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Identification of Milk Origin and Process-Induced Changes in Milk by Stable Isotope Ratio Mass Spectrometry

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ABSTRACT: Stable isotope values were used to develop a new analytical approach enabling the simultaneous identification of milk samples either processed with different heating regimens or from different geographical origins. The samples consisted of raw, pasteurized (HTST), and ultrapasteurized (UHT) milk from different Italian origins. The approach consisted of the analysis of the isotope ratio of $\delta^{13}C$ and $\delta^{15}N$ for the milk samples and their fractions (fat, casein, and whey). The main finding of this work is that as the heat processing affects the composition of the milk fractions, changes in $\delta^{13}C$ and $\delta^{15}N$ were also observed. These changes were used as markers to develop pattern recognition maps based on principal component analysis and supervised classification models, such as linear discriminant analysis (LDA), multivariate regression (MLR), principal component regression (PCR), and partial least-squares (PLS). The results give proof of the concept that isotope ratio mass spectroscopy can discriminate simultaneously between milk samples according to their geographical origin and type of processing.

KEYWORDS: *milk, IRMS, stable isotope, milk processing, authentication, origin*

INTRODUCTION

The main purpose of milk processing is to ensure its microbiological safety and stability. Heat treatments yield a variety of products characterized by different shelf lives and different physical and chemical properties. Moreover, the severity of treatments applied to milk (e.g., heating, homogenizing, filtering, etc.) can also affect the functionality of spray-dried milk products, for example, in terms of dispersibility and taste. Furthermore, milk nutritional and economic value is often associated with its geographical origin. As such, the origin of milk is a quality criterium of great importance for manufacturers. Milk produced in specific geographical regions has found favorable acceptance among customers, who are willing to pay more for a product perceived as genuine, typical, and of higher quality. In this respect, it is important for the legislator to have methods enabling the recognition of either the processing conditions used or the origin of milk.

Currently, there are several methods specifically developed for the determination of heating loads. One of the most common methods is the whey protein nitrogen index (WPNI).[1] More recently, nondenatured soluble whey proteins have been quantified by HPLC[2,3] and by immunochemical methods.[4] Other methods are based on the detection of Maillard reaction products, which are indicative of severe heat treatment such as furosine and lactulose.[5] Furosine is an amino acid formed during the Maillard reaction as a result of the acid hydrolysis of lysine and lactose.[6] It can be determined by HPLC[5,7,8] or by capillary electrophoresis.[9,10] Lactulose is also formed during heating of milk by isomerization of lactose.[11,12] Finally, a sensitive indicator for heat treatment of milk formed during the Maillard reaction is lysinoalanine (LAL), which has been separated and detected by RP-HPLC. LAL was proven to be a suitable and sensitive indicator to assess the heat treatment intensity given to milk.[13] Unfortunately, all of these methods are useful only to evaluate the heating load applied to milk, but they fail to discriminate milk origins.

For the identification of the geographical origin of milk, a number of methods have been developed. One of the most promising approaches makes use of isotope ratio mass spectrometry (IRMS). This approach has been applied to the characterization of the geographical origin of milk,[14−18] butter,[19] and cheese[20] thanks to the use of several stable isotope ratios (such as carbon, nitrogen, hydrogen, oxygen, sulfur, and strontium). Two of these isotope ratios, $\delta^{13}C$ and $\delta^{15}N$, are of special interest because $\delta^{13}C$ values of milk vary as a function of the animal feed and, especially, due to the presence of C4 or C3 plants within the forages.[21] Conversely, $\delta^{15}N$ values are influenced by factors such as soil conditions and the use of industrial fertilizers.[22] These provide proof of a correlation between stable isotopes of milk and its geographical origin.

However, to the best of our knowledge, there is no study showing the connection between the isotope values of $\delta^{13}C$ and $\delta^{15}N$ in milk or milk fractions (casein, fat, and whey) and the processing conditions used during milk processing. Thus, the aim of this work is to provide proof of concept that $\delta^{13}C$ and $\delta^{15}N$ of

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the main fractions of milk can be used to identify simultaneously the geographical origin and the processing conditions applied. The work is based on the assumption that physical changes occurring during processing result in a change in the stable isotope ratio in individual fractions of the milk, which allows a joint distinction between geographical origin and treatments applied.

MATERIALS AND METHODS

Milk Sampling. The sampling plan consisted of raw, pasteurized (HTST), and ultrahigh-temperature processed (UHT) milk from intensive and mountain extensive breeding systems, collected in 2011. Milk from the intensive breeding system was obtained by Despar Industry (Mantova; latitude, 45° 9′ 37″; longitude, 10° 47′ 52″) and from Latterie Friuli (Udine; latitude, 46° 1′ 11″; longitude, 13° 9′ 38″). Milk from the mountain extensive breeding system was obtained by Mila Milkon (Bolzano, Italy) using milk from two local farmers. The farmers were located in Sarntal (latitude, 46° 38′ 40″; longitude, 11° 21′ 25″) and Seiser Alm, Kastelruth (latitude, 46° 34′ 3″; longitude, 11° 33′ 43″). The overall sampling plan is summarized in Table 1.

Table 1. Sampling Plan of Milk Samples[a]

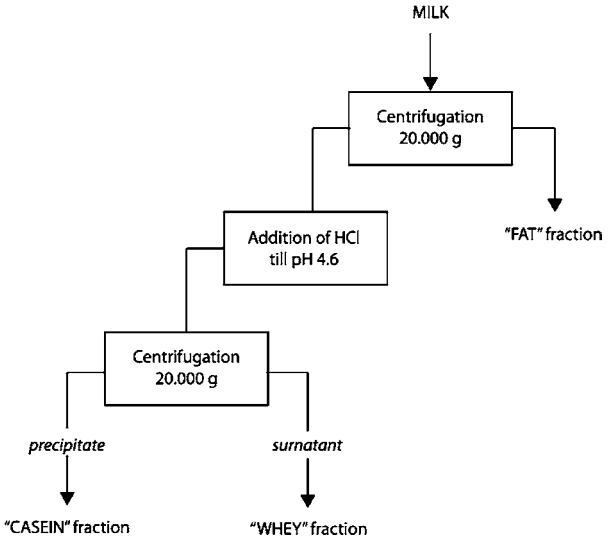
	origin		
processing	Bolzano	Udine	Mantova
raw	16	8	nd
HTST	8	8	8
UHT	8	8	8

[a]Raw and processed samples labeled as "Bolzano" were obtained by Mila Milkon, Bolzano (Italy). Processed samples labeled as "Udine" and "Mantova" were purchased in a local grocery. Raw samples labeled as "Udine" were obtained by local farmers located in Udine.

Forage. Alfalfa and maize silages have been collected form South Tyrol and Udine (Italy), respectively.

Milk Sample Fractionation. Fractionation of the milk sample is described in Scheme 1. Briefly, the fat content was removed by a centrifugation method, adapted from that of Feng and Luna.[23,24] Milk (1.5 mL) was centrifuged at 20000*g* for 30 min at 4 °C. The upper layer was removed and labeled "fat", although it may contain also some protein bonded with the fat droplets. Thereafter, casein was separated according to the current International Dairy Federation (IDF) procedure, which is based on the precipitation of casein at its isoelectric point (pH 4.6). In practice, the defatted sample was precipitated by the addition of 60 μL of 6.0 M HCl, vortexed for 5 s, and allowed to stand for 10 min at room temperature. Then, casein micelles (and the denatured β-lactoglobulin) were separated by centrifugation at 20000*g* for 30 min at 4 °C. In this second centrifugation step, the precipitate residue was labeled "casein". Any fat layer eventually present as supernatant was discharged. Thus, the supernatant was transferred into a vial and labeled "whey". Control experiments showed that native β-lactoglobulin remains in the "whey fraction" after the second centrifugation step. Whole milk and its casein, whey, and fat fractions were all frozen for 24 h at −20 °C, then lyophilized, and finally stored at room temperature in a desiccator.

Scheme 1. Steps for the Fractionation of Milk Samples



Isotope Ratio Mass Spectroscopy. Approximately 1 mg of freeze-dried sample of forage, casein, whey, and fat, respectively, was weighed in a tin capsule for the measurement of $\delta^{13}C$ and $\delta^{15}N$ in one run. The analysis was carried out using a Delta V isotope ratio mass spectrometer (Thermo Scientific, Germany) equipped with a Flash EA 1112 elemental analyzer (Thermo Scientific). The isotope ratios were expressed in δ‰ versus Vienna−Pee Dee Belemnite (V-PDB) for $\delta^{13}C$ and air for $\delta^{15}N$ according to the formula

$$\delta‰ = [(R_{sample} - R_{standard})/R_{standard}] \times 1000$$

where R_{sample} is the isotope ratio measured for the sample and $R_{standard}$ is the isotope ratio of the international standard. R is the abundance ratio of the minor, heavier isotope of the element to the major, lighter isotope (e.g., $^{13}C/^{12}C$). The isotopic values were calculated against international reference materials: L-glutamic acid USGS 41 and ammonium sulfate IAEA-N-2 (IAEA-International Atomic Energy Agency, Vienna, Austria). All analyses were performed in duplicate.

Statistical Analysis. Principal component analysis (PCA) was used to display the samples in an unsupervised pattern recognition map (score plot) and observe relationships between samples and variables (from the comparison of the score and loading plot). PCA was developed starting from the correlation matrix to autoscale the data and obtain variables with zero mean and unit standard deviation.[25]

Linear discriminant analysis (LDA) was used to develop linear functions of the variables (also called classification functions) able to assign a milk sample into one of the predetermined groups.[25] Variables used in this classification model were chosen according to the results of the PCA. Validation of the model was performed with a new set of milk samples.

Regression models such as multiple linear regression (MLR), principal component regression (PCR), and partial least-squares (PLS) consisted of equations made by a number of predictors equal to the number of variables used plus one constant (the intercept). The outcome of each model was approximated to the nearest categorical number (1, 2, or 3) to predict the type of treatment (raw, HTST, or UHT) or the origin (Bolzano, Udine, Mantova) of the unknown milk sample. Cross-validation was used to validate regression models.[25]

RESULTS AND DISCUSSION

Effect of Processing. Preliminary experiments aimed to explore the feasibility of $\delta^{15}N$ and $\delta^{13}C$ to discriminate milk samples processed with different heat treatments. Raw, HTST, and UHT whole milks of the same origin were analyzed (Table 2). One-way ANOVA of $\delta^{15}N$ values showed that the three milk samples are significantly different. However, the pairwise comparison of treatments indicates that only the difference

Table 2. $\delta^{15}N$ Values of Whole Milk Samples (Unfractionated) Obtained by Mila Milkon, Bolzano (Italy)[a]

treatment	$x \pm s$ ($n = 8$)
raw	4.9 ± 0.1b
HTST	5.3 ± 0.1a
UHT	5.4 ± 0.1a

[a]Letters indicate significant differences at $P < 0.05$.

between unprocessed (raw) and processed (HTST and UHT) milk samples is significant. The same conclusion was obtained with the analysis of the $\delta^{13}C$ values (not shown). Thus, the $\delta^{15}N$ or $\delta^{13}C$ values are unable to discriminate between the processed samples (HTST vs UHT); they can discriminate only between the raw and processed milk samples.

The same experiment was conducted with the $\delta^{15}N$ or $\delta^{13}C$ values of the milk fractions, consisting of casein, whey, and fat, as described under Materials and Methods (Table 3). A two-way ANOVA performed on the sample from Bolzano confirms shows that the milk fractions and the treatments are significantly different in terms of $\delta^{13}C$ and $\delta^{15}N$ values. However, as for the (unfractionated) milk sample analysis (vide supra), such differences are observed only between unprocessed (raw) and processed (HTST and UHT) milk samples. Such a difference is consistent with the changes in composition of the fat globules after homogenization.

Table 3. Results of $\delta^{15}N$ and $\delta^{13}C$ Values of Fractionated Raw and Processed Milk Samples from Bolzano, Udine, and Mantova (Italy)[a]

				fractions		
	isotope ratio	treatment	n	casein	whey	fat
Bolzano	$\delta^{15}N$	raw	16	5.5	3.6	−0.1
		HTST	8	5.3	3.7	4.0
		UHT	8	5.4	2.9	4.5
	$\delta^{13}C$	raw	16	−23.9	−24.7	−26.7
		HTST	8	−24.0	−23.7	−25.9
		UHT	8	−24.0	−23.7	−25.4
Udine	$\delta^{15}N$	raw	8	3.1	1.9	−0.5
		HTST	8	4.3	3.2	4.0
		UHT	8	3.0	5.0	4.2
	$\delta^{13}C$	raw	8	−18.6	−17.7	−19.6
		HTST	8	−17.2	−20.8	−19.7
		UHT	8	−21.1	−21.1	−23.1
Mantova	$\delta^{15}N$	raw		nd	nd	nd
		HTST	8	5.9	4.0	4.3
		UHT	8	5.3	3.3	4.6
	$\delta^{13}C$	raw		nd	nd	nd
		HTST	8	−19.4	−18.3	−21.3
		UHT	8	−18.2	−17.1	−19.5

[a]Precision of $\delta^{13}C$ and $\delta^{15}N$ measurements was ±0.2‰; nd, not determined.

A general limit of ANOVA is that the responses of each isotope ratio of the fractions are analyzed individually; this is a problem especially in the presence of correlated responses. A better understanding of the data set can be obtained by PCA, which combines all of the variables (isotopic values of the milk fractions) down to a small number of components. The PCA of the milk samples from Bolzano is shown in Figure 1. Using all of the variables, the samples are clearly grouped along the first principal component, from left to right, following the order of processing treatment severity (raw < HTST < UHT). Moreover, the raw milk samples originating from two distinct farms within the province of Bolzano are separated, although both are located at the left side of the first principal component, opposite the other processed samples.

The trend can be explained by observing that the repartition of $\delta^{13}C$ and $\delta^{15}N$ in the milk fractions changes according to the treatment used. In particular, the whey fraction of HTST or UHT milk samples from Bolzano had $\delta^{15}N$ values lower than the corresponding raw milk (Table 3). Conversely, the isotopic ratios of the fat fraction were higher in the processed milk samples than in the raw ones. Apparently, as the milk treatments become more severe, part of the whey proteins irreversibly denature[26] and bind via disulfide bonds to the proteins, stabilizing the fat globules. As a consequence, $\delta^{15}N$ values of the whey fraction decrease, whereas theose of the fat fraction increase (Table 3). This concept is illustrated in Scheme 2, which depicts the main effects of heating and homogenization on the milk components together with their potential interactions.

Effect of the Origin. The effect of the origin on the isotope ratio of milk samples was considered. Figure 2 shows the PCA obtained with the combination of the $\delta^{13}C$ and $\delta^{15}N$ values from the milk fractions (casein, whey, and fat) of Udine and Bolzano. A great difference between the two regions is observed along the first principal component. Moreover, the samples are also separated along the second principal component, following the order of severity of the processing. This is consistent with the results reported in the previous section.

The capability of $\delta^{13}C$ and $\delta^{15}N$ values to determine the geographical origin of milk samples has previously been reported by Camin et al.[27] This finding has been also verified here in connection with the analysis of forages collected in the areas in

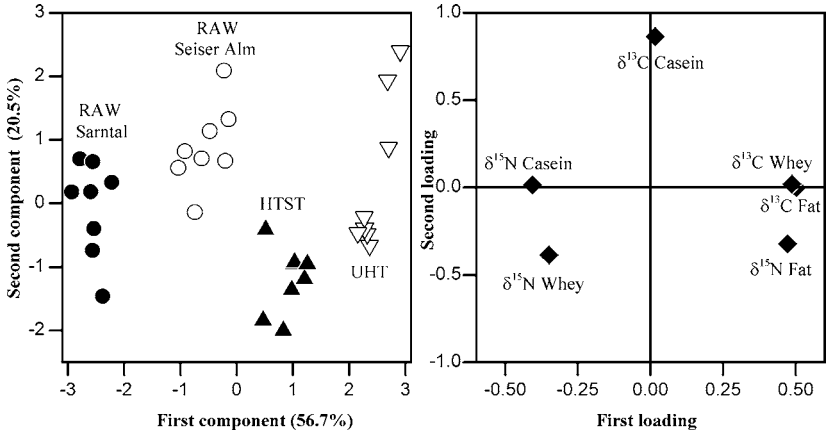


Figure 1. Score (left) and loading (right) plots of the principal component analysis of the data set containing processed and unprocessed milk samples from Bolzano (Italy). The data set is composed of 32 samples (n = 16 raw, n = 8 HTST, and n = 8 UHT).

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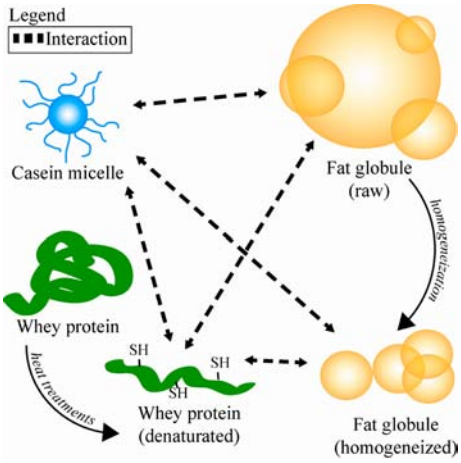
Scheme 2. Sketch of the Main Effects and Interactions between Whey, Casein, and Fat during Heat and Homogenization Processing[a]



[a]Milk components are affected by the type of processing, explaining the different repartition of the isotopic values in the milk fractions.

which milk samples were produced. The $\delta^{13}C$ value of forages from Bolzano is $-28.1 \pm 0.2‰$ ($n = 2$), whereas the one from Udine is $-27.7 \pm 0.2‰$ ($n = 4$, Table 4). Also, $\delta^{13}C$ for alfalfa (*Medicago sativa* L.), one of the most well-known and widely used forage crops in the world, collected from Bolzano is $-29.2 \pm 0.2‰$ ($n = 4$), whereas that from Udine is $-28.2 \pm 0.2‰$ ($n = 2$). In both cases, $\delta^{13}C$ values of Udine are slightly higher than the one of Bolzano (Table 4). However, a great difference of the $\delta^{13}C$ values appears when these forages are compared with maize silages, the $\delta^{13}C$ value of which is $-11.1 \pm 0.1‰$ ($n = 2$). The reason for this difference can be ascribed to the different photosynthetic CO_2 fixation pathways used by C3 (e.g., forage, alfalfa, etc.) and C4 plants (e.g., maize). This difference reflects the $\delta^{13}C$ found in milk samples of different origins. In fact, milk produced by cows fed C3 plants has, in general, a $\delta^{13}C$ lower than the one obtained from cows fed C4 plants. Accordingly, raw milk (unfractionated) from Udine (with $\delta^{13}C$ of $-18.6 \pm 0.1‰$, $n = 8$), which is obtained from cows fed a mixture of C3 and C4 plants, can be easily separated from the milk samples from Bolzano (with an average $\delta^{13}C$ value of $-25.2 \pm 0.4‰$, $n = 16$) because the latter is obtained only by C3 plants (Table 4).

Table 4. $\delta^{15}N$ and $\delta^{13}C$ Values of Forages from Two Different Regions, Udine and Bolzano, Respectively[a]

	Udine		Bolzano	
sample	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$
maize silage	2.3	−11.1	nd	nd
alfalfa	−1.0	−28.2	0.7	−29.2
forage	0.4	−27.7	1.1	−28.1

[a]Precision of $\delta^{13}C$ and $\delta^{15}N$ measurements was ±0.2‰. Each sample was analyzed in double; nd, not determined.

Simultaneous Effect of the Processing and the Origin.

The feasibility of $\delta^{15}N$ and $\delta^{13}C$ to discriminate simultaneously milk samples from different origins and processings was finally explored. Milk samples from Udine, Mantova, and Bolzano were analyzed for $\delta^{13}C$ and $\delta^{15}N$ values. A total of $n = 72$ whole milk samples, comprising $n = 24$ raw, $n = 24$ HTST, and $n = 24$ UHT milk samples were fractionated into casein, whey, and fat and analyzed.

Pattern recognition techniques were used to reveal similarities within groups and develop classification models. The left panel of Figure 3 shows the scores of the principal component analysis. Samples located in the same geographical origin can be grouped together along with the first principal component (53% of total variance). Moreover, raw, HTST, and UHT milk samples can be clustered along the second principal component (23.6% of total variance).

From the analysis of the loadings (Figure 3, right panel), the most important variables enabling the separation of the samples along the first PC (or according to the origin) are both the $\delta^{13}C$ and $\delta^{15}N$ of all the fractions, except $\delta^{15}N$ of fat. The importance of $\delta^{13}C$ values was expected because it is connected with the geographical origin through the isotope values of the feed used in the animal diet (vide supra).

According to the loading of the first PC, also $\delta^{15}N$ contributes to milk discrimination with respect to the origin. This is particularly evident when the results of the raw milk samples are observed. The raw milk sample collected from Bolzano (Italy) show a higher $\delta^{15}N$ value ($4.9 \pm 0.1‰$, Table 2) than the one from Udine ($3.0 \pm 0.2‰$). This difference can be attributed to the different feedstuff used in the diet of the cows, for example, the presence of leguminous species and/or the agronomic practice adopted for the feed cultivation, for example, different

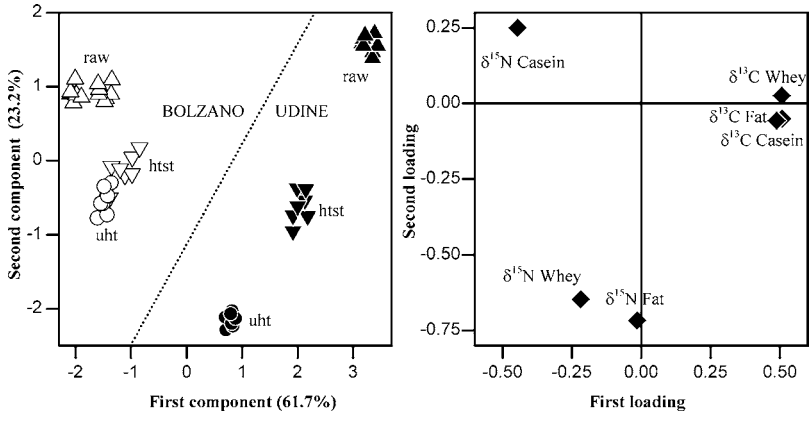


Figure 2. Score (left) and loading (right) plots of the principal component analysis of the data set containing processed and unprocessed milk samples from Bolzano and Udine. Data set is composed of 56 samples ($n = 32$ from Bolzano and $n = 24$ from Udine).

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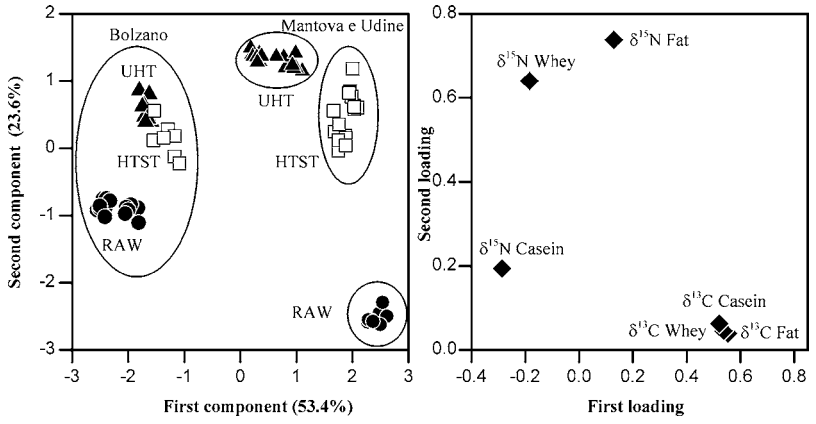

Figure 3. Score (left) and loading (right) plots of the principal component analysis (a) based on a data set containing 72 samples from Bolzano, Udine, and Mantova (n = 24 raw, n = 24 HTST, and n = 24 UHT).

fertilization practices.[27] Therefore, also δ^{15}N can contribute to discriminating the production area.

Considering the loading of the second PC, the most important variables are mainly the δ^{15}N values of the whey and fat fractions. The δ^{15}N values of the whey fraction in heat-treated milk are significantly different mainly because of the whey protein denaturation, which drives the interaction between caseins or fat globules (see Scheme 2). As a result, δ^{15}N values of the whey and fat fractions are good markers for discriminating between treatments applied to milk.

A classification model based on LDA was developed to test the ability of δ^{13}C and δ^{15}N as markers of the processing level used. The model was built on the samples from Bolzano and with three of the six original variables (δ^{15}N of whey and fat and δ^{13}C of casein). Results of the cross-validation gave 100% successful recognition, enabling the identification of raw, HTST, or UHT milk samples. The rationale to choose a reduced model with only three variables is that it minimizes "overfitting". Figure 4 shows

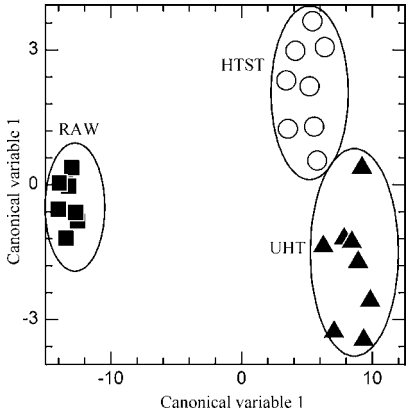

Figure 4. Score plot of the canonical classification method based on a data set containing 24 milk samples (n = 8 raw, n = 8 HTST, and n = 8 UHT).

the distribution of the samples according to the classification functions. The LDA classification model was also applied to a new set of data: n = 8 raw milk samples obtained by a local milk producer located in South Tyrol, n = 2 commercial HTST samples, and n = 2 UHT samples purchased from a local market. All samples were correctly classified (error rate in prediction for new unknown samples 0%).

Finally, regression models based on MLR, PCR, and PLS were also applied on the full data set comprising milk samples from the other geographical locations (Bolzano, Udine, and Mantova) as well as the three different process types (raw, HTST, and UHT). For each sample, all six variables were used, namely, the fractions casein, whey, and fat analyzed each for δ^{13}C and δ^{15}N values. The equations of these three regression models for the prediction of the treatment are reported in Table 5 and for the prediction of the origin in Table 6.

Table 5. Regression Coefficients for the Classification of Milk Samples According to Their Treatments (Regression Model: $y = k_0 + \delta^{15}N_{fat} \times k_1 + \delta^{15}N_{casein} \times k_2 + \delta^{15}N_{whey} \times k_3 + \delta^{13}C_{fat} \times k_4 + \delta^{13}C_{casein} \times k_5 + \delta^{13}C_{whey} \times k_6$)[a]

	coefficients		
predictor	MLR	PCR	PLS
constant (k_0)	2.12	2.04	2.08
$\delta^{15}N_{fat}$	0.38	0.30	0.38
$\delta^{15}N_{casein}$	0.05	−0.02	0.01
$\delta^{15}N_{whey}$	−0.33	0.06	−0.34
$\delta^{13}C_{fat}$	0.03	0.05	0.04
$\delta^{13}C_{casein}$	0.10	0.08	0.04
$\delta^{13}C_{whey}$	−0.13	−0.09	−0.09
PRESS	5.1	10.1	5.4

[a]Results are y = 1 for raw milk, y = 2 for HTST milk, and y = 3 for UHT milk. MLR, multivariate linear regression; PCR, principal component analysis; PLS, partial least-squares regression; PRESS, prediction error sum of squares.

The coefficients of the models used to predict either milk treatment or the origin show coefficients with the same sign and comparable absolute values. The predictive capability of the models, as measured by the predicted residual sums of squares (PRESS) statistic obtained with the leave-one-out validation method, shows that MLR and PLS are superior to PCR. In the prediction of milk treatments, the most important terms are the δ^{15}N values of the whey and fat fractions. Instead, in the case of the prediction of the origin, δ^{15}N of the whey and δ^{13}C of the casein fractions dominate. These results are consistent with the previous discussion and highlight the different importance of the original variables to the discrimination of the origin or the process type. Accordingly, for prediction purposes, models with just a reduced number of coefficients, among the ones reported in Table 5, should be used.

Table 6. Regression Coefficients for the Classification of Milk Samples According to Their Origin (Regression Model: $y = y = k_0 + \delta^{15}N_{fat} \times k_1 + \delta^{15}N_{casein} \times k_2 + \delta^{15}N_{whey} \times k_3 + \delta^{13}C_{fat} \times k_4 + \delta^{13}C_{casein} \times k_5 + \delta^{13}C_{whey} \times k_6$)[a]

	coefficients		
predictor	MLR	PCR	PLS
constant (k_0)	4.01	4.16	4.06
$\delta^{15}N_{fat}$	0.08	0.18	0.08
$\delta^{15}N_{casein}$	−0.16	−0.14	−0.15
$\delta^{15}N_{whey}$	0.57	0.04	0.58
$\delta^{13}C_{fat}$	0.07	0.13	0.09
$\delta^{13}C_{casein}$	0.28	0.21	0.27
$\delta^{13}C_{whey}$	−0.18	−0.23	−0.18
PRESS	2.1	12.5	2.1

[a]Results are y = 1, Bolzano; y = 2, Mantova; y = 3, Udine. See Table 5 for abbreviations.

In conclusion, milk samples from different origins produced under different heat treatments have been discriminated and classified on the basis of the content of $\delta^{13}C$ and $\delta^{15}N$. Through the analysis of the $\delta^{13}C$ and $\delta^{15}N$ values among the three main fractions of milk, namely, casein, whey, and fat, the type of process and origin can, in principle, be simultaneously recognized. Further work is required to better understand and model the effect of each individual treatment (such as heating, homogenizing, filtering) on the stable isotope values of the milk fractions.

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Notes

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