Characterization of the Geographical Origin of Pecorino Sardo Cheese by Casein Stable Isotope ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) Ratios and Free Amino Acid Ratios

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The stable isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of casein measured by isotope ratio mass spectrometry (IRMS) and some free amino acid ratios (His/Pro, Ile/Pro, Met/Pro, and Thr/Pro) determined by HPLC in samples of ewes' milk cheese from Sardinia, Sicily, and Apulia were found to be parameters independent of ripening time. Multivariate data treatments performed by applying both unsupervised (principal component analysis and cluster analysis) and supervised [linear discriminant analysis (LDA)] methods revealed good discrimination possibilities for the cheeses according to place of origin. In this respect, particularly significant were the variables Ile/Pro, Thr/Pro, $^{13}C/^{12}C$, and $^{15}N/^{14}N$ ratios on which basis 100% discrimination and classification of the samples by LDA was obtained.

Keywords: *Ewes' milk cheese; free amino acid ratios; casein stable isotope ratios; geographical origin; statistical analysis*

INTRODUCTION

Pecorino Sardo is a cheese traditionally made from ewes' milk in Sardinia and protected under European Union rules (EC Regulation 2081/92—Denomination of Protected Origin). This designation guarantees that the quality of the product is closely linked to its geographical origin. However, at present no analytical parameters exist that enable the actual origin of the cheese to be verified and Pecorino Sardo to be distinguished from similar products from other regions. Reliable parameters for the identification of the place of origin would guarantee the authenticity of the cheese produced on the island with consequent economic benefits.

Research carried out on typical milk products has shown that it is possible to characterize a cheese according to its free amino acid content by creating mathematical−statistical models on the basis of which the period of production and/or its typical characteristics can be determined (*1*−*8*).

Free amino acids are essential for developing the aroma and taste of cheeses (*9*). They are formed by complex proteolytic phenomena during ripening, caused by a specific set of enzymes (*10*, *11*) that can determine a characteristic amino acid profile for each cheese (*12*, *13*). One example is Parmigiano Reggiano, which has a particular free amino acid composition when it reaches an optimal degree of ripening (*14*). In the case of Pecorino Sardo, also, it has been possible to identify the free amino acids and the ratios most closely related to the age of the cheese, using regression procedures (*15*).

Recent studies published in the literature have shown that determining the ratios of stable isotopes of bioelements in foodstuff components, widely used to check the genuineness and geographical origin of various products such as fruit juices (*16*, *17*), wines (*18*, *19*), and honey (*20*, *21*), can also be applied to milk products (*22*, *23*).

This analytical technique is based on the small but significantly different ratios of the stable isotopes of bioelements, mainly hydrogen (D/H), oxygen ($^{18}O/^{16}O$), carbon ($^{13}C/^{12}C$), and nitrogen ($^{15}N/^{14}N$), present in certain organic molecules, due to kinetic−chemical and physical factors that can be correlated with the botanical−metabolic and/or geographical origin of a product, respectively. In particular, the $^{13}C/^{12}C$ ratio in plant material depends on isotopic fractionation, which takes place in the biosynthetic reactions of organic compounds, in relation to the different photosynthetic cycles for CO_2 fixation by the plants, that is, the Calvin or C_3 cycle and the Hatch and Slack or C_4 cycle (*24*). The different range of isotopic values is also transmitted to the animals in their diet and consequently to their products (*25*), providing interesting information on the vegetable composition of the diet, particularly with regard to maize, a C_4 cycle plant. Differences in $^{18}O/^{16}O$ of the water of milk from alpine regions were observed, possibly seasonal, due to the different source of the animals' main supply of drinking water in winter and in summer, that is, ground or tap water or water in vegetation, respectively. On the other hand, no significant $^{15}N/^{14}N$ variation was found in the casein of the same milk samples but only between the milk produced in mountain and nonmountain areas, which could be due to a different input of nitrogen fertilizer and different climatic conditions or even different feed supplements, which can alter the $^{13}C/^{12}C$ values, as in the case of feeding with maize silage (*23*).

In fact, the $^{15}N/^{14}N$ ratio in the biomolecules depends on the ^{15}N content of the inorganic nitrogen present in the soil (the primary source of nitrogen for the plants).

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This in turn depends on the type of fertilizer used, because, for example, organic fertilizers and intensive farming methods increase the level of ^{15}N in the soil (*26*). Other factors include water stress (*27*) and the pedoclimatic conditions of the location (the characteristics of the soil, altitude, and humidity), which affect the biological turnover of the nitrogen, causing a higher or lower number of processes in the soil accompanied by significant isotopic fractionation such as mineralization, nitrification, nitrogen assimilation or denitrification, and leaching (*28*). The organic material of leguminous plants, which have a low ^{15}N content, is a case apart because these plants also utilize the nitrogen in the air by an enzymatic system of nitrogenase (*29*) and grow more abundantly in less fertilized soil.

As with carbon, the isotopic abundance of nitrogen in the compounds of animal products reflects the isotopic composition of the diet with an increase of ~3−5% (*30*) and can contribute to the identification of the geographical origin of products such as milk and cheese.

In light of the above considerations, a study was conducted on ewes' milk cheeses of different southern Italian geographical origins such as Sardinia, Sicily, and Apulia with the aim of determining whether variables such as casein $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios and some free amino acid ratios, which seem to be uninfluenced by the ripening period (*15*, *31*), can be used to identify the area of production, using multivariate statistical procedures [principal component analysis (PCA), linear discriminant analysis (LDA), and cluster analysis].

MATERIALS AND METHODS

Cheeses. The study was carried out on 40 samples of cheese collected from cheese factories; 27 were from Sardinia, 9 from Sicily, and 4 from Apulia.

The Sardinian cheeses, all Pecorino Sardo, came from different parts of the island, mostly in the north and central north. The Sicilian samples came from the province of Enna and the Apulia samples from the Foggia area. Pecorino Sardo is a half-cooked cheese made from heat-treated milk inoculated with a starter culture and coagulated with calf rennet. Pecorino Siciliano and Pecorino Pugliese are both made from raw milk without a starter culture and coagulated with lamb rennet. The samples, produced at different times between January and July 1996, were taken at different stages of ripening ranging from 30 to 360 days.

Reagents. Catalyzers and adsorbent substances supplied by Costech (Valencia, Spain) were used in the process of CO_2 and N_2 production for the analyses of the isotopic ratios. The primary standards were supplied by the International Atomic Energy Agency (IAEA), Vienna, Austria. Working standards were used in the analyses, measured against the international standards for $\delta^{13}C$ (commercial grade flour) and $\delta^{15}N$ (commercial grade powdered milk) in solids.

All of the solvents used in the extraction and purification of the casein and free amino acids were supplied by Carlo Erba (Milan, Italy).

Preparation of Samples To Determine Isotope Ratios. The $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic ratios of the cheeses were determined in the casein fraction obtained as follows: the cheese was grated, stored at −18 °C, and freeze-dried (Lyophilizer Edwards, West Sussex, U.K.). The fat content was removed by treating 6 g of the freeze-dried sample with 3 × 50 mL of diethyl ether. The suspension obtained was homogenized with Ultraturrax equipment (model X-620, Staufen, Germany) at 20500 rpm for 2 min and centrifuged at 2000 rpm for 4 min (centrifuge ALC 3229) to separate the ether fraction containing the fats. The ether residue in the casein fraction was eliminated in an oven for 1 h at 40 °C, and then the casein fraction was resuspended in 30 mL of distilled water that had been acidified to pH 4.3 with 5 M HCl, shaken for 30 min with a magnetic agitator, and then centrifuged at 2000 rpm for 4 min; the fluid was decanted and the residue washed again with 5 mL of water, decanted, and freeze-dried.

Measurement by Isotopic Ratio Mass Spectrometry (IRMS). Quantities of ~0.5 and ~1 mg of casein for the measurement of, respectively, $\delta^{13}C$ and $\delta^{15}N$ were weighed in tin containers and introduced by means of an autosampler into the elemental analyzer (Nitrogen Analyzer 1500, Carlo Erba Strumentazione, Rodano, Italy), where, in the presence of O_2 and CuO, it was burnt quantitatively to CO_2 and NO_x; the latter was then reduced to N_2 with copper. The formed gases were separated on a gas chromatographic column and analyzed in the isotopic mass spectrometer (Sira II-VG Fisons, Rodano, Italy).

The values of the isotopic ratios are expressed in δ ‰ and correspond to an international standard (V-PDB per $\delta^{13}C$, Air per $\delta^{15}N$) according to the following general formula:

$$\delta \text{‰} = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 1000$$

where R represents in general the ratio between the less abundant and more abundant isotopes, in particular $^{13}C/^{12}C$ and $^{15}N/^{14}N$.

On the basis of the repeatability of the measurements in such experimental conditions as shown in international ring tests, each sample was analyzed twice and the values averaged; the measurement was repeated if the deviation found was >0.2‰ for $\delta^{13}C$ and >0.3‰ for $\delta^{15}N$.

Determination of the Free Amino Acids. A previously described method (*15*) was used to extract and identify the amino acids in HPLC. The method includes extraction by citrate buffer at pH 2.2, treatment with trifluoroacetic acid, centrifugation, filtration through 0.22 μm membrane filters, and derivatization with phenylisothiocyanate.

The values of all the free amino acids quantified in samples of Pecorino Sardo were reported in the above-mentioned work.

Statistical Analyses. The multivariate analysis of the data was performed using the SPSS 8.0 package for Windows (SPSS Inc., Chicago, IL). The procedures were PCA as descriptive of the system, cluster analysis to show similarity among the objects and detect any subgroupings, and LDA for classification.

By PCA it is possible to reduce the multidimensionality of the system to a few functions such as linear combinations of the initial variables (eigenvectors) that synthesize the information contained in the complete data set. The cluster analysis was done by the average between groups method, using the Euclidean distances. The LDA was carried out to construct a model capable of identifying the classes.

RESULTS AND DISCUSSION

Table 1 shows the mean values and standard deviations of the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios of the casein of the cheese samples, expressed in δ ‰, and the free amino acid ratios: threonine/proline (Thr/Pro), isoleucine/proline (Ile/Pro), methionine/proline (Met/Pro), and histidine/proline (His/Pro), these last ratios chosen as possible variables independent of cheese ripening time.

With regard to the isotope values, the scatter plot (Figure 1) shows that those of $\delta^{13}C$ are independent of the place of origin of the samples. Apart from three samples, all are in the range of −26.5 to −24.5‰.

A comparison between the $\delta^{15}N$ values shows that the Apulian samples have values very close to those of the Sardinian samples and both tend to have higher values than the Sicilian samples.

Table 1. Mean and Standard Deviation of the Values of the Stable Isotope Ratios and Free Amino Acid Ratios in Ewes' Milk Cheeses

	δ^{13}C (‰) vs V-PDB	δ^{15}N (‰) vs air	Met/Pro	Thr/Pro	Ile/Pro	His/Pro
Pecorino Sardo (27 samples)						
mean	−25.70	6.29	0.38	0.26	0.40	0.39
SD	0.68	0.72	0.19	0.07	0.18	0.16
Pecorino Siciliano (9 samples)						
mean	−25.62	4.54	1.36	0.79	1.48	1.31
SD	0.88	0.59	0.79	0.35	0.62	0.58
Pecorino Pugliese (4 samples)						
mean	−26.04	5.97	1.02	0.43	1.25	0.60
SD	0.90	0.72	0.05	0.11	0.04	0.35

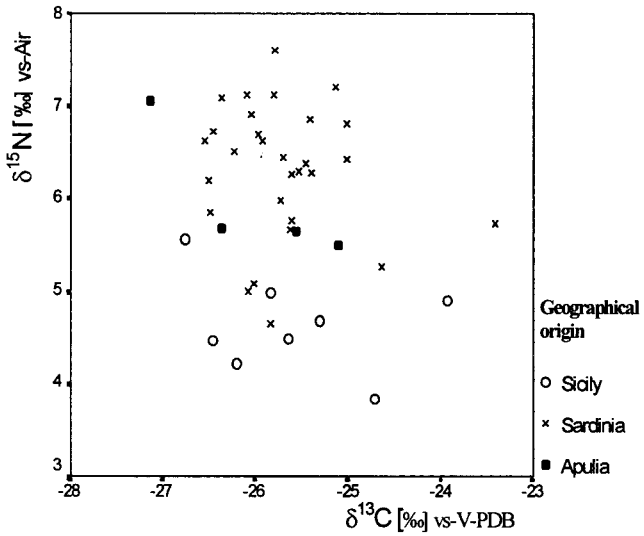

Figure 1. Score plot of δ^{15}N versus δ^{13}C in cheeses of different geographical origins.

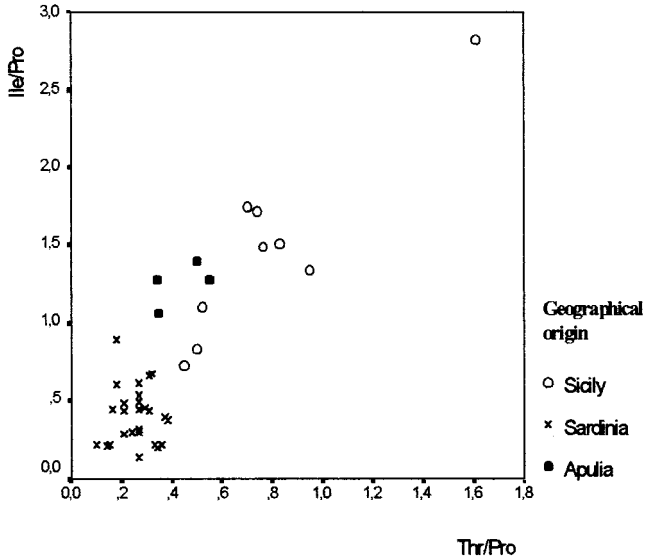

Figure 2. Score plot of Thr/Pro and Ile/Pro ratios in cheeses of different geographical origins.

In particular, the Sardinian samples were analyzed to determine whether the variability of the parameters could be influenced by geographical location (north/south) and/or the season of production. Statistical analysis based on regression lines was carried out to discover any correlation between the nitrogen isotope ratio and the location of the cheese factory. The regression line described by the equation $Y = aX + b$, where Y is the value of δ^{15}N and X is the meridian distance (in kilometers) of the cheese factories in relation to the parallel passing through the city of Cagliari, showed no correlation between the two variables.

The previously mentioned study (*15*) on the evolution of free amino acids during the ripening of Pecorino Sardo showed that most of the single amino acids, some of the ratios between them, and their total content are correlated with the time of ripening. An analysis of the free amino acid ratios revealed that some had a variability that was not correlated to the age of the cheese; indeed, no significant variations were found at different ripening stages. They were therefore tested as possible codescriptors of the typicality of Pecorino Sardo (Table 1).

The data in the scatter plots on the free amino acid ratios (Figure 2) indicate that the Thr/Pro and Ile/Pro ratios allow the Sardinian samples to be identified more clearly, whereas no distinction was found between the Sicilian and Apulian samples.

Because single isotope ratios and single ratios between free amino acids were alone not sufficient to provide a clear differentiation between samples of different geographical origins, such variables were submitted to multivariate statistical procedures.

Table 2. Variable Loadings on Different Components

	components		
variable	I	II	III
δ^{13}C	−0.074	0.956	0.285
δ^{15}N	−0.759	−0.429	0.489
Ile/Pro	0.904	−0.180	0.253
Thr/Pro	0.926	−0.099	0.176
eigenvalue	2.255	1.139	0.415
variance %	56.38	28.48	10.38
cumulative variance %	56.38	84.86	95.24

PCA, applied to a data set of 6 variables and 40 objects, demonstrated that the first three components were responsible for 94% of the total variance; the score plot on the first and second components showed a nonoptimal separation between the Sicilian and Apulian samples, whereas the Sardinian samples were totally differentiated by the first component.

An improved separation between the Apulian and Sicilian samples was possible by optimizing the data set by eliminating one outlier among the Sicilian samples because its amino acid ratios diverged remarkably from the mean. Moreover, the variable number was reduced after the elimination of those that were less correlated with the components. The new data set comprised 39 objects and 4 variables (δ^{13}C, δ^{15}N, Thr/Pro, and Ile/Pro). The new PCA showed how the first three components explained 95% of the total variance; the Thr/Pro and Ile/Pro ratios had a major weight on the first component, as did δ^{15}N with an opposite sign of association, whereas the δ^{13}C ratio had more influence on the second component (Table 2).

The score plot of the first and second principal

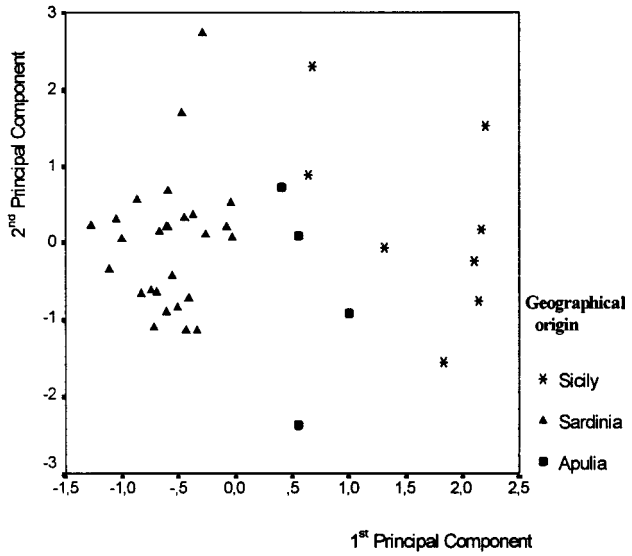

Figure 3. Score plot of the first and second components.

components (Figure 3) showed the presence of three distinct groups in which the separation of the Sardinian samples compared with the others was again due to the first component, with the highest loadings for the two amino acid ratios chosen and δ^{15}N. Both components were needed to differentiate the Sicilian samples from the Apulian samples.

At this point, the cluster analysis was applied to the same data set (39 objects and 4 variables), using the average linkage method (between groups) to optimize the formation of the groups through the hierarchy of similarity between the objects considered. The result is shown in the dendrogram in Figure 4, which presents a separation between the products from the three different regions. Taking as an arbitrary cutoff point a similarity level <5, four groups are identifiable, one Sardinian (one outlier is present), two Sicilian, and one Apulian, the greatest homogeneity, however, being among the Sardinian samples.

Finally, the PCA and the cluster analysis indicated the possibility of distinguishing among the regions. We then proceeded to separate the groups by LDA on the basis of the regional origin, using the data set that included 39 objects and 4 variables. The results showed two discriminant functions: in particular, the first function, correlated with the amino acid ratio (Ile/Pro and Thr/Pro), which explained 93.5% of the variance, allowed the groups to be differentiated according to geographic origin (Figure 5). On the other hand, the isotope ratios were correlated most with the second discriminant function.

The classification results can be seen in Table 3, where the number of samples correctly classified is shown diagonally. We obtained 100% correct classification, whereas with the leave-one-out method the classification percentage fell to 89.7%. By selecting the variables (the stepwise method) the variable δ^{13}C was eliminated, thus bringing the predictive capacity of the model to 92.3%.

CONCLUSIONS

The δ^{15}N and δ^{13}C ratios of casein and the ratios between some free amino acids such as Thr/Pro, Ile/Pro, Met/Pro, and His/Pro in ewes' milk cheeses produced in Sardinia, Sicily, and Apulia (parameters that were found to be independent of ripening time) were successfully used to obtain an identification of the place of origin. Although the nitrogen isotope ratio and the amino acid ratios Ile/Pro versus Thr/Pro could discrimi-

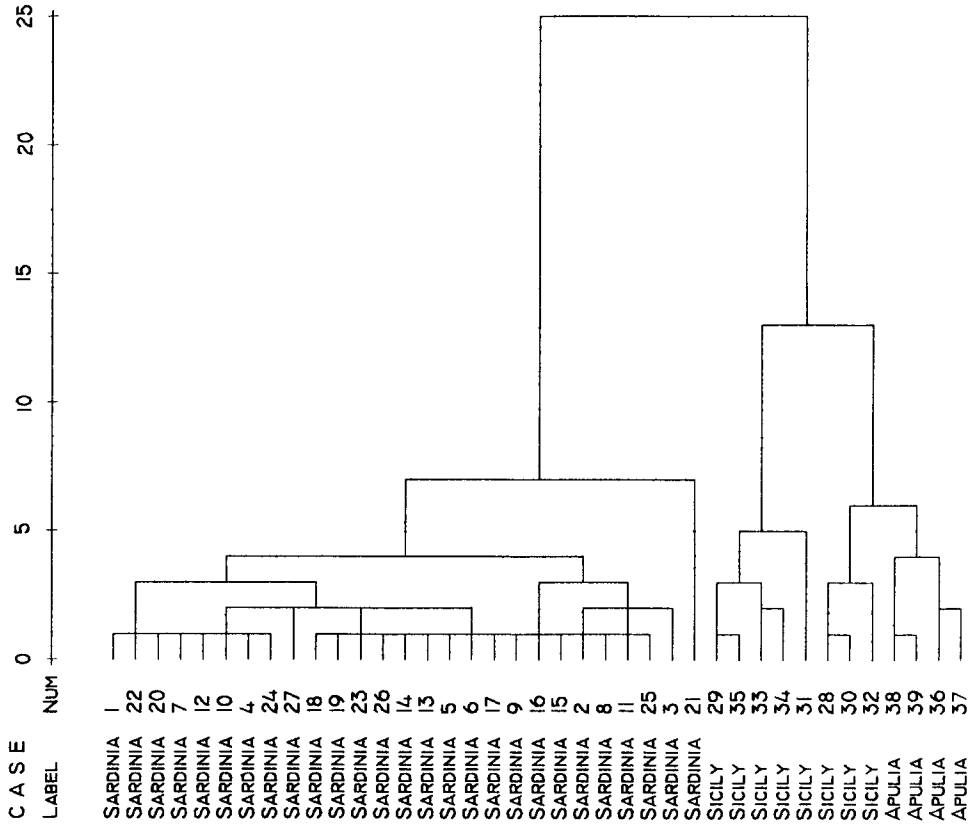

Figure 4. Dendrogram of the results of the cluster analysis subdividing the samples according to place of origin.

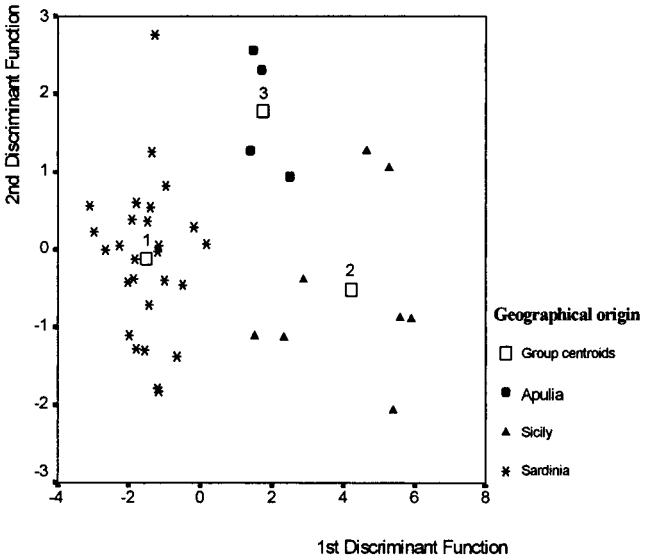

Figure 5. Distribution of the samples expressed as discriminant points between the two functions, according to place of origin.

Table 3. Classification of the Three Groups from the Three Different Regions

	actual group	predicted group			
		Sardinia	Sicily	Apulia	total
original[a]	Sardinia	27	0	0	27
	Sicily	0	8	0	8
	Apulia	0	0	4	4
	%	100	100	100	100
cross-validated[b]	Sardinia	25	0	2	27
	Sicily	0	6	2	8
	Apulia	0	0	4	4
	%	92.6	75.0	100	89.7

[a] 100% of original grouped cases correctly classified. [b] 89.7% of cross-validated grouped cases correctly classified.

nate only Pecorino Sardo cheeses from the Sicilian and Apulian types, regional distinctions could be achieved by applying multivariate statistical analytical procedures. In particular, PCA allowed separation by means of four variables ($\delta^{13}C$, $\delta^{15}N$, Thr/Pro, and Ile/Pro). Cluster analysis demonstrated that the Sardinian samples were more homogeneous than the Sicilian samples, which were found to be separated into two groups.

The use of LDA allowed the separation of samples in three groups with 100% classification and a percentage of classification by the leave-one-out method of 89.7%.

Supporting Information Available: Table reporting the data of the set of variables considered for the cheeses of different geographical origins. This material is available free of charge via the Internet at http://pubs.acs.org.

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