

Milk Identification of Different Species: ^{13}C -NMR Spectroscopy of Triacylglycerols from Cows and Buffaloes' Milks

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ABSTRACT

Triacylglycerols from cows and buffaloes' milk fat were investigated by ^{13}C nuclear magnetic resonance (NMR) spectroscopy. By the addition of pure triacylglycerols standards, we identified the resonances of both milk fats, and the peaks were used for qualitative and quantitative analysis of acyl groups. Multivariate analysis treatment of triacylglycerols distribution and composition parameters enabled us to identify milk. This study shows that NMR can safely be used to quantitate milk fatty acid content, providing unique information for milk identification of different animal species.

(Key words: buffaloes' milk, milk fat, triacylglycerols, ^{13}C -nuclear magnetic resonance)

Abbreviation key: FID = free induction decay, GC = gas chromatography, NMR = nuclear magnetic resonance, PCA = principal component analysis.

INTRODUCTION

Milk is a fundamental dietary constituent of many societies and is an exceptionally complex biological fluid. It contains several components (fat globule, somatic cells, serum, and casein micelles) that have been studied separately by many techniques (Walstra and Jenness, 1984; Jensen et al., 1991). Recent investigations have explored the possibility of altering milk composition to improve its functional properties and increase its marketability (Grummer, 1991; Gibson, 1991; Karatzas and Turner, 1997), as well as to search for parameters that could unequivocally identify the animal species from which milk comes. The latter ability is very important in many countries, particularly those bordering the Mediterranean, where a large number of

dairy products are made with milk obtained from species other than cows.

In the last decade the use of nuclear magnetic resonance (NMR) in food science has consistently grown (Belton et al., 1993; Gil et al., 1996). This noninvasive technique preserves food structure and extracts useful information from such a chemically complex and highly heterogeneous system. NMR also supports the food industry in its increasing need to understand and be innovative in products and process and provides a new method to enforce legislation and control quality. A well-known example is the authentication of olive oil by using ^{13}C NMR spectroscopy (Sacchi et al., 1992, 1997). From a single ^{13}C NMR spectrum, the fatty acid amount, the saturated, monounsaturated and polyunsaturated fatty acid ratios can be determined. In addition, the presence of unsaturated *trans* isomers and the distribution of fatty acids on the glycerol chain can also be detected (Wellenberg, 1990; Sacchi et al., 1995; Lie Ken Jie and Mustafa, 1997, and references therein).

Although widely studied, because of its complexity, milk and dairy products have not yet been subjected to thorough examination by NMR (Belton et al., 1993; Gil et al., 1996). For example, the use of ^1H and ^{13}C NMR has been limited to the description of the nonrandom distribution of butyric acyl group in triacylglycerols from butter oil fraction (Pfeffer et al., 1977; Gunstone, 1993; Kalo et al., 1996; Van Calsteren et al., 1996).

In this paper we report on the composition and the distribution of fatty acids in triacylglycerols from cows and Italian river buffaloes' (*Bubalus bubalis*) milks as obtained by ^{13}C NMR spectroscopy. We examined the 13 most abundant fatty acids (Jensen et al., 1991), showing that NMR can safely be applied to measure milk fatty acid content, affording data reliable as those obtained by other techniques, without requiring extensive manipulation of the sample. Furthermore, we show that the composition and distribution of the fatty acids in cows and buffaloes' milk triacylglycerols can be used to distinguish the two milks, providing a potential solution to the milk authenticity issue. This issue is particu-

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larly important in the region around Naples. Here, there is an urgent demand for quality control due to the high profit from selling "Mozzarella di bufala" cheese exclusively produced from buffaloes' milk. This is a typical "Appellation d'Origine Protégée" cheese of Campania ("Mozzarella di bufala campana").

MATERIALS AND METHODS

Preparation of the Samples

Spring cows and buffaloes' bulk milks were obtained from local farms. The raw samples were collected during 2 mo. Triacylglycerols were extracted from 250 mg of fat from both milks with chloroform and methanol (ratio of 2:1 by volume) by using the modified Folch method (Hamilton et al., 1992). The final samples had the same amount of triacylglycerols, with the volume adjusted to 0.5 ml. In the sample preparation procedure only deuterated solvents were used.

NMR Spectra of Triacylglycerols

High-resolution ^{13}C NMR spectra were obtained at 75.5 MHz on a Bruker DPX-300 spectrometer with a 5-mm dual $^{13}\text{C}/^1\text{H}$ probe. Each free induction decay (FID) was acquired over a spectral width of 220 ppm at 27°C using a 90° pulse of 5.1 μs , and inverse-gated decoupling to avoid a nuclear Overhauser effect to the signals. To avoid signal saturation, we used a delay of 20 s between acquisitions; the estimated spin-lattice relaxation rate of the slowest relaxing nuclei (the carbonyls) is about 5 s. The FID, acquired with 64 K complex data points were zero-filled to 128 K, baseline corrected, and Fourier transformed without apodization functions. Chemical shifts were referred to internal chloroform, assumed to resonate at 77.01 ppm. For the 13 most abundant fatty acids, resonance assignment was obtained by adding pure triacylglycerols standards (tributylin, tricaprinn, tricaproin, tricapyrillin, trilaurin, trilinolein, trimyristin, trimyristolein, triolein, tripalmitin, tripalmitolein, tripentadecanoin, and tristearin) purchased from Sigma Chemical (St. Louis, MO).

Quantitative Spectral Analysis

Fourier-transformed spectra were phased and then baseline corrected by spline interpolation of 14 baseline selected points. The spectral signals were fit to a sum of Lorentzian curves by a nonlinear least-square algorithm. The calculated area of each NMR signal was used to determine the relative concentration of each fatty acid. The total area of signals in the region of interest was used as normalization parameter. Data

analysis was performed with the software MacFID 1D 5.3 (Tecmag Inc., Houston, TX).

Multivariate Analysis

Principal component analysis (PCA) was applied to 10 parameters derived from the spectra. In particular, we considered the overlapping ω_3 carbon signal of myristoleic and oleic acyl groups and the contents of several fatty acids, namely, butyric, caproic, and linoleic (averaged on ω_1 , ω_2 , and ω_3 resonances), caprylic (averaged on ω_1 and ω_3 resonances), myristoleic (averaged on ω_1 and ω_2 resonances), capric and palmitoleic (ω_3 resonance only). The ratio between the areas of C1 signals, referring to saturated and unsaturated fatty acids in the β -position, and the ratio obtained from overlapping C9 signals of oleic, myristoleic, and palmitoleic α - and β -positions, were also included in the PCA analysis. The first three principal components, which accounted for the 52.9, 19.0, and 12.3% of the total variance, were considered. The PCA was carried out with Matlab version 5.3.0 (The MathWorks, Inc.) routines.

RESULTS AND DISCUSSION

Spectral Analysis of Triacylglycerols

The ^{13}C resonances of cows and buffaloes' milk triacylglycerols were assigned by the addition of triacylglycerols standards (see Materials and Methods) to both samples. Comparisons between spectra indicated that they are qualitatively very similar. Figure 1 reports the C1, C2, and olefinic regions of triacylglycerols from buffaloes' milk fat. The signal at 173.04 ppm (panel A, C1 region) corresponds to the butyric acyl group in *sn*-1,3 position of the glycerol backbone (also called α -position). The signal relative to *sn*-2 position (also called β -position), expected to resonate at 172.66 ppm as observed upon tributyrin addition, was not detected. The nonrandom distribution of butyric acid was also observed in the ^{13}C NMR spectrum of cows' milk fat (not shown). This result is in agreement with pancreatic lipase deacylation (Breckenridge and Kuksis, 1968), previous ^1H and ^{13}C NMR studies (Pfeffer et al., 1977; Gunstone, 1993; Kalo et al., 1996; Van Calsteren, 1996), and Grignard deacylation thin-layer chromatography (Angers et al., 1998) performed on cows' fat.

Two clusters of peaks, centered at 173.21 and 172.80 ppm, accounted for the α - and β -positions, respectively, of the remaining fatty acids. In each cluster the carbonyls were identified by the addition of triacylglycerol standards. Being more deshielded, saturated chains (labeled S in Figure 1) resonated at lower field than those of the unsaturated ones (labeled U). However, the presence of as many as 13 different acyl groups hampered

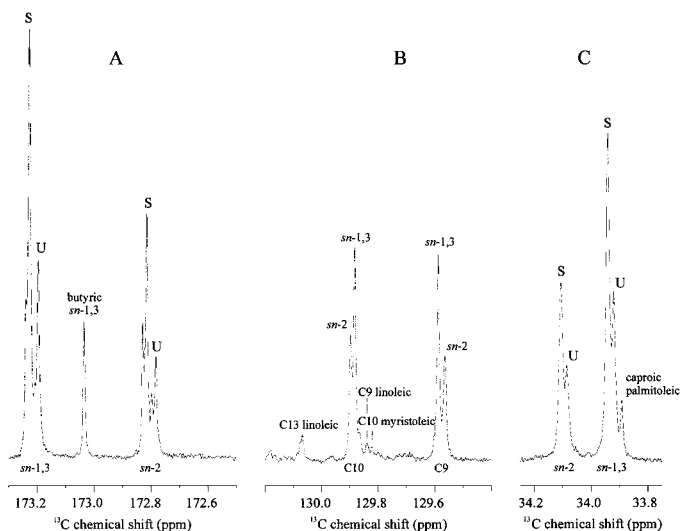


Figure 1. 75.5 MHz ^{13}C nuclear magnetic resonance (NMR) spectrum of the C1 (A), olefinic (B), and C2 (C) regions of triacylglycerols from buffaloes' milk fat in CDCl_3 at 27°C. Saturated, S, and unsaturated, U, fatty acids signals are labeled referring to the glycerol backbone, i.e., position *sn*-1,3 and position *sn*-2. In (A) the C1 signal for butyric acyl group in *sn*-1,3 position is labeled. In (B), C10 labels corresponding oleic and palmitoleic acyl groups resonances, and C9 refers to oleic, palmitoleic, and myristoleic acyl groups; peaks originating from C9 and C13 of linoleic, and C10 of myristoleic acyl groups are also indicated. The C2 signal for caproic and palmitoleic acyl groups in *sn*-1,3 position is labeled in (C).

a unique assignment of resonances. Furthermore, because the carbonyl chemical shift was also affected by neighboring fatty acid chains (Van Calsteren et al., 1996), no qualitative or quantitative parameters could be extracted from the carbonyl region.

In the olefinic region, we identified the most abundant monounsaturated fatty acids (oleic, palmitoleic, and myristoleic) and the most abundant diunsaturated fatty acid (linoleic) (Figure 1B). For unsaturated carbons (C9–C10 for monounsaturated, and C9–C10 and C12–C13 for diunsaturated fatty acids) we observed characteristic pairs of signals according to the α - or β -position. As shown by Ng (1984), the chemical shift between the peaks in a pair became smaller for the olefinic carbon nearer the methyl end of the fatty acid chain. The C9 signals of oleic and palmitoleic as well as the C10 signals were coincident (129.57 and 129.89 ppm, respectively), while myristoleic C9 overlapped with C9 signals of oleic and palmitoleic acids (at 129.57 ppm), and C10 partially superimposed with linoleic acid C9. Linoleic C10 and C12 (not shown) and C13 (at 130.07 ppm) signals were all isolated.

In Figure 1C the signals relative to the C2 carbons of buffaloes' milk fat are reported. We observed two clusters centered at 34.09 and 33.92 ppm, corresponding to the β - and α -positions, respectively. Within each

cluster the saturated and unsaturated fatty acids signals could easily be identified. The observed peaks did not include butyric acid, which gave a signal at 35.80 ppm corresponding to the α -position. Again, no signal referred to its β -position (expected to resonate at 35.65 ppm, as from the standard) was detected (data not shown). Caproic ($\text{C}_{6:0}$) and palmitoleic ($\text{C}_{16:1}$) acyl chains showed a preferential distribution in the α -position, having a single overlapping signal at 33.89 ppm. In cows' milk, caproic acid was mostly found in α -position, whereas palmitoleic acid was predominantly located in the β -position in triacylglycerols of lower size, but in α -position in longer triacylglycerols (Angers et al., 1998).

The presence of strongly overlapping signals, and the effect on chemical shift of the neighboring chains (Van Calsteren et al., 1996) also render the C2 region unsuitable for the analysis of fatty acids composition.

The ω 1, ω 2, and ω 3 regions seemed to be more suitable for qualitative and quantitative analysis, showing well-separated signals (Figure 2). In the ω 1 region (Figure 2C) we identified five separated acyl chain signals [butyric ($\text{C}_{4:0}$) at 13.47 ppm, caproic ($\text{C}_{6:0}$) at 13.75 ppm, caprylic ($\text{C}_{8:0}$) at 13.92 ppm, myristoleic ($\text{C}_{14:1}$) at 13.86 ppm and linoleic ($\text{C}_{18:2}$) at 13.94 ppm], while all the others gave a cluster of peaks at lower field (13.98 ppm). Isolated signals were also observed for the ω 2 carbons (Figure 2B), and they referred to caproic (22.18 ppm), caprylic (22.49 ppm), myristoleic (22.23 ppm), linoleic (22.47 ppm), and lauric acid ($\text{C}_{12:0}$) (22.53 ppm). Palmitoleic ($\text{C}_{16:1}$) and oleic ($\text{C}_{18:1}$) acids gave a single signal at 22.56 ppm, partially separated from the cluster at 22.58 ppm, accounting for all the other acyl chains examined. The ω 2 signal of butyric acid in α -position resonated at 18.23 ppm (data not shown).

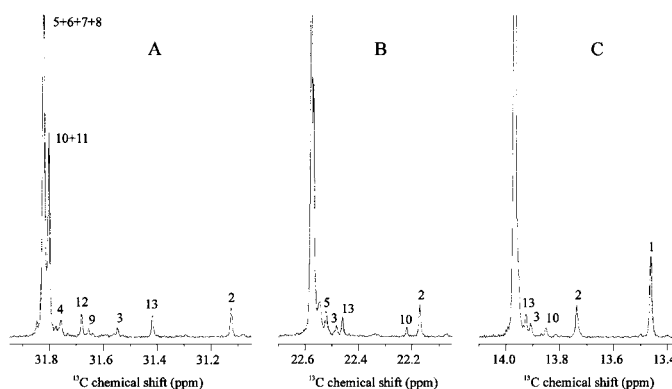


Figure 2. 75.5 MHz ^{13}C NMR spectrum of the ω 3 (A), ω 2 (B), and ω 1 (C) regions of triacylglycerols of buffaloes' milk fat in CDCl_3 at 27°C. Peak 1 in the ω 1 region and peak 2 in the ω 3 region belong respectively to butyric and caproic acyl groups in *sn*-1,3 position. All the other peaks belong to saturated and unsaturated fatty acids analyzed and are not dependent from their position in the glycerol backbone. Numbers used are the same as those shown in Table 1.

Table 1. Fatty acid composition (mol %) of triacylglycerols from cows and buffaloes' milk fats.

Id. number	Acyl group	Composition (mol %)							
		Buffalo ¹³ C NMR				Cow ¹³ C NMR			
		ω 3 ¹	ω 2 ¹	ω 1 ¹	Average	ω 3 ¹	ω 2 ¹	ω 1 ¹	Average
1	4:0	10.9 ± 0.4	11.6 ± 1.3	12.3 ± 1.4	11.6 ± 1.3	10.4 ± 0.6	10.5 ± 0.6	11.5 ± 1.2	10.8 ± 0.9
2	6:0	4.5 ± 0.3	4.2 ± 0.7	4.9 ± 0.7	4.5 ± 0.7	4.7 ± 0.4	4.5 ± 0.2	5.1 ± 0.4	4.8 ± 0.3
3	8:0	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.6	1.5 ± 0.4	2.0 ± 0.5	2.0 ± 0.6	1.8 ± 0.6	1.9 ± 0.5
4	10:0	1.8 ± 0.6	1.8 ± 0.6	3.2 ± 0.6	3.2 ± 0.6
5	12:0	54.0 ± 1.3 ²	4.2 ± 0.6	...	4.2 ± 0.6	53.5 ± 3.1 ²	3.9 ± 0.5	...	3.9 ± 0.5
6	14:0	49.8 ± 1.4 ⁴ (54.0–4.2)	49.6 ± 3.1 ⁴ (53.5–3.9)
7	16:0
8	18:0
9	15:0	0.8 ± 0.2	0.8 ± 0.2	1.5 ± 0.5	1.5 ± 0.5
10	14:1	20.6 ± 1.2 ³	1.0 ± 0.2	1.5 ± 0.7	1.2 ± 0.6	19.0 ± 1.8 ³	0.9 ± 0.3	1.3 ± 0.4	1.1 ± 0.4
11	18:1	19.4 ± 1.2 (20.6–1.2)	17.9 ± 1.8 (19.0–1.1)
12	16:1	2.6 ± 0.6	2.6 ± 0.6	2.0 ± 0.5	2.0 ± 0.5
13	18:2	2.6 ± 0.5	2.7 ± 0.6	4.1 ± 1.2	3.1 ± 1.1	3.1 ± 0.9	2.8 ± 0.6	3.0 ± 1.1	3.0 ± 0.7

¹Each value averages at least 7 samples. A dotted line (. . .) indicates that the corresponding acyl group has not been determined as a single species.

²Values refer to the sum of (C_{12:0}), (C_{14:0}), (C_{16:0}), and (C_{18:0}) acyl groups, as shown in Figure 2A.

³Values refer to the sum of (C_{14:1}) and (C_{18:1}) acyl groups, as shown in Figure 2A.

⁴Values refer to the sum of (C_{14:0}), (C_{16:0}) and (C_{18:0}) acyl groups.

Figure 2A (ω 3 region) showed six clearly resolved peaks, assigned to capric (31.77 ppm), caprylic (31.56 ppm), caproic (31.14 ppm), linoleic (31.43 ppm), palmitoleic (31.69 ppm), and pentadecanoic (C_{15:0}) (31.66 ppm) acyl chains. The two intense signals at 31.83 and 31.81 ppm originated from overlapping stearic (C_{18:0}), palmitic (C_{16:0}), myristic (C_{14:0}), and lauric (C_{12:0}) acids, and to oleic and myristoleic acids, respectively. The ω 3 signal of butyric acid corresponded to the C2 signal discussed previously, and resonated outside the shown ω 3 region at 35.80 ppm. Caproic acid in standard triacylglycerols gave two different signals at 31.14 and 31.09 ppm, depending on whether the chain is present in the α - or β -position. In both cows and buffaloes' milks we found this acid mostly in α -position. Similar distribution was been obtained for bovine milk fat by Grignard deacylation thin-layer chromatography (Angers et al., 1998).

Fatty Acid Composition of Triacylglycerols

The resonances in the ω 1, ω 2, and ω 3 regions were simulated, and the area of each peak was used for a quantitative analysis of the fatty acid contents in both milks. The results are summarized in Table 1. For isolated acyl groups in the three regions [butyric (C_{4:0}), caproic (C_{6:0}), caprylic (C_{8:0}), and linoleic (C_{18:2}), Figure 2] we obtained three composition (mol %) values. For some acyl groups, one (C_{10:0}, C_{12:0}, C_{15:0}, and C_{16:1}) or two (C_{14:1}) values were obtained from corresponding isolated peaks in one or two regions (Figure 2). In these

cases, the average is certainly reliable for quantitative analysis as values reported for each region referred to at least seven milk samples. Some overlapping peaks were also evaluated. The oleic (C_{18:1}) and the myristoleic (C_{14:1}) acyl groups were determined as an overlapping peak in the ω 3 region. On the other hand, the myristoleic was obtained as a single peak in ω 1 and ω 2 regions, and the average value obtained from the latter measurements allowed the determination, as a difference, of the oleic percentage. Peaks from lauric (C_{12:0}), myristic (C_{14:0}), palmitic (C_{16:0}), and stearic (C_{18:0}) acyl chains were all superimposed in a single resonance in ω 3, while in ω 2 the lauric acyl group was isolated. Accordingly, we report the percentage for the lauric and, as a difference, the value for the myristic, palmitic, and stearic envelope.

The NMR data of Table 1 indicate that the triacylglycerol fatty acid composition of buffaloes' milk is almost identical with cows' milk. For example, we determined 11.6, 4.5, and 1.5% for butyric, caproic, and caprylic acids, respectively, for buffaloes, and 10.8, 4.8 and 1.9% for cows. Considering the standard deviation, the notable exception between cows and buffaloes' milk fat is the capric (C_{10:0}) acyl group with following values: 1.8 ± 0.6 and 3.2 ± 0.6%, respectively.

The reported NMR values can be compared with the percentages derived from gas chromatography (GC) analysis. In Table 2 we report our data and GC values for bovine milk fatty acids adapted from Christie and Clapperton (1982) (third column) and from Angers et al. (1998) (fourth column). For example, NMR percent-

ages of caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), and pentadecanoic ($C_{15:0}$) acyl groups are in excellent agreement with both GC data sets. Exceptions were butyric ($C_{4:0}$), caproic ($C_{6:0}$), palmitoleic ($C_{16:1}$), and linoleic ($C_{18:2}$) acyl groups, for which there is excellent agreement with the data from Christie and Clapperton (1992). The discrepancy with Angers et al. (1998) is linked to the fact that their analysis excluded triacylglycerols with partition number <32 and those with partition number equal to 52, most likely containing short and long chain acyl groups, respectively. The single NMR value (49.6%) obtained for myristic, palmitic, and stearic groups compares well with 48.8%, obtained by adding the corresponding Angers values (11.1, 28.9, and 8.8%). On the other hand, oleic NMR percentage (17.9%) also agrees with Angers (18.1%). In both cases, we hypothesize that the discrepancy with Christie and Clapperton data (42.1 and 24%) is due to changes in the diet of animals. In fact, such a dependence was described for long chain fatty acids (Grummer, 1991; Christie and Clapperton, 1982). The myristoleic acid percentage (1.1%), not described by Christie and Clapperton, is in agreement with the value derived from Angers (1.0%).

Distinguishing Cows' from Buffaloes' Milks

The spectral analysis and the fatty acid composition of triacylglycerols showed $C_{10:0}$ content as the single appreciable difference between cows and buffaloes' milks. From a thorough comparison between the milks,

Table 2. Comparison of fatty acid composition (mol %) of triacylglycerols from cow milk fat as determined by different techniques.

Acyl group	Composition (% mol)		
	NMR ¹	GC ²	GC ³
4:0	10.8 ± 0.9	11.8	8.6
6:0	4.8 ± 0.3	4.6	3.6
8:0	1.9 ± 0.5	1.9	1.8
10:0	3.2 ± 0.6	3.7	3.3
12:0	3.9 ± 0.5	3.9	3.7
14:0		11.2	11.1
16:0	49.6 ± 3.1 ⁴	23.9	28.9
18:0		7.0	8.8
15:0	1.5 ± 0.5	2.1	1.2
14:1	1.1 ± 0.4	... ⁵	1.0
18:1	17.9 ± 1.8	24.0	18.1
16:1	2.0 ± 0.5	2.6	1.3
18:2	3.0 ± 0.7	2.5	1.3

¹Data from Table 1.

²Gas chromatography data adapted from Christie and Clapperton (1982).

³Gas chromatography data adapted from Angers et al. (1998).

⁴This number refers to the sum of ($C_{14:0}$), ($C_{16:0}$) and ($C_{18:0}$) acyl groups.

⁵Not reported.

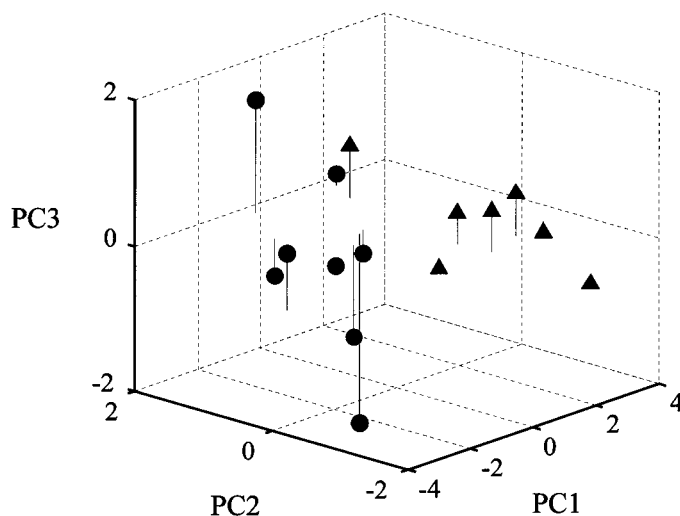


Figure 3. Score plot of the first three principal components (PC1, PC2, and PC3) obtained from principal component analysis (PCA) of 10 NMR parameters from 15 milk fat samples. The filled circles represent the cows' samples, whereas the buffaloes' samples are indicated by triangles.

two important parameters could be obtained from the distribution of acyl group in glycerol backbone. From overlapping C9 signals of oleic, myristoleic, and palmitoleic α - and β -positions (at 129.39 and 129.35 ppm, respectively, Figure 1B), we obtained a ratio between the areas of 2.50 ± 0.33 for cows' and 1.87 ± 0.14 for buffaloes' milks. The ratio between the areas of C2 signals referring to saturated and unsaturated fatty acids in the β -position (centered at 34.09 ppm, Figure 1C) gave 2.58 ± 0.40 for buffaloes' and 3.85 ± 0.69 for cows' milk.

For each milk sample, collected NMR data were analyzed by multivariate statistics. In particular, the PCA was applied to the average acyl group composition ($C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{14:1}$, $C_{16:1}$, $C_{18:2}$, $C_{14:1} + C_{18:1}$), including the two distribution parameters described above. To discriminate between cows and buffaloes' milks, the first three principal components, which account, respectively, for the 52.9, 19.0, and 12.3% of the total variance, were considered. The score plot of the three factors is reported in Figure 3, where it can be seen that there is a clear discrimination between the milks.

CONCLUSIONS

It is well known that the composition of milk from individual animals is affected by stage of lactation, diet, breed, and other factors. We have shown here that the use of bulk milks and multivariate statistics helps to differentiate milks from cows and buffaloes. ¹³C NMR spectroscopy was used to study the triacylglycerols from

cows and buffaloes' milk fat. The resonances were assigned and quantified to differentiate milks from different species. In particular, the multivariate analysis treatment of the acyl group composition and of some distribution parameters, clearly separated the two milks, thus yielding a potential suitable approach to the solution of milk authenticity issue. Furthermore, it must be pointed out that the NMR analysis, although inherently much less sensitive than other techniques, does not require extensive chemical manipulation.

To assess the contributions of the different factors affecting the natural variability of milk fat content, we are in the process of analyzing much larger data sets covering variations of milks linked to breed, season, and diet.

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