^{1}H NMR Characterization of Milk Lipids: A Comparison Between Cow and Buffalo Milk

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ABSTRACT: Characterization of the lipid fraction of raw cow and buffalo milk samples, collected in different breeding areas in Apulia, a region of southern Italy, were performed by means of ^{1}H NMR. The aim of this work was to establish whether FA composition data obtained by ^{1}H NMR can be used in the differentiation of buffalo and cow milk samples according to species. A complete assignment of the signals present in the spectrum was attempted by COSY, heteronuclear multiple-quantum coherence, and heteronuclear single-quantum coherence spectra. Quantification of FA was carried out by inserting the integrals of particular peaks in suitable calculations. Multivariate statistical analysis, conducted on the results of the quantification, permitted buffalo and cow milks to be distinguished.

Paper no. J10560 in *JAOCS 81,* 431–436 (May 2004).

KEY WORDS: Characterization, milk species, multivariate analysis, ^{1}H NMR, triacylglycerols.

In Italy, buffalo milk mozzarella is considered a very high-quality cheese. Indeed, it has obtained the Protected Designation of Origin (PDO) label "Mozzarella di Bufala Campana" in the southern Italian regions of Campania and Latium. Here the presence of ample grazing grounds and stagnant water throughout the year facilitates buffalo breeding. Many producers in other areas of southern Italy are beginning to request PDO recognition. Therefore, the problem of analytical authentication will represent a major challenge in the near future both for producers and for people responsible for the control of misrepresentations.

The production regulation for buffalo milk mozzarella provides for the exclusive use of buffalo milk; in this way, addition of any milk obtained from other species has to be forbidden, otherwise it should be declared in the list of ingredients.

For example, because buffalo milk is more expensive than cow milk, the latter is sometimes fraudulently added at present, and the resulting products are sold at the same price as the authentic buffalo mozzarella.

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Analytical methods are necessary to detect the addition of cow milk in buffalo mozzarella and thus to protect the consumer. The methods that are the most promising are based on the analysis of the protein fraction—in particular, of the casein or whey protein fraction. Various electrophoretic methods based on the identification of casein αs1 (1), β-casein (2), and γ2-casein (3) have been proposed. Use of HPLC of whey cheese proteins soluble at pH 4.6 permitted the detection and quantification of low amounts of cow milk added to buffalo milk in buffalo milk mozzarella. Indeed, since cow milk whey contains β-lactoglobulin A, which is absent in buffalo milk, it is possible to distinguish as little as 1% of cow milk in buffalo mozzarella (4).

The official analytical method of the European Community (5) for the detection of cow milk in cheeses made from goat or buffalo milk is based on isolating caseins from cheeses and submitting them to plasmin cleavage. Plasmin-treated caseins are subjected to isoelectric focusing in the presence of urea and to protein staining. Afterward, the stained casein patterns are evaluated by comparing the pattern obtained from the sample with those obtained in the same gel from the reference standards, containing 0 and 1% cow milk. The use of capillary zone electrophoresis to determine the presence of cow milk in buffalo milk and mozzarella cheese also has been evaluated recently (6).

Although these methods have given good results, detecting the presence of cow milk at levels as low as 0.5–1.0% in buffalo milk samples, they can be very slow and laborious since they require various preparation and analysis steps and/or large amounts of reagents and solvents. However, the issue of adulteration should be viewed in light of the fact that frauds become economically advantageous when the adulterant (i.e., cow milk) is added in much higher quantities than 1%. Therefore, a method for detecting adulteration does not need to have very low detection limits. Rather, it should be rapid, cheap, and simple so as to permit the analysis of a high number of samples. Recently, methods based on innovative techniques have been proposed (7–9). Among these, ^{13}C NMR spectroscopy has been used to identify different milk species through the determination of the TAG composition (10).

A similar approach has never been applied using ^{1}H NMR spectroscopy, although it has more promise as a screening method compared with ^{13}C NMR, because the much higher sensitivity of ^{1}H NMR spectra should allow much shorter times for analyses. Thus, ^{1}H NMR would have a lower cost than ^{13}C NMR, which is very important in the analysis of a large number of samples. The information contained in a ^{13}C NMR spectrum is necessary to perform a complete assignment of the compounds present in a mixture, but once the assignment of proton spectra has been performed, ^{1}H NMR should be used for routine analysis. In this work, it was necessary to perform ^{13}C NMR experiments on one sample to achieve additional information about the structure of the compounds present in the analyzed mixture.

The TAG fraction of samples of buffalo and cow milk was analyzed by means of ^{1}H NMR spectroscopy. Signal assignment was carried out with the help of 2-D NMR spectroscopy, and a quantitative determination of the FA content was performed. The principal aim of this work was to apply multivariate statistical analysis to the ^{1}H NMR data to differentiate cow and buffalo milk samples according to species.

MATERIALS AND METHODS

Milk samples. Fourteen samples of raw buffalo milk and 22 samples of raw cow milk were supplied by local farms in the Apulia region. Cows and buffaloes were in the same phase of lactation. All samples were collected in polypropylene bottles and stored at –80°C until the moment of analysis.

^{1}H NMR. TAG were obtained from milk by extraction with chloroform/methanol (2:1 vol/vol) (11). Spectra were acquired at 500 MHz by using a DRX500 Avance Bruker spectrometer. The following experimental conditions were applied: spectral width = 3500 Hz (~7 ppm); time domain = 32K points; number of transients = 256, pulse width = 7.2 μs (90° pulse), recycle delay = 5 s.

The sample temperature in the probehead was kept at 300 K for all the samples. Spectra were processed by applying an enhancement multiplication with a 0.6 Hz line-broadening factor. Fourier-transformed spectra were phased and then baseline-corrected by spline interpolation of 14 baseline selected points. Signal integration was carried out by the Bruker XWINNMR integration routine, manually defining the regions of integration and setting to 1000 the intensity of the signal resonating at 1.27 ppm due to $–(CH_2)_n–$ groups of FA. The integration regions were the same for all the spectra.

The 2-D gradient-COSY spectrum (cosygs pulse sequence in Bruker software) was obtained using 1024 × 256 time domain data points; 7 ppm of spectral width; 16 dummy scans; 8 transients for each increment (F1 dimension). Gradient-heteronuclear single-quantum coherence (HSQC) 2-D spectrum (invieagssi pulse sequence in Bruker software) was acquired by using 2048 × 512 time domain data points; 7 (F2 dimension) and 140 ppm (F1 dimension) of spectral width; 32 transients for each increment (F1 dimension). It was processed by using 2048 × 1024 data points and no shifted square sine bell functions in both dimensions. The gradient-heteronuclear multiple-bond coherence (HMBC) 2-D spectrum (inv4gplplrnd pulse sequence in Bruker software) was acquired by using the same parameters employed for gradient-HSQC, apart from an additional delay of 0.06 s for evolution of long-range couplings.

Statistical analysis. Principal component analysis (PCA), hierarchical clustering analysis (HCA), discriminant analysis (DA), and ANOVA were carried out on the obtained data by using Statistica software (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Quantification of the principal compounds and statistical analysis. The ^{1}H NMR spectrum of the TAG fraction of milk (Fig. 1) consists of two series of signals having very different intensities. It is possible to distinguish principal and secondary compounds. The assignment of the main resonances, whose pattern is typical of the ^{1}H NMR spectrum of a fat sample (12), was performed with the help of COSY (Fig. 2), HMBC, and heteronuclear multiple-quantum coherence (HMQC) experiments (Table 1).

FA composition of milk was determined by calculating the areas of the NMR signals labeled A–F in the spectrum (Fig. 1). To evaluate the linoleic and linolenic acid concentrations, the integrals of the signals due to the diallylic moieties (Fig. 3), resonating, respectively, at 2.79 and 2.83 ppm (13), were calculated. The following equations were derived:

$$\text{linoleic acid (\%)} = 3B/2(E + F) \quad [1]$$

$$\text{linolenic acid (\%)} = 3A/4(E + F) \quad [2]$$

The percentage of monounsaturated FA (MUFA) can therefore be obtained by subtracting the percentages of these two acids from the total content of unsaturated acids, determined as follows:

$$\text{unsaturated acids (\%)} = \text{MUFA} + \text{linoleic acid} + \text{linolenic acid} = D/2C \quad [3]$$

The signal resonating at 0.96 ppm (signal E in Fig. 1) has been attributed to the CH_3 of linolenic acid and to the CH_3 of butyric acid, as confirmed by COSY and HSQC and by comparing the chemical shifts of the ω_1 carbon with those already reported in the literature (14). The percentage of this acid can be determined with the following equation:

$$\text{butyric acid (\%)} = E/(E + F) – 3A/2(E + F) \quad [4]$$

Saturated FA content is obtained by subtracting the unsaturated FA content from the total FA:

$$\text{saturated FA (\%)} = F/(E + F) + E/(E + F) – D/2C \quad [5]$$

The FA composition was calculated for all the samples, and a data set was built with all these values. Multivariate statistical analysis was conducted on the FA percentages determined to evaluate whether they can be used to discriminate the milk species. The height of the peak resonating at 0.697 ppm (signal 5 in Fig. 1), attributable to cholesterol, was added to the data set for each sample.

The means and SD of the parameters determined for cow and buffalo milks are summarized in Table 2. ANOVA was applied

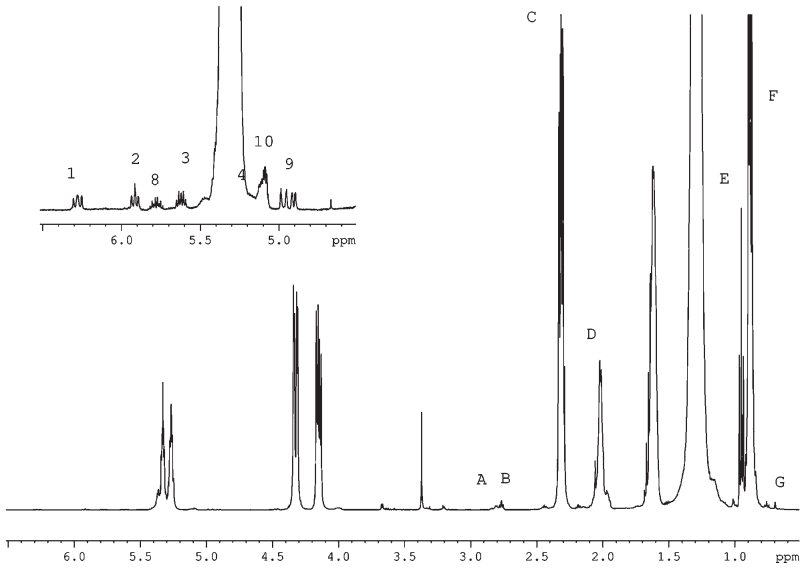


FIG. 1. 500 MHz ^{1}H NMR spectrum of milk fat TAG. The inset shows signals due to secondary components.

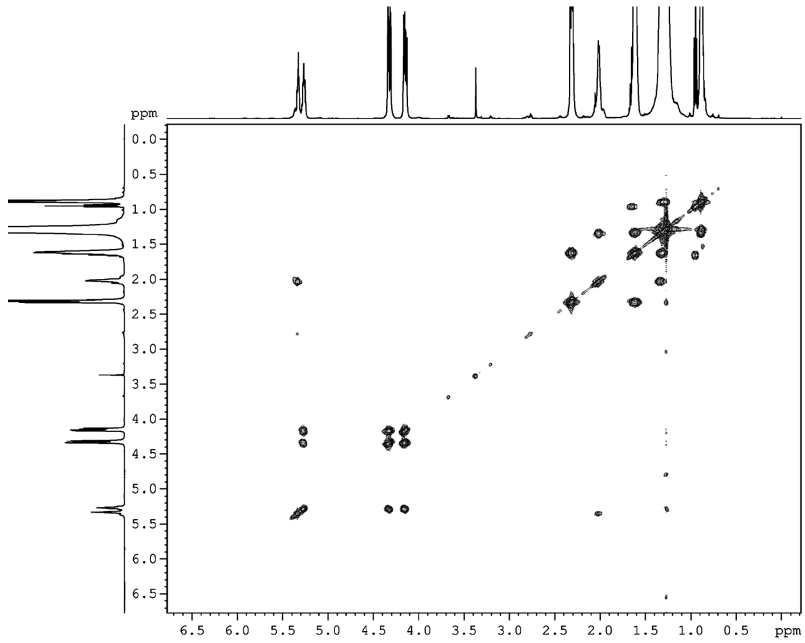


FIG. 2. 500 MHz COSY spectrum of milk fat TAG.

to test whether the differences in the average values relative to the considered species were significant. The *F*-critical parameter represents the ratio of the between-group variance over the within-group variance. *F*-critical was calculated by the software as 1.34. The calculated values for the considered variables are listed in Table 2, and the relative *P*-level tends toward zero in all cases, indicating that all the NMR data were significantly different for the species being considered.

The concentrations of saturated and unsaturated FA were eliminated from the data set, being highly correlated with MUFA concentrations. For this reason they were not subjected to multivariate statistical analysis. Butyric acid was eliminated because it was highly correlated with linolenic acid concentration. PCA was applied to a data set of 36 samples. In examining the plot of the samples in the space defined by the first two principal components, which cover 70% of the variance (Fig. 4), a separation between milk samples according to their species was found on PC1. All the analyzed variables had high loadings on the first principal component. The numerical value of the loading of each variable on a given principal component shows how much the variable has in common with that component. Hence, the loadings can be

TABLE 1
Assignment of TAG Resonances in 500 MHz ^{1}H NMR Spectrum of Milk Fat[a]

δ ^{1}H (ppm)	δ ^{13}C ^{1}H-^{13}C HMQC	δ ^{13}C ^{1}H-^{13}C HMBC	Assignment
0.88 *t*	14.23	39.0; 34.6–34.0; 32.3; 30.0–29.0; 27.34; 27.30; 23.00	CH_3 FA
0.96 *t*	13.65	18.44; 36.2	CH_3 linolenic and butyric acids
1.27 *m*	22.84 29.9 32.12 32.10	35.0–20.0	–$(CH_2)_n$–
1.32 *m*	29.9–29.2		–$(CH_2)_n$–
1.62 *m*	25.02	174; 36.2; 34.6–34.0; 32.12; 30.0–29.0	–C*H*$_2$–CH_2–COO–
1.65 *m*	18.44		
2.02 *m*	27.30 27.34	129.7–130.1	–C*H*$_2$–CH=CH–
2.31 *t*	36.2	173.6–173.0	–C*H*$_2$–COO– butyric acid
2.33 *t*	34.12 34.28 35.96	173.6–173.0	–C*H*$_2$–COO–
2.79 *t*	25.96	129.7–130.1	–CH=CH–C*H*$_2$–CH=CH– linoleic acid
2.83 *t*	25.96	129.7–130.1	–CH=CH–C*H*$_2$–CH=CH– linolenic acid
4.15 *dd*	62.28		CH_2 glycerol
4.33 *dd*	62.58		CH_2 glycerol
5.26 *m*	69.25		CH glycerol
5.33 *m*	129.76		–CH=CH– FA

[a]HMQC, heteronuclear multiple-quantum coherence; HMBC, heteronuclear multiple-bond coherence.

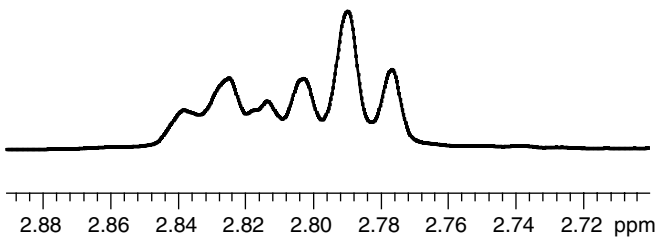


FIG. 3. Expansion of the 2.7–2.9 ppm region in the 500 MHz ^{1}H NMR spectrum of milk fat TAG. The methyl groups of linolenic acid and linoleic acid appear at 2.83 and 2.79 ppm, respectively.

TABLE 2
***F*-Statistic, Means (%), and SD of the Parameters Determined for Cow and Buffalo Milks**

	F	Cow		Buffalo	
Variables	(1.34)	Mean	SD	Mean	SD
Linolenic acid	15.45	0.78	0.12	0.58	0.19
MUFA[a]	17.45	25.00	4.60	19.68	1.43
Linoleic acid	10.89	2.12	0.69	1.46	3.56
Unsaturated FA	19.28	27.92	5.10	21.73	1.51
Saturated FA	20.21	72.08	4.98	78.27	1.51
Butyric acid	32.27	10.39	1.07	12.57	1.26
Cholesterol	20.07	1.34	0.23	1.03	0.15

[a]Monounsaturated FA.

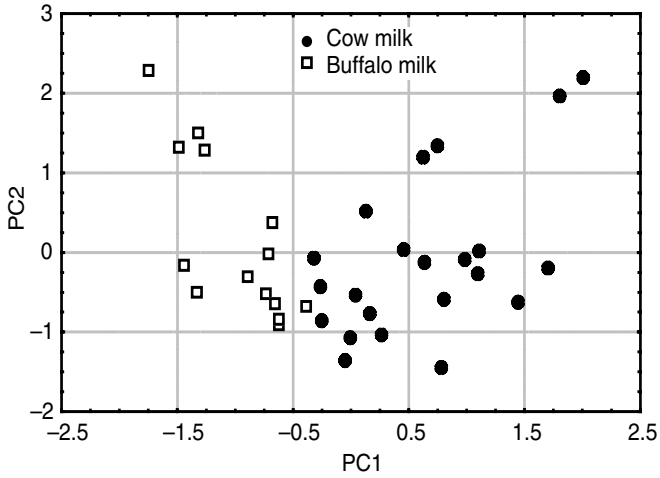


FIG. 4. Score plot of the milk samples on the first two principal components (PC) obtained from ^{1}H NMR data.

interpreted as correlations between the variables and the components. In the present case, PC1 loadings were high and positive for all the determined parameters. This reflects the relevant influence of the FA composition in achieving a discrimination between samples according to species.

HCA was carried out on the same variables. In this case, a matrix consisting of the Manhattan distances was used as a similarity matrix. A hierarchical agglomerative method was employed to obtain clusters: the complete linkage method. In this method, the distances between clusters are determined by the greatest distance between any two objects in the different clusters. The dendrogram in Figure 5 shows the results of this analysis. At a similarity level of 6.5, four clusters were found: The first three clusters consisted of 21 samples of cow milk; the fourth cluster was composed of all buffalo milks and one cow milk.

Both PCA and HCA are unsupervised statistical methods and give complementary information on the similarities and groupings of the considered samples. Since the considered variables permitted discrimination of samples according to species, it was interesting to evaluate whether a classification of samples could be obtained.

DA was applied on the first three principal components using the species of the animals from which samples were collected as classes for assignment. The recognition ability of the two classes was highly satisfactory since all samples were correctly classified.

Afterward, to verify the prediction ability of the model obtained, the data set was split into a training set, for developing a discriminant model, and a test set (made of four samples of buffalo milk and eight samples of cow milk). The obtained model assigned 97% of the training set samples correctly and 94.5% of the test set samples.

Analysis of minor compounds. To carry out a complete analysis of the ^{1}H NMR spectrum of a milk sample, the identification of minor intensity signals was attempted. The first group of minor intensity signals was indicated with the labels 1–4 (Fig. 6). These signals correlate with

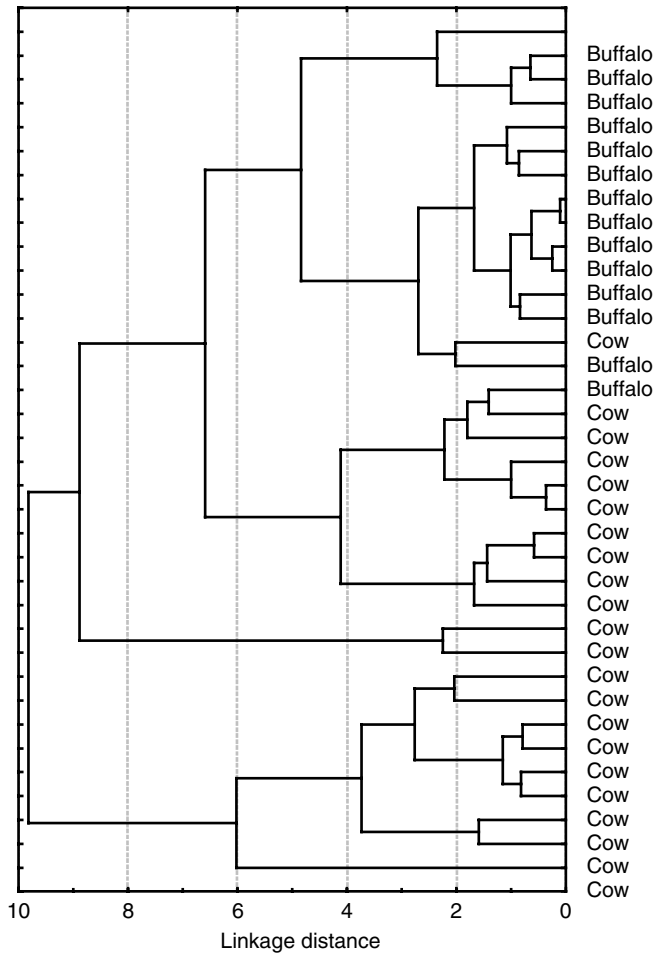
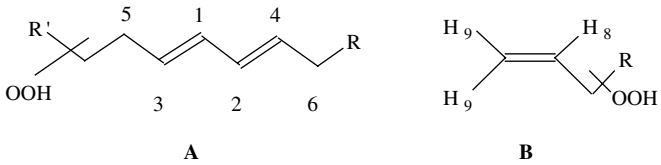


FIG. 5. Dendrogram of milk samples obtained from ^{1}H NMR data.

TABLE 3
Assignment of Secondary Resonances in 500 MHz ^{1}H NMR Spectrum of the TAG of Milk Fat

Signal	δ ^{1}H (ppm)	δ ^{13}C ^{1}H-^{13}C HMQC[a]	COSY	Assignment
1	6.28 *dd*	126	2, 3	
2	5.92 *t*	129	4	–CH=CH– in peroxides with A or B structure
3	5.61 *dt*	135		
4	5.25 —	—		
5	2.09 —	26	1, 3	$–CH_2$ in peroxides with A or B structure
6	2.15 —	26	2, 4	
7	2.04 —		8, 9	$–CH_2$ in peroxides with A or B structure
8	5.78 *mt*	130	7, 9	–CH=CH– in peroxides with A or B structure
9	4.95 *dd*	115		
10	5.10 *m*	73	11	–CH– group of glycerol of 1,2-DAG
11	3.68 *d*	61		α′ $–CH_2$– group of glycerol of 1,2-DAG

[a]For abbreviation see Table 1.



SCHEME 1

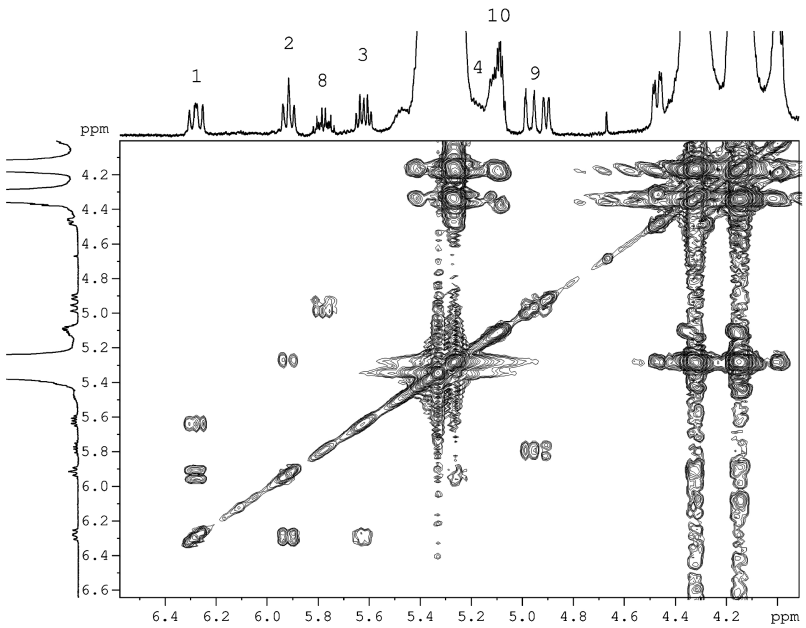


FIG. 6. Slice of the 3.9–6.6 ppm region in the 500 MHz COSY spectrum of the TAG of milk fat.

CH_2 groups, indicated as 5 and 6 in Table 3. The protons labeled as 8 and 9 are both correlated with a CH_2 group indicated as 7 (Table 3). On the grounds of their chemical shifts and multiplicities and according to References 15 and 16, these signals could hypothetically belong to hydroperoxides of A or B structure (Scheme 1). As far as signals 10 and 11 are concerned, COSY correlations and ^{13}C are in agreement with Reference 12 and are due to glycerol protons of 1,2-DAG. Minor peaks located between 3.1 and 3.8 ppm were not assigned.

This investigation showed that the composition of the FA in cow and buffalo milk TAG, determined by NMR, can be used to distinguish the two milks. ^1H NMR spectroscopy was used to study the TAG of milk, and a quantitative measure of the most important classes of FA was obtained. The assignment of the minor compounds may carry information for milk species discrimination.

ACKNOWLEDGMENTS

Many thanks are due to Drs. Pasquale D'Amico and Dr. Christoforo Carrino for supplying the samples.

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[Received February 3, 2004; accepted March 31, 2004]