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Italian Cheeses Discrimination by Means of $\delta^{13}C$ and $\delta^{15}N$ Isotopic Ratio Mass Spectrometry

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Abstract
Protection of the quality products and particularly Protected Designation of Origin (PDO)/Protected Geographical Indication (PGI) foods is a strategic issue in the EU economy, in terms of protection of market competition and safety. Having reliable tools for the assessment of key parameters useful for the identification of authenticity and/or frauds is therefore of main interest. In this work, the isotope ratios of stable elements variability of four PDO cheeses (Taleggio PDO, Asiago PDO, Pecorino Toscano PDO, and Provolone Valpadana PDO) were investigated with the aim to find discrimination among different kinds of cheeses. The specificity of isotope ratios of stable elements can be profitably used when sample characteristics, conditions, or degradation strongly suggest looking directly at the atoms rather than to the molecules. We analyzed five isotopic parameters: δ13C and δ15N on casein fraction and on cheese as a whole; δ13C on the fat fraction of the cheese. The dataset was composed by 118 cheese samples coming from five different Italian regions and collected over a 3-year period. Data elaboration showed that beyond interesting differences already observed on each individual cheese on the basis of some parameters taken into account (year, season, province, altitude), the characteristic isotopic ratios of each cheese are stable within a narrow range of δ‰. Univariate analysis showed that single parameters were not enough to provide a clear discrimination between each cheese, while principal component analysis (PCA) and partial least squares regression-discriminant analysis (PLS-DA) showed a good separation between cheese classes, particularly for the Pecorino Toscano cheese type. This data suggested a positive indication to the possibility of introducing in the production disciplinary of the concerned cheeses also a range of isotopic ratios of C and N as a further tool for the protection of this four types of PDO cheeses.

Keywords Stable isotope ratio analysis · IRMS · Cheese discrimination

Introduction

The quality of traditional food products is related both with organoleptic properties and nutritional characteristics; UE 1151/2012 Regulation sets the rules for the geographical indications to protect the "quality brand" regional foods (Regulation (EU) No. 1151/2012).

The peculiarities of traditional food have been granted using the labeling Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI), which represent distinct regimes of geographical indications within the legal framework of the Protected Geographical Status defined by European Union to protect the quality brand regional foods. For both PDO/PGI products, the production area, including human-related factors such as know-how, is very important. The major difference between the two categories is that in PDO products, the entire production and transformation chain is carried in one geographical area while in the term in PGI products, only a part of the food chain can be typically carried in an area (e.g., primary production area is not the area where the food is transformed).

Italy has the greatest number of PDO/PGI products: at the moment, 291 are the registered products; 51 of which are cheeses (DOOR: EU database of agricultural products and food).

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Protection of the quality products and particularly PDO/PGI foods is a strategic issue in the EU economy, in terms of protection of market competition and safety.

In this respect, reliable tools for the assessment of key parameters useful for the identification of authenticity and/or frauds are of main interest.

In the last years, many isotopic parameters have been used for the geographical assignment, not only for wine and fruit derivatives, but also for dairy products (Rossmann 2007; Camin et al. 2017).

The evaluation of isotopic ratios is very helpful for the identification of the geographic origin of raw material; however, there is a natural variability in the isotopic ratios, making difficult to obtain accurate references. In fact, significant differences related to multiple variables may occur in the same geographical area in different years (Manca et al. 2001; Pillonel et al. 2003; Camin et al. 2004; Renou et al. 2004; Brescia et al. 2005; Crittenden et al. 2007; Bontempo et al. 2012; Camin et al. 2012; Silva et al. 2014; Camin et al. 2015; Capici et al. 2015; Stevenson et al. 2015; Nečemer et al. 2016).

Isotopic ratio mass spectrometry (IRMS) methods have been used in previous works for the geographical characterization of various products. For instance, in the case of olive oil (Camin et al. 2010, 2016; Portarena et al. 2017), this technique has been found to produce output data that are practically independent of the employed cultivar and the production technique.

Different studies on cheese using multivariate analysis have been reported such as, the screening of various cheese-related bacteria for their ability to produce aroma compounds (Pogačić et al. 2015; Afzal et al. 2017; Pisano et al. 2016; Pogačić et al. 2016) or NMR metabolomics to classify cheese samples (Piras et al. 2013; 2013; Afzal et al. 2017; Pisano et al. 2016).

Moreover, in some cases, IRMS has already been introduced as an official method (AOAC method 991.41-1996; EC Regulation 822/97; EC No: IT-PDO-0217-0011-26.07.2006; AOAC method 2004.01-2004).

The aim of this paper was to study the $\delta^{13}C$ and $\delta^{15}N$ isotopic variability in four different PDO cheeses to verify the possibility of classifying, each individual cheese, and using the characteristic range of variability as a new discriminant tool.

The IRMS technique was chosen because, even if a PDO cheese authentication can be accomplished with methods involving metabolic profiling, the use of isotope ratios of stable elements has the advantage of being independent from events that can influence results, such as sample conditions or degradation.

To achieve this goal, the unsupervised pattern recognition method called principal component analysis (PCA) has been initially used to evaluate the intrinsic variation in a cheese data set using all $\delta^{13}C$ and $\delta^{15}N$ isotopic parameters. As reported in many works on food, this unsupervised pattern recognition method is a very useful tool to explore the main information contained in the initial variables in a lower number of uncorrelated variables (Eriksson et al. 2001; Speranza et al. 2015; Sliwinska et al. 2014; Faberi et al. 2014; Solieri et al. 2014). To further assess the discriminatory capacities of this approach, data improvement classification was then demonstrated with a partial least squares-discriminant analysis (PLS-DA). The PLS-DA is a well-known classification model applied in many scientific fields (Pierna et al. 2011; Maltas et al. 2013; Gan et al. 2016). This chemometric approach has been used in this study because it allows straightforward control of data.

Experimental

Samples

Samples were collected by the relevant protection consortiums of each cheese and shipped to the laboratory of the Central Inspectorate for the protection of the quality and fraud repression of food products (ICQRF). A total of 118 cheeses was analyzed:

- No. 56 Taleggio cheeses PDO
- No. 23 Asiago cheeses PDO
- No. 15 Pecorino Toscano cheeses PDO
- No. 24 Provolone Valpadana cheeses PDO

For each sample, traceability metadata consisting of site of production and trimester and year of manufacturing were also provided.

Reagents and Materials

All reagents were of analytical grade and were supplied by Sigma-Aldrich (St. Louis, MO, USA). High purity gases were supplied by SIAD Spa (Bergamo, Italy).

Extraction of the Casein Fraction

The casein fraction was obtained by applying the procedure in the Regulation (EC) No. 273/2008- All. IX, section 6.1.1 (reference method for the detection of casein in cow's milk and sheep's milk, goat, or buffalo). Briefly, about 8 g of cheese were homogenized with a dispersing homogenator (Ultra-Turrax, IKA-Werke GmbH, Germany) in a 100-ml centrifuge tube after the addition of 30 ml of ultrapure water. The pH level was brought to 4.6 by the addition of a few drops of dilute acetic acid and homogenized again. The sample was then centrifuged at 4000 rpm for 5 min and the fat and the

serum supernatant were removed. The residue was re-homogenized after the addition of 20 ml of acidic water and 10 ml of dichloromethane. The suspension obtained was centrifuged again and finally, the layer of casein—positioned between the aqueous phase and the organic phase —was recovered with a spatula. The latter phase process was repeated three times until the two extraction phases appeared completely colorless. Finally, the protein residue was homogenized with 25 ml of acetone, filtered through medium-speed filter paper, washed on the filter with 25 mL of acetone, and allowed to dry in the air.

Extraction of the Fat Fraction

The cheese was milled by means of a bench homogenizer and freeze-dried for at least 24 h (T condenser − 55 °C; final pressure minimum 50 mtorr).

The lyophilized cheese, after being grinded again by hand with a stainless steel mortar and pestle, was transferred into a cellulose extraction thimble, previously degreased with *n*-pentane; the thimble was extracted with a Soxhlet apparatus using 250 ml of *n*-pentane at a temperature of 65 °C for 6 h.

At the end of the process, the extracting solvent was removed under vacuum and the fat was placed in an oven at 50 °C for 30 min, to remove the last traces of solvent.

Isotopic Analysis of ^{13}C and ^{15}N in the Case in Fraction

The carbon isotope ratios were determined according to the elemental analysis EA/IRMS technique. The experiments were carried out by a flash combustion on an elemental analyzer EA-Flash 2000 HT (Thermo Fisher, Bremen, Germany), connected to a Delta V Plus (Thermo Fisher, Bremen, Germany) isotope ratio mass spectrometer operating in continuous helium flow mode, via a Conflo IV interface (Thermo Fisher, Bremen, Germany).

For the analysis of the isotopic ratios $^{13}C/^{12}C$ and $^{15}N/^{14}N$ on casein fractions, about 0.8 mg of sample was weighed into a tin capsule and introduced in the reactor for combustion of the elemental analyzer by means of an auto sampler for solids. The latter oxidizes all organic matter under synchronized helium flow and oxygen pulse flow (3 s) through combustion flash which occurs in a quartz reactor at 920 °C. The reactor contains packaged redox catalysts Cr_2O_3 and $(Co_3O_4)Ag$. The combustion gas (CO_2, N_2, NO_x, and H_2O) was passed through a layer of metallic Cu allowing elimination of the O_2 excess and reduction of nitrogen oxides to N_2. The water was instead removed by using a trap filled with $Mg(ClO_4)_2$, anhydrous. N_2 and CO_2 are at this point separated on a gas chromatographic column Pora-PLOT Q, $L = 5$ m, thermostated at the temperature of 45 °C, and sent through the ConFlo IV to the IRMS, to determine the isotopic composition.

CO_2 and N_2 reference gases were inserted in the continuous He flow gas pure standard pulses. The carbon isotope ratio ($^{13}C/^{12}C$) was determined from the ionic currents m/z 44 ($^{12}C^{16}O^{16}O$), m/z 45 ($^{13}C^{16}O^{16}O$), and m/z 46 ($^{12}C^{16}O^{18}O$ or $^{13}C^{16}O^{17}O$) (the latter ratio m/z is used by the software to take a small correction $\delta^{13}C$, according to Craig (Craig 1957), because of the contribution of ^{17}O produced by CO_2. The nitrogen isotope ratio $^{15}N/^{14}N$ was calculated from the ionic currents m/z 28 ($^{14}N^{14}N$), m/z 29 ($^{14}N^{15}N$), and m/z 30 ($^{15}N^{15}N$).

In each sequence of analysis, two different reference materials have been used, one for the calibration of the instrument (calibration materials) and one for the quality control of the analysis (control material). As reference materials two different kinds of protein derivatives of dried milk products were used (skimmed milk protein powder by ultra filtration and whey protein powder) whose reference values of $\delta^{13}C$ and $\delta^{15}N$ have been previously standardized against certified reference materials (IAEA-NBS-22, IAEA-CH-6, USGS-40 for $\delta^{13}C$ and IAEA-NO-3, USGS-40 for $\delta^{15}N$) are used.

Isodat 3.0 software (Thermo Scientific) management tool calculated the values of $\delta^{13}C$ in the samples by comparison of the signal ratios of the masses 45 and 44 corrected according to Craig with reference CO_2 gas. $\delta^{15}N$ was calculated similarly using the ratio of the masses 29 and 28.

Stable isotope ratios are calculated the conventional δ – notation (Brand et al. 2014).

$$\delta^{i}E = \frac{{}^{i}\mathrm{RSA} - {}^{i}\mathrm{RREF}}{{}^{i}\mathrm{RREF}}$$

where

E	is the element considered
i	is the mass number of the heavier isotope of element E (for example, ^{13}C);
RSA	is the respective isotope ratio of a sample;
RREF	is the relevant internationally recognized reference material.

Delta values are expressed multiplied by 1000 in units “per mil” (‰).

The delta obtained for the samples was processed, through an Excel that verifies the repeatability limit of the MR used as “reference material” (MR1) and only if it is below 0.3‰ δ units (usually accepted for this analysis), the values of $\delta^{13}C$ and $\delta^{15}N$ are averaged; then, the difference between the resulted value and the “true value” (by setting) is calculated and all the other data of $\delta^{13}C$ and $\delta^{15}N$ of the series, including MR used as “control material” (MR2), are corrected for the difference. Samples are analyzed in duplicate and the $\delta^{13}C$ and $\delta^{15}N$ average of the measurement checked for the error and corrected as reported earlier.

The value obtained for $\delta^{13}C$ and $\delta^{15}N$ of MR used as "control" (MR2) should be compatible with the theoretical one in accordance with the following formula:

$$\left|\bar{y}-\mu\right| \leq 2\sqrt{u_y^2 + u_\mu^2}$$

where:

- $\bar{y}$ MR2 average value obtained from the laboratory during the analysis;
- μ reference value of MR2 obtained in the laboratory during the standardization of the MR2;
- u_y $0 = 0.3‰$ uncertainty of ;
- u_μ uncertainty of μ.

Statistical Evaluation

Univariate analysis was performed using Excel 2013 (Microsoft Office). Multivariate statistical analysis was performed using two different approaches, PCA and PLS-DA by means of MatLab R2011b (Mathworks, Natick, MA, USA) integrated with a classification toolbox for MATLAB obtained from Milano Chemometrics and QSAR Research Group (version 3.0). The data set consisted of a 118 × 5 matrix, in which rows represented the samples (118 cheese samples), and columns, the five isotopic variables. Data have been autoscaled (zero mean and unitary variance). Data vectors belonging to the same cheese type were firstly analyzed by unsupervised PCA. This technique gives the possibility to project data from a higher to a lower dimensional space having a data overview without any preliminary assumptions (Stanimirova et al. 2007). Then, the supervised technique PLS-DA was applied to the autoscaled data matrix of the five isotopic profiles in order to improve the separation between cheese classes. PLS-DA was used as a supervised deterministic classification technique capable of discriminating the observations on the basis of a class membership categorical matrix (Gromski et al. 2015). PLS-DA was performed on the dataset using also a cross validation of the model by using "venetian blinds" cross validation with number of cv groups equal to 2.

Using confusion matrices, the reliability of the classification models achieved was studied in terms of recognition ability (percentage of the members of the training set correctly classified) and prediction ability (percentage of the members of the test set correctly classified using the rules developed in the training step).

Results and Discussion

The work examined five isotopic parameters: $\delta^{13}C$ and $\delta^{15}N$ on casein fraction and on cheese as a whole; $\delta^{13}C$ on the fat fraction of the cheese.

The dataset was composed of 118 cheese samples coming from five different Italian regions and collected over a 3-year period. As reported in Table 1, the cheese samples were from four different types Asiago, 23 samples; Pecorino Toscano, 15 samples; Provolone Valpadana, 24 sample; and Taleggio, 56 samples. The variables selected were the five isotopes: namely the δ isotopic composition of $\delta^{13}C$ in cheese, fat, and casein

Table 1 Cheese types used to divide the data set (118 samples) of the work. Each classification included the geographical origin ("Region" column) and the sampling year ("Year" column) of the samples

Cheese types	No. of samples	Region	No. of samples	Year	No. of samples	Label
Asiago	23					
		Veneto	*23*			
				2010	6	A1-A6
				2011	17	A7-A23
Pecorino Toscano	15					
		Tuscany	*15*			
				2010	15	B1-B15
Provolone Valpadana	24					
		Lombardy	*14*			
				2010	14	C1-C14
		Trentino	*2*			
				2010	2	C15-C16
		Veneto	*8*			
				2010	8	C17-C24
Taleggio	56					
		Lombardy	*54*			
				2010	12	D1-D12
				2011	28	D13-D40
				2012	14	D41-D54
		Piedmont	*2*			
				2010	1	D55
				2011	1	D56

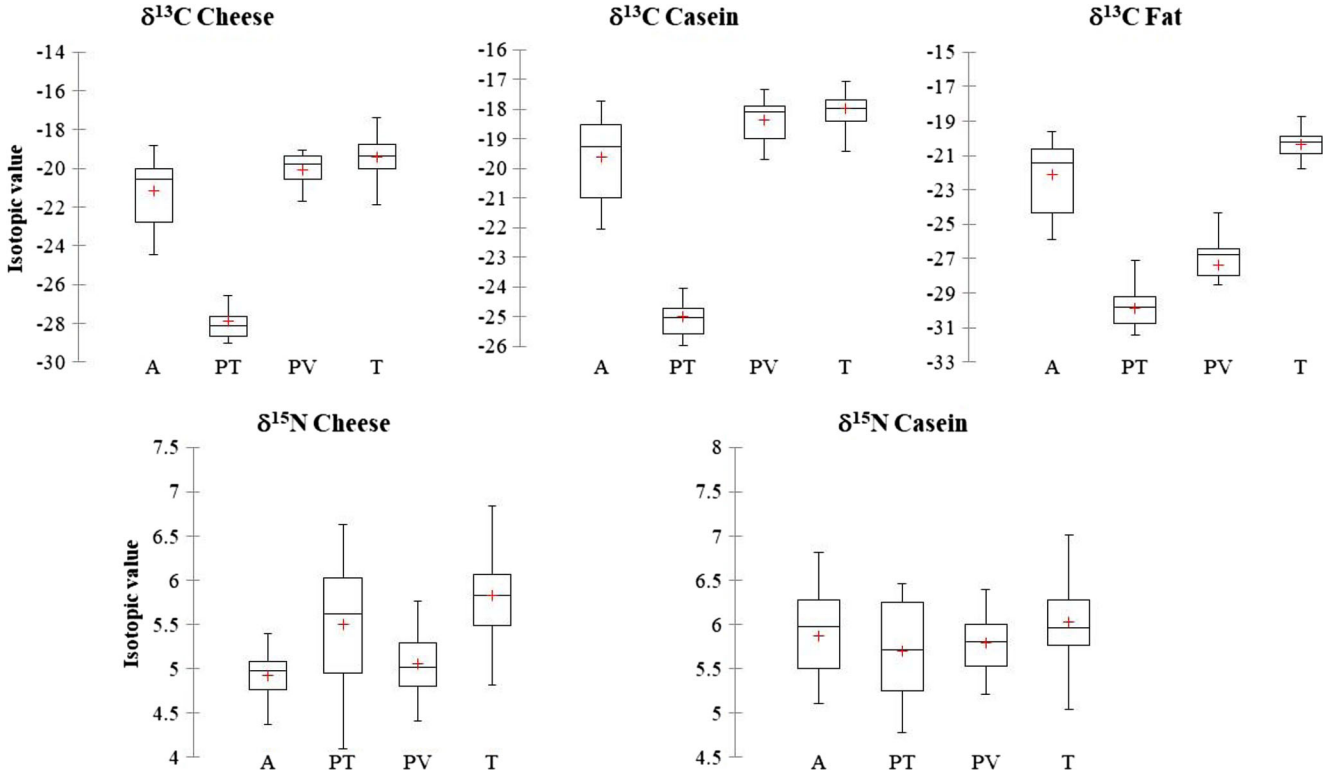

Fig. 1 Isotopic data of δ^{13}C cheese, δ^{13}C casein, δ^{13} C fat, δ^{15}N cheese, δ^{15}N casein for the four types of cheese are reported in a form of box and whisker plot. A Asago, PT Pecorino Toscano, PV Provolone Valpadana, T Taleggio

and the isotopic composition of δ^{15}N in cheese and casein. Fifty-eight samples were collected in the first year, 46 samples in the second year, and only 14 samples in the third year. The sampling year was reported to check the variability due to sampling; however, no variability between different years was found.

Data vectors belonging to the same cheese category were firstly analyzed by the univariate procedure of the analysis of variance and showing the shape of the distribution via the graphical representation of box and whisker plot, and afterwards, by two multivariate techniques, the unsupervised PCA and supervised PLS-DA. The univariate analysis of the differences between the cheese classes with a confidence interval of 95% highlighted no statistical differences using both δ^{15}N isotopic composition or δ^{13}C variables. Only the Pecorino Toscano class was statistically different with respect to the other cheese classes, with the exception of δ^{13}C value in fat for which also Provolone Valpadana class was significantly distinguishable from the other classes.

Since single parameters were not enough to provide a clear discrimination, such variables were treated with multivariate statistical procedures. The three δ^{13}C and the two δ^{15}N isotopic composition, determined for the 118 Italian cheese samples represented the entire dataset used for multivariate analysis (Fig. 1).

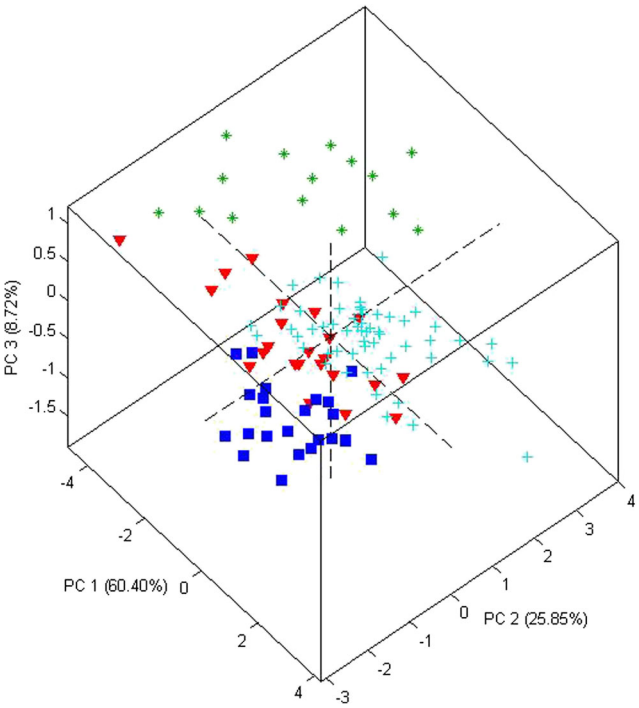

Fig. 2 PCA on the 118 cheese samples with all the five isotopic variables. Score plots of the first three components (explained variance = 94.97%). Data have been linearly normalized and autoscaled (zero mean and unitary variance) in order to remove concentration effects and different signals ranges. The four cheese classes are marked with different symbols and colors: red triangle Asiago, green star Pecorino Toscano, dark blue square Provolone Valpadana, blue cross Taleggio

Table 2 PCA loadings associated to each variable on the five principal components

PC	1	2	3	4	5
Variance	60.40%	25.85%	8.72%	4.69%	0.34%
Variable	Loadings				
δ^{13}C cheese	0.52	−0.33	−0.24	−0.21	0.72
δ^{13}C casein	0.52	−0.33	−0.29	−0.26	−0.69
δ^{13}C fat	0.48	−0.16	0.73	0.47	−0.04
δ^{15}N cheese	0.32	0.66	0.33	−0.59	0.01
δ^{15}N casein	0.37	0.56	−0.48	0.56	0.00

As reported in Fig. 2, PCA was applied to the 118 cheese samples; before applying the PCA algorithm, data were linearly normalized and autoscaled (zero mean and unitary variance) in order to remove concentration effects and different signal ranges. The PCA evidenced that for all variables tested, the first three principal components accounted for 94.97% of the total variance. The score plot on the first three components showed a good separation between cheese classes, particularly for the Pecorino Toscano cheese type (marked with green stars). The scores data suggested that the selected isotopes can clearly distinguish between the four cheese classes considered, even if a partial overlap was observed between Asiago, Provolone Valpadana, and Taleggio.

As usual with multivariate data analysis, each sample corresponds to a vector in multidimensional space. It is worth, then, to discuss the position of the loadings as they are a measure of the contribution of the individual parameter to the principal components and, then, the contribution of the measurement to the representation of PCA. In particular, loadings associated to each variable on the five principal components allow to appreciate the corresponding correlations. In fact, for two variables with different loadings, correlation is lower because they are orthogonally distributed and orthogonality in PCA plots means uncorrelated contributions (Jolliffe 2002). Looking at the isotopic variable contribution (Table 2), the isotope δ^{13}C in cheese and casein had very close loading values in the first four principal components but not for the fifth. Different behaviors were observed for the isotope δ^{13}C in fat that was partially correlated in the first principal component but very different in the second component with respect to cheese and casein changing sign in the third component. The δ^{15}N value of cheese and casein has a certain degree of correlation with respect to first and second principal components but they are anticorrelated starting from the third component. Despite the correlation looking at the first principal component, the isotopes δ^{13}C and δ^{15}N have different trends. This demonstrates that isotopes independently contribute to the dataset discrimination particularly in separating cheese classes in the second and third component projection.

To evaluate the real efficiency of the discrimination among the different cheese classes, based on the multi-isotopic analysis, a supervised multivariate discriminant analysis was applied. As described earlier, the set of samples (total number 118; see Table 1 for details on the sample set) was used for this approach. As in any supervised classification techniques, the classes have to be chosen a priori. The natural choice for the samples in this case was to choose the cheese classes. With this classification scheme, a PLS-DA model has been built. Data have been linearly normalized and then autoscaled (zero mean and unitary variance) before analysis.

As reported in Table 3, a very good PLS-DA sensitivity and specificity in both calibration and cross validation was found for Pecorino Toscano and Provolone that gave the lower RMSEC, RMSECV, and classification errors. Asiago cheese class presented the highest classification errors, but keeping sensitivity and specificity in both calibration and cross validation higher than 0.85. A PLS-DA graphical representation is reported in Fig. 3, respectively, for the first three latent variables representing 93.8% of the

Table 3 Statistics for each cheese class. PLS-DA model was carried out using X block of 118 by 5 and Y block of 118 by 4 with autoscaled preprocessing. Cross validation venetian blinds method was used with the number of cv groups equal to 2

Modeled cheese classes	Asiago	Pecorino Toscano	Provolone Valpadana	Taleggio
Sensitivity (Cal)	0.91	1.00	1.00	0.98
Specificity (Cal)	0.88	1.00	0.98	0.95
Sensitivity (CV)	0.87	1.00	1.00	0.96
Specificity (CV)	0.88	1.00	0.98	0.95
Class. Err (Cal)	0.10	0.00	0.01	0.03
Class. Err (CV)	0.12	0.00	0.01	0.04
RMSEC	0.27	0.13	0.15	0.25
RMSECV	0.30	0.13	0.16	0.26
Bias	−4.2E−16	5.6E−16	−1.0E−15	1.1E−15
CV bias	5.3E−03	6.5E−04	−6.3E−04	−5.3E−03
R^2	0.53	0.85	0.86	0.75
CV R^2	0.46	0.84	0.85	0.72

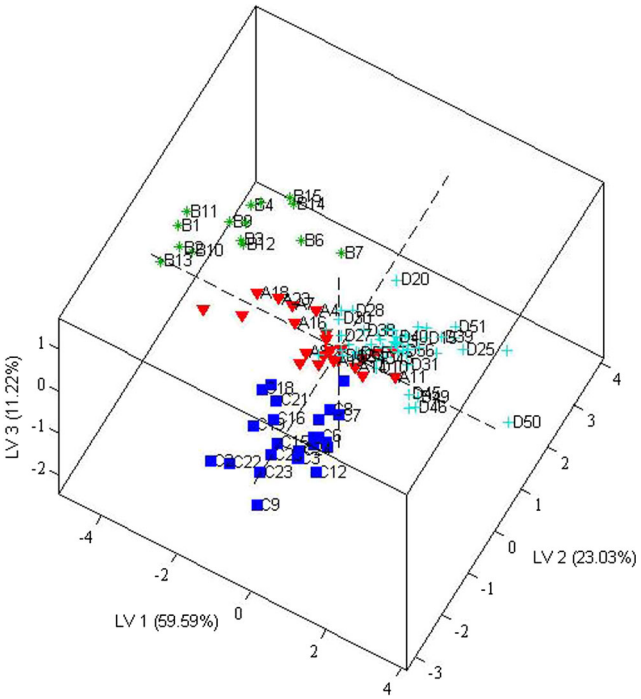

Fig. 3 Score plot of PLS-DA model built to classify the 118 cheese samples using all the five isotopic variables. Data have been linearly normalized and autoscaled (zero mean and unitary variance) in order to remove concentration effects and different signal ranges. The four cheese classes are marked with different symbols and colors: red triangle Asiago, green star Pecorino Toscano, dark blue square Provolone Valpadana, blue cross Taleggio

percentage variance captured by the regression model on the X block.

A good separation between the data related to the four classes is observed using both $\delta^{13}C$ and $\delta^{15}N$ variables even if a partial overlapping was observed between Asiago and Taleggio classes.

A numerical evaluation of the classification properties can be obtained by considering both fitting the cross validation of the PLS-DA method according to the "venetian blinds" technique. The results are shown in Table 4, in a form of a confusion matrix. A total of 95% of the samples have been correctly classified by isotopic variables in fitting and 92% in cross validation.

In fitting, Pecorino Toscano and Provolone showed 100% of correspondences between real and predicted samples. This percentage decreased to 91% only for Pecorino Toscano in cross validation. It should be noted that only the Taleggio class in cross validation was lower than 90% confirming that isotopic variables can be used for discriminating Italian cheese types.

Conclusions

Data elaboration showed that beyond interesting differences already observed on each individual cheese on the basis of some parameters taken into account (year, season, province, altitude), the characteristic isotopic ratios of each cheese are stable within a narrow range of δ‰.

Furthermore, the correlation between isotopic ratios allows a precise identification of characteristic areas of each cheese. In some cases, overlapping is observable on the featured areas that do not allow to identify the individual cheese.

In fact, for each cheese considered, there are intervals of values sufficiently narrow to classify each specific PDO cheese.

In addition, the obtained indications showed a substantial constancy in time for the ranges if values, which would suggest the possibility to maintain the isotopic database for an indefinite period, without the need for an extensive annual reconstitution, provided that just periodic reconfirmation of validity is made.

Unfortunately, having cheese samples pretending to belong to the studied cheese categories with different origins to be

Table 4 Confusion matrix of the PLS-DA classification model with all the variables (fitting and validation results are both reported). Cross validation venetian blinds method was used with the number of cv groups equal to 2. True classes are read along the columns and estimated classes along the rows. The total accuracy was also reported

Real/predicted	Asiago	Pecorino Toscano	Provolone Valpadana	Taleggio	Row (%)
Fitting					
Asiago	22	1	0	0	*96*
Pecorino Toscano	0	15	0	0	*100*
Provolone Valpadana	0	0	24	0	*100*
Taleggio	5	0	0	51	*91*
Total (%)	*95*				
Cross validation					
Asiago	21	2	0	0	*91*
Pecorino Toscano	1	14	0	0	*93*
Provolone Valpadana	0	0	24	0	*100*
Taleggio	6	0	1	49	*88*
Total (%)	*92*				

The % of correct classifications is indicated in italic

used in cross validation is not possible because of the specificity and unicity of these Italian cheese categories.

In any case with the basic assumption that this study needs to be further continued and confirmed, we can formulate the hypothesis as in the case of the isotopic wine database that the present work could be used as a starting point of introducing in the production disciplinary of the concerned cheeses, a range of isotopic ratios of C and N as a further tool for the protection of the PDO cheeses.

However, the variability of other isotopic parameters (such as δ^2H, $\delta^{18}O$, and $\delta^{34}S$) has to be studied as further authentication markers.

Compliance with Ethical Standards

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

Conflict of Interest Angelo Faberi declares that he has no conflict of interest. Dario Compagnone declares that he has no conflict of interest. Fabio Fuselli declares that he has no conflict of interest. Alessandro La Mantia declares that he has no conflict of interest. Marcello Mascini declares that he has no conflict of interest. Camilla Montesano declares that she has no conflict of interest. Rachele Rocchi declares that she has no conflict of interest. Author Manuel Sergi declares that he has no conflict of interest.

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