isocratic (20 min), 35 to 70% (65 min), and final isocratic (20 min).
[e] Gradient 3: 0 to 35% (50 min), isocratic (20 min), and final gradient from 35 to 85% (80 min).
[f] Gradient 4: 0 to 35% (50 min), isocratic (20 min), 35 to 80% (75 min), and final isocratic (10 min).
[g] Gradient 5: 0 to 35% (50 min), isocratic (20 min), 35 to 70% (35 min), and final isocratic (50 min).

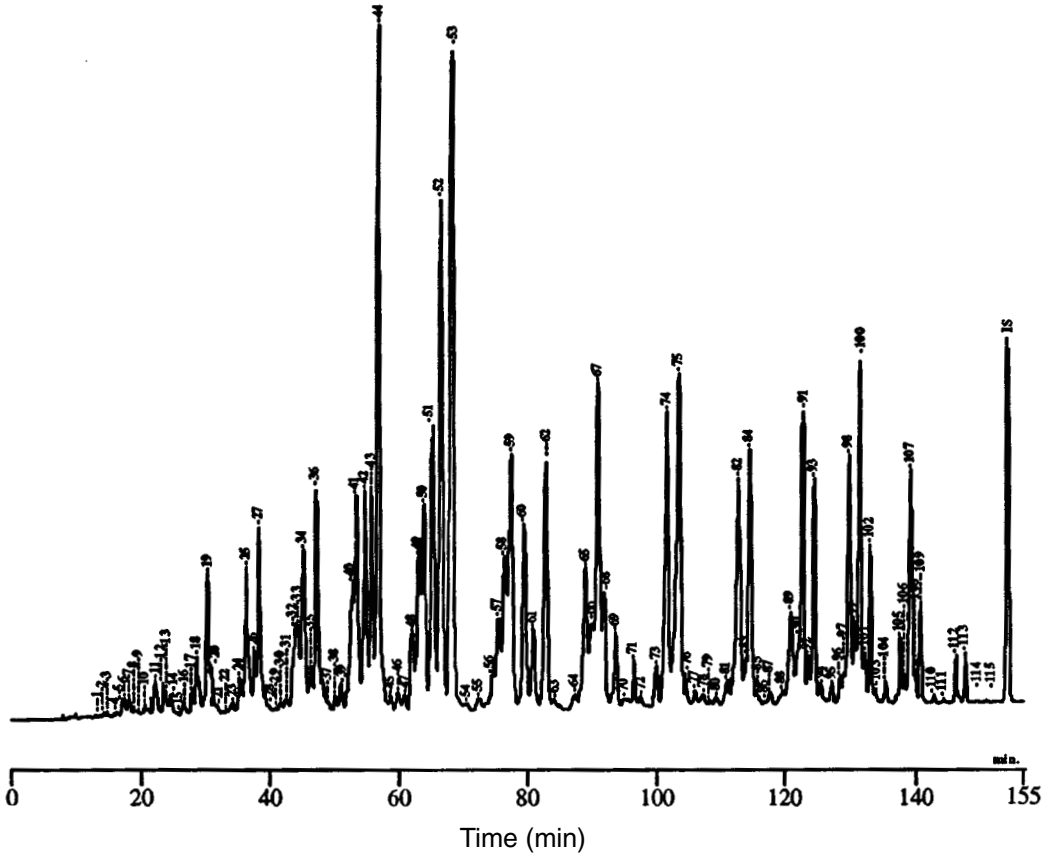

FIG. 1. High-pressure liquid chromatogram for Idiazabal cheese triglycerides employing the following conditions of analysis: two columns (20 cm × 4.6 mm) connected in series packed with Nucleosil 120 C-18 with a particle size of 3µm; gradient elution with a mobile phase starting at 0% acetone in acetonitrile and rising to 35% acetone over 50 min, followed by isocratic mobile phase conditions for 20 min, then renewed gradient conditions raising the proportion of acetone to 80% over 75 min, and finally isocratic conditions again for 10 min; flow rate: 1.0 mL/min; analysis temperature: 30°C; injection solvent: dichloromethane 5 µL. IS = internal standard (tristearin).

most highly saturated triglycerides on the column. After evaluating the effects of gradient type on retention, selectivity, efficacy, and solubility of triglycerides on the chromatographic system, gradient 4 was chosen as the most suitable mobile phase for analyzing cheese triglycerides.

Table 2 presents the results for the effect of analysis temperature on the retention time and α values for the pairs of peaks considered. Comparing the different temperatures under isothermal conditions (30, 35, and 40°C), the retention times decreased substantially as temperature increased, with

TABLE 2
Retention Time and Selectivity Values in the HPLC Analyses of Certain Pairs of Idiazabal Cheese Triglycerides Using Different Column Temperatures[a]

Peak number[a]	PN[b]	rt (min)					α				
		30°C	35°C	40°C	T_1[b]	T_2[c]	30°C	35°C	40°C	T_1[b]	T_2[c]
42	32	50.88	41.98	37.53	49.01	48.11	0.990	0.984	0.981	0.988	0.987
43	34	51.36	42.60	38.19	49.55	48.68					
51	36	60.78	50.52	45.37	58.94	58.04	1.000	0.989	0.990	1.000	1.000
52	36	60.78	51.00	45.78	58.94	58.04					
58	38	69.77	57.59	51.23	67.50	66.89	1.000	1.000	0.981	1.000	1.000
59	38	69.77	57.59	52.15	67.50	66.89					
74	42	93.94	79.73	69.52	84.62	80.29	0.967	0.963	0.965	0.980	0.987
75	42	9707	82.64	71.91	86.31	81.32					
97	48	119.11	109.29	100.51	101.87	91.78	0.983	0.984	0.984	0.983	0.986
98	48	121.06	110.99	102.13	103.57	93.07					

[a]For abbreviations see Table 1.
[b]T_1, gradient from 30 to 45°C, at a rate of 2°C/min starting at 65 min.
[c]T_2, gradient from 30 to 65°C, at a rate of 2°C/min starting at 65 min.

the greatest effect on the peaks with the longest retention times, as was to be expected (40). Contrary to expectations, the α values for the pairs improved at temperatures above 30°C (Table 2). This was because at the higher temperatures the triglycerides eluted at lower proportions of acetone in the mobile phase (as compared to the temperature of 30ºC for analysis times longer than 70 min, approximately 9.5% lower at 35°C and 15.5% lower at 40°C). The effect of the proportion of acetone outweighed the effect of analysis temperature. Accordingly, the values of *N* for the pairs considered were always higher at the temperature of 30°C (420,000 plates for peak 98 at 30°C as opposed to 230,000 plates at 35°C and 270,000 plates at 40°C).

As proposed by certain researchers (4,7), temperature gradients were tested with a view to lowering the retention times and increasing the solubility of the triglycerides with the highest PN. Temperature gradients T_1 and T_2 (Table 2) resulted in sizable decreases in the retention time and α values, particularly for the pairs with the longest retention times. Conversely, the values of *N* were much higher than for the isothermal analyses (700,000 and 940,000 plates for peak 98 using gradients T_1 and T_2, respectively). Thus, unlike the case for the isothermal analysis temperatures, the effect of the temperature gradients on efficacy (number of plates) and selectivity outweighed the effect of the proportion of acetone in the mobile phase.

In view of these results, it was decided to carry out the analyses under isothermal conditions. The isothermal temperature of 30°C was chosen, because even though better selectivity values were achieved for certain pairs at 40°C (Table 2), a large number of the peaks in the rest of the chromatogram were wide and poorly defined. In addition, the temperature of 30°C ensured good solubility of the most highly saturated triglycerides in the mobile phase without requiring overly long analysis times.

In order to resolve peak pairs 51–52 and 58–59, which were not resolved under the chromatographic conditions chosen above, a new packing, Nucleosil 120 C-18, with a particle size of 3 µm and an uneven particle shape, was tested. Table 3 sets out the retention time and α values for the pairs of peaks considered on the Spherisorb ODS-2 and Nucleosil 120 C-18 stationary phases. The higher retention time values for the unevenly shaped stationary phase contributed to resolution of the peaks for peak pairs 51–52 (α = 0.980) and 58–59 (α = 0.989). The *N* values for the Nucleosil 120 C-18 stationary phase were approximately three times higher than the values for the Spherisorb ODS-2 stationary phase over the entire chromatogram. Consequently, Nucleosil 120 C-18 was chosen as the more suitable stationary phase for analysis of the cheese triglycerides. It was observed that after approximately 100 injections using the chromatographic conditions described, the ODS column lost selectivity for many critical pairs.

Figure 1 shows the HPLC chromatogram for the Idiazabal cheese triglycerides under the chosen optimal conditions as described above. Analysis time was around 155 min, and the analysis yielded 115 peaks with estimated PN values ranging from 22 to 53. These results represent an advance in the resolution of triglycerides from milk fat over the results published by other workers. Frede and Thiele (6) and Weber *et al.* (4) separated 46 and 49 peaks in analysis times of 40 and 80 min, respectively. Spanos *et al.* (41) resolved 58 peaks with PN values of between 30 and 52, and Barron *et al.* (28) improved on that result by separating 62 peaks with PN values of from 22 to 50. The findings closest to the results presented in this paper were published by Hierro *et al.* (17), who reported 93 peaks in an analysis time of 120 min, and recently by Ruiz-Sala *et al.* (18), who reported 111 peaks with PN values ranging from 24 to 54 in an analysis time of 160 min.

TABLE 3
Retention Time and Selectivity Values in the HPLC Analyses of Certain Pairs of Idiazabal Cheese Triglycerides Using Two 3 µm Stationary Phases: Spherisorb ODS-2 and Nucleosil 120 C-18[a]

Peak number	PN	rt (min)		α	
		Sp	Nu	Sp	Nu
42	34	50.88	55.50	0.990	0.979
43	34	51.36	56.60		
51	36	60.78	66.58	1.000	0.980
52	36	60.78	67.85		
58	38	69.77	78.32	1.000	0.989
59	38	69.77	79.16		
74	42	93.94	103.04	0.967	0.981
75	42	97.07	104.98		
97	48	119.11	128.23	0.983	0.979
98	48	121.06	130.93		

[a]Sp, Spherisorb; Nu, Nucleosil. For other abbreviations see Table 1.

To validate the quantitative method, the linearity of the response of the triglycerides in the light-scattering detector, the detection and quantification limits, and the reproducibility of the method were calculated. Table 4 presents the calculations for the linearity of response for the different pure triglyceride standards. Logarithmic functions of chromatographic peak area (log *A*) and the quantity of the triglycerides injected (log *c*) were used to calculate the linear response of the triglycerides in the light-scattering detector. The slopes of the regression lines for the triglycerides considered exhibited significant differences ($P \leq 0.05$) and ranged from 1.23 for trimyristin to 1.85 for tricaprylin, indicative of differing detector calibration sensitivities. Ruiz-Sala *et al.* (18) published similar findings.

The noise recorded experimentally for blanks grew considerably higher after analysis times of 120 min, when the percentage of acetone in the mobile phase was greater than

TABLE 4
Estimated Equations for Standard Triglycerides in the Linearity Study of the Light-Scattering Detector[a]

TG	PN	Equation	R^2	SE
Tricaprylin	24	$\log A = 0.409 + 1.85 \log c$	0.955	0.236
Trimyristin	42	$\log A = 1.158 + 1.23 \log c$	0.946	0.236
Triolein	48	$\log A = 0.770 + 1.50 \log c$	0.945	0.236
Tripalmitin	48	$\log A = 1.018 + 1.29 \log c$	0.963	0.236

[a]TG, triglycerides; R^2, coefficient of correlation; SE, standard area; *c*, concentration. For other abbreviation see Table 1.

TABLE 5
Mean and Standard Deviation Values for the Detection and Quantification Limits for the Zones Bordering the Analysis Time of 120 min in the Chromatograms, Estimated for Pure Triglycerides[a]

	Prior 120 min			Post 120 min		
Noise (mV × min)		$\bar{x}$ ± SD 0.013 ± 0.002			$\bar{x}$ ± SD 0.058 ± 0.014	
	Tricaprylin (rt = 16.4 min)	Trimyristin (rt = 104.1 min)	$\bar{x}$ ± SD	Triolein (rt = 128.9 min)	Tripalmitin (rt = 133.6 min)	$\bar{x}$ ± SD
Detection limit (mg)	0.116	0.039	0.077 ± 0.054	0.204	0.157	0.180 ± 0.033
Quantification limit (mg)	0.173	0.071	0.122 ± 0.072	0.396	0.340	0.368 ± 0.039

[a]Noise was estimated as the mean area (mV × min) of the strongest signal obtained on analyzing blanks. $\bar{x}$ ± SD, mean ± standard deviation; for other abbreviation see Table 1.

65%. For that reason, the detection and quantification limits for the two zones on either side of that analysis time on the chromatogram were calculated. Table 5 gives the results of the calculations for the different pure triglycerides. The mean detection and quantification limit values were similar to those estimated by other researchers for RP-HPLC analysis of triglycerides using light-scattering detectors (35,42–44). Detectable peaks were defined as peaks with an area at least twice the noise, and quantifiable peaks were defined as peaks with an area at least six times the noise (35–38).

Because of the enormous complexity of the milk fat triglyceride mixture, it is quite difficult to achieve an accurate quantitation of the different triglyceride molecular species. In most of the previous papers on milk fat triglycerides analyzed by HPLC, the percentage of peak area has been used to estimate the percentage of the triglycerides in milk fat samples (4,18,45). However, the accuracy of the quantitation method can be significantly improved by means of an internal standard, so mean response factors were employed using tristearin as the internal standard. This triglyceride was selected as internal standard because it was not detected in the fat fraction of Idiazabal cheese. The cheese sample chromatogram shown in Figure 1 indicates that the only triglyceride molecular species that could be used as internal standard should have PN values either lower than 22 or higher than 52. Also, tristearin was selected because it did not present problems relating to its response to the evaporative light-scattering detector at 40°C, as the triglycerides with PN < 22 did because of their volatility.

The mean response factor (rf) values were calculated for ranges of estimated PN values for the peaks for the triglycerides from the Idiazabal cheeses using the equation

$$\text{PN} = 6.9290 + 25.0052 \log k' \quad [1]$$
$$(\text{SE} = 2.5106)$$

Table 7 presents the estimated PN values for the 115 peaks separated in the analysis of the Idiazabal cheese triglycerides.

The mean rf values applied were chosen according to the PN values of the peaks in the HPLC analyses. To improve the reproducibility of the method, three significantly different ($P \leq 0.05$) mean rf were established for three different PN ranges. The mean rf values were calculated based on the rf values for pure triglycerides with PN values of between 18 and 52 relative to the rf value for tristearin obtained experimentally (Table 6). On the whole, all the rf values were close to 1, although there were differences according to the PN values of the triglycerides. The mean rf values were 1.336 for PN values less than or equal to 36, 1.202 for PN values greater than 36 and less than or equal to 44, and 1.027 for PN values greater than 44.

Replications were used to measure the reproducibility of the quantitative method. Except for three peaks, the coefficient of variation values were less than 11.5% (Table 7), a value regarded as acceptable bearing in mind the nature of the

TABLE 6
Mean Response Factor Values Relative to the Value for the Internal Standard (tristearin) for Pure Triglycerides[a]

TG	PN	rf	CV (%)
CoCoCo	18	1.149	5.5
ClClCl	24	1.208	4.5
NoNoNo	27	1.253	4.7
CaCaCa	30	1.430	5.7
LnLnLn	36	1.462	4.1
MiMiMi	36	1.320	2.6
LaLaLa	36	1.494	3.8
LaLaM	38	1.381	2.3
DeDeDe	39	1.287	2.4
MMLa	40	1.222	3.3
LLL	42	1.181	2.7
MMM	42	1.226	2.5
LLO	44	1.143	2.9
MMO	44	1.076	1.5
MMP	44	1.105	2.5
PnPnPn	45	1.031	3.8
MOP	46	1.091	1.1
PPM	46	1.016	1.3
OOO	48	1.035	1.5
OOP	48	1.225	0.6
PPO	48	1.074	1.0
PPP	48	1.147	0.8
OSO	50	0.851	1.7
POS	50	1.053	1.3
SSM	50	0.968	1.2
HeHeHe	51	0.981	1.0
SOS	52	0.918	1.3
SSP	52	0.965	1.0

[a]CV = coefficient of variation for five replications. Co = caproic; Cl = caprylic; No = nonanoic; Ca = capric; Ln = linolenic; Mi = myristoleic; La = lauric; M = myristic; De = tridecanoic; L = linoleic; O = oleic; P = palmitic; Pn = pentadecanoic; S = stearic; He = heptadecanoic. For other abbreviations see Tables 1 and 4.

extraction process applied to the fat fraction and the complexity of chromatographic analysis of cheese triglycerides.

Table 7 sets out the mean concentration values (g triglycerides/kg cheese) for the peaks in the HPLC analyses of the triglycerides from the Idiazabal cheeses after 90 d of ripening. The main peaks were 44 (PN = 34), and 53 and 52 (in both cases, PN = 36), which accounted for 8.79, 8.39, and 5.24% of the total triglyceride content, respectively. The peaks for the triglycerides with odd PN and for the triglycerides with the even PN 22, 24, 26, and 52 were very small, with individual percentage contributions not exceeding 1%. Grouping together the peaks with the same PN values, the main groupings were

TABLE 7
Mean Triglyceride Content of Idiazabal Cheese (g TG/kg cheese) Obtained from Five Replicate Analyses[a]

HPLC[b] peak	PN	g TG/kg cheese $\bar{x}$	CV	HPLC peak	PN	g TG/kg cheese $\bar{x}$	CV	HPLC peak	PN	g TG/kg cheese $\bar{x}$	CV
1	22	0.038	3.13	40	34	14.340	11.37	79	44	0.179	2.94
2	22	0.171	2.94	41	34	14.598	0.83	80	44	0.374	0.21
3	22	0.099	8.84	42	34	15.702	6.00	81	44	1.430	5.18
				43	34	20.054	4.78	82	44	6.774	1.38
4	23	0.047	1.84	44	34	51.038	3.93	83	44	1.036	8.12
								84	44	6.732	11.18
5	24	0.134	3.50	45	35	1.250	9.05				
6	24	0.384	1.06	46	35	1.612	4.26	85	45	0.376	2.93
7	24	0.324	3.47					86	45	0.105	4.17
				47	36	1.076	9.45	87	45	0.522	10.27
8	25	0.148	0.84	48	36	7.487	2.17				
9	25	0.074	1.65	49	36	13.552	4.32	88	46	1.037	0.38
10	25	0.385	1.73	50	36	15.827	3.26	89	46	3.266	3.93
				51	36	22.684	2.97	90	46	1.387	6.14
11	26	0.974	6.00	52	36	30.440	7.99	91	46	6.424	5.51
12	26	0.736	4.82	53	36	48.697	6.77	92	46	1.261	2.22
13	26	0.751	4.29					93	46	4.482	5.44
				54	37	0.769	17.50				
14	27	0.765	0.63	55	37	1.035	0.93	94	47	0.550	6.29
15	27	0.344	4.48					95	47	0.658	3.78
				56	38	2.859	4.72				
16	28	0.462	6.87	57	38	7.415	2.36	96	48	0.814	1.17
17	28	0.892	1.37	58	38	11.603	10.73	97	48	0.886	1.59
18	28	0.494	1.62	59	38	15.698	1.08	98	48	4.943	8.50
19	28	5.621	5.44	60	38	12.279	1.77	99	48	1.820	0.93
20	28	2.097	11.44	61	38	5.528	11.45	100	48	6.837	5.92
				62	38	14.427	4.52	101	48	0.967	6.46
21	29	0.277	11.13					102	48	2.801	3.94
				63	39	0.588	19.86	103	48	0.379	0.37
22	30	0.028	16.52								
23	30	0.770	3.90	64	40	1.371	0.20	104	49	0.472	4.08
24	30	1.794	8.82	65	40	9.838	4.18				
25	30	5.338	3.82	66	40	5.360	10.70	105	50	1.738	2.65
26	30	2.660	5.69	67	40	21.628	4.85	106	50	1.072	2.09
27	30	9.841	1.19	68	40	6.202	7.96	107	50	4.509	5.46
				69	40	4.745	6.48	108	50	0.834	10.34
28	31	0.115	1.12					109	50	1.861	2.90
29	31	0.113	8.98	70	41	1.109	9.59				
30	31	0.345	10.42					110	51	0.235	5.22
31	31	1.191	1.56	71	42	2.857	4.33	111	51	0.111	9.12
				72	42	0.576	11.27				
32	32	2.698	0.47	73	42	2.544	4.87	112	52	1.111	3.80
33	32	8.237	6.10	74	42	18.341	8.83	113	52	0.848	1.26
34	32	7.281	1.59	75	42	23.292	8.70				
35	32	3.117	2.55	76	42	1.329	4.37	114	53	Not quantifiable	
36	32	19.569	9.95					115	53	Not quantifiable	
				77	43	0.770	4.31				
37	33	0.486	2.89	78	43	0.667	5.13				
38	33	0.684	2.80								
39	33	1.102	0.65								

[a]For abbreviations see Tables 1, 4, and 6.
[b]Peak numbers from Figure 1.

for PN values of 36, 34, and 38, with percentage contributions of 24.07, 19.93, and 12.02, respectively. The groupings for the odd PN of 23 and 29 had the lowest percentage contributions, 0.008 and 0.048, respectively (Table 7).

These results were very similar to the findings reported for milk fat by other researchers, according to which the main triglycerides had PN values of 34 and 36 (18,28,41,45). However, the accuracy of the quantitative results was significantly improved because of the use of tristearin as internal standard added to the cheese samples.

In conclusion, the RP-HPLC method presented here yielded satisfactory results on analyzing a complex mixture of triglycerides spanning a broad range of PN values. The method improved the selectivity of critical pairs of milk fat triglycerides, providing good resolution for the 115 peaks with PN values between 22 and 53 obtained from the analysis of triglycerides from an aged cheese. The use of an internal standard, such as tristearin, and the relative response factors calculated for different PN ranges made it possible to more accurately estimate the weight of triglycerides in each chromatographic peak.

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