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Application of Multielement Stable Isotope Ratio Analysis to the Characterization of French, Italian, and Spanish Cheeses

FEDERICA CAMIN,*,† KARINE WIETZERBIN,‡ ANAISABEL BLANCH CORTES,§ GEORG HABERHAUER,# MICHÉLE LEES,‡ AND GIUSEPPE VERSINI†

Laboratorio di Analisi e Ricerche, Istituto Agrario di San Michele all'Adige, via E. Mach 1, 38010 S. Michele all'Adige, Trento, Italy; Eurofins Scientific, Rue Pierre Adolphe Bobierre, Site de la Gèraudiére, B.P. 42301, 44323 Nantes Cedex 3, France; Laboratorio Arbitral Agroalimentaria, Ministerio de Agricultura, Pesca y Alimentacion, Paseo Infanta Isabel, 28014 Madrid, Spain; and Department of Environmental Research, ARC Seibersdorf research GmbH, A-2444 Seibersdorf, Austria

The stable isotope ratios (δ^{13}C, δ^{15}N, and δ^{34}S of casein and δ^{13}C and δ^{18}O of glycerol) measured by IRMS of French, Italian, and Spanish cheeses are presented and discussed. Variability factors such as animal-feeding regimen, geographical origin, and climatic and seasonal conditions were studied to check the possibilities of cheese characterization offered by each isotopic parameter. δ^{13}C values of both casein and glycerol appeared to be strongly correlated to the amount of maize in the animal diet. δ^{15}N and δ^{34}S of casein proved to be mostly influenced by the geoclimatic conditions of the area (aridity, closeness to the sea, altitude). δ^{18}O of glycerol was more dependent on the geographical origin of the cheeses and on climatic/seasonal parameters. By applying a multivariate stepwise canonical discriminant analysis, good discrimination possibilities for the different European cheeses were obtained, confirmed by the classification analysis, when >90% of the samples were correctly reclassified.

KEYWORDS: PDO European cheeses; stable isotope ratios; geographical origin; animal-feeding regimen; statistical analysis

INTRODUCTION

Consumers are increasingly attracted to foodstuffs that declare not only their composition but also their geographical origin, such as products with a Protected Denomination of Origin (PDO). As these products command a premium price, origin mislabeling is a tempting fraudulent practice. To protect consumers against such types of fraud, efficient analytical tools able to demonstrate and characterize the typicality of PDO products, for example, PDO cheeses, are required.

In this context, a European Project designated "Development and Validation of Methods to Determine the Geographical Origin of Milk, Butter and Cheese" (SMT4 CT98 2236) was carried out in 1998−2000 and involved six European countries (Austria, France, Germany, Italy, Spain, and the United Kingdom). An analytical approach including the measurements of stable isotopic ratios of bioelements and the analysis of trace elements was developed and applied to different European dairy products to check its efficiency in geographical characterization.

* Author to whom correspondence should be addressed (telephone +39 0461 615261; fax +39 0461 615288; e-mail federica.camin@ismaa.it).
† Istituto Agrario di San Michele all'Adige.
‡ Eurofins Scientific.
§ Ministerio de Agricultura, Pesca y Alimentacion.
# ARC Seibersdorf research GmbH.

In this work, the results of stable isotope analyses of French, Italian, and Spanish cheeses are presented and discussed. It is worth remembering that within the past few years, such kinds of analyses, widely used in authenticity control and origin determination of food and food ingredients, notably wine (*1*, *2*) and fruit juice (*3*−*5*), have gained increasing importance in the geographical characterization of milk (*6*−*8*), butter (*9*), and cheese (*10*−*12*).

The present study focused on the following isotopic ratios measured in different cheese components and chosen because they may provide useful information in origin discrimination:

^{13}C/^{12}C of Casein and Glycerol. It is well-known that this ratio discriminates between C_3 and C_4 plant materials (*13*). For animal products, such as meat, milk, and cheese, δ^{13}C increases with the amount of maize (a C_4 plant) included in the diet (*6*−*8*, *11*, *12*, *14*, *15*).

^{15}N/^{14}N of Casein. In dairy products this isotopic ratio reflects, through the plants consumed, the isotopic composition of the original soil (*6*, *7*, *10*, *11*, *12*, *16*), which is influenced by many factors such as agricultural practices (intensive or extensive cultivation) (*17*) and climatic and geographical conditions (*18*). The use of massive quantities of organic fertilizers and other factors such as aridity, salinity, and closeness to the sea, tend to increase this isotopic ratio in soil, plants (*18*), and animal products (*6*, *7*, *10*−*12*). Moreover, for animal

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products, the presence of nitrogen-fixing plants in the diet can lead to lower values, because these plants use both atmospheric and soil nitrogen as a nitrogen source (*19*), which results in a lower ^{15}N content with respect to the other plants relying only on soil nitrogen (enriched in ^{15}N compared to the atmospheric nitrogen).

$^{18}O/^{16}O$ of Glycerol (Obtained from Lipid Hydrolysis). Only very few data on this parameter in dairy products are available in the literature (*12*, *20*), and there has never been a wide scale investigation of the influence of cheese origin on the $\delta^{18}O$ of glycerol. The ^{18}O content of milk water, on the other hand, has been studied in more detail (*6*, *7*). $\delta^{18}O$ of milk water reflects the isotopic composition of the ground water drunk by the animals, and in turn the ^{18}O content of the ground water is influenced by geographical factors such as altitude, latitude, and distance from the sea (*21*). Moreover, seasonal effects are also noticeable: in summer, milk water exhibits higher ^{18}O content because animals eat fresh plants containing ^{18}O-enriched water due to evapotranspiration phenomena in leaves. Milk organic compounds such as lactose show $\delta^{18}O$ values that are correlated to those of the milk water, even if greatly enriched (*22*), and they are therefore influenced by the same factors. It is expected that $^{18}O/^{16}O$ of glycerol would show a similar variability and that this ratio would be a good tracer of the geoclimatic origin of dairy products.

$^{34}S/^{32}S$ of Casein. In milk casein this parameter is considerably influenced by the geology of the area from which the animal feed originates (igneous or sedimentary, acid or basic) (*7*). Other important factors could be the fertilization procedure, mineralization and demineralization reactions, leaching, climatic conditions, and closeness to the sea (*23*).

The aim of this work was to establish the isotopic signature of different cheeses in order to identify and discuss the most important factors that account for the variability of isotopic ratios in cheese components and to verify if a multi-isotopic approach could be an efficient tool for cheese origin characterization. To our knowledge, such a multi-isotopic approach with so many European Union (EU) cheeses has never been reported.

MATERIALS AND METHODS

Cheeses. The cheese samples were purchased directly from the dairies at regular intervals over a 2 year period to take into account the seasonal variation and changes in feeding regimen.

The sampling locations are shown in **Figure 1**.

French Cheeses. (a) Reblochon (Cow PDO Cheese). The 16 samples were supplied by the Syndicat Interprofessionnel du Reblochon (Thônes) between November 1998 and November 2000. The samples were produced on different farms around Thônes in the French Alps (latitude of ∼46°). The farms were located at altitudes ranging between 650 and 1650 m high. In agreement with the regulation associated with this PDO, the cattle diet did not contain silage. The cows were essentially fed on grass, hay, and mineral additives. Up to 20% of the hay can originate from other regions. The herd usually moves to high-mountain pasture during the summer season.

(b) Camembert de Normandie (Cow PDO Cheese). The 10 samples were obtained from the Laiterie de Benières (Bernières d'Ailly) between January 1999 and November 2000. The production area was around the city of Falaise (Normandie, western France, latitude of ∼49°). The feeding regimen included fodder, maize, and grass silage (mostly in winter) and fresh grass (in summer).

Italian Cheeses. (a) Grana Padano (Cow PDO Cheese). The 13 samples were collected between December 1998 and October 2000 in dairies located in the southern part of the region of Lombardia (northwestern Italy, latitude of 45−46°). The production zone was located in a plain and in an area of intensive agricultural treatment. The soil is deep, calcareous, and highly drained. The feeding regimen

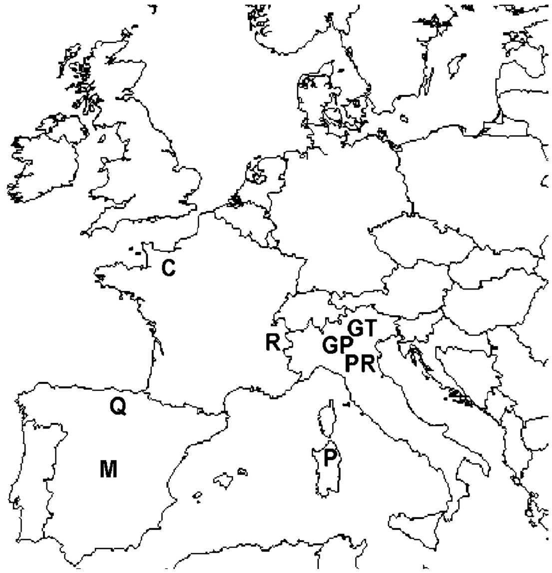

Figure 1. Map of Europe with sampling locations [France, Reblochon (R) and Camembert (C); Italy, Grana Padano (GP), Grana Trentino (GT), Parmigiano Reggiano (PR), and Pecorino Sardo plus Sardinia cow cheese (P); Spain, Quesuco (Q) and Manchego (M)].

was constant throughout the year and was based on Unifeed, a mixture of maize silage with hay and fodder, not necessarily grown in Lombardia. The PDO regulation allows a large content of maize silage (up to 50%) in the animal diet.

(b) Grana Trentino (Cow PDO Cheese). This product belongs to the "Consortium of Grana Padano" but has gained a typical commercial trademark and denomination. The 12 samples were collected between December 1998 and October 2000 and were produced in the Non Valley (Trentino-Alto Adige region, northern Italy, latitude of 46−47°), which is the main production area for this cheese. The sampling location (mostly Sporminore) was at ∼500 m above sea level (asl); the soil is lixiviated and calcareous with subalkaline pH values (7.2−8.4). Hay and fodder were usually the main animal feed, and the hay could be produced in other areas, for example, Padana Plain. The animal diet cannot contain silage, according to the PDO regulation.

(c) Parmigiano Reggiano (Cow PDO Cheese). Thirteen samples were mostly collected in the Modena area (Emilia-Romagna region, north-central Italy, latitude of 44−45°) between December 1998 and October 2000. The production area was located in a plain, in an area under intensive cultivation. The soil is deep, with reduced oxygen availability and a thin texture, resulting in a highly calcareous and moderately alkaline layer. The animal feed did not change during the year and was based on Unifeed, that is, a mixture of hay and fodder, which came from the same region. The PDO regulation does not allow silage in the animal diet.

(d) Pecorino Sardo (Sheep PDO Cheese). The eight samples were produced on the island of Sardinia between November 1998 and July 2000. The sampling locations (Siligo and Ittireddu) lie in the north-western part of Sardinia (latitude of 40−41°) at ∼400 m asl with Mediterranean scrub as the main flora. The soil presents a composition derived from basaltic rock and a calcareous-marly one. It is poor in humus, soft, and porous with spontaneous vegetation. The animals were pastured all year round: the diet could be complemented with fodder, oats, and maize, predominately in winter. The animals did not produce milk from July until November because of pregnancy and soil aridity.

(e) Sardinia Cow Cheese (Submitted for PDO Recognition). The eight samples were produced in Benettutti and Nulvi, in the central northern part of Sardinia (latitude of 40−41°), between November 1998 and July 2000. The production area was located in a mountain district at ∼400 m asl with Mediterranean scrub as the main flora. The soil derives from intrusive granite rock. The fertility and the nitrogen content of the ground are low. As is the case of Pecorino Sardo, the animals pasture and the diet could be mixed with fodder, oats, and maize. The cheese is not produced in summer.

Table 1. Results Obtained by the Different Laboratories for the Isotopic Measurements of Common Samples of Pure Casein and Glycerol (Number of Repetitions in Parentheses) and of a Common Sample of Cheese

	pure casein				pure glycerol				casein from cheese		glycerol from cheese	
lab	δ^{13}C [‰]$_{V-PDB}$	SD	δ^{15}N [‰]$_{AIR}$	SD	δ^{13}C [‰]$_{V-PDB}$	SD	δ^{18}O [‰]$_{V-SMOW}$	SD	δ^{13}C [‰]$_{V-PDB}$	δ^{15}N [‰]$_{AIR}$	δ^{13}C [‰]$_{V-PDB}$	δ^{18}O [‰]$_{V-SMOW}$
1	–23.3 (n = 11)	0.11	6.2 (n = 10)	0.12	–28.1 (n = 7)	0.08	27.2 (n = 7)	0.23	–18.0	5.5	–22.1	21.2
2	–23.5 (n = 23)	0.13	6.0 (n = 20)	0.23	–28.2 (n = 9)	0.15	22.6 (n = 9)	0.96	–18.1	5.4	–21.9	16.9
3	–23.4 (n = 16)	0.07	6.4 (n = 19)	0.41	–28.1 (n = 8)	0.09	24.1 (n = 17)	0.79	–18.4	5.7	–21.8	17.8
mean	23.40		6.20		–28.16		24.60		–18.17	5.53	–21.93	18.63
SD	0.10		0.20		0.06		2.35		0.21	0.15	0.15	2.27

Spanish Cheeses. (a) Manchego (Sheep PDO Cheese). The 12 samples were obtained in the region of La Mancha, situated in the center of Spain (latitude of 39−40°), between November 1998 and December 2000. This region is located in the South Peninsular subplateau and is a relatively high plane, set on calcareous-clay soil from the Miocene. The pasture is formed by substrates rich in limestone. The region has a dry continental climate with great oscillation of temperatures between day and night and between summer and winter. In summer temperatures often reach 40 °C, whereas in winter subzero values are not exceptional. The amount of rainfall is very low, and the atmosphere is dry, with a relative humidity of ∼65%. The animal diet included ears left after harvesting cereals, stubble from leguminous plants with high protein contents such as chickpea and lentil, lupine, beet, and some feed supplement. Maize is allowed by the PDO regulation, but it is not a common feed.

(b) Quesuco de Liebana (Cow PDO Cheese). Ten samples were produced in Camaleño in southwestern Cantabria province (latitude of 43−44°), between November 1998 and October 2000. The region corresponds to a circle tectonic trough. The landscape is quite rugged. There are narrow and extended valleys and eroded terrains. The soil of the region has Palaeozoic slate in the lower part and Palaeozoic calcareous material in the upper part and conglomerates clay and sandstone. The region has a very mild climate, plenty of rainfall with an average temperature of 14.5 °C and average rainfall between 900 and 1200 mm. The cows were essentially fed on grass, hay, and some feed supplement. Maize is allowed by the PDO regulation, but it is not a common feed.

Drinking Water and Animal Feed. In each sampling location samples of feed water and animal feed were collected to check the relationship between the isotopic values of the animal diet and those of the relevant cheese.

Methods. *Isolation of Glycerol from Cheese Lipids.* The method was adapted from the procedure described in Weber et al. (*24*).

The lipids were extracted with diethyl ether from 40−50 g of freeze-dried ground cheese, using a Soxhlet device (8 h of extraction) or a homogenizer (Ultraturrax equipment). After organic phase evaporation, 15−20 g of the resulting lipids was saponified at 60 °C in 150 mL of 2 N NaOH for 2 h. The solution was then acidified with HCl, and the fatty acids were removed by repeated extractions with diethyl ether/petroleum ether 1:1 (v/v). The aqueous phase was concentrated by rotary evaporation under vacuum and the dried residue suspended in ethanol. The mixture was filtered to eliminate NaCl. Ethanol was removed from the filtrate by rotary evaporation to obtain crude glycerol. It was then purified by distillation under reduced pressure using a microdistillator (e.g., Büchi B-580), which avoids any isotopic fractionation by ensuring a very high yield of distillation.

Isolation of Casein (10). After the lipid extraction, the skimmed cheese was warmed to 40 °C to remove any possible residual ether and then resuspended in 50 mL of water. The pH was then adjusted to 4.3 with HCl to completely precipitate the casein. After centrifugation, the casein fraction was collected, washed with water, and centrifuged again. The resulting casein was freeze-dried to obtain a fine white powder.

Isotopic Measurements. (a) IRMS Measurements. δ^{13}C (glycerol, casein, animal feed), δ^{15}N (casein, animal feed), δ^{18}O (glycerol, feed water), and δ^{34}S (casein) were measured by isotopic ratio mass spectrometry (IRMS) against an international standard [Vienna-Pee Dee Belemnite (V-PDB) for δ^{13}C, air nitrogen (AIR) for δ^{15}N, Vienna-Standard Mean Ocean Water (V-SMOW) for δ^{18}O, and Canyon Diablo Triolite (CDT) for δ^{34}S] according to the following formula:

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

where R represents the ratio between the heavy and light isotopes.

δ^{13}C, δ^{15}N, and δ^{18}O measurements of French, Italian, and Spanish products were performed at Eurofins Scientific, Istituto Agrario of San Michele all'Adige, and Laboratorio Arbitral Agroalimentario of Madrid (Ministerio de Agricultura, Pesca y Alimentacion), respectively. δ^{34}S analysis was carried out at the Department of Environmental Research at the Austrian Research Centre of Seibersdorf.

For the determination of carbon and nitrogen isotope ratios, the samples were placed in tin containers and submitted to a flash combustion in an elemental analyzer coupled to a mass spectrometer.

The δ^{18}O value of feed water was measured after equilibration for 5 h with standard CO_2 (*25*).

The oxygen isotope ratio of glycerol was determined by on-line pyrolysis of the samples. The glycerol was carefully dried in a desiccator over phosphorus pentoxide before the analysis and rapidly introduced into silver capsules, which were thoroughly sealed to avoid water contamination. The capsules, stored in the desiccator until their allocation into the autosampler, were dropped into the elemental analyzer, where the pyrolysis took place under a helium flow at a temperature of 1060 or 1170 °C over nickelized carbon or glassy carbon, depending on the laboratory. CO was separated from the other pyrolysis gases through a GC column packed with molecular sieves.

Determination of δ^{34}S was carried out on methionine separated from hydrolyzed casein according to the published protocols (*7*, *26*).

(b) Precision of the Measurements. (1) Repeatability. The extraction of casein followed by $^{13}C/^{12}C$ and $^{15}N/^{14}N$ measurement was repeated 4, 5, and 10 times in the three laboratories in order to determine the overall repeatability. The standard deviations obtained were 0.3‰ and 0.1‰ for δ^{13}C and δ^{15}N, respectively, in two of the laboratories and 0.1‰ for both parameters in the third one. Glycerol was extracted from the same cheese samples and measured seven times, and the standard deviations were 0.2‰ and 0.5‰ for δ^{13}C and δ^{18}O, respectively.

With regard to δ^{34}S measurement of casein, an organic sample was analyzed 10 times and a standard deviation of 0.3‰ was found.

(2) Reproducibility. As the sample preparation and analysis were carried out in different laboratories, it was important to check the reproducibility of the measurements to be sure that results were comparable. To this end, common samples of pure casein and glycerol were analyzed, and one cheese sample was extracted and analyzed by the three laboratories involved in δ^{13}C, δ^{15}N, and δ^{18}O IRMS measurements (**Table 1**). Regarding the measurements of pure samples, the interlaboratory standard deviation was 0.1‰ for δ^{13}C and 0.2‰ for δ^{15}N (**Table 1**), corresponding to a reproducibility ($R = 2 \cdot SD \cdot \sqrt{2}$) of 0.3‰ and 0.6‰, respectively. The reproducibility was therefore very good for carbon and nitrogen isotopic ratios. With regard to oxygen isotopic ratio, the standard deviations obtained in each laboratory were 0.2‰, 1.0‰, and 0.8‰, respectively, and the interlaboratory standard deviation was 2.3‰. Therefore, the reproducibility was better for δ^{13}C and δ^{15}N measurements than for δ^{18}O. It is important to note here that δ^{18}O measurement by on-line pyrolysis is a relatively new technique compared to the measurements of δ^{15}N and δ^{13}C by combustion, and

it is still being improved. The measurement protocol is not standardized and different instruments and pyrolysis catalysts were used in these laboratories. Unsatisfactory reproducibility, when using on-line $\delta^{18}O$ measurements, has been observed for other organic compounds such as sugar, as reported in the framework of the European Project SMT4-CT98-2219, which dealt with the determination of $\delta^{18}O$ in fruit juice sugars. In addition, the hygroscopic nature of glycerol makes the measurement very sensitive to water contamination. To carry out a multivariate discriminant analysis, a systematic correction on the $\delta^{18}O$ values was applied. The correction was calculated from the values obtained with the reference glycerol sample: 27.2‰ (obtained by laboratory 1) was taken arbitrarily as the true value, and 4.5‰ and 3‰ were therefore added to the values obtained by the other two laboratories (where the values obtained for the reference glycerol were 22.6‰ and 24.1‰, see **Table 1**). The interlaboratory standard deviations obtained for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ measured on the relevant components after extraction from the cheese sample (**Table 1**) were similar to those obtained for the measurement of pure casein and glycerol. Moreover, the systematic correction applied to the $\delta^{18}O$ values (additions of 4.5‰ for laboratory 2 and 3‰ for laboratory 3) fits well with the results. This shows that the extraction procedure in the different laboratories does not add uncertainty to the isotopic measurements of cheese components.

Statistical Analysis. For each variable of each cheese product, descriptive statistics were computed [mean, standard deviation (SD), minimum, and maximum].

The existence of differences was verified through representation of variables in box−whisker diagrams (*centerpoint* = median, *box* = 25−75° percentile, *whisker* = minimun nonoutlier − maximum nonoutlier, *symbols out of the box-whisker* = outliers and extreme values) or through analysis of variance and subsequent means comparison by Unequal *N* Tukey HSD (Honestly Significantly Different) test at a confidence level of 95%.

The existence of statistically significant correlation among variables was checked by computing the coefficients r and r^2 and verifying their significance by a t test for the number of data points used.

To assess the discrimination efficiency for cheese origin, a multivariate analysis of the data was carried out by discriminant analysis, which maximized the differences between the groups by means of a linear combination of the variables. The selection of the most significant isotopic ratios was performed by forward stepwise analysis (F to enter = 5; T = 0.01; number of steps = 5): the variables were included in the model one by one, choosing at each step the variable that made the most significant additional contribution to the discrimination (with the largest F value). The variable was excluded from the model if it was shown to be redundant ($T < 0.01$). On the basis of the selected variables, different independent discriminant functions (RAD) were computed by canonical discriminant analysis. Their maximum number is equal to either the number of variables or the number of groups minus one, if the latter is smaller. The statistical significance of each discriminant function was evaluated on the basis of the *Wilks' Lambda factor* after the function was removed. This parameter ranges from 1.0 (no discriminatory power) to 0.0 (perfect discriminatory power). The separation among groups in the discriminant space was checked by plotting the first and the second two RAD. Finally, to verify the power and the stability of the model, a classification discriminant analysis was applied, looking at the classification matrix. Known samples were used as unknowns to validate the model built on the basis of a reduced set of cases. In detail, different sets of cheese randomly selected (one or two samples for each group) were removed from the data, the model was calculated again, and the excluded cheeses were introduced into the statistical treatment as unknowns (*27*). The system is stable and could be applied in the future to commercial cases if the unknown samples are correctly classified.

The data were processed by means of the statistical software package Statistica for Windows, version 6.1.144.0, StatSoft Italia S.r.l.

RESULTS AND DISCUSSION

Isotopic Results of Feed Water ($\delta^{18}O$) and Animal Feed ($\delta^{13}C$ and $\delta^{15}N$). As the stable isotopic ratios of animal products depend on the nature of the feeding regime (*6*, *7*, *9*), samples of feed water and animal feed corresponding to the quoted cheese have been analyzed. Consistent with the literature (*21*), the $\delta^{18}O$ values of tap water (**Figure 2**) roughly vary according to latitude even if other geoclimatic parameters appear also to have a significant influence, such as the altitude for Trentino and Haute Savoie or the closeness to the sea for the island of Sardinia and for Normandy.

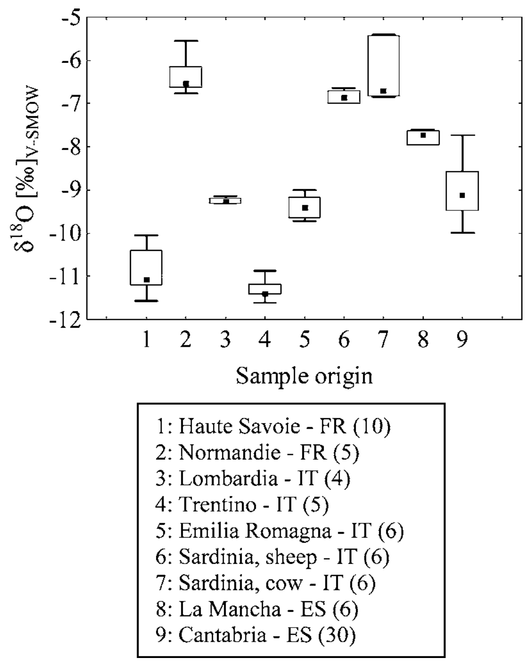

Figure 2. Box–whisker diagrams of $\delta^{18}O$ values of feed water. The numbers along the abscissa indicate the origin of the samples. The corresponding area is given in the legend (number of samples in parentheses).

Figure 3 shows the plot of $\delta^{13}C$ versus $\delta^{15}N$ values for some of the different constituents of the animal diet. Except for the feeding regime in Veneto (Grana Padano) and Emilia Romagna (Parmigiano Reggiano), which is a mixture of hay and supplement, the proportion of each fraction in the diet is not known exactly, and therefore these values do not represent the real values of the total feed intake but give, however, some information about it. Pure maize—a C_4 plant—has a $\delta^{13}C$ around −12‰ (see the samples of pure maize silage from Normandy and Sardinia), whereas the $\delta^{13}C$ value of pure hay and grass lies in the range between about −26 and −30‰ independent of geographical origin. Consequently, according to the percentage of maize content, animal feed is more or less depleted in ^{13}C. For example, the supplement given in Trentino contains about 30% of maize and exhibits a mean $\delta^{13}C$ value of −19‰, intermediate between those of pure grass and pure maize. The fact that the Cantabria supplement is more depleted in ^{13}C suggests that the maize proportion is lower than 30%. It results therefore that this isotopic parameter is more dependent on the botanical origin of the products (C_3 or C_4 plants) than on the geographical provenance. In contrast, it appears that the $\delta^{15}N$ values of hay and grass are more influenced by the location. The lowest values (about 0‰) were obtained for hay given in Trentino, Cantabria, and Haute Savoie (mountain areas), whereas the highest ones were obtained from Sardinia and la Mancha. The depletion in ^{15}N could be due to the presence of leguminous plants and the absence of intensive agricultural practices, whereas the dry and hot climate in Sardinia and La Mancha

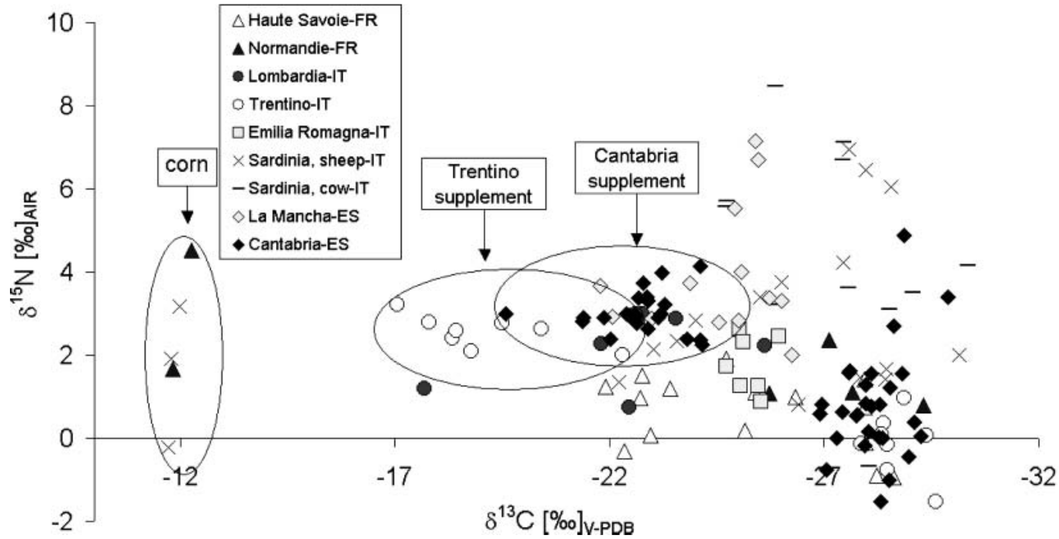

Figure 3. Animal feed: distribution of δ^{15}N versus δ^{13}C on total material.

could be the cause of the higher δ^{15}N values obtained for these areas.

Isotopic Results of Cheeses. Table 2 summarizes mean, standard deviation, minimum, and maximum obtained for δ^{13}C, δ^{15}N, and δ^{34}S of casein and for δ^{13}C and δ^{18}O of glycerol, expressed in δ‰. Moreover, the results of pairwise comparisons among means by Unequal *N* Tukey HSD test at a confidence level of 95% are shown.

δ^{13}C of Casein and Glycerol. As expected, an enrichment in ^{13}C relative to diet is observed for casein: comparing the mean δ^{13}C value of animal feed (mixture of hay and supplement) with the relevant mean value of casein for Grana Padano and Parmigiano Reggiano highlights an increase of about 3‰ (for the other cheeses, this comparison is not as clearly defined due to the unclear contribution of grass/hay or supplement to the diet). This enrichment is very similar to that observed in milk casein (*8*).

It appears that δ^{13}C values of casein and glycerol vary quite similarly according to the feeding regimen of the animal (**Table 2**). Where the animal-feeding rules allow large amounts of maize silage, as for Camembert and Grana Padano, the δ^{13}C values of casein and glycerol are significantly higher. On the other hand, cheeses related to an animal diet based on hay and grass (Reblochon, Sardinia cow cheese, and Quesuco) present significantly lower ^{13}C content, whereas Grana Trentino, Parmigiano Reggiano, and Manchego show intermediate values, due to a reduced maize content in the animal feed. In addition, for a given cheese, the variation of the δ^{13}C value throughout the year indicates a possible change of feeding regime. δ^{13}C mean values and standard deviation of cheese casein for summer and winter samples are summarized in **Table 3**. Camembert show the largest significant seasonal effects, the winter products presenting higher values than the summer ones. This is due to the fact that in winter the proportion of maize silage in the feed is greater because grass is less available than in summer. In contrast, products such as Grana Padano, Grana Trentino, and Parmigiano Reggiano (their values are not quoted because the production dates are not exactly known), for which it is known that the feeding regimen does not change throughout the year, do not show any seasonal variability (in fact, they present the narrowest ranges of δ^{13}C during the 2 years of study).

The mean casein δ^{13}C value of Pecorino Sardo (−23.2‰) is significantly higher ($p = 0.05$) in comparison to the ones reported in the literature (*10*, *11*). This variation could be due to the different climatic conditions of the years being studied: with a very arid climate, lack of food can occur and the addition of maize in the feed can become necessary throughout the year. Moreover, such extreme climatic conditions can lead to an increase in the ^{13}C content of plants (*28*).

The mean difference between glycerol and casein δ^{13}C values is 5‰, the casein fraction being always enriched in ^{13}C compared to glycerol. This is in agreement with earlier observations (*9*, *12*) and might be due to the depletion in ^{13}C occurring during the synthesis of glycerol, as it occurs in plants (*24*). This difference changes according to the type of cheese: it is lower for Camembert (4.19‰), Pecorino Sardo (3.76‰), and Manchego (3.98‰), that is, cheeses relating to feeding regimen based on C_4 plants, and higher for Reblochon (6.11‰) and Sardinia cow cheese (6.63‰). A possible explanation could be that the C_3 plants give a higher difference between casein and glycerol compared to the C_4 ones. Nevertheless, the δ^{13}C values of casein and glycerol are significantly correlated ($r = 0.94$, $r^2 = 0.88$; $t = 26.30$; $N = 95$), consistent with the literature (*12*).

In conclusion, the δ^{13}C values permit the respective cheeses to be distinguished on the basis of the relevant animal feeding regimen.

δ^{15}N Values of Casein. Similarly to the δ^{13}C parameter, an increase in δ^{15}N of about 3‰ is noticeable between the feed of cattle producing milk used for the production of Grana Padano and Parmigiano Reggiano and the relevant cheese casein. This value is consistent with previous observations on milk (*29*). In the case of both Italy and Spain, δ^{15}N values of casein distinguish the products of the southern area from those of the north (**Table 2**). Sardinia cow cheese and Manchego, followed by Pecorino Sardo, show the highest values, probably due to the dry climate of both locations (*18*). In France, the Savoie cheese (Reblochon), produced in a cooler mountain area characterized by extensive agricultural practices, presents the significantly lowest ^{15}N content. A rather low content of ^{15}N in casein fraction was observed also in Emmental cheeses from Savoie (*12*) and in mountain cheeses from northern Italy (*16*).

No significant seasonal differences are observed for this isotopic parameter (**Table 3**) except for Sardinia cow cheese.

We can conclude from these observations that δ^{15}N of cheese casein is strongly linked to the geoclimatic origin of the cheese.

δ^{18}O Values of Glycerol. As explained under Materials and Methods, the interlaboratory reproducibility achieved up to now

Table 2. Isotopic Results of Cheeses (Mean, Standard Deviation, Minimum, and Maximum) and Results of HSD Unequal *N* Tukey's Test ($p = 0.05$)[a]

	$\delta^{13}C$ [‰]$_{V-PDB}$ casein	$\delta^{13}C$ [‰]$_{V-PDB}$ glycerol	$\delta^{15}N$ [‰]$_{AIR}$ casein	$\delta^{18}O$ [‰]$_{V-SMOW}$ glycerol[b]	$\delta^{34}S$ [‰]$_{CDT}$ casein
Reblochon (Haute-Savoie, France)					
no. of determinations	16	16	16	16	9
mean	−25.02 a	−31.13 a	3.79 a	19.92	2.99 a
SD	1.09	1.23	0.60	2.21	0.99
min	−26.7	−32.9	2.8	16.4	1.5
max	−23.5	−29.5	4.7	23.1	4.1
Camembert (Normandy, France)					
no. of determinations	10	10	10	10	5
mean	−19.70 d	−23.89 c	5.08 bc	22.62	4.58 bc
SD	1.99	2.62	0.51	1.04	0.61
min	−22.3	−27.7	4.3	20.7	3.8
max	−16.9	−20.4	5.9	24.1	5.3
Grana Padano (Lombardia, Italy)					
no. of determinations	13	13	13	12	10
mean	−19.62 d	−24.50 c	5.22 cd	15.52	3.42 ab
SD	0.77	1.08	0.45	0.99	0.53
min	−20.9	−25.9	4.5	14.1	2.7
max	−18.1	−21.9	6.0	17.0	4.5
Grana Trentino (Trentino, Italy)					
no. of determinations	12	12	12	12	9
mean	−22.46 bc	−27.98 b	4.48 b	13.35	3.68 abc
SD	0.48	0.93	0.27	0.84	0.65
min	−23.1	−29.3	4.0	12.3	2.8
max	−21.7	−26.2	5.0	15.2	4.9
Parmigiano Reggiano (Emilia Romagna, Italy)					
no. of determinations	13	11	13	11	10
mean	−21.78 c	−26.61 b	5.15 bcd	14.53	3.12 a
SD	0.89	1.07	0.59	1.13	0.64
min	−23.6	−28.1	3.7	12.4	2.1
max	−20.8	−25.1	6.0	15.3	4.0
Pecorino Sardo (Sardinia, Italy)					
no. of determinations	8	7	8	7	8
mean	−23.20 bc	−26.97 b	5.97 d	19.62	7.68 d
SD	1.62	1.78	0.54	2.05	1.08
min	−26.9	−30.7	5.5	17.2	6.3
max	−21.3	−24.9	6.8	22.4	9.8
Sardinia cow cheese (Sardinia, Italy)					
no. of determinations	8	6	8	6	8
mean	−26.07 a	−32.70 a	6.87 e	19.76	8.81 d
SD	1.02	1.33	0.72	1.40	1.13
min	−27.1	−34.0	5.5	18.3	6.4
max	−24.1	−30.8	7.7	21.8	10.0
Manchego (La Mancha, Spain)					
no. of determinations	12	10	12	12	7
mean	−23.54 b	−27.58 b	7.52 e	19.89	7.83 d
SD	0.91	0.99	0.59	2.30	0.69
min	−25.0	−29.0	6.9	14.3	6.4
max	−22.1	−25.5	8.9	22.6	8.3
Quesuco (Cantabria, Spain)					
no. of determinations	10	10	10	10	6
mean	−25.47 a	−30.38 a	5.06 bc	18.34	4.95 c
SD	1.09	1.61	0.38	1.60	0.44
min	−26.9	−32.0	4.6	15.0	4.3
max	−24.4	−27.1	5.9	20.1	5.5

[a] Means with the same letter in the same column are not significantly different ($p = 0.05$). [b] Original values without correction.

for this analysis is not satisfactory, probably due to systematic errors, and therefore no general considerations could be established.

When the $\delta^{18}O$ values of feed water are compared with those of cheese glycerol, remarkable enrichments of 25.4‰, 27.5‰, and 29.9‰ appear for Italian, Spanish, and French products, respectively. This enrichment was also observed in glycerol samples from vegetable products and from animal and milk fat (*22*, *30*). It is due to the isotopic effects occurring in the metabolic pathways involved in the biosynthesis of carbohydrates, the biochemical precursors of glycerol (*22*). The different increments observed for the different countries are comparable to the intralaboratory shifts observed in the reproducibilty test, confirming the presence of systematic errors for this measurement.

Considering separately the cheeses of the different countries, the trend observed is very similar to that described for $\delta^{18}O$ of feed water: $\delta^{18}O$ values of glycerol changed according to the latitude, altitude, and climatic conditions (**Table 2**). Among the Italian products, Grana Trentino (highest altitude and latitude)

Table 3. Mean Values and Standard Deviations of δ^{13}C, δ^{15}N, and δ^{34}S of Casein and of δ^{18}O of Glycerol for Summer and Winter Samples (Number of Samples in Parentheses) and Results of HSD Unequal *N* Tukey's Test ($p = 0.05$)[a]

	Reblochon, France		Camembert, France		Pecorino, Italy		Sardinia cow, Italy		Manchego, Spain		Quesuco, Spain	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
δ^{13}C [‰]$_{V-PDB}$	winter (6)		winter (6)		winter (4)		winter (5)		winter (8)		winter (6)	
casein	−24.16 a	0.51	−18.32 a	1.05	−22.36 a	0.76	−26.21 a	1.23	−23.81 a	0.92	−25.62 a	1.28
	summer (10)		summer (4)		summer (4)		summer (3)		summer (4)		summer (4)	
	−25.53 b	1.03	−21.78 b	0.69	−24.05 a	1.91	−25.75 a	0.70	−23.01a	0.71	−25.23 a	0.84
δ^{15}N [‰]$_{AIR}$	winter (6)		winter (6)		winter (4)		winter (5)		winter (8)		winter (6)	
casein	4.01 a	0.69	4.89 a	0.49	5.89 a	0.60	7.28 a	0.43	7.38 a	0.65	5.25 a	0.38
	summer (10)		summer (4)		summer (4)		summer (3)		summer (4)		summer (4)	
	3.65 a	0.52	5.38 a	0.43	6.05 a	0.55	6.19 b	0.59	7.80 a	0.38	4.78 a	0.16
δ^{18}O [‰]$_{V-SMOW}$	winter (6)		winter (6)		winter (4)		winter (4)		winter (8)		winter (6)	
glycerol	17.37 a	0.76	22.28 a	1.22	19.32 a	2.26	19.23 a	1.22	19.31 a	2.57	18.53 a	1.1
	summer (10)		summer (4)		summer (3)		summer (2)		summer (4)		summer (4)	
	21.46 b	0.92	23.11 a	0.43	20.02 a	2.21	20.82 a		21.06 a	1.15	18.06 a	2.35
δ^{34}S [‰]CDT	winter (4)		winter (3)		winter (4)		winter (5)		winter (5)		winter (4)	
casein	3.85 a	0.33	4.40 a	0.66	6.90 a	0.43	9.10 a	0.57	7.70 a	0.79	4.73 a	0.33
	summer (5)		summer (2)		summer (4)		summer (3)		summer (2)		summer (2)	
	2.30 b	0.73	4.85 a		8.45 b	0.97	8.33 a	1.81	8.15 a		5.40 a	

[a] Means with the same letter in the same column are not significantly different ($p = 0.05$).

shows the lowest values (significantly lower than the other Italian cheeses, except for Parmigiano Reggiano) and Grana Padano and Parmigiano Reggiano intermediate ones, whereas the highest ones ($p = 0.05$) were obtained for the Sardinian cheeses (lowest latitude, closeness to the sea, arid climate). The glycerol from Reblochon (French Alps) has a significantly low ^{18}O content ($p = 0.05$) compared to Camembert, probably due to the higher altitude and to the colder climate of this location. A not significant difference ($p = 0.05$) is observed between the two Spanish products, even if the highest values are obtained for the cheese originating from the location with the driest and hottest climate (Manchego).

As observed for δ^{18}O of milk water (*6*), δ^{18}O of glycerol seems to depend, even if with less variation, on seasonal factors, especially when the animal-feeding regimen changes during the year from dry to fresh plants. In fact, due to evapotranspiration phenomena, the water of fresh plants is enriched in ^{18}O (*6*). δ^{18}O glycerol mean values and standard deviation are summarized in **Table 3** for summer and winter samples except for Grana Padano, Parmigiano Reggiano, and Grana Trentino (see above). Reblochon shows the greatest seasonal effect.

All of these results confirm the importance of the δ^{18}O parameter for origin authentication.

δ^{34}S Values of Casein. This measurement was performed on a reduced number of samples (**Table 2**) and was carried at the Department of Environmental Research at the ARC Seibersdorf research GmbH.

Factors such as climatic conditions and closeness to the sea (seaspray sulfur) appear to highly influence the δ^{34}S values of casein of the quoted cheeses (**Table 2**). Similarly to δ^{15}N, samples from warm regions, especially if they are close to the sea (e.g., Sardinia products and Quesuco), show the significantly highest ^{34}S content, whereas products from mountain areas remote from the ocean, such as Reblochon, exhibit the lowest δ^{34}S mean value.

Rossmann et al. (*7*) reported that δ^{15}N and δ^{34}S values of soil compounds (and therefore of plant and animal products) should be correlated, because their isotopic fractionation is influenced by similar factors (fertilization procedure, mineralization and demineralization reactions, leaching, climatic conditions, and closeness to the sea). However, they did not observe this trend for milk (*7*), and they explained that the soil geology might influence more importantly the ^{34}S content. In our work, a significant correlation ($r = 0.77$; $r^2 = 0.59$; $t = 10.12$; $N = 72$) was obtained for relating δ^{15}N and δ^{34}S values (**Figure 4**). The geology influence on this parameter is not discussed here because a more detailed investigation and knowledge about the geological situation of the relevant sampling locations would have been necessary.

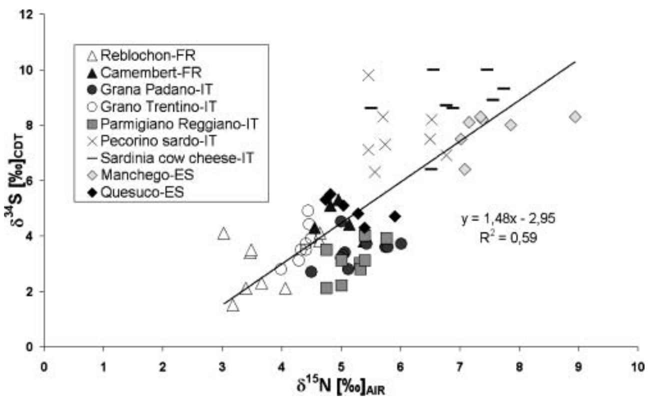

Figure 4. Correlation between δ^{15}N and δ^{34}S values of casein.

However, with regard to the existence of seasonal variability, Reblochon and Sardinia cow cheese show statistically significant differences with opposite trend.

In conclusion, δ^{34}S values of cheese casein for the considered cheeses are correlated with δ^{15}N, as they are influenced by the same variability factors.

Geographical Discrimination of the Cheeses by Multivariate Data Treatment. To evaluate the real efficiency of discrimination among the different types of cheeses, based on the multi-isotopic analysis, a multivariate discriminant analysis was applied. As described under Materials and Methods, the δ^{18}O results were corrected versus an arbitrary value due to a systematic error among laboratories. A reduced set of samples (total number = 66; see **Table 4** for details on the sample set) was used for this approach, because cases with missing data were ignored.

Before any multivariate treatment was carried out, the distribution of the variables and their intercorrelations were checked, considering both box−whisker plots and histograms

Table 4. Results of Classification Matrix (66 Samples)[a]

cheese and origin	%	Reblochon	Camembert	Grana Padano	Grano Trentino	Parmigiano Reggiano	Pecorino	Sardinia cow	Manchego	Quesuco
Reblochon, France (9)[b]	100	9	0	0	0	0	0	0	0	0
Camembert, France (5)	100	0	5	0	0	0	0	0	0	0
Grana Padano, Italy (9)	78	0	0	7	0	2	0	0	0	0
Grana Trentino, Italy (9)	100	0	0	0	9	0	0	0	0	0
Parmigiano Reggiano, Italy (8)	88	0	0	0	1	7	0	0	0	0
Pecorino, Italy (7)	100	0	0	0	0	0	7	0	0	0
Sardinia cow, Italy (6)	100	0	0	0	0	0	0	6	0	0
Manchego, Spain (7)	100	0	0	0	0	0	0	0	7	0
Quesuco, Spain (6)	100	0	0	0	0	0	0	0	0	6

[a] Mean classification error = 5%. [b] The number of samples for each cheese type employed in discriminant analysis is given in parentheses.

Table 5. Results of Classification Matrix (102 Samples)[a]

cheese and origin	%	Reblochon	Camembert	Grana Padano	Grana Trentino	Parmigiano Reggiano	Pecorino	Sardinia cow	Manchego	Quesuco
Reblochon, France (16)[b]	88	14	0	0	0	0	0	0	0	2
Camembert, France (10)	100	0	10	0	0	0	0	0	0	0
Grana Padano, Italy (13)	85	0	0	11	0	2	0	0	0	0
Grana Trentino, Italy (12)	100	0	0	0	12	0	0	0	0	0
Parmigiano Reggiano, Italy (13)	92	0	0	0	1	12	0	0	0	0
Pecorino, Italy (8)	88	0	0	0	0	0	7	1	0	0
Sardinia cow, Italy (8)	88	0	0	0	0	0	1	7	0	0
Manchego, Spain (12)	100	0	0	0	0	0	0	0	12	0
Quesuco, Spain (10)	100	0	0	0	0	0	0	0	0	10

[a] Mean classification error = 7%. [b] The number of samples for each cheese type employed in discriminant analysis is given in parentheses.

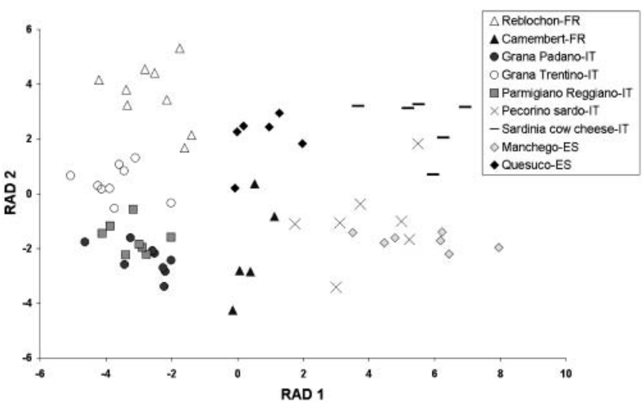

Figure 5. Scatterplot of the first two canonical variables.

of frequency distribution. We concluded that the considered variables were basically normally distributed within most of the groups.

The forward stepwise discriminant analysis (F to enter = 5; T = 0.01; N steps = 5) pointed out that all of the variables contributed significantly to the discrimination, the most significant variable being δ^{34}S, followed by δ^{13}C and δ^{18}O of glycerol and by δ^{15}N and δ^{13}C of casein.

By applying canonical discriminant analysis, five different independent discriminant functions (RAD) were computed by linear combination of the isotopic variables. The first four discriminant functions were statistically significant for the discriminantion (Wilks' lambda < 0.3), principally the first three (Wilks' lambda < 0.08).

The combination of the first two canonical variables RAD1 (60%) and RAD2 (22%) accounted for 82% of variability (scores plot shown in **Figure 5**). The first canonical axis (RAD1), loaded with all of the parameters (mostly with δ^{34}S and δ^{13}C of casein), separated Manchego (Spain), Sardinia cow cheese (IT), and most Pecorino samples (Italy) from Quesuco (Spain) and Camembert (France) and from the other cheeses. The second one (RAD2), mainly determined by δ^{13}C of glycerol, improved the discrimination between Sardinia cow cheese (Italy), Manchego (Spain), and Pecorino (Italy), between Camembert (France) and Quesuco (Spain), between Reblochon (France) and Grana Trentino (Italy), and between Grana Padano (Italy) and Parmigiano Reggiano (Italy). The third axis (RAD3), which accounts for 11% of the total variability, was mainly loaded with δ^{18}O of glycerol and improved the separation between Sardinia cow cheese (Italy) and Manchego (Spain) with Pecorino (Italy), whereas RAD4 (5% of the total variability, determined by δ^{13}C of casein and glycerol) increases the separation between Grana Padano (Italy) and Parmigiano Reggiano (Italy).

The good separation of the different groups demonstrated by the canonical discriminant analysis was confirmed by the reclassification discriminant analysis, where about 95% of the 66 samples were correctly reclassified with 100% correct reclassification for all of the cheeses except Grana Padano and Parmigiano Reggiano (**Table 4**).

To check the predictive discrimination power and the stability of the model, some of the analyzed samples were used as unknowns to validate the model built on the basis of the remaining cases. In detail, three different sets of cheese randomly selected (one sample for Camembert and two for the other cheeses) were removed from the data, and the model was calculated on the remaining 49 cases and was validated with all 66 samples (including the excluded cheeses).

In all analyses, all of the samples were correctly classified except the three samples (two Grana Padano and one Parmigiano Reggiano) misclassified also above, two samples of Reblochon classified as Quesuco, and one of Pecorino classified as Manchego. However, the mean classification error never exceeded 9%, confirming that the system is quite stable.

To apply the multivariate discriminant analysis to a larger set of samples, missing values were substituted with the mean values of the relevant group and the statistical procedure described above was carried out (total number of samples = 102). Very similar results and considerations were observed for either stepwise or canonical or classification discriminant analyses. The classification matrix is shown in **Table 5**. The mean classification error was 7%. When the model was validated using known samples as unknown (see above), it increased to 10%. Besides the samples misclassified in classification analysis (**Table 5**), one sample of Reblochon was classified as Grana Trentino and two samples of Manchego were classified as Sardinia cow cheese.

Conclusions. The original results described in this work on different European cheeses confirm the high potential of multi-isotopic analyses for the characterization of not only vegetable products, as already largely demonstrated, but also animal products such as cheese. In the future, these analyses, complemented with other isotopic ratios such as D/H or $^{87}Sr/^{86}Sr$ of casein (*9*, *12*), could be efficient tools to fight against fraud, in particular, origin mislabeling and counterfeiting of PDO products. The detection of such frauds is essential to maintain a fair trade and honest competition between producers, as well as to preserve the confidence of consumers toward those products

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