A METHOD TO INVESTIGATE GLYCERIDES IN COW'S MILK AND DAIRY PRODUCT FATS

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Abstract

The official Italian legislation describes the milk fat analysis of triglycerides by assuming the result mathematically elaborated as characterizing parameter.

This paper proposes an alternative HPLC method of qualitative and quantitative analysis, easier, faster and more suitable than the official Italian method.

For this purpose, samples of raw and pasteurised milk and cheeses obtained from the same milk were analysed. According our method, the fat is dissolved at room temperature in the eluente used for HPLC. A gradient elution with evaporative light-scattering detector (ELSD) yields good results in short time.

The proposed method performs qualitative and quantitative analysis. Di- and tri- glycerine esters, cholesterol and cholesteryl derivates were determined. Limit of detection (LOD = 4 ppm) and limit of quantification (LOQ = 8 ppm) were also obtained. Chromatograms obtained for cheese qualitatively coincide with those of milk of provenience.

Riassunto

La legislazione ufficiale Italiana riporta l'analisi dei trigliceridi del grasso del latte assumendo il risultato elaborato matematicamente come parametro caratterizzante.

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Questo lavoro propone un metodo HPLC di analisi qualitativa e quantitativa alternativo, più semplice, più veloce, e più adatto del metodo ufficiale Italiano. A questo scopo, erano analizzati campioni di latte crudo e pastorizzato e formaggio ottenuto dallo stesso latte. Secondo il metodo qui proposto, il grasso è sciolto a temperatura ambiente nell'eluente usato per HPLC. Una eluizione a gradiente con il rivelatore "evaporative light-scattering detector" (ELSD), diede buoni risultati in tempi brevi.

Il metodo proposto è in grado di fare l'analisi qualitativa e quantitativa. Erano determinati di- e tri- esteri della glicerina, colesterolo, e colesterin derivati. Sono stati anche ottenuti i limiti di rivelabilità (LOD = 4 ppm) e di quantificazione (LOQ = 8 ppm). Cromatogrammi relativi ai gliceridi del grasso del formaggio coincidono con quelli del latte da cui sono stati preparati.

Keywords: glycerides, cow milk, dairy product fats, HPLC

Introduction

The fat of milk and its analysis was a subject of particular interest, because the nutritional and commercial value of milk and dairy products is very often referred to the percentage of fat and to its composition. Furthermore, manipulations can occur so that strange substances are added to the fat of milk and cheese.

As glycerides are the prevailing constituents of fat, several methods were proposed for their analysis. The some time ago used periodic acid method is abandoned being time consuming (1). The GC (gas chromatographic) method is more diffused, but it requires derivatization (2-6).

Simple HPLC (high performance liquid chromatography) methods were developed to separate some mono- and di- saturated and unsaturated glycerides. However, the use of the UV detector represented a limit of the method.

The evaporative light – scattering detector (ELSD) was used in the analysis of triglicerides in edible oil and fat samples (7-8). It is an universal detector and its response is a function of the solute particles mass regardless of their chemical identities. In addition, the ELSD is compatible with the gradient elution carried out also by using most of organic solvents. Recently, the fat extracted from human milk was analysed for triglycerides by HPLC by using ELSD as detector (9).

The analysis of glycerides, without any differentiation among mono-, di- and triglycerides, was recommended by a D.M. of the Italian Ministero delle Politiche Agricole e Forestali (10) to evaluate the fat quality of milk and cheese. The procedure describes a GC analysis, carried out at 350 °C by using a packed glass silanized column, connected with a FID, modified for these chromatographic drastic conditions. Chromatograph and column had to be suitable to operate at 400 – 450 °C and particular care of the apparatus is requested (10).

The aim of our paper is to propose an easier, faster and accurate method of analysis of cow's milk and dairy product fat.

Experimental

Samples

Raw caw milk, mozzarella and scamorza (south Italian cheese) produced from the same milk were obtained from the same factory. Two breeds of cattle were represented in factory and a veterinary doctor oversaw them. All samples of milk were obtained from a mixture of two milking (morning and evening) at the end feed during 1 day.

The samples were stored in the fridge (about + 4 °C) at home and during transport to the laboratory were maintained at the same temperature.

Butter and pasteurised milk were commercial samples each of the same firm and also maintained at the same temperature.

Reagents

The solvents used, such as HPLC – grade dichloromethane and HPLC – grade acetonitrile, diethyl ether (BHT stabilized) were purchased by C. Erba (Milano, Italia).

Other reagents employed for the extraction of the fat, such as petrol ether, methanol, 96% ethanol, 30% NH_3, 37% HCl and KOH pellets, were RP grade obtained from C. Erba.

To prepare stock solutions injectable in HPLC, known amounts of standards were dissolved in the suitable solvent (dichloromethane for HPLC).

The purity grade of standards and solvents was checked before the use in HPLC, and resulted satisfactory for our purposes.

The following standards, all RP reagents, were used without further purification.

Monoglycerides (Fluka, Buchs, Switzerland): DL-α-Monoolein, DL-α-Palmitin, DL-α-Laurin and DL-α-Miristin.
Diglycerides (Fluka): DL-α,β-Distearin, 1,2-Dipalmitoyl-sn-Glycerol, Glycerol 1,3-Didecanoate, 2,3-Dipalmitoyl-sn-glycerol, 2,3-Dimiristoyl-sn-glycerol, Diolein.
Triglycerides (Sigma-Aldrich, Milano, Italia): Glyceryl trioleate, Glyceryl tripalmitelaidate, Glyceryl tripalmitoleate, 1,2-Dioleoyl-3-Palmitoyl-rac-glycerol, 1,2-Dilinoleoyl-3-oleoyl-rac-glycerol, Glyceryl Tripalmitate (PPP), Glyceryl Tristearate (SSS), Glyceryl Trimyristate (MMM), 1-Lauroyl-2-Oleoyl-3-Palmitoyl-rac-glycerol, 1,3-Dioleoyl-2-Palmityl-rac-glycerol, 1,2-Dioleoyl-3-Stearoyl-rac-glycerol,
Sterols (Sigma-Aldrich): Cholesterol, Cholesteryl Butyrate, Cholesteryl Pelargonate, Cholesteryl Palmitate, Cholesteryl Stearate, Cholesteryl Oleate

Detail on experimental apparatus

HPLC analyses were performed by using a Varian mod. 9010 pump system, an evaporative light – scattering detector (ELSD) Alltech mod. 500 and an acquisition data system DATA APEX CSW 32 v. 1. 3. All the analyses were carried out at room temperature. The analytical column used was Alltima (C 18, 150 mm, × 4.6 i.d., 3 μm particle size) and a guard-column (Alltima C18, 7.5 × 4.6 mm, 5 μm particle size).

HPLC chromatographic separation was carried out using a gradient acetonitrile – dichloromethane from (80 : 20, v/v) to (70 : 30, v/v) from 0 to 10 min and to (55 : 45, v/v) from 10 to 20 min. Finally, from 25 to 30 min., the (80 : 20, v/v) mixture flowed for 10 min more. After a few runs, the column was cleaned with n – hexane during 20 min. The eluent flow – rate was 1.5 ml / min. The volume of the sample injected was 20 μl. The temperature of ELSD was set at 75 °C and the gas flow (N_2 from cylinder) was 2.0 SLPM.

Chromatograms obtained by injecting standards at known concentration allowed to perform qualitative and quantitative analyses. As usually, the retention time (RT) was used for qualitative analysis and the peak area for the quantitative one.

Fat extraction

Known quantities of solid samples (i.e. mozzarella and scamorza) mixed with Na_2SO_4 were extracted for 6 hours with diethyl ether in a Soxhlet (10). After the total evaporation of the solvent at room temperature,

the sample could be analysed, by means of HPLC. Samples of butter were analysed by HPLC without preliminary treatment.

The extraction of fat from liquid samples was carried out according to the procedure proposed by Rose, modified by Gottlieb (11). Fat was extracted from five aliquots each of 10 ml of raw milk or pasteurised commercial milk. Extraction was performed by adding to the sample for three times, fresh quantities of 25 ml of petrol ether and 25 ml of diethyl ether. Fat was obtained by evaporating the solvent at room temperature, till constant weight. Known amounts of milk fat and butter were used for HPLC procedures.

Sample preparation for HPLC

Using a 500 mg/8.0 ml Silica Alltech extraction SPE, according to the following procedure, purified known amounts of fat dissolved in n-hexane.

After the activation, with 4.5 ml of eluent A (n-hexane: methylterbuthylic ether, 96 : 4 v/v), the SPE was charged by the sample dissolved in n-hexane. Eluent B (n-hexane: methylterbuthylic ether, 200 : 3 v/v) was added gradually till 5 ml. The obtained eluate was discarded. According to ref. (10), the eluate for the analysis was collected by adding eluents B (3 ml) and A (8 ml).

Results and Discussion

To carry out this research, the extraction of the fat fraction from each dairy product taken into account was performed, as before described (10-11).

Table 1 collects the results of the average percentages of the extracted fat.

The reported percentages represent the average of the results obtained by analysing 30 samples of raw milk (RM) produced by the same factory, 30 samples of pasteurised commercial milk (PA) of the same firm, 10 commercial samples of butter (BT) of the same firm, 10 samples of mozzarella (MZ) and 10 samples of scamorza (SA). Raw milk, mozzarella and scamorza were produced by the same factory, this means that both cheeses were made with the same milk.

TABLE 1

**AVERAGE PERCENTAGES OF EXTRACTED FAT FROM THE INVESTIGATED DAIRY PRODUCTS.
SYMBOLS HAVE THE MEANING EXPLAINED IN THE TEXT.
THE ERROR LIMITS REPRESENTS THE MAXIMUM DEVIATION OF THE RESULTS WITH RESPECT TO THE AVERAGE.**

DAIRY PRODUCT	% FAT FRACTION
RM	3.9 ± 0.1
PM	3.8 ± 0.1
MZ	46 ± 1
SA	46 ± 1
Butter	87 ± 1

Every obtained sample of fat was investigated by HPLC analyses for glycerides. The obtained results are presented, discussed and described in different sections.

HPLC results

In Figure 1, an example of HPLC chromatogram of fat obtained from pasteurized milk (PM) is reported. Several peaks are present in all the chromatograms, more than 30 for milk and more than 20 for mozzarella. Resolution is good and the time for a complete run never is more than 30 minutes. Chromatographic trends of fat extract from RM, butter, MZ and SA are similar.

The compounds corresponding to the chromatographic peaks were identified by comparing their retention times (RT) with those of suitable standards. An example of a HPLC chromatogram of standards is shown in Figure 2.

Quantitative analysis was performed by comparing the areas of the peaks of identified compounds with those of standards.

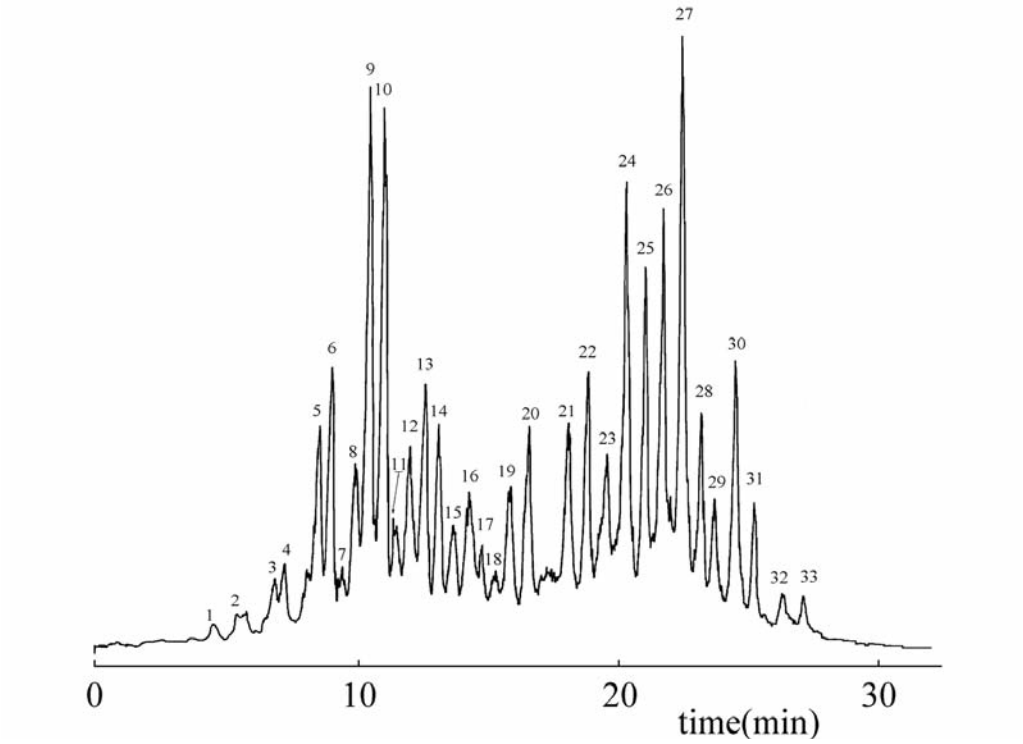

Fig. 1 - An example of HPLC chromatogram of a sample of pasteurized milk fat. 33 peaks, well resolved, can be observed. The pick numbers correspond to the compounds collected in Table 1.

For this purpose, it was necessary to determine LOD (Limit of Detection) and LOQ (Limit of Quantification) and to check the linearity of the detector response. It was found that LOD generally was about 4 ppm and LOQ 8 ppm. Only for a few compounds, higher values (8 and 15 ppm, respectively) were found.

Table 2 shows the average values of the percentage of each compound present in the fat fraction of raw milk, pasteurised milk, butter, mozzarella (MZ) and scamorza (SA).

The compounds listed in Table 2 are present in all the analysed samples (i.e. RM, PM, butter, MZ and SA) and correspond to the peaks identified in Figure 1.

Most of the identified compounds are diglycerides and triglycerides, but also the percentage of cholesterol and cholesteryl derivates is remarkable.

The chromatographic trend of Figure 2, as expected, shows that the diglycerides have RT values less than those of triglycerides. No appreciable amount of mono glycerides was detectable.

The type of group bond to cholesterol seems to be determinant on the RT value. A bigger group bond to cholesterol causes a higher value of RT.

Although the same compounds are present in all 5 types of samples, their percentages often are different.

It can be observed that it was identified and determined little less than 80% of RM and about 61% of PM. The percentages of identified compounds for butter, MZ, SA are lower than the others, i.e. they are about 50%.

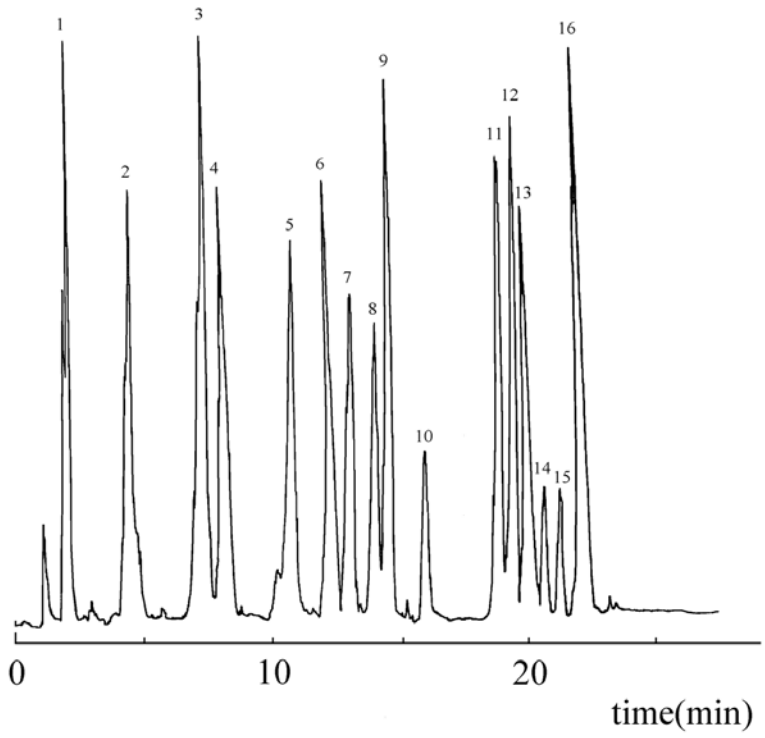

Fig. 2 - An example of chromatographic run for some selected standards.
The peaks correspond to the following standards: 1)= Glycerol 1,3-Didecanoate, 2)= 2,3-Dimiristoyl-sn-glycerol, 3)= 1,2-Dipalmitoyl-sn-Glycerol + 2,3-Dipalmitoyl-sn-glycerol, 4)= Cholesteryl Butyrate, 5)= DL-α,β-Distearin, 6)= Cholesteryl Pelargonate, 7)= Glyceryl tripalmitoleate, 8)= Glyceryl tripalmitoleate +1,2-Dilinoleoyl-3-oleoyl-rac-glycerol, 9)= Trimyristin, 10) =1-Lauroyl-2-

Oleoyl-3-Palmitoyl-rac-glycerol, 11)= Glyceryl trioleate, 12)= 1,2-Dioleoyl-3-Palmitoyl-rac-glycerol, + 1,3-Dioleoyl-2-Palmityl-rac-glycerol, 13)= Cholesteryl Palmitate, 14) = 1,2-Dioleoyl-3-Stearoyl-rac-glycerol, 15)= Glyceryl Tripalmitate, 16)= Cholesteryl Stearate.

Data of Table 2 show that the qualitative composition of RM, MZ, SA (obtained from the same milk) is not modified in the production process of MZ and SA.

That means that the same compounds are found in the samples RM, MZ and SA.

On the contrary, the percentages of several compounds are modified. It has to be taken present that the percentages of Table 2 refer to the fat content.

To compare the numeric values it is necessary to consider that the values of the third and fourth column of Table 2 (RM) and (PM) respectively refer to the 3.9 percent of extracted fat, whereas the values of the other columns refer to 87% (butter), and 46% (MZ) and (SA) of extracted fat. By taking into account this consideration it can be deduced that in the cheeses the percentages of the corresponding compounds generally increase from RM to MZ or SA. As expected, also the pasteurisation process modifies the percentages of constituents and, except few cases, the percentages decrease from RM to PM, even if it is hard to compare the data RM and PM because they are not referred to the same milk.

From an inspection of Table 2, it could be deduced that cholesterol percentage seems to be higher in milk than in solid corresponding products.

This result is not correct because the percentage of the RM column is referred to the 3.9% of fat extracted from milk, while the percentage of the MZ and SA column is referred to the 46% of fat extracted from each of the cheeses. If the percentages are referred to the original products (milk and cheeses), it can be calculated that the percentage of cholesterol is about 0.35% in milk and about 1.8 % in both cheeses.

TABLE 2

AVERAGE PERCENTAGES AND STANDARD DEVIATIONS (% ± σ) OF THE COMPOUNDS IDENTIFIED BY MEANS OF HPLC ANALYSIS ON RAW MILK (RM), PASTEURISED MILK (PM), BUTTER, MOZZARELLA (MZ) AND SCAMORZA (SA).

Peak		**RM**	**PM**	**Butter**	**MZ**	**SA**
		% ± σ.	% ± σ.	% ± σ.	% ± σ.	% ± σ.
1	Glycerol-1,3- Didecanoate	0.36 ± 0.11	0.06 ± 0.01	0.37 ±0.18	0.33 ±0.15	0.42 ± 0.24
3	2,3-Dimiristoyl-sn-glycerol	0.24 ± 0.04	0.17 ± 0.02	0.32 ± 0.20	0.23 ± 0.13	2.20 ± 0.43
4	1,2-Dipalmitoyl-sn-glycerol	1.21 ± 0.14	0.95 ± 0.20	4.98 ± 0.51	3.09 ± 0.03	6.80 ± 1.46
5	2,3-Dipalmitoyl-sn-glycerol	1.93 ± 0.19	1.55 ± 0.18	4.15 ± 0.71	6.21 ± 0.10	4.52 ± 1.37
7	Cholesteryl-butyrate	4.42 ± 0.16	3.70 ± 0.46	3.70 ± 0.73	4.14 ± 0.63	4.11 ± 0.11
13	DL-α,β-Stearin	12.21 ± 0.33	7.44 ± 0.52	4.75 ± 2.24	4.48 ± 0.93	5.57 ± 2.48
15	Cholesterol	8.47 ± 0.30	7.11 ± 0.24	3.90 ± 1.82	2.90 ± 1.19	2.96 ± 1.88
16	Cholesteryl-Pelargonate	3.06 ± 0.09	2.19 ± 0.21	1.30 ± 0.90	1.00 ± 0.14	1.50 ± 0.69
17	Glyceryl Tripalmitoleate	4.12 ± 0.18	2.72 ± 0.04	-	-	-
19	Glyceryl Tripalmitelaidate + LLO	10.10 ± 1.97	6.51 ± 0.45	4.59 ± 3.53	6.03 ± 0.02	6.89 ± 1.05
20	MMM	2.16 ± 0.10	1.60 ± 0.02	2.81 ± 0.77	3.66 ± 0.27	5.37 ± 0.87
25	OOO	4.99 ± 0.09	4.86 ± 0.17	4.11 ± 0.55	4.63 ± 1.59	4.57 ± 1.36
26	OOP + OPO	4.75 ± 0.10	4.59 ± 0.26	4.54 ± 1.38	5.54 ± 0.07	4.05 ± 3.03
27	Cholesteryl-Palmitate	9.51 ± 0.23	8.67 ± 0.47	1.24 ± 0.73	2.03 ± 0.52	1.28 ± 0.74
28	OOS	5.48 ± 0.05	5.72 ± 0.27	-	-	-
29	PPP	2.40 ± 0.13	2.56 ± 0.16	2.43 ± 0.41	2.97 ± 0.43	3.58 ± 1.67
30	Cholesteryl-Stearate	1.65 ± 0.13	1.18 ± 0.08	0.26 ± 0.05	0.54 ± 0.02	0.98 ± 0.08
	Total	**77.06**	**61.58**	**43.45**	**47.78**	**54.80**

The samples MZ and SA are produced from the same factory of RM, but their quantitative composition changes. It seems reasonable to explain the change both by means of the above explained calculations and for the different approach followed to obtain fat and with the wide enzymatic transformation involved with production process. Vice versa, the qualitative composition of RM, MZ and SA, deducible from the Table 2, is similar for all three type of products.

It seems to be impossible to try to obtain a balance for the obtained compounds also because loss of fat, proteins, lactose and other material is confirmed by the results of a previous investigation (12) that showed as whey is reach of organic material.

Furthermore, by comparing the percentages found for RM and PM, it can be observed that, although the two types of milk came from different firms, the same compounds are present in the corresponding columns and many values of the reported percentages agree within a reasonable range. In the same way, the columns relative to MZ and SA can be compared because both cheeses are obtained from the same milk. Also in this case, it can be observed that many values agree within a reasonable range, often within the standard deviation. Differences can be due also to the different process followed to produce MZ or SA.

It seems probable that the difference from data of columns 3, 6, and 7 with respect to those of columns 4 and 5 could be attributed to the origin (race of cows and their feeds) of the milk.

Data of Table 2 can represent the first step fundamental to characterize raw milk of a factory, a race and type of alimentation. This investigation must be extended to other factories, other races and other alimentation of cattle to deduce how breed and alimentation can change the composition of the produced milk.

Although only 50% of the compounds are identified and determined in MZ and SA, it seems reasonable to evaluate the experimental evidence given by superimposing the chromatograms obtained for RM, MZ and SA. By superimposing the three chromatograms a good fit is obtained, so that they are coinciding qualitatively. As before explained, this evidence agrees very well with our knowledge that MZ and SA are produced by the same RM, obtained by the same factory.

The method here proposed is very easy to perform, because the preparation of the sample for analysis is the slowest point of the procedure. It can be obtained or by means of the Rose – Gottliebe (11) method (for milk) or by extraction by Soxhlet (9) (for cheese).

The preparation of the sample for the analysis is similar in our and the official procedure. In our procedure, the sample is directly injected in HPLC connected with ELSD as detector. A commercial HPLC column is able to carry out the analysis, while the official procedure (10) foresees a glass packed column with a particular preparation that must be pre -treated for silanization. The preparation of the gas chromatograph with connected column, injector and detector requires a sophisticated technique and an enormous waste of time.

Even the official method (10) claims the use of GC suitable to support temperatures till 450 °C and detector modified for a high carrier gas power. Furthermore, the glass part of detector must be cleaned very often and GC must be equipped with septa suitable for high temperatures.

Finally, the official method performs qualitative and quantitative analysis by mean of a very sophisticated treatment of the experimental trend of the chromatograms, based on mathematic assumption and relative equations.

It is hard to obtain directly an answer about the required composition of the glycerides present in investigated milk or in cheese. In the official method (10) LOD and LOQ are not given, because it is supported by the sequence of a series of peaks hardly intelligible.

Conclusion

The main aim of this investigation was to develop a method alternative to the official one.

The here obtained results, mainly represented by Figures and Tables, show that the proposed method, applied to milk and cheese, gives performances better than those of the official method (10). Next to the direct evaluation of the obtained chromatograms, other important advantages are represented by instrumental point of view, i.e. the absence of manipulation of the sample, the rapidity of the analysis and the ELSD detector use.

In the proposed method, the sample has to be dissolved directly in the eluent and, after a very easy purification, injected in the HPLC chromatograph.

The very drastic increasing of temperature (400 – 450 °C), the column and its silanization present in the official method, are avoided.

In our method, the gradient elution carried out at room temperature assures the integrity of the fat. The ELSD use gives also an important contribute to the good performance of the analysis.

Finally, the glycerides composition seems to be of great importance under a further point of view. From an inspection of Table 1, it can be seen that the column MZ and SA are qualitatively similar. By taking into account that MZ = mozzarella and SA = scamorza, are obtained from the same milk (RM), the glycerides composition could represent a marker to identify the raw material for the production of cheese. This paper can represent a first investigation on this path.

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REFERENCES

(1) Official and Tentative Methods of the American Oil Chemists' Society, Vol. I and II, 3rd edn., American Oil Chemists'Society, Champaign, 1966, Method Cd 11 – 57.

(2) R. Watts, R. Dils, "Separation of mono- and diglycerides by gas-liquid chromatography", *J. Lipid Res. 1969*, 10, 33-40.

(3) J. Blum, W.R. Koehler, "Quantitative determination of glycerol, mono and diglycerides by gas liquid chromatography", *Lipids 1970*, 5, 601-606.

(4) E. Bottari, M.R. Festa, "Analysis of lipids in milk", *Ann. Chim. (Rome) 1988*, 78, 153-162.

(5) T. Lee, C. Hastilow, K. Smith, "Simple method for derivatization of monoglycerides and diglycerides", *J. Assoc. Off. Anal. Chem, 1988*, 71, 785-788.

(6) A. J. Palmer, F.J. Palmer, "Rapid analysis of triacyglycerols using high performance liquid chromatography with light scattering detection", *J. Chromatogh. 1989*, 465, 369-377.

(7) A. Stolyhwo, H. Colin, G. Guiochon, "Analysis of triglycerides in oils and fats by liquid chromatography with light scattering detector", *Anal. Chem. 1985*, 57, 1342-1354.

(8) C. H. Winter, E. B. Hoving, F. A. J. Muskiet, "Fatty composition of human milk triglycerides species. Possible consequences for optimal structures of infant fromula triglycerides., *J. Chromatogh. 1993*, 616, 9-24.

(9) M. Romeu-Nadal, S. Morera-Pons, A. I. Castellote, M.C. López-Sabater, "Comparison of two methods for the extraction of fat from human milk", *Anal. Chim. Acta 2004*, 513, 457-461.

(10) Gazzetta Ufficiale Repubblica Italiana n. 90, 17 April 2003, D.M. of 28 Mars 2003 and G.U.C.E. l 37 of 7/2/2001 p. 51 – 59.

(11) Norme FIL – IDF, Vol. I Ed., Associazione Italiana Tecnici del Latte

(12) L. Allegrucci, E. Bottari, M.R. Festa, *Proceeding 1st Conf. Eur. Cust. Chem., Fiuggi Fonte (FR, Italy), 18-20 /10/2000*, pag. 59-69.