^{13}C NMR Spectra of TAG: An Easy Way to Distinguish Milks from Different Animal Species

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ABSTRACT: In order to differentiate milks from different species, we carried out a comparative analysis of TAG from cow, buffalo, goat, and sheep milk fat based on ^{13}C NMR experiments. NMR spectroscopy, although less sensitive than other techniques, does not require an extensive chemical manipulation of samples and can easily highlight the differences in the content of short-chain acyl groups in the four milk species. The resonances were assigned and quantified, and by using only three NMR parameters in data clustering with fuzzy logic analysis, we were able to distinguish goats' milk from sheep's milk, and both of these milks from cows' and buffaloes' milks. This appears to be an important result, considering the ease and rapidity with which milk identification can be obtained. From ^{13}C NMR spectra of TAG, the positional distribution of FA chains on the glycerol backbone can also be easily evaluated. In particular, analysis of the positional distribution of monounsaturated FA revealed that it may be species-specific, and we are currently analyzing larger data sets in order to evaluate the use of this parameter as a suitable approach to address the issue of milk authenticity.

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Key words: ^{13}C NMR, goats' milk, milk fat, TAG from milk, sheep's milk.

Milk and dairy products are fundamental dietary constituents of many societies; therefore, the search for new and rapid methods to enforce control quality is of primary importance. In recent years, the application of NMR spectroscopy to milk has constantly increased, especially because it is a nondestructive technique that avoids extensive manipulation of the sample. It has successfully been applied to milk for structural characterization of several components, to monitor the biological transformations induced by microorganisms responsible for the organoleptic characteristics of dairy products, and to investigate the physical state of water and milk fat (1–3).

In milk, about 98% of total lipids, which occur as globules emulsified in the aqueous phase, are TAG (4). We have recently investigated TAG from cows' and buffaloes' milks by ^{13}C NMR spectroscopy (5) and showed that, although inherently less sensitive than other techniques, NMR can safely be used to quantitate milk FA content, providing data as reliable as those obtained by other techniques such as GC. In particular, by analyzing ^{13}C NMR parameters of several resonances, we obtained the FA composition and information on the distribution of some FA on the glycerol backbone. We also showed that principal component analysis applied to 10 parameters derived from the spectra of each milk resulted in an effective separation of the two milks, thus affording a new way to address the milk authenticity issue.

As part of a long-running project on milk characterization, we have applied this method to goat, sheep, and bovine milks to investigate the possibility of finding a few characteristic NMR spectral parameters that could unequivocally identify the milk. This aspect is very important because in many countries a large number of dairy products are made with milk obtained from species other than cows. For a number of reasons, these products have a much higher commercial value than those made from cows' milk, and there is obviously the temptation for the latter to be added to increase production volume.

In the present study, we have investigated ^{13}C NMR spectra of TAG from goat, sheep, and bovine milks. Fuzzy logic analysis using only three parameters obtained from the ω3 region of the spectrum (in particular, the content of butyryl and capryl groups and the overlapping signal of lauryl, myristyl, palmityl, and stearyl groups) has been found to afford a quick method for distinguishing goats' milk from sheep's milk, and both of these milks from cows' and buffaloes' milks. As previously reported (5), statistical analysis of a larger number of NMR parameters from TAG spectra also allows cows' milks to be distinguished from buffaloes' milks.

EXPERIMENTAL PROCEDURES

Sample preparation. Raw milks from spring goats, sheep, cows, and buffaloes were obtained from local farms. The extraction method of Folch *et al.* with chloroform/methanol (2:1, vol/vol) was performed to obtain TAG from 250 mg of each sample of milk fat (6). The final samples had almost the same amount of TAG, with the volume adjusted to 0.5 mL. In

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the sample preparation procedure, only deuterated solvents were used.

^{13}C NMR spectroscopy. High-resolution ^{13}C NMR spectra were obtained at 75.5 MHz on a Bruker DPX-300 spectrometer (Karlsruhe, Germany) equipped with a 5-mm dual $^{13}C/^{1}H$ probe. Each free induction decay (FID) was acquired over a spectral width of 220 ppm at 300 K, using a 90° pulse of 5.1 μs and inverse gated decoupling to eliminate the nuclear Overhauser effect. To avoid signal saturation, a delay of 20 s, corresponding to four times the estimated spin–lattice relaxation rate of the slowest-relaxing carbonyl carbon, was used. The FID, acquired with 64 K complex data points, were zero-filled to 128 K, Fourier-transformed without apodization functions, and baseline-corrected. Chemical shifts were measured relative to the resonance of chloroform, for which a value of 77.01 ppm was assumed. Resonances were assigned by adding pure TAG standards (tributyrin, tricaprin, tricaproin, tricaprylin, trilaurin, trilinolein, trilinolenin, trimyristin, trimyristolein, triolein, tripalmitin, tripalmitolein, tripentadecanoin, and tristearin), obtained from Sigma Chemical Co. (St. Louis, MO).

Quantitative spectral analysis. NMR signals were fitted to a sum of Lorentzian curves by a nonlinear least-squares algorithm. The relative concentration of each FA was calculated from the area of the corresponding NMR signal, the total area of the glycerol signals at 68.8 and 62.0 ppm being used as a normalization parameter. MacFID 1D 5.3 software (Tecmag Inc., Houston, TX) was used for data analysis.

Fuzzy logic analysis. Fuzzy logic analysis was applied to 17 data points (milk samples), and for each of them we considered three NMR parameters obtained from the ω3 region of the spectrum. In particular, we considered the butyryl and capryl content and the overlapping signal of lauryl, myristyl, palmityl, and stearyl groups. The Matlab (version 5.3.0; The MathWorks Inc., Natick, MA) function used for data analysis is an iterative algorithm that minimizes the distance of any data point from a cluster center, weighted by the membership grade of each data point. During the iteration process, both the cluster centers and the membership grades for each data point are varied.

RESULTS AND DISCUSSION

Qualitative analysis. Goats' and sheep's milk TAG were analyzed by ^{13}C NMR spectroscopy, and 14 acyl groups were assigned by addition of TAG standards. In order to obtain comparable data sets, NMR experiments on cows' and buffaloes' milks, although previously reported (5), were again carried out, and the spectra analyzed in parallel with those of goats' and sheep's milks. In particular, we considered nine saturated FA (butyric, capric, caproic, caprylic, lauric, myristic, pentadecanoic, palmitic, and stearic), three 9*Z*-monounsaturated FA (myristoleic, oleic, and palmitoleic), and one 9*Z*,12*Z*-diunsaturated (linoleic) FA, these being the most abundant FA in bovine milk. In addition, α-linolenic acid (a 9*Z*,12*Z*,15*Z*-triunsaturated acid) was also considered. Comparison between the

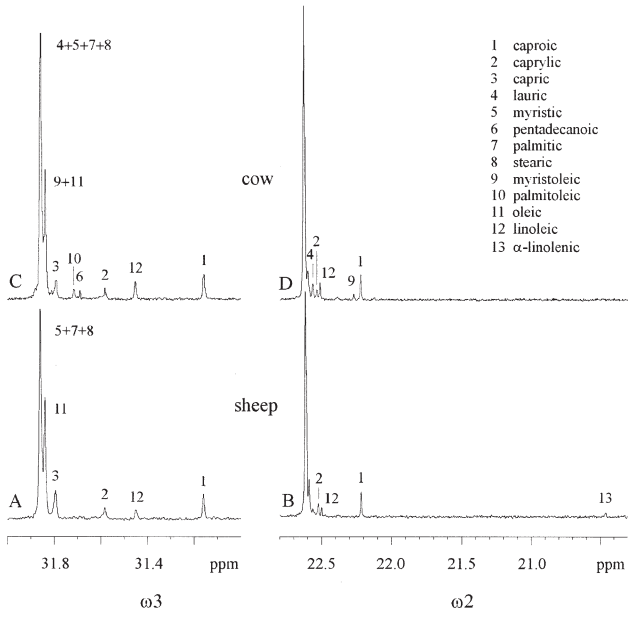

FIG. 1. The ω3 (A and C) and ω2 (B and D) regions in the 75.5 MHz ^{13}C NMR spectrum of TAG of cows' (C and D) and sheep's (A and B) milk fat in $CDCl_3$ at 300 K. Identification of peaks as obtained by addition of standards is shown, and they are labeled according to the legend above spectrum D. The intensities of the spectra were adjusted in order to match the caproic acid signals.

spectra of goat and sheep samples indicates that they are very similar, as are those of the cow and buffalo samples. Therefore, while the discussion covers all four types of milks, only the spectra of cows' and sheep's milk fat are presented below.

The ^{13}C NMR spectra of acylglycerols contain signals arising from the acyl chains and the glycerol carbon atoms. Resonances arising from the acyl chains can be divided into several groups, such as those of the C1, the C2, the olefinic, and the ω1, ω2, and ω3 carbons (7). In the case of milk fat, the ω3 region should contain six peaks originating from caproyl, caprylyl, capryl, pentadecanoyl, palmitoleyl, and linoleyl groups. All these signals were clearly observed in the spectra of cows' milk samples (Fig. 1; the above signals are labeled 1, 2, 3, 6, 10, and 12, respectively; refer to the legend on the figure) and buffaloes' milk samples. In the spectra of goats' milk samples and one out of three sheep's milk samples, palmitoleyl (peak 10) and pentadecanoyl (peak 6) signals were not detectable (Fig. 1A), indicating that these groups most likely represent less than 0.5% of the acyl groups. Butyryl (4:0) and caproyl (6:0) groups in standard TAG give two different signals, stemming from the *sn*-1,3 and *sn*-2 positions on the glycerol backbone (also termed α- and β-positions, respectively). The butyryl ω3 signal corresponding to the C2 signal was observed at 35.80 ppm, outside the region shown in Figure 1. Similarly to cows' and buffaloes' milk samples (Fig. 1, spectrum C), both goats' and sheep's milk samples showed butyric and caproic acids mostly in the α-position (Fig. 1, spectrum A), in agreement with the find-

ings of Angers *et al.* (8), who reported that in bovine milk fat short-chain FA (4:0 to 8:0) are located at the α-position in high proportions, irrespective of TAG size.

In Figure 1C, the intense signal at 31.86 ppm originates from overlapping resonances of lauric, myristic, palmitic, and stearic acids (labeled 4, 5, 7, and 8, respectively), whereas the signal at 31.84 ppm is the envelope of the resonances of myristoleic and oleic acids (labeled 9 and 11, respectively). The myristoleyl group gave rise to a single peak in both the ω1 (not shown) and ω2 regions. However, since the corresponding peak was not detected in the ω2 region in the spectra of goats' and sheep's milk samples (Fig. 1B), we suggest that the signal at 31.84 ppm (Fig. 1A) arises only from the oleyl group (labeled 11). Analogously, since the resonance of the lauryl group in the ω2 region was not detectable in the spectra of both goats' and sheep's (Fig. 1B) milk samples, we conclude that the signal at 31.86 ppm (Fig. 1A) represents myristyl, palmityl, and stearyl groups (labeled 5 + 7 + 8). The ω3 carbon of the α-linolenyl group resonates outside the region shown because it is an olefinic carbon. The linolenyl ω2 carbon resonates at 20.45 ppm (labeled 13 in Fig. 1B); this signal was clearly identified in the spectra of sheep's milk samples, whereas it was not detectable in the spectra of all cows' milk samples, nor in the spectra of at least one of the buffaloes' and goats' milk samples.

In both the C1 (carbonyl carbons) and C2 (α-carbons) regions, the signals were separated into two clusters, corresponding to the α- and β-positions. In each cluster, saturated and unsaturated groups may be identified (5,9). Notwithstanding these separations, the presence of strongly overlapping signals and the effect of the neighboring chains did not allow any qualitative or quantitative results to be otained from analysis of these regions (9), except regarding the nonrandom distribution of the butyryl group (5, 9–11). In particular, as already observed for cows' and buffaloes' milks, only the signal assigned to the α-position of the butyryl group was detected in goats' and sheep's milk samples (data not shown), in agreement with the analysis performed on the ω3 region (see above).

In the olefinic region, the unsaturated carbons (C9–C10 for oleic, palmitoleic, and myristoleic acids, C9–C10 and C12–C13 for linoleic acid, and C9–C10, C12–C13, and C15–C16 for α-linolenic acid) give characteristic pairs of signals arising from the acyl groups in the α- and β-positions, although the chemical shift between peaks in a pair becomes smaller when the olefinic carbon is nearer the methyl end of the FA chain (12). The C9 signals of oleyl, palmitoleyl, and myristoleyl groups were coincident (Fig. 2). In the case of goats' milk samples, the signals of myristoleyl and palmitoleyl groups were not detected in the ω regions; thus, we conclude that the signal centered at 129.58 ppm originates from oleic acid only. Therefore, the ratio between the α and β areas represents the distribution of oleic acid between the two positions.

The comparative analysis of ^{13}C NMR spectra of TAG from cows', buffaloes', goats', and sheep's milks can be summarized as follows: (i) only 5 (butyryl, caproyl, caprylyl, capryl, and linoleyl) of the 14 acyl groups investigated were clearly detectable in goats' and sheep's milk samples; (ii) butyryl and caproyl groups showed a nonrandom distribution, being mostly located in the α-position in each type of milk; (iii) lauryl, pentadecanoyl, myristoleyl, and palmitoleyl groups are present in goats' and sheep's milk samples with an abundance of less than 0.5%; (iv) no information could be gained about the relative abundance of myristyl, palmityl, and stearyl groups in each milk sample; and (v) α-linolenic acid was detectable in the sheep's milk samples examined, whereas the abundance of this acyl group in cows', buffaloes', and goats' milk samples was less than 0.5%.

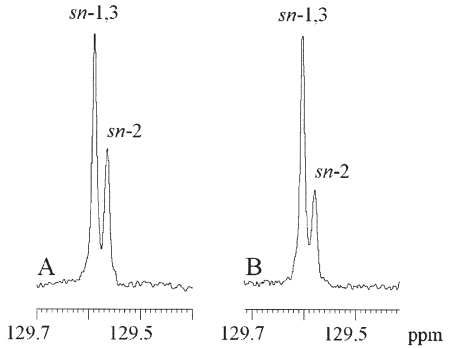

FIG. 2. C9 signals of the monounsaturated acyl groups in TAG from cows' (A) and sheep's (B) milk fat. Spectra were acquired in $CDCl_3$ at 300 K. The intensities were adjusted in order to show the different distribution of the monounsaturated acyl groups between *sn*-1,3 and *sn*-2 positions.

Quantitative analysis. On the basis of the above considerations, a quantitative analysis of cows', buffaloes', goats', and sheep's TAG was performed. From the spectral simulation of the C9 signals (Fig. 2), we evaluated the distribution of the monounsaturated FA between the α- and β-positions. Independently of the total amount of the monounsaturated FA, the α/β ratio, as derived from the C9 signals in the spectra of TAG samples from cows' (Fig. 2A), buffaloes', sheep's (Fig. 2B), and goats' milks, amounts to 2.5 ± 0.3, 1.9 ± 0.1, 1.6 ± 0.2, and 1.9 ± 0.1, respectively. Certainly, a larger data set is required in order to take into account the natural variability of milk fat content. However, as suggested for bovine and human milk lipids (13), it can be hypothesized that differences in TAG patterns specific for each species may be linked to differences in activity of acylglycerol transferases.

The resonances in the ω3 region were simulated, and the area of each peak was used to calculate the relative concentration of each FA. The total area of the glycerol signals at 68.80 and 62.00 ppm was used as a normalization parameter because it is proportional to the total content of the three glycerol carbons and, consequently, to the total content of acyl groups. In addition, the α-linolenyl content was determined from the ω2 signal at 20.45 ppm. The results are summarized in Table 1, in which the average value and the range of variability for each acyl group are shown.

TABLE 1
Fatty Acid Composition of TAG from Cows', Buffaloes', Goats', and Sheep's Milk Fats as Determined by ^{13}C NMR Spectroscopy[a]

FA	Composition (mol%)[b]			
	Cow[c]	Buffalo[d]	Sheep[e]	Goat[e]
Butyric	10.1 (9.3–10.4)	10.7 (9.9–11.1)	9.3 (8.7–11.0)	6.6 (6.5–6.7)
Caproic	4.6 (3.9–4.9)	3.9 (3.7–4.3)	4.7 (4.4–5.1)	5.0 (4.4–6.1)
Caprylic	2.0 (1.2–2.5)	1.5 (1.2–1.8)	3.0 (2.7–3.4)	4.1 (2.9–5.3)
Capric	3.1 (2.4–4.1)	1.6 (0.8–2.5)	8.6 (7.6–9.6)	12.8 (10.6–16.3)
Pentadecanoic	1.4 (0.8–2.2)	0.9[f] (<0.5–1.9)	1.2[f] (<0.5–2.2)	<0.5
Lauric, myristic, palmitic, stearic	52.0 (47.9–53.7)	52.3 (50.4–54.3)	42.8 (40.8–44.0)	45.1 (42.5–47.9)
Palmitoleic	1.9 (1.3–2.7)	2.6 (2.0–3.4)	1.0[f] (<0.5–2.0)	<0.5
Oleic, myristoleic	18.5 (15.4–20.4)	20.7 (17.7–23.6)	21.6 (20.8–22.8)	18.2 (17.0–19.8)
Linoleic	3.3 (2.4–4.4)	2.7 (2.2–3.7)	1.9 (1.5–2.1)	1.8 (1.5–1.9)
Linolenic	<0.5	1.0[f] (<0.5–1.6)	2.4 (2.2–2.6)	0.7[f] (<0.5–1.1)

[a]See text for more details on FA composition.
[b]Data are mean values and range (in parentheses).
[c]$n = 5$.
[d]$n = 6$.
[e]$n = 3$.
[f]At least one of the examined samples showed an abundance less than 0.5%.

The most important differences in the FA composition of milk fat among the four species concern the short-chain FA (butyric, caproic, caprylic, and capric), which are provided by the rumen activity of the animals and are mostly responsible for the milk flavor. In particular, both sheep's and goats' milk samples showed a higher content of caprylyl and capryl groups in comparison with cows' and buffaloes' milk samples. The higher content of capric and caprylic acids in goats' milk samples (12.8 and 4.0%) in comparison with cows' (3.1 and 2.0%) and buffaloes' (1.6 and 1.5%) milk samples is only partially balanced by a lower content of butyric acid (6.6, 10.1, and 10.7%, respectively). In addition, a lower content of saturated FA containing at least 12 carbons has been observed in sheep's (42.8%) and goats' (45.1%) milk samples compared with cows' (52.0%) and buffaloes' (52.3%) milk samples. Linoleic acid is more abundant in cows' and buffaloes' milk samples than in sheep's and goats' milk samples (3.3, 2.7, 1.9, and 1.8%, respectively). α-Linolenic acid ranges between 2.2 and 2.6% in sheep's milk whereas in all cows' milk samples, and in at least one of the buffaloes' and goats' milk samples, it was not detectable.

Fuzzy logic analysis. In order to differentiate the milk samples by using a limited number of NMR parameters, the butyryl and capryl content, as determined from the ω3 region, and the overlapping signal of myristyl, palmityl, and stearyl groups, as determined from the ω3 region, were considered. These data were used for clustering by fuzzy logic analysis. The purpose of data clustering is to identify natural groupings of samples to produce a concise representation of a system's behavior. In fuzzy clustering, each data point belongs to a cluster to some degree, specified by a membership grade. Fuzzy logic analysis gave the best result when asked to group the data in three clusters (Fig. 3). We found that this procedure correctly distinguishes goats' from sheep's milk samples, and each of them from cows' and buffaloes' milk samples; the latter are clustered in a single group as their differentiation requires a larger number of NMR parameters (5). Each sample was characterized by a membership grade. In particular, for the samples belonging to the caprine group, the estimated grades were 0.86, 0.86, and 0.88, whereas for those belonging to the bovine group we obtained 0.69, 0.81, and 0.81. In the ovine group, values ranged from 0.92 to 0.99, except for one sample, for which a value of 0.57 was obtained.

The results reported in this paper indicate that it is possible to use ^{13}C NMR spectroscopy to distinguish goats' milk from sheep's milk, and both of these milks from cows' and buffaloes' milks. This appears to be an important result considering the ease and rapidity with which milk identification can be made with the use of this technique.

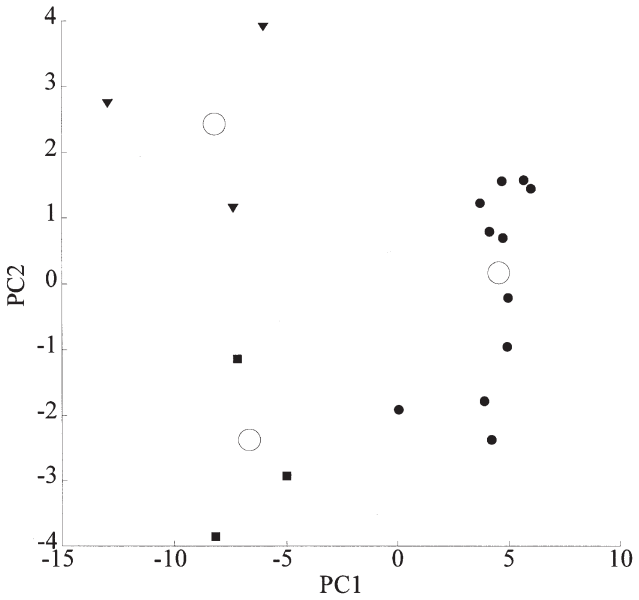

FIG. 3. Fuzzy logic clustering based on the signals of butyryl and capryl groups and the overlapping signal of myristyl, palmityl, and stearyl groups in the ^{13}C NMR spectra of TAG extracted from cows' and buffaloes' (●), goats' (▼), and sheep's (■) milks. The first two principal components (PC1 and PC2) of the experimental data are plotted. Open circles represent the center of each of the three clusters obtained from fuzzy logic analysis.

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