**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 mono-unsaturated 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 non-destructive 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
the sample preparation procedure, only deuterated solvents were used.

\textit{\textsuperscript{13}C NMR spectroscopy.} High-resolution \textsuperscript{13}C NMR spectra were obtained at 75.5 MHz on a Bruker DPX-300 spectrometer (Karlsruhe, Germany) equipped with a 5-mm dual \textsuperscript{13}C/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 \textmu s 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, tricaproyl, tricaprylin, trilaurin, trilinolein, trilinolenin, trimyristin, trimyristolein, triolein, tripalmitin, tripalmitolein, tripentadecanoin, and tristearin), obtained from Sigma Chemical Co. (St. Louis, MO).

\textbf{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.

\textbf{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 \textit{o3} 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.

\section*{RESULTS AND DISCUSSION}

\textbf{Qualitative analysis.} Goats’ and sheep’s milk TAG were analyzed by \textsuperscript{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 9Z-monounsaturated FA (myristoleic, oleic, and palmitoleic), and one 9Z,12Z-diunsaturated (linoleic) FA, these being the most abundant FA in bovine milk. In addition, \textit{\alpha}-linolenic acid (a 9Z,12Z,15Z-triunsaturated acid) was also considered. Comparison between the 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 \textsuperscript{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 \textit{o1}, \textit{o2}, and \textit{o3} carbons (7). In the case of milk fat, the \textit{o3} region should contain six peaks originating from caproil, capryl, 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 caproil (6:0) groups in standard TAG give two different signals, stemming from the \textit{sn}-1,3 and \textit{sn}-2 positions on the glycerol backbone (also termed \textit{\alpha}- and \textit{\beta}-positions, respectively). The butyryl \textit{o3} 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 \textit{\alpha}-position (Fig. 1, spectrum A), in agreement with the find-