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Characterization of animal products according to geographic origin and feeding diet using nuclear magnetic resonance and isotope ratio mass spectrometry: cow milk

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Abstract

Six groups of six cows each, on two distinct sites, were fed on pasture and silage. Two analytical methods were employed, NMR to determine the proportions of polyunsaturated (PUFA), monounsaturated (MUFA) and saturated fatty acids (SFA) in milk fat and IRMS to measure the ^{18}O enrichment of milk water. The fatty acid composition was sensitive to the diet while IRMS results were more influenced by the production area. A discriminant analysis showed that high resolution NMR and IRMS are two complementary methods suitable for the authentication of milk according to the feeding diet and geographic origin.

Keywords: Milk; Lipids; Water; Fatty acids; Nuclear magnetic resonance (NMR); Isotope ratio mass spectrometry (IRMS); Authentication; Geographic origin; Feeding diet

1. Introduction

Food characterization represents an important strategic issue for the food industry. Milk products from mountain areas are reputed to have specific organoleptic and nutritional qualities (Bosset et al., 1999; Coulon & Priolo, 2002). Environmental protection is also strengthened by keeping cows grazing in the mountains. The tracing of milk production sites is therefore essential to avoid fraud.

The authentication of plant derived products, such as oils, coffee or vanilla, is often carried out by ^{2}H and/or ^{13}C nuclear magnetic resonance (NMR) (Martin & Martin, 1995). Milk fat, which represents 4% of milk content, is essentially composed of lipids (99%), consisting of 95–96% triglycerides. The proportions of saturated and mono- and polyunsaturated fatty acid chains can be determined by ^{13}C NMR (Bonnet, Denoyer, & Renou, 1990). The diet of an animal strongly modifies the composition of its milk fat and, in particular the ratio of unsaturated to saturated fatty acids (Chilliard, Ferlay, & Doreau, 2001). Hence ^{13}C NMR spectroscopy can be used to identify milk according to the feeding diet by determining the relative proportions of polyunsaturated (PUFA), monounsaturated (MUFA) and saturated fatty acids (SFA).

Isotope ratio mass spectrometry (IRMS) is another suitable method for the authentication of food products. Determination of the geographic origin of milk (Kornexl, Werner, Rossmann, & Schmidt, 1997; Rossmann, Kornexl, Versini, Pichlmayer, & Lamprecht, 1998) and milk products (Camin, Coloru, Dependori, Franco, Manca, & Versini, 2001; Manca et al., 2001) has recently become possible, by measuring the stable

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isotope ratios of oxygen ($\delta^{18}O$) in milk water and the nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotope contents of specific milk fractions in samples from different regions of Europe. The $\delta^{18}O$ values could be assigned to mountain or non-mountain regions. The $\delta^{13}C$ ratio was found to be highly dependent on the composition of the diet, particularly with regard to maize, a C4 cycle plant. Finally, the $\delta^{15}N$ ratio was influenced by the intensity of agricultural use.

The aim of the present study was to establish that a combination of NMR and IRMS allows more precise identification of the geographical origin of milk.

2. Materials and methods

2.1. Samples

Milk was taken from Holstein cows raised on two distinct INRA (Institut National de la Recherche Agronomique) sites differing in their geographical situations and altitudes: Rennes is located in Brittany and will be referred to as the *Plain* site (altitude 200 m), while Marcenat is located in the Massif Central and will be referred to as the *Mountain* site (altitude 1100 m). On both sites, 12 Holstein cows in mid-lactation were used during two experimental periods, each of 4 weeks, in winter and spring. The cows were in pasture during spring on both sites. At Marcenat during the winter, six cows were fed on a hay/concentrate diet (70/30, DM basis) and six on a grass silage/concentrate diet (65/30). During spring (10 weeks after the end of the first experimental period), six cows (three from each winter group) were in pasture (natural grassland) with access to a 2 kg/day concentrate supply. At Rennes, during the winter, six cows were fed on a maize silage/concentrate diet (70/30) and six on a hay/concentrate diet (65/35). During spring (nine weeks after the end of the first experimental period), six cows were in pasture (perennial ryegrass) with access to a 3 kg/day concentrate supply. The milk produced during the last week of each period was collected individually and drinking water was also collected on each site for 4 weeks in winter and summer.

2.2. Sample preparation and techniques

2.2.1. NMR

Milk samples (20 ml) were collected and frozen at −20 °C. Triglycerides were extracted from the milk cream by the Folch method and the chloroform phase was removed, dried over anhydrous sodium sulfate and filtered. Chloroform was eliminated at 50 °C in a rotary evaporator, triglycerides were dissolved in $CDCl_3$ (EURISO-TOP, France) and the lipid samples were frozen at −20 °C until analysis.

2.2.2. IRMS

This technique was used to determine the ^{18}O and ^{2}H isotope ratios in milk water. Rennet was added to full milk and the samples were left overnight at room temperature, after which milk water was collected by filtration and frozen at −20 °C until analysis.

2.3. NMR measurements

Aliquots (400 μl) of deuterated lipids were put into 5-mm glass NMR tubes (Aldrich, USA) and ^{13}C NMR spectra were recorded at 298 °K on a Brüker AM400 spectrometer (Brüker, Germany) at a frequency of 100.6 MHz. The entire ^{13}C spectrum was recorded with a sweep width of 20,000 Hz (32k). A pulse of 5 μs corresponded to a 60° pulse, the recycle time was 1.786 s and proton decoupling was achieved by using an inverse gate decoupling mode to suppress the nuclear Overhauser effect. The relaxation times T_1 were measured with an inversion-recovery (180-τ-90) pulse sequence. Areas of interest in the ^{13}C spectra were identified and the proportions of fatty acids determined according to previous results (Bonnet et al., 1990).

2.4. IRMS measurements

^{18}O enrichment values were determined by gas chromatography–isotope ratio mass spectrometry (VG Isochrom-μgas, VG Isotech, Cheshire, UK). Samples (1 ml) were introduced into 10 ml vacutainers (100×16 mm, Becton Dickinson sterile vacutainers with no additive) previously filled with a 5% CO_2/He gas mixture at atmospheric pressure. The vacutainers were then placed in a shaker and equilibrated at 25 °C for a minimum of 10 h, by which time at least 99% equilibrium had been reached. Results were expressed as the isotope ratio in ppm relative to that of International Standard Vienna Mean Ocean Water (SMOW) :

$$\partial = \left(\frac{R_s}{R_{SMOW}} - 1\right) \times 10^3,$$

where R_S and R_{SMOW} are the heavy and light isotope ratios in the sample and SMOW respectively. The $^{18}O/^{16}O$ isotope ratio in SMOW, is 2005.2 ppm.

$^{2}H/^{1}H$ isotope ratios were measured in duplicates by mass spectrometry using the Indiana Zn technique (Biogeochemical Laboratories, Indiana University, Bloomington, IN, USA) as described by Wong, Lee, and Klein (1987).

2.5. Data analysis and statistics

The factors studied in this work were the production region and the feeding diet which depended directly on the time of year. For each factor, the results obtained by

^{13}C NMR and IRMS, expressed in percentage (PUFA, MUFA and SFA) and ppm ($^{18}O/^{16}O$ and $^{2}H/^{1}H$) respectively, were considered as variables for the data analysis (ANOVA). One sample from a cow fed hay at the plain site was not taken into account due to clinical mastitis during the experimental period.

Selection of the three best variables giving the most efficient classification was performed by stepwise discriminant analysis (SDA) of the five variables. A discriminant analysis (DA) was also performed and 'leave-one-out' cross validation to evaluate the discriminating power of the two methods. Statistica V.5.5 software (Statsoft, France) was employed for these analyses.

3. Results and discussion

3.1. ^{18}O and ^{2}H IRMS

Table 1 shows the $\delta^{18}O$ and $\delta^{2}H$ values for drinking and milk water. The values for drinking water differed significantly between sites ($P<0.001$) but not between winter and summer, in either the plain or the mountain area.

Milk enrichments differed significantly between sites for both ^{18}O and ^{2}H ($P<0.001$). On the plain, the ^{18}O enrichments were significantly higher ($P<0.001$) for grazing cows than for those fed on maize silage or hay (Table 1) whereas, for these latter two diets, no significant differences were observed in $\delta^{18}O$ or $\delta^{2}H$. At the mountain site, the ^{18}O and ^{2}H enrichments both varied between cows fed on grass, grass silage or hay. These differences may be explained by a variable isotope intake with food since, although the drinking water enrichments were similar within a site, the animals were fed on different diets. Water represents up to 85% of grass, 75% of silage and 15% of hay and this is the source of a substantial variation in isotope entries (Ritz, Cole, Davies, Goldberg, & Coward, 1996). The ^{2}H enrichments displayed wide variability within each group, which could explain why the differences in $\delta\ ^{2}H$ were not significant for animals fed on maize silage or hay on the plain.

There was a significant correlation between ^{18}O and ^{2}H enrichments in milk water ($R^2=0.79$; $P<0.001$) and the slope of the regression line was 7.2, very close to analogous slopes in human biological fluids (Ritz et al., 1996) and to that of meteoric water. These relationships reflect the complex fractionation mechanisms occurring within the body and the fact that meteoric water goes through cycles of evaporation and condensation.

3.2. ^{13}C NMR

The relative proportions of PUFA, MUFA and SFA, as determined by ^{13}C NMR spectroscopy, are shown in Table 2. In milks from grazing cows, the percentage of PUFA was significantly greater in mountain than in plain milk ($P<0.001$), whereas MUFA and SFA did not differ significantly between the two sites. The flora of mountain pasture lands is distinctly different from that of the lowlands. Mountain pastures are very rich in non-leguminous herbaceous dicotyledons, while plain pastures are mainly composed of graminae and leguminous plants (Collomb, Bütikofer, Spahni, Jeangros, & Bosset, 1999). This difference in botanical composition leads to a greater proportion of PUFA in mountain milks than in plain milks (Chilliard et al., 2001).

The milk fat from cows in pasture contained more MUFA than that from animals fed on grass ensilage or dehydrated grass (Table 2) and a grass silage diet increased the percentage of SFA at the expense of MUFA (Table 2). These results are in agreement with previous work (for a recent review see Chilliard et al., 2001). Thus, the fatty acid contents, measured by ^{13}C NMR on the two sites, highlight the influence of the diet on the composition of milk fat.

3.3. Discriminant analyses

Discriminant analyses were performed to distinguish milks according to the two factors of production site and feeding diet. $\delta^{18}O$ was found to be a powerful parameter to discriminate between the different milks. This parameter was influenced by both the production

Table 1
$\delta^{18}O$ and $\delta^{2}H$ of drinking water at the two sites and of milk water for different diets

	Mountain			Plain			Effect	
	Pasture	Grass silage	Hay	Pasture	Hay	Maize silage	Site	Diet
Drinking water								
$\delta\ ^{18}O$	−10.50±0.46	−10.66±0.08		−5.21±0.30	−5.21±0.16		***	NS
$\delta\ ^{2}H$	−45.3±6.1	−50.4±3.4		−17.4±5.6	−21.8±5.4		***	NS
Milk water								
$\delta\ ^{18}O$	−4.93±0.37 (a)	−7.85±0.37 (b)	−9.41±0.25 (c)	−2.02±0.35 (a)	−4.37±0.19 (b)	−4.43±0.18 (b)	***	***
$\delta\ ^{2}H$	1.6±15.5 (a)	−27.6±6.3 (b)	−35.1±15.4 (b)	11.5±8.3 (a)	−0.9±5.0 (a)	1.5±8.2 (a)	***	NS

Different letters indicate a significant difference between groups ($P<0.05$). *t*-test: *** $P<0.001$; ** $P<0.01$; * $P<0.05$; NS $P>0.1$.

Table 2
Relative proportions of PUFA, MUFA and SFA in milk fat according to the site and diet

	Mountain			Plain			Effect	
	Pasture	Grass silage	Hay	Pasture	Hay	Maize silage	Site	Diet
PUFA (%)	4.3±2.4	6.2±1.1	5.8±0.7	1.8±1.5	1.7±0.4	1.8±0.9	***	NS
MUFA (%)	22.0±3.5 (a)	17.3±2.4 (b)	21.0±5.9 (ab)	26.0±5.4 (a)	19.7±1.2 (b)	21.3±1.8 (ab)	NS	*
SFA (%)	73.7±4.5	76.5±2.4	73.3±6.0	72.3±6.0	78.7±1.4	77.0±2.1	NS	NS

PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids. Different letters indicate a significant difference between groups ($P<0.05$). *t*-test: *** $P<0.001$; ** $P<0.01$; * $P<0.05$; NS $P>0.1$.

site and the diet, whereas the fatty acid proportions were more sensitive to the diet. A very efficient classification of milks could be obtained using $\delta^{18}O$. At the mountain site, the different diets were clearly distinguished (hay, grass silage or pasture) and 100% of the milks were well classified. Furthermore, for a pasture diet, the two sites (plain and mountain) were clearly separated and plain milks from grazing cows were also well distinguished from other milks. The only source of confusion was plain milk in winter where the diets of maize silage and hay were mismatched. Addition of the ^{3}C NMR parameters PUFA and MUFA allowed a slightly better classification (86% of milks well classified versus 78%).

4. Conclusion

The present study clearly demonstrates that it is possible to distinguish milks from different geographical locations and feeding diets. Since IRMS analyses were performed on the aqueous phase while NMR gave the fatty acid composition, the two methods are complementary. Each is focussed on one milk fraction, the composition of which could be influenced by either the geographic origin or the feeding diet. However, $\delta^{18}O$ appears to be the most discriminant parameter for both the geographic origin and the diet. Hence this study represents a first encouraging step toward food authentication using IRMS and NMR. To confirm these results, research is now being carried out at various geographic sites in France before extending the study to other parts of Europe. The variability of the sites and the analysis of milk from different breeds of cow and different herds will be used to validate our results and create a data base.

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