

Detection of cow milk in donkey milk by chemometric procedures on triacylglycerol stereospecific analysis results

Lina Cossignani, Francesca Blasi, Ancilla Bosi, Gilda D'Arco, Silvia Maurelli, Maria Stella Simonetti and Pietro Damiani*

Dipartimento di Scienze Economico-Estimative e degli Alimenti, Sezione di Chimica Bromatologica, Biochimica, Fisiologia e Nutrizione, Università degli Studi di Perugia, Via San Costanzo, 06126, Perugia, Italy

Received 14 January 2011; accepted for publication 5 May 2011

Stereospecific analysis is an important tool for the characterization of lipid fraction of food matrices, and also of milk samples. The results of a chemical-enzymatic-chromatographic analytical method were elaborated by chemometric procedures such as linear discriminant analysis (LDA) and artificial neural network (ANN). According to the total composition and intrapositional fatty acid distribution in the triacylglycerol (TAG) backbone, the obtained results were able to characterize pure milk samples and milk mixtures with 1, 3, 5% cow milk added to donkey milk. The resulting score was very satisfactory. Totally correct classified samples were obtained when the TAG stereospecific results of all the considered milk mixtures (donkey-cow) were elaborated by LDA and ANN chemometric procedures.

Keywords: Donkey milk, cow milk, triacylglycerol stereospecific analysis, linear discriminant analysis, artificial neural network.

Dairy products sold for public consumption must be accurately labelled in accordance with the contained milk species. Fraudulent labelling which may not be detected still exists. It affects the quality of the product and moreover species identification has a remarkable importance on account of frequent human adverse reactions, such as allergies (Commission Regulation, 1992, 1996, 2001; Mayer, 2005). In the last few years, many studies have been made to identify the best natural substitutes for human milk (Businco et al. 2000). In the case of IgE-mediated and non-IgE-mediated protein allergy, donkey's milk was found to be a valid alternative to cow's milk, also considering the favourable palatability (Monti et al. 2007). The high lactose content, the characteristic protein profile and the relevant percentage of essential amino acids make this milk a potential new dietetic food and a valid breast milk substitute (Guo et al. 2007).

Methods enabling detection and quantitative determination of cow milk in mixtures with other milks have been studied for a long time. The principal frauds involve the undeclared addition of cow milk to goat or ewe milks, the main motivation being due to the lower yield and the higher

price of ewe or goat milks (Ramos & Juráez, 1986; Rodriguez-Nogales, 2006).

As species identification in animal products has received great attention in recent years, many methods for milk speciation have been set up. For this purpose different analytical approaches could be used: lipid and protein analysis or DNA-based methods for example.

As regards the protein fraction either electrophoresis or immunoassays have been applied (Hurley et al. 2004); capillary electrophoresis (Molina et al. 1999; Rodriguez-Nogales, 2006), two-dimensional electrophoresis (Chianese et al. 1990), isoelectric focusing (Moio et al. 1990), chromatographic techniques (Haza et al. 1995; De Noni et al. 1996; Ferriera & Caçote, 2003) and enzyme linked immunosorbent assay methods (Ritcher et al. 1997) were worth mentioning. Recently, DNA-based methods such as polymerase chain reaction (Bottero et al. 2003; López-Calleja et al. 2007) have been used.

The lipid fraction has been used less for milk species identification. The most commonly used methods were based on milk fat composition such as some fatty acid (FA) ratios or triacylglycerol (TAG) profile, but these do not always allowed detection of milk adulteration. Lipid analysis was based on chromatographic techniques (Goudjil et al. 2003) or on nuclear magnetic resonance (NMR) spectroscopy. Andreotti et al. (2000) have investigated the TAG from cow and buffalo milk fat by ¹³C-NMR, a useful

*For correspondence; e-mail: chimbrom@unipg.it; coslina@unipg.it

technique to provide unique information for milk identification of different animal species. Brescia et al. (2004) have characterized and differentiated buffalo and cow milk samples by means of $^1\text{H-NMR}$; multivariate statistical analysis, carried out on the results of the lipid analysis, allowed buffalo milk to be distinguished from cow milk.

In a previous paper (Blasi et al. 2008) various samples of milk fat from mammalian non-ruminant (donkey) as well as ruminant (cow, ewe, goat, buffalo) species were studied. Donkey milk appeared interesting for its potential biological properties. In fact, it represented an important dietary source of essential FA, esterified in high percentage in the *sn*-2-position. The different distribution of the FA on the glycerol backbone of TAG has suggested this non-random distribution could provide some procedures to detect possible adulteration of one milk type with another of minor commercial value.

Among multivariate statistical data analysis, linear discriminant analysis (LDA) is probably the best known method (Fisher, 1936) and can be used not only to examine multivariate differences between groups, but also to determine which variables are the most useful for discriminating between groups (Leotta, 2004). LDA is considered an important classical parametric method for grouping samples when the sample allocation is previously known. It has been successfully used for the identification/differentiation of various foods, such as oils, wines and others (Damiani et al. 1997; Arvanitoyannis et al. 1999; Cocchi et al. 2007). Discriminant analysis has been used to distinguish the milk and cheese of various species (Fresno et al. 1995; Rodriguez et al. 1999; Rodriguez-Nogales, 2006; Karoui et al. 2008).

The objective of this study was to apply LDA to the experimental data of TAG stereospecific analysis to select the best variables to characterize the matrixes according to animal species origin and to provide unique information for identification, characterization and differentiation of donkey and cow milk and of their mixtures.

The analyses were also carried out using an artificial neural network (ANN), technique widely used for analyzing data for food quality prediction (Ni & Gunasekeran, 1998).

Materials and Methods

Samples and reagents

Five milk samples of cow and donkey, randomly selected from Italian farms, were collected at morning milking in sterile bottles by direct manual milking, from animals in mid-lactation. The samples were immediately stored at 4 °C for the subsequent analyses. All animals, free from mastitis or any other inflammatory diseases, were multiparous; the number of lactation was 2-3 for both animal species; they grazed in the morning and in the afternoon were reared in stables and fed with hay, fodder grass and vegetables.

In order to verify the possibility of a group separation on the basis of the analytical data, the milk samples were

assigned to five groups indicated as: D (pure donkey milk, $n = 5$), D/C 99:1 (99% donkey milk-1% cow milk, $n = 5$), D/C 97:3 (97% donkey milk-3% cow milk, $n = 5$), D/C 95:5 (95% donkey milk-5% cow milk, $n = 5$) and C (pure cow milk, $n = 5$). Group D contained separate samples from each of the five donkeys and group C contained separate samples from each of the five cows. The D/C groups contained five separate donkey milks, each mixed with one representative pool of five cow milk samples. The data of TAG, relative to total and intrapositional FA compositions, of the considered mixtures were obtained using software developed at the University of Perugia, that is able to calculate their compositions from the experimental data of the stereospecific analysis of cow and donkey pure milk samples.

All solvents and reagents were of analytical grade and were purchased from Carlo Erba Reagents (Milano, Italy). Phospholipase A_2 from hog pancreas (561 U/mg) was from Fluka. A standard mixture from Supelco (Bellefonte, PA, USA), Supelco 37 component fatty acid methyl esters (FAME) mix, containing the methyl esters of 37 FA (Catalog No. 47885-U) was used; the FA contents ranged between 2% and 4%, while the palmitic acid methyl ester was 6%.

Lipid extraction

Lipid extraction was carried out according to the procedure reported in a previous work (Blasi et al. 2008). In brief, lipid fraction was extracted with a mixture of chloroform/methanol (2:1, v/v). The chloroform phase was collected, while the aqueous phase was washed with a mixture chloroform/methanol/water (86:14:1, v/v/v). The collected organic phases were washed with water, totally dried in a rotary evaporator and then dissolved in hexane.

Stereospecific analysis of TAG

TAG fraction was isolated from total fat by thin layer chromatography (TLC) as previously reported (Blasi et al. 2008); in the cited article the phospholipase A_2 procedure for stereospecific analysis of TAG has been detailed. In brief, *sn*-1,3/*sn*-1,2(2,3)-diacylglycerols (DAG) were prepared by partial chemical deacylation of TAG using ethyl magnesium bromide in anhydrous ethyl ether; *sn*-1,2(2,3)-DAG were isolated by TLC and then subjected to chemical synthesis to obtain *sn*-1,2(2,3)-phosphatidylcholines (PC). Finally *sn*-1,2(2,3)-PC, isolated by TLC, were reacted with phospholipase A_2 to obtain *sn*-1-lysophosphatidylcholines (LPC) and free FA; these hydrolysis products were separated by TLC, derivatized and analyzed by HRGC analysis, as reported in the following Section.

HRGC analysis

FAME preparation and their HRGC analysis were reported in previous work (Blasi et al. 2008). The FAME of total TAG and *sn*-1-LPC were prepared by transmethylation using hexane and methanolic 2M-KOH while the esterification of free FA

Table 1. Total and intrapositional FA composition of TAG fraction of cow and donkey pure milks

(% mol mean values \pm standard deviations, n=5)

FA	Cow				Donkey			
	Total	<i>sn</i> -1-	<i>sn</i> -2-	<i>sn</i> -3-	Total	<i>sn</i> -1-	<i>sn</i> -2-	<i>sn</i> -3-
C4:0	6.7 \pm 0.8	1.6 \pm 1.2	0.3 \pm 0.2	18.3 \pm 1.8	2.2 \pm 1.1	1.0 \pm 1.1	0.6 \pm 0.7	5.1 \pm 3.2
C6:0	2.9 \pm 0.6	0.3 \pm 0.1	1.0 \pm 0.2	7.6 \pm 1.9	0.5 \pm 0.5	0.5 \pm 0.6	0.1 \pm 0.1	1.0 \pm 1.1
C8:0	1.7 \pm 0.2	0.3 \pm 0.1	0.2 \pm 0.1	4.5 \pm 0.6	4.4 \pm 0.5	1.1 \pm 0.8	1.5 \pm 1.5	10.6 \pm 3.6
C10:0	3.5 \pm 0.5	1.1 \pm 0.4	1.3 \pm 0.6	8.0 \pm 1.4	10.1 \pm 3.6	4.4 \pm 0.8	7.2 \pm 8.7	18.7 \pm 12.6
C12:0	3.8 \pm 0.6	2.3 \pm 0.4	4.3 \pm 0.8	4.7 \pm 1.4	9.5 \pm 5.3	7.2 \pm 4.4	12.7 \pm 7.3	8.6 \pm 7.2
C14:0	13.8 \pm 1.0	11.2 \pm 1.3	22.2 \pm 0.7	8.0 \pm 1.7	7.9 \pm 3.5	8.0 \pm 5.7	12.1 \pm 4.9	3.7 \pm 2.6
C16:0	33.1 \pm 1.0	45.7 \pm 0.8	42.5 \pm 2.8	11.0 \pm 1.0	22.3 \pm 1.4	28.6 \pm 4.0	30.3 \pm 3.1	7.9 \pm 7.2
C16:1n-7	1.9 \pm 0.4	1.4 \pm 0.1	2.5 \pm 0.5	1.8 \pm 0.7	5.7 \pm 2.1	5.5 \pm 0.4	5.6 \pm 2.7	6.1 \pm 5.9
C18:0	7.9 \pm 0.5	11.6 \pm 0.7	5.9 \pm 0.4	6.1 \pm 1.1	1.5 \pm 0.8	2.0 \pm 1.3	1.7 \pm 0.7	0.9 \pm 1.2
C18:1n-9	22.6 \pm 2.9	22.8 \pm 2.1	17.6 \pm 2.7	27.3 \pm 4.2	20.9 \pm 4.7	26.4 \pm 5.8	14.9 \pm 7.7	21.4 \pm 9.3
C18:2n-6	2.0 \pm 0.2	1.7 \pm 0.1	1.9 \pm 0.4	2.5 \pm 0.3	9.5 \pm 3.1	9.0 \pm 3.0	9.1 \pm 5.6	10.4 \pm 3.8
C18:3n-3	0.2 \pm 0.1	0.2 \pm 0.0	0.2 \pm 0.0	0.3 \pm 0.1	5.4 \pm 4.0	6.3 \pm 4.2	4.3 \pm 3.8	5.7 \pm 5.1

was performed using diazomethane. A standard solution containing 37 FAME was used to identify the individual FA; the same solution was used to calculate some analytical parameters (linearity parameters, limits of detection (LOD) and limits of quantification (LOQ)). Correlation coefficients were found to be higher than 0.9947 in all cases, indicating a good linearity; the LOD value (ng/ml) ranged from 10 for linolenic acid to 36 for butyric acid; the LOQ value (ng/ml) ranged from 30 for linolenic acid to 110 for butyric acid. The percentage of each FA was calculated using the peak area of the samples corrected with the respective correction factors, as reported by Christie (2003). The data were normalized considering only the main reported FA (% mol mean values greater than 0.1). Each HRGC analysis was carried out in duplicate.

Statistical analysis

The considered FA were the following: butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3); the variables were the total (TAG) and the intrapositional (INTRA) FA compositions (%mol) in the three *sn*- positions (*sn*-1-, *sn*-2-, *sn*-3-) of the TAG glycerol backbone of the milk mixtures.

The variables entered in the chemometric analysis were the ones selected by means of the multiple regression method, rejecting the variables linearity associated to the others already in the equation (Lachenbruch, 1979).

The data were elaborated with the classical multivariate statistical analysis methods (LDA), according to the Mahalanobis distance criterion and using the SPSS Professional Statistics version 9.0 software.

The ANN data elaboration was carried out using the Neural Connection software from SPSS Inc., Chicago, IL, USA. The structure of the ANN used for TAG data elaboration consisted of one input layer (analytical results) with 12 nodes, which corresponded to the FA, two hidden layers (with 7 and 3 nodes, respectively) and one output layer for the required output (differentiation of milk samples). The structure of the ANN used for INTRA data elaboration consisted of one input layer (analytical results) with 36 nodes, which corresponded to the FA in the three *sn*-glycerol positions, two hidden layers (with 18 and 9 nodes, respectively) and one output layer for the required output. The ANN training was terminated when the root mean square error reached the minimum value.

Results and Discussion

Table 1 shows the total and intrapositional FA compositions (mean values and standard deviations) of TAG fraction of cow and donkey pure milks. Both milks contained high percentage of saturated FA with some differences relative to the individual FA; in particular donkey milk showed lower % contents of short and long chain FA and higher content of medium chain FA relative to cow milk. Moreover the donkey milk had the higher amount of polyunsaturated FA, represented by linoleic and linolenic acids, both essential FA. These data confirmed that donkey milk is a very interesting food matrix from a nutritional point of view. The stereospecific analysis data showed differences in the FA distribution; donkey milk presented a higher % value of essential FA in all the three *sn*- positions, while the short chain saturated FA preferred the *sn*-1- and the *sn*-3- positions.

Table 2 shows the data of the total and intrapositional FA compositions of the three D/C mixtures (99:1, 97:3, 95:5),

Table 2. Total and FA intrapositional composition of TAG fraction of donkey and cow milk mixtures

(% mol mean values \pm standard deviations, n = 5)

FA	Donkey-Cow 99:1			Donkey-Cow 97:3			Donkey-Cow 95:5					
	Total	sn-1-	sn-2-	sn-3-	Total	sn-1-	sn-2-	sn-3-	Total	sn-1-	sn-2-	sn-3-
C4:0	2.6 \pm 0.1	1.0 \pm 0.1	0.6 \pm 0.0	4.0 \pm 0.2	3.2 \pm 0.2	1.1 \pm 0.3	0.6 \pm 0.1	6.3 \pm 0.4	3.7 \pm 0.3	1.1 \pm 0.4	0.5 \pm 0.1	8.1 \pm 0.6
C6:0	0.8 \pm 0.1	0.3 \pm 0.0	0.5 \pm 0.1	1.7 \pm 0.2	1.2 \pm 0.2	0.3 \pm 0.0	0.6 \pm 0.1	2.8 \pm 0.5	1.5 \pm 0.3	0.3 \pm 0.1	0.6 \pm 0.1	3.6 \pm 0.8
C8:0	3.9 \pm 0.0	0.9 \pm 0.1	0.8 \pm 0.0	8.8 \pm 0.1	3.6 \pm 0.1	0.8 \pm 0.1	0.8 \pm 0.1	8.1 \pm 0.2	3.3 \pm 0.1	0.7 \pm 0.0	0.7 \pm 0.0	7.6 \pm 0.2
C10:0	9.0 \pm 0.1	3.4 \pm 0.0	2.6 \pm 0.1	26.6 \pm 0.2	8.1 \pm 0.2	3.0 \pm 0.1	2.4 \pm 0.1	23.6 \pm 0.4	7.4 \pm 0.2	2.7 \pm 0.1	2.2 \pm 0.2	21.4 \pm 0.7
C12:0	8.5 \pm 0.1	5.8 \pm 0.1	7.9 \pm 0.1	8.0 \pm 0.1	7.7 \pm 0.2	5.2 \pm 0.1	7.4 \pm 0.3	7.5 \pm 0.4	7.2 \pm 0.3	4.8 \pm 0.2	7.0 \pm 0.5	7.2 \pm 0.5
C14:0	8.2 \pm 0.1	8.4 \pm 0.1	14.3 \pm 0.1	6.4 \pm 0.1	9.2 \pm 0.3	9.0 \pm 0.4	15.7 \pm 0.3	6.7 \pm 0.4	9.9 \pm 0.4	9.3 \pm 0.5	16.8 \pm 0.5	7.0 \pm 0.5
C16:0	22.2 \pm 0.3	25.5 \pm 0.2	28.6 \pm 0.3	3.1 \pm 0.2	24.2 \pm 0.7	29.1 \pm 0.6	31.2 \pm 1.0	4.6 \pm 0.6	25.8 \pm 0.9	31.7 \pm 0.9	33.1 \pm 1.4	5.7 \pm 0.8
C16:1n-7	5.1 \pm 0.1	9.8 \pm 0.0	10.4 \pm 0.0	3.7 \pm 0.0	4.6 \pm 0.1	8.4 \pm 0.1	9.1 \pm 0.1	3.4 \pm 0.1	4.2 \pm 0.1	7.4 \pm 0.1	8.1 \pm 0.1	3.2 \pm 0.2
C18:0	2.1 \pm 0.2	3.8 \pm 0.1	2.6 \pm 0.1	2.0 \pm 0.2	2.9 \pm 0.4	5.0 \pm 0.2	3.0 \pm 0.4	2.5 \pm 0.5	3.6 \pm 0.5	5.9 \pm 0.4	3.4 \pm 0.5	2.9 \pm 0.7
C18:1n-9	19.9 \pm 0.4	19.4 \pm 0.3	14.5 \pm 0.4	14.4 \pm 0.6	20.1 \pm 1.1	19.8 \pm 0.8	14.7 \pm 1.1	16.2 \pm 1.4	20.2 \pm 1.6	20.0 \pm 1.1	14.9 \pm 1.6	17.5 \pm 2.0
C18:2n-6	8.4 \pm 0.1	12.5 \pm 0.0	8.9 \pm 0.0	9.3 \pm 0.0	7.3 \pm 0.1	10.8 \pm 0.1	7.8 \pm 0.1	8.2 \pm 0.1	6.5 \pm 0.1	9.4 \pm 0.1	6.9 \pm 0.1	7.4 \pm 0.2
C18:3n-3	4.6 \pm 0.0	6.2 \pm 0.0	3.8 \pm 0.0	3.7 \pm 0.0	3.9 \pm 0.0	5.2 \pm 0.0	3.2 \pm 0.0	3.2 \pm 0.1	3.3 \pm 0.1	4.4 \pm 0.0	2.8 \pm 0.1	2.7 \pm 0.0

obtained by elaboration of experimental results of the stereospecific analysis of cow and donkey pure milk samples and used as variables in the successive statistical analyses.

Among the various multivariate techniques, the simplest seems to be the LDA method, a widespread parametric method of classification, where the distinction between two categories is a linear function. The parametric LDA techniques are based on the assumption that the data obey a multivariate normal distribution and that the covariance matrix of each category (dispersion of the category) is not significantly different from one case to another. The Mahalanobis distances of each object from the centroids of the categories are computed and the resulting object assigned to the category with lowest distance. When performing a multiple group discriminant analysis some optimal combinations of variables can automatically be determined so that the first function provides the most overall discrimination between groups, the second provides second most, and so on.

Table 3 shows the values of Fisher's linear discriminant functions (eigenvalues, their respective percentages of variance and significance tests). They have been obtained carrying out the chemometric analysis on the samples grouped according to the following classification parameters: TAG (total FA compositions of TAG fraction, %mol) and INTRA (intrapositional FA compositions of TAG fraction, %mol) of milk mixtures (D/C 99:1, 97:3, 95:5). The first discriminant function (function 1) explained 99.9% of the variance in the case of TAG; in the case of INTRA the function 1 and the function 2 explained respectively 90.0% and 9.9% of the variance. Wilks's lambda test showed that only the first function was significant ($P < 0.05$) for TAG data, while the first two functions were the most important for INTRA data.

Initially, the samples were analyzed according to the total FA composition (%mol) and subsequently according to the intrapositional FA composition (%mol), obtained by stereospecific analysis of TAG fraction. Since each lipid matrix, both animal and vegetable, has a non-random characteristic distribution of FA among the glycerol positions (Parodi, 1983), these last data could be useful to determine the origin of the fat as well as to study a possible diversification of the milk mixtures in order to detect possible adulteration of dairy products.

Tables 4a and 4b show the unstandardized canonical discriminant function coefficients of mixtures, elaborated according to TAG and INTRA data, respectively. These coefficients can be used to compare the relative importance of the independent variables. The unstandardized discriminant functions of each considered system appear as:

$$\text{Group} = K_{\alpha} + \alpha_1 \cdot x_1 + \alpha_2 \cdot x_2 + \dots + n\alpha_n \cdot x_n$$

where K_{α} is a constant of the model, x_1, \dots, x_n are the analytical parameters of each sample entered in the model,

Table 3. Fisher's linear discriminant functions and functions at group centroids obtained from LDA analysis using TAG and INTRA acidic compositions

Function	Eigenvalue	% of variance	Cumulative (%)	Canonical correlation	Test of function	Wilk's lambda	Chi-square	df	Signif.
TAG									
1	777.164†	99.9	99.9	0.999	1–4	0.001	97.476	24	0.000
2	0.582†	0.1	100.0	0.607	2–4	0.569	7.607	15	0.939
3	0.110†	0.0	100.0	0.020	3–4	0.901	1.413	8	0.994
4	0.000†	0.0	100.0	0.020	4	1.000	0.005	3	1.000
	Centroids	Function 1	Function 2						
	D	–22.305	1.088						
	D/C 99:1	–16.978	–0.249						
	D/C 97:3	–6.891	–0.450						
	D/C 95:5	0.558	–0.769						
	C	45.616	0.380						
INTRA									
1	418.713†	90.0	90.0	0.999	1–4	0.000	127.640	32	0.000
2	46.260†	9.9	99.9	0.989	2–4	0.015	52.145	21	0.000
3	0.302†	0.1	100.0	0.482	3–4	0.729	3.949	12	0.984
4	0.053†	0.0	100.0	0.225	4	0.949	0.649	5	0.986
	Centroids	Function 1	Function 2						
	D	–11.822	11.079						
	D/C 99:1	–15.517	–5.579						
	D/C 97:3	–6.062	–3.259						
	D/C 95:5	–0.548	–2.921						
	C	33.950	0.679						

†for the analyses were used the first four canonical discriminant functions
D, pure donkey milk; C, pure cow milk; D/C, donkey/cow milk mixtures

Table 4a. Unstandardized canonical discriminant function coefficients obtained from LDA analysis using TAG acidic compositions

	Function			
	1	2	3	4
C4:0t	5.366	1.778	2.543	1.184
C6:0t	0.784	–2.467	4.495	1.864
C8:0t	13.174	12.416	8.301	–1.297
C10:0t	–9.886	–6.960	–13.103	–0.034
C12:0t	3.020	3.812	11.285	1.109
C14:0t	4.700	0.947	–3.874	–1.198
(Constant)	–58.042	–29.084	11.470	1.325

t, total FA% content in TAG fraction

Table 4b. Unstandardized canonical discriminant function coefficients obtained from LDA analysis using INTRA acidic compositions

	Function			
	1	2	3	4
C4:0-sn-1	6.890	5.822	2.857	3.020
C6:0-sn-1	11.187	17.807	14.328	1.860
C8:0-sn-1	0.418	6.831	–21.781	20.815
C10:0-sn-1	–9.762	–17.136	–3.235	–15.504
C12:0-sn-1	–1.346	8.757	–0.933	5.328
C14:0-sn-1	3.734	–1.864	1.930	0.123
C16:0-sn-1	0.916	0.722	–0.913	–0.226
C8:0-sn-2	17.662	18.455	15.983	4.683
(Constant)	–54.370	–32.772	21.380	1.128

sn-1, FA% content in sn-1- position of TAG fraction

sn-2, FA% content in sn-2- position of TAG fraction

$\alpha_1, \dots, \alpha_n$ are the regression coefficients of these parameters and n is the number of predictive variables.

As regards TAG data, only six of twelve FA were considered, among which were all the short and medium chain acids. Caprilic acid was the variable that contributed most the first two canonical functions, accounting for most of the discrimination between milk mixtures (99.9%), while capric acid showed the lowest values. In the case of INTRA data, eight FA were considered as variables: all the saturated

acids in sn-1- position, with the exception of stearic acid, and only the caprilic acid for the sn-2- position. This last variable was the most important discriminant parameter, together with caproic acid in sn-1- position. The data of FA compositions in the sn-3- position were never considered.

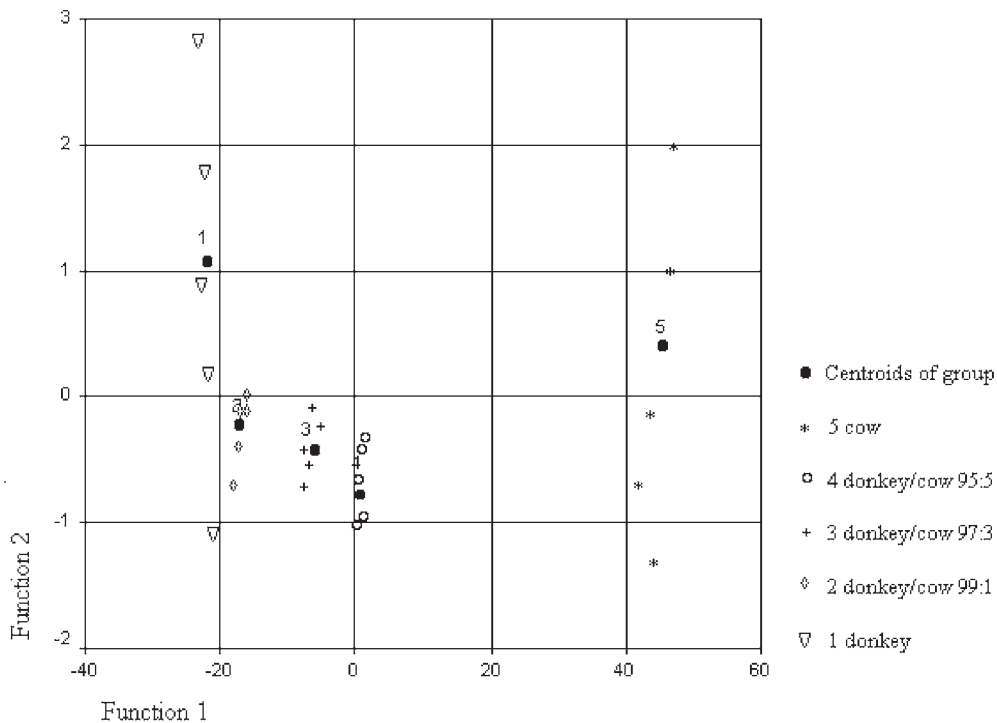


Fig. 1. Discriminant function plot of the first two functions obtained from LDA analysis using TAG acidic compositions.

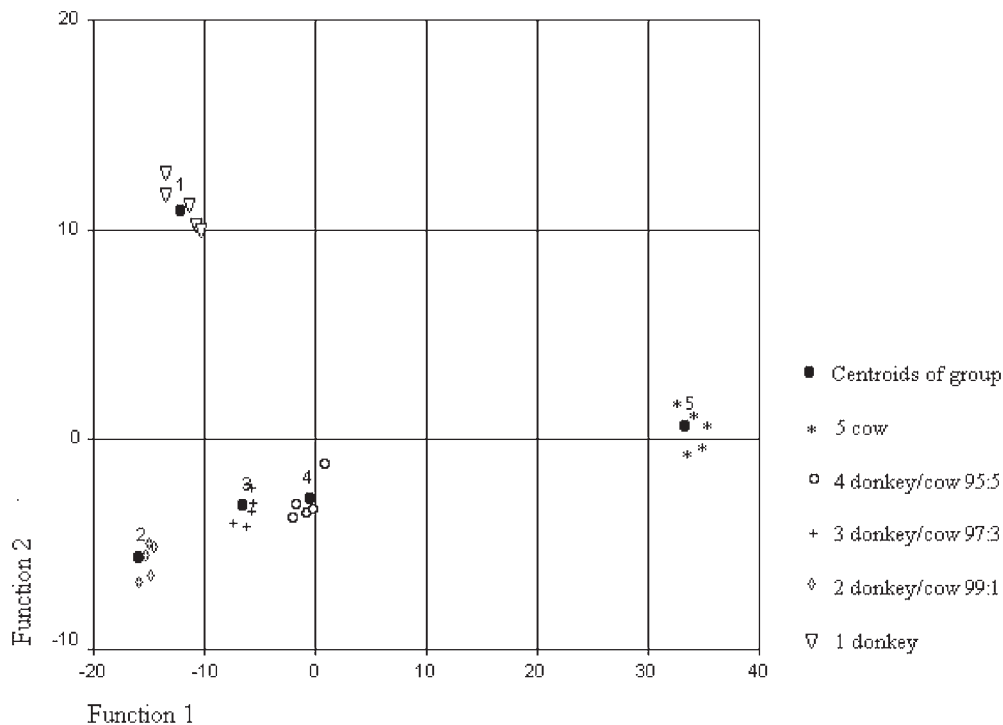


Fig. 2. Discriminant function plot of the first two functions obtained from LDA analysis using INTRA acidic compositions.

The plot of results of known samples can be obtained using the values of the first two discriminant functions, especially when these explain almost the entire variance.

Fig. 1 and **2** show the graphical distribution of the milk samples on the plots, for the TAG and INTRA data elaboration respectively. It is possible to observe that the

Table 5. Classification results obtained from LDA analysis using TAG and INTRA acidic compositions

	%‡	1	2	3	4	5	Total
Original	TAG						
	D	100-0					100-0
	D/C 99:1		100-0				100-0
	D/C 97:3			100-0			100-0
	D/C 95:5				100-0		100-0
	C					100-0	100-0
	INTRA						
	D	100-0					100-0
	D/C 99:1		100-0				100-0
	D/C 97:3			100-0			100-0
D/C 95:5				100-0		100-0	
C						100-0	

‡samples correctly classified
For abbreviations, see Table 3

INTRA data permitted a better separation of different groups, in fact both pure and mixture samples were close to the group centroid. Using these functions, the coordinates of the centroids of the D/C samples (using the group means, as reported in Table 3) were calculated.

Table 5 shows the classification of results; it reports the percentages of samples correctly classified into each group (on the diagonal of the matrices). The study, carried out to correlate the total and the intrapositional FA (%mol) compositions of milk samples with the percentage of cow milk in donkey milk, showed that a completely effective classification was always possible by LDA, using both the data of total and intrapositional compositions; in fact from these results it was evident that all the samples were correctly classified (100.0%).

The results showed that this statistical approach can be used to evaluate the differences between milk samples, in fact the LDA always gave satisfactory results, even if the milk samples were not numerous. Nevertheless, the stereospecific analysis data were useful to correctly discriminate even the mixture with the 1% of bovine milk, because, as known, total and intrapositional TAG compositions are related to the biosynthetic pathway.

As alternative approach to the classical and more usual LDA, in this research the data of TAG total and intrapositional FA compositions of the pure and mixture milk samples were elaborated by ANN. This system analyzes the information coming from the interconnections among the nodes of the various layers which constitute the net (Zupan & Gasteiger, 1993) and offer the possibility of classifying/characterizing the examined samples according to parameters of the net used.

Table 6 shows the parameters for the ANN analysis used for the classification of the same milk sample groups considered for LDA analysis. The ANN results showed that the classification was in all cases well defined, in fact low error and good correlation have been obtained using both

Table 6. Parameters and results of ANN analysis using TAG and INTRA acidic compositions

	TAG	INTRA
Iterations	30,000	30,000
Training error	0.001821	0.000920
Learn rate	0.075183	0.288120
Momentum factor	0.800	0.800
Training data		
Bias	1.2273E-05	6.1661E-06
Max error	0.01963	0.01495
Correlation	0.99997	0.99999

TAG and INTRA data. However, as already observed with LDA, the ANN results were slightly better when the INTRA data were used.

Conclusions

The results reported in this work showed that FA intrapositional compositions were able to characterize donkey milk and identify mixtures containing very small amounts of cow milk. The results showed that the considered statistical approaches permitted discrimination among pure and adulterated milks; also the separation of the samples containing different percentages (1, 3 and 5%) of cow milk added to donkey milk has been obtained. The elaborations carried out by LDA were completely significant for the differentiation/classification of the milk mixtures; the elaborations by ANN confirmed the results obtained by LDA. For a practical application of this method, as the classification of new samples of unknown mixtures, a broader calibration set-up would probably be necessary.

References

- Andreotti G, Trivellone E, Lamanna R, Di Luccia A & Motta A 2000 Milk identification of different species: ^{13}C -NMR spectroscopy of triacylglycerols from cows and buffaloes' milks. *Journal of Dairy Science* **83** 2432–2437
- Arvanitoyannis IS, Katsota MN, Psarra EP, Soufleros EH & Kallithraka S 1999 Application of quality control methods for assessing wine authenticity: Use of multivariate analysis (chemometrics). *Trends in Food Science and Technology* **10** 321–336
- Blasi F, Montesano D, De Angelis M, Maurizi A, Ventura F, Cossignani L, Simonetti MS & Damiani P 2008 Results of stereospecific analysis of triacylglycerol fraction from donkey, cow, ewe, goat and buffalo milk. *Journal of Food Composition and Analysis* **21** 1–7
- Bottero MT, Civera T, Numerà D, Rosati S, Sacchi P & Turi RM 2003 A multiplex polymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. *International Dairy Journal* **13** 277–282
- Brescia MA, Mazzilli V, Sgaramella A, Ghelli S, Fanizzi FP & Sacco A 2004 ^1H NMR characterization of milk lipids: A comparison between cow and buffalo milk. *Journal of the American Oil Chemists' Society* **81** 431–436
- Businco L, Giampietro PG, Lucenti P, Lucaroni F, Pini C, Di Felice G, Iacovacci P, Curadi C & Orlandi M 2000 Allergenicity of mare's milk in children with cow's milk allergy. *Journal of Allergy and Clinical Immunology* **105** 1031–1034
- Chianese L, Laezza P, Smaldone LA, Stingo C, Del Giovine L & Addeo F 1990 Evaluation of bovine milk in the buffalo mozzarella cheese by two-dimensional electrophoresis. *Scienza e Tecnica Lattiero-Casearia* **41** 315–326
- Christie WW 2003. *Lipid Analysis*, third ed. The Oily Press, Bridgwater
- Cocchi M, Durante C, Marchetti A, Armanino C & Casale M 2007 Characterization and discrimination of different aged 'Aceto Balsamico Tradizionale di Modena' products by head space mass spectrometry and chemometrics. *Analytica Chimica Acta* **589** 96–104
- Commission Regulation 1992 Reference method for the detection of cows' milk casein in ewes' milk cheese EEC no 690/92 of 19 March 1992. *Official Journal of the European Communities* **L74** 23–32
- Commission Regulation 1996 Reference method for the detection of cows' milk and caseinate in cheeses from ewes' milk, goats' milk and buffaloes' milk or mixtures of ewes', goats' and buffaloes' milk EC no 1081/96 of 14 June 1996. *Official Journal of the European Communities* **L142** 15–25
- Commission Regulation 2001 Reference method for the detection of cows' milk and caseinate in cheeses from ewes' milk, goats' milk and buffaloes' milk or mixtures of ewes', goats' and buffaloes' milk EC no 213/2001 of 9 January 2001. *Official Journal of the European Communities* **L37** 51–60
- Damiani P, Cossignani L, Simonetti MS, Campisi B, Favretto L & Gabrielli Favretto L 1997 Stereospecific analysis of the triacylglycerol fraction and linear discriminant analysis in a climatic differentiation of Umbrian extra-virgin olive oils. *Journal of Chromatography A* **758** 109–116
- De Noni I, Tirelli A & Masotti F 1996 Detection of cows' milk in non-bovine cheese by HPLC of whey protein: Application to goat milk cheese. *Scienza e Tecnica Lattiero-Casearia* **47** 7–17
- Ferriera IMPLVO & Caçote H 2003 Detection and quantification of bovine, ovine and caprine milk percentages in protected denomination of origin cheeses by reversed-phase high-performance liquid chromatography of beta-lactoglobulins. *Journal of Chromatography A* **1015** 111–118
- Fisher RA 1936 The use of multiple measurements in taxonomic problems. *Annals of Eugenics* **7** 179–188
- Fresno JM, Prieto B, Urdiales R, Sarmiento RM & Carballo J 1995 Mineral content of some Spanish cheese varieties. Differentiation by source of milk and by variety from their content of main and trace elements. *Journal of Science Food Agricultural* **69** 339–345
- Goudjil H, Fontecha J, Fraga J & Juárez M 2003 TAG composition of ewe's milk fat Detection of foreign fats. *Journal of the American Oil Chemists' Society* **80** 219–222
- Guo HY, Pang K, Zhang XY, Zhao L, Chen S, Dong ML & Ren FZ 2007 Composition, physicochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk. *Journal of Dairy Science* **90** 1635–1643
- Haza AI, Morales P, Martín R, García T, Anguita G, González I, Sanz B & Hernández PE 1995 Immunoreactivity of goats' milk casein fractionated by ion exchange chromatography. *Journal of Agriculture and Food Chemistry* **43** 2025–2029
- Hurley IP, Ireland HE, Coleman RC & Williams JHH 2004 Application of immunological methods for the detection of species adulteration in dairy products. *International Journal of Food Science and Technology* **39** 873–878
- Karoui R, De Baerdemaeker J & Dufour E 2008 A comparison and joint use of mid infrared and fluorescence spectroscopic methods for differentiating between manufacturing processes and sampling zones of ripened soft cheeses. *European Food Research and Technology* **226** 861–870
- Lachenbruch PA 1979 *Discriminant analysis*, Hafner Pree, New York
- Leotta R 2004 Use of linear discriminant analysis to characterise three dairy cattle breeds on the basis of several milk characteristics. *Italian Journal of Animal Science* **3** 377–383
- López-Calleja I, González I, Fajardo V, Martín I, Hernández PE, García T & Martín R 2007 Real-time TaqMan PCR for quantitative detection of cows' milk in ewes' milk mixtures. *International Dairy Journal* **17** 729–736
- Mayer HK 2005 Milk species identification in cheese varieties using electrophoretic, chromatographic and PCR techniques. *International Dairy Journal* **15** 595–604
- Moio L, Sasso ML, Chianese L & Addeo F 1990 Rapid detection of bovine milk in ovine, caprine and water buffalo milk or cheese by gel isoelectric focusing on phastsystem. *Italian Journal of Food Science* **3** 185–190
- Molina E, Martín-Álvarez PJ & Mercedes R 1999 Analysis of cows', ewes' and goats' milk mixtures by capillary electrophoresis: quantification by multivariate regression analysis. *International Dairy Journal* **9** 99–105
- Monti G, Bertino E, Muratore MC, Coscia A, Cresi F, Silvestro L, Fabris C, Fortunato D, Giuffrida MG & Conti A 2007 Efficacy of donkey's milk in treating highly problematic cow's milk allergic children: an in vivo and in vitro study. *Pediatric Allergy and Immunology* **18** 258–264
- Ni H & Gunasekaran S 1998 Food quality prediction with neural networks. *Food Technology* **52** 60–65
- Parodi PW 1983 Positional distribution of fatty acids in the triglyceride classes of milk fat. *Journal of Dairy Research* **49** 73–80
- Ramos M & Juárez M 1986 Chromatographic, electrophoretic and immunological methods for detecting mixtures of milk from different species. *Bulletin International of Dairy Federation* **202** 175–190
- Ritcher W, Krause I, Graf C, Sperrer I, Schwarz C & Klostermeyer H 1997 An indirect competitive ELISA for the detection of cows' milk and caseinate in goat's and ewes' milk and cheese using polyclonal antibodies against bovine γ -caseins. *Zeitschrift für Lebensmittel-Untersuchung und-Forschung* **204** 21–26
- Rodríguez EMR, Alaejos MS & Romero CD 1999 Chemometric studies of several minerals in milks. *Journal of Agricultural and Food Chemistry* **47** 1520–1524
- Rodríguez-Nogales JM 2006 Approach to the quantification of milk mixtures by partial least-squares, principal component and multiple linear regression techniques. *Food Chemistry* **98** 782–789
- Zupan J & Gasteiger J 1993 *Neural networks for chemist: an introduction*, VCH-Verlagsgesellschaft, Weinheim