Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 89 (2005) 139–147
Food Chemistry
www.elsevier.com/locate/foodchem

Characterisation of the geographical origin of buffalo milk and mozzarella cheese by means of analytical and spectroscopic determinations

M.A. Brescia [a,*], M. Monfreda [a], A. Buccolieri [b], C. Carrino [c]

[a] *Dipartimento di Chimica, Università degli Studi di Bari, Via Orabona 4, 70126 Bari, Italy*
[b] *Dipartimento di Scienza dei Materiali, Università degli Studi di Lecce, via Arnesano, 73100 Lecce, Italy*
[c] *via Marchese De Rosa 40, 71100 Foggia, Italy*

Received 16 October 2003; received in revised form 5 February 2004; accepted 5 February 2004

Abstract

Appreciation of qualified national products, together with a guaranteed reference for consumers, has become necessary in the field of dairy products. Indeed, the *Protected Designation of Origin* (PDO) trademark has been assigned to numerous cheeses, such as buffalo milk mozzarella. In order to receive this designation, the raw materials have to be produced and processed in the specified region from which the product gets its name. Therefore, in order to determine the authenticity of typical dairy products it is necessary to determine the geographical origin of the milk and of the finished product obtained from it. Classical techniques, high performance ion chromatography (HPIC), inductively coupled plasma emission spectroscopy (ICP-AES), nuclear magnetic resonance (NMR) and isotope ratio mass spectrometry (IRMS) were used for determining different compounds in combination with chemometric methods for the geographical characterization of buffalo milk mozzarella cheeses originating from two areas of Southern Italy. Isotopic ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) and other variables were affected by the specific area of origin of milk samples, while NMR data, together with isotopic ratios, were useful for the discrimination of mozzarella samples.

Keywords: Buffalo milk mozzarella; NMR; Multivariate statistical analysis; IRMS; Geographical origin

1. Introduction

The typicality of a food product can be correlated with its geographical localisation, the quality of raw material, and the production techniques. The environmental conditions set up in a geographical area impart specific characteristics to the product, becoming a factor of primary importance in determining its typicality. The production technique is of primary importance for agricultural products and for transformed products, where culture, the instruments used, the ability and experience of the operator, and the addition of particular ingredients create a unique product. EC regulation no. 2081/92 (E.U., 1992) regulates the protection of the Protected Designation of Origin (PDO) of food products in the community market. The PDO mark is assigned to products strictly linked to a characteristic area. The production of the raw materials and their transformation into the final product have to be carried out in the region that gives its name to the product. For example, a PDO cheese must be obtained from milk of animals bred in the PDO area and the geographical environment of the area of origin must essentially determine its characteristics.

Buffalo mozzarella is a fresh and stringy-textured Italian cheese made from buffalo milk. The appreciation of this product by consumers is demonstrated by the "Mozzarella di Bufala Campana" PDO recognition obtained in 1996. Mozzarella di Bufala Campana is produced according to a disciplinary approved by the European Union, describing the origin and the conditions of transformation of buffalo milk (E.U., 1996).

* Corresponding author. Tel.: +39-080-5442042; fax: +39-080-5442129.
E-mail address: brescia@lgxserve.ciseca.uniba.it (M.A. Brescia).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.02.016

Fig. 1. Map of Southern Italy.

The PDO territory currently includes some areas in the Italian regions of Campania and Lazio (Fig. 1). In February 2002 the Italian Ministry of Agriculture approved the extension of PDO to 12 towns in the North Apulia region, around the city of Foggia. When the European Union approves this extension, the products obtained in Apulia will carry the "Mozzarella di Bufala Campana" denomination with a "Protected Geographical Indication" (PGI), certifying the Apulian origin of the milk.

Many investigations have been carried out to characterise Buffalo mozzarella. They deal with establishing methods to detect the addition of cow's milk to buffalo milk in mozzarella preparation, which is forbidden by the disciplinary (Addeo et al., 1995), and with establishing its microbiological profile (Coppola, Parente, Dumontet, & La Peccerella, 1988; Romano, Ricciardi, Salzano, & Suzzi, 2001). Very few works are dedicated to geographical origin of mozzarella or other cheeses. Mauriello, Moio, Genovese, and Ercolini (2003) found that the flavouring capabilities and the microbial diversity of the natural whey cultures, used for traditional water–buffalo mozzarella cheese manufacture, proved to be closely linked and as well as related to their geographical origin. Pillonel et al. (2002) carried out a determination of geographical origin of Emmentaler cheese from six regions in Europe, based on some chemical parameters. Isotope ratio mass spectrometry (IRMS) has been used in the problem of geographical origin differentiation of milk samples (Brescia, Caldarola, Buccolieri, Dell'Atti, & Sacco, 2003; Kornexl, Werner, Rossmann, & Schmidt, 1997; Rossmann, Kornexl, Versini, Pichlmayer, & Lamprecht, 1998). This technique has given promising results in application to dairy products (Manca et al., 2001; Rossmann et al., 2000).

In the present work, we compare the use of analytical and spectroscopic techniques for determining different compounds in mozzarella. The results were evaluated, with chemometric methods, for the classification of buffalo mozzarella samples from Foggia and Caserta provinces. Since mozzarella is a transformed product, its characteristics are basically determined by two factors, the raw milk and the production technology. Therefore, the characterisation work was also conducted on the milk with the purpose of evaluating how the differences among milk samples were transferred to the final product.

The experimental work was carried out by physical–chemical determinations, ^{1}H nuclear magnetic resonance (NMR) for the semi quantitative determination of some amino acids, organic acids, alcohols and sugars, and IRMS for determination of isotopic ratios $^{13}C/^{12}C$ and $^{15}N/^{14}N$.

The data obtained on mozzarella samples by classical analytical methods and those obtained by ^{1}H NMR were treated separately by multivariate analysis in order to compare the discriminating potential of each methodological approach.

2. Materials and methods

2.1. Materials

Fourteen samples of buffalo milk and fourteen samples of water buffalo mozzarella were supplied by producers in Southern Italy. In detail, seven samples of milk and mozzarella, numbered from 1 to 7, came from Caserta

province (Campania) and seven samples, numbered from 8 to 14, came from Foggia province (northern Apulia).

All samples were collected in polypropylene boxes and stored in a freezer at −80 °C prior to analysis.

2.2. Routine analyses

Fat, lactose and protein contents of milk samples were determined using a Milko Scan FT 120 (Foss Italia, Padova, Italy) while pH was measured with a pH meter. The dry extract was obtained by heating the sample to 102 °C and the ash content was obtained by heating the dry extract to 600 °C.

For mozzarella samples, pH was measured with a pH meter on the solution obtained by dissolving 10 g of cheese in 100 ml of water. Fat content was measured with a Soxhlet extractor; moisture and ash contents were determined by the same treatment used for milk and total nitrogenated substances, using the Kjeldhal method.

2.3. Chromatographic analyses

Lithium, sodium, potassium, magnesium and calcium contents were measured by means of high performance ion chromatography (HPIC, DX 120 EX, Dionex, Sunnyvale CA, USA, equipped with a conductometric detector), following the procedure described in a previous paper (Brescia et al., 2002b).

For these determinations, milk ash and mozzarella samples were dissolved in 50 ml of 0.1 N HCl.

2.4. Atomic emission spectrometric measurements

The concentrations of Al, Cr, Cu, Fe, Mn, Ni, Zn, Pb, Se and Ba were measured, only on mozzarella samples, by inductively coupled plasma atomic emission spectrometry inductively coupled plasma emission spectroscopy (ICP-OES) on a Varian Liberty 110 (Varian Inc., Palo Alto, USA) instrument equipped with an ultrasonic nebulizer U-5000AT$^+$ (Cetac Technologies Inc., Omaha, Nebraska, USA).

Before analysis, the samples were freeze-dried and subjected to a mineralization process: 0.5 g of sample was dissolved in 6 ml of 70% HNO_3 Ultrapure Reagent (J.T. Baker, Phillipsburg, USA) and 1 ml of 30% H_2O_2 (J.T. Baker, Phillipsburg, USA) and digested in a microwave system MLS-1200 MEGA (Milestone, Bergamo, Italy).

Calibration was obtained with an external standard: standard solutions were prepared by diluting a 1000 mg l^{-1} standard solution for inductively coupled plasma (J.T. Baker, Phillipsburg, USA). High-purity water (electrical resistivity >10 MΩ cm) was produced with a Milli-*Q* RG system (Millipore, MA, USA). Glassware was cleaned by soaking overnight in a 10% nitric acid solution and then rinsed with deionized water.

2.5. Isotopic ratio determinations

Isotopic contents ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) were determined by using an isotopic mass spectrometer (Finnigan MAT delta S mass, Thermo Finnigan, San Jose, USA). A Carlo Erba 1110 elemental analyzer (Carlo Erba, Milano Italy) was connected to the spectrometer to convert carbon and nitrogen to CO_2 and N_2, respectively. Isotopic ratios were expressed as isotopic deviations δ, defined as $\delta = (R_s - R_{ref})/R_{ref} \times 1000$, where R_s is the isotopic ratio measured for the sample and R_{ref} is the isotopic ratio of the reference. The latter is the PDB (Pee Dee Belemnite) for

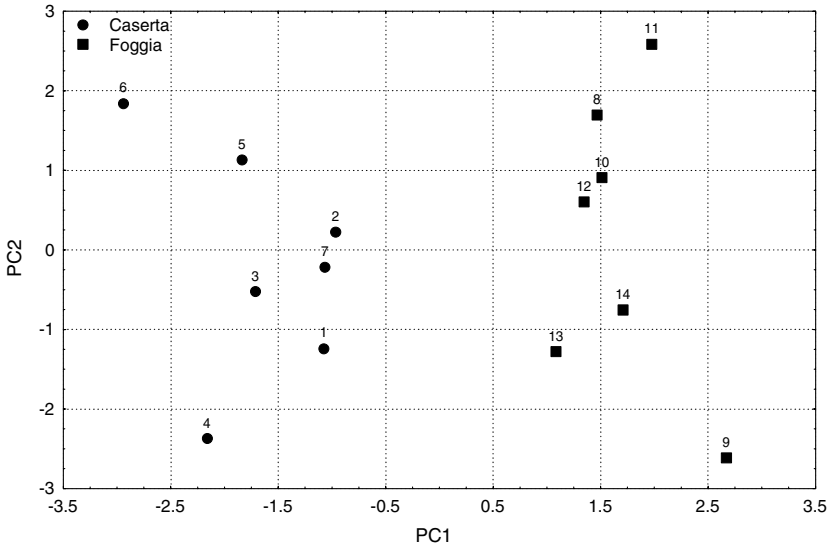

Fig. 2. Scatter plot of the scores of the milk samples from the first two principal components PC1 and PC2 obtained using analytical data.

carbon and atmospheric N_2 for nitrogen (Bréas, Reniero, & Serrini, 1994).

2.6. NMR determinations

^{1}H NMR spectra were realized only on freeze-dried mozzarella samples; 2 g of sample were dissolved in 7 ml of D_2O. The mixture was placed in an ultrasonic bath and, after filtration, the solution was placed in a 5 mm NMR sample tube. Spectra were obtained on a Bruker Avance 500 MHz spectrometer (Bruker Analytik GMBH, Rheinstetten, Germany), using a presaturation sequence for water suppression. The following conditions were used: 65000 data points, 200 scans, spectral width of 9.76 ppm, line broadening of 0.3 Hz.

2.7. Multivariate statistical analyses

Results of routine and chromatographic analyses on milk samples were collected in one data set.

For mozzarella samples, two data sets were obtained: the first contained results of routine, chromatographic analyses and the emission spectrometric measurements; the second data set contained results of isotopic ratios and NMR determinations.

Multivariate statistical analysis was applied to all the data sets; chemometric methods were principal component analysis (PCA), hierarchical clustering analysis (HCA) and discriminant analysis (DA) that were described in a previous paper (Brescia et al., 2002a).

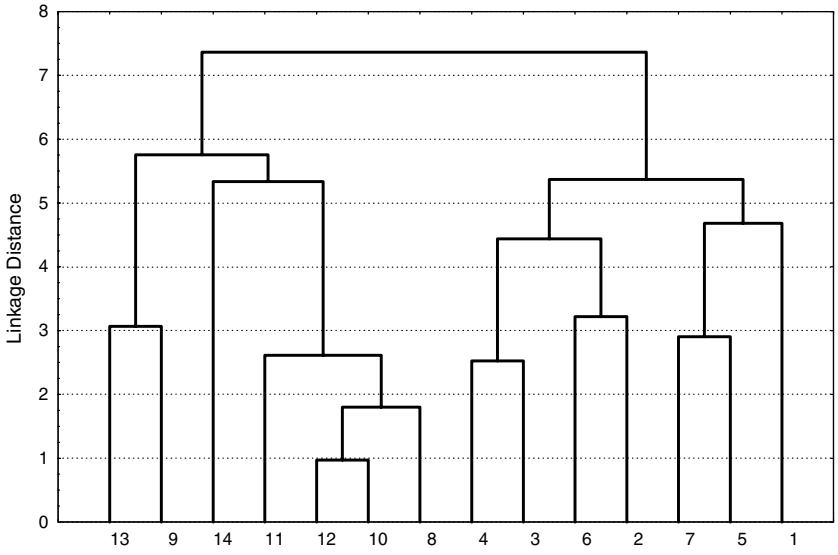

Fig. 3. Dendrogram of the milk samples obtained using analytical data.

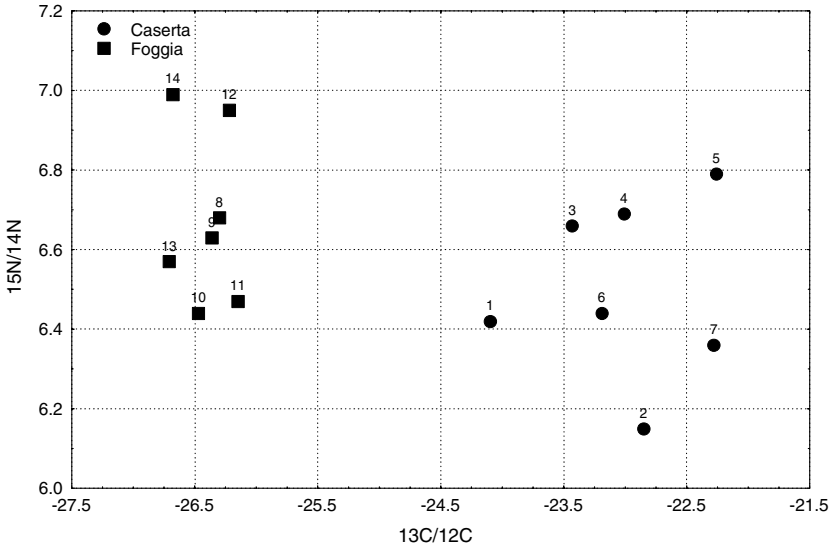

Fig. 4. Scatter plot of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ for the analysed milk samples.

3. Results and discussion

3.1. Milk samples

PCA was applied to a matrix of 11 analytical parameters for 14 samples. Four PCs were extracted explaining 82% of the total variance. In Fig. 2 the scatter plot of PC2 vs. PC1 (describing 53% of the sample variability) is reported. From this plot, a separation of samples in accordance with geographical origin was realized on PC1: samples from Campania have negative scores on this component, whereas samples from Apulia have positive scores. The examination of the loadings associated with each PC allows distinction of the most

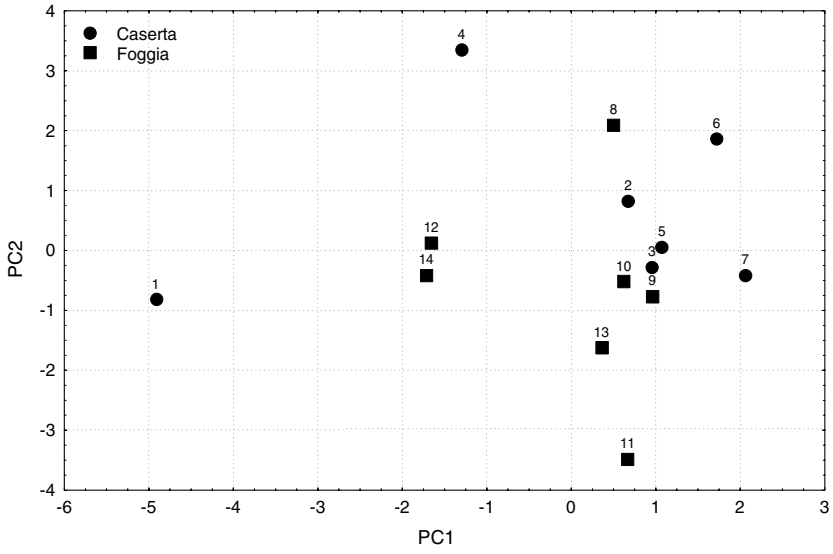

Fig. 5. Scatter plot of the scores of the mozzarella samples from the first two principal components PC1 and PC2 obtained using analytical data.

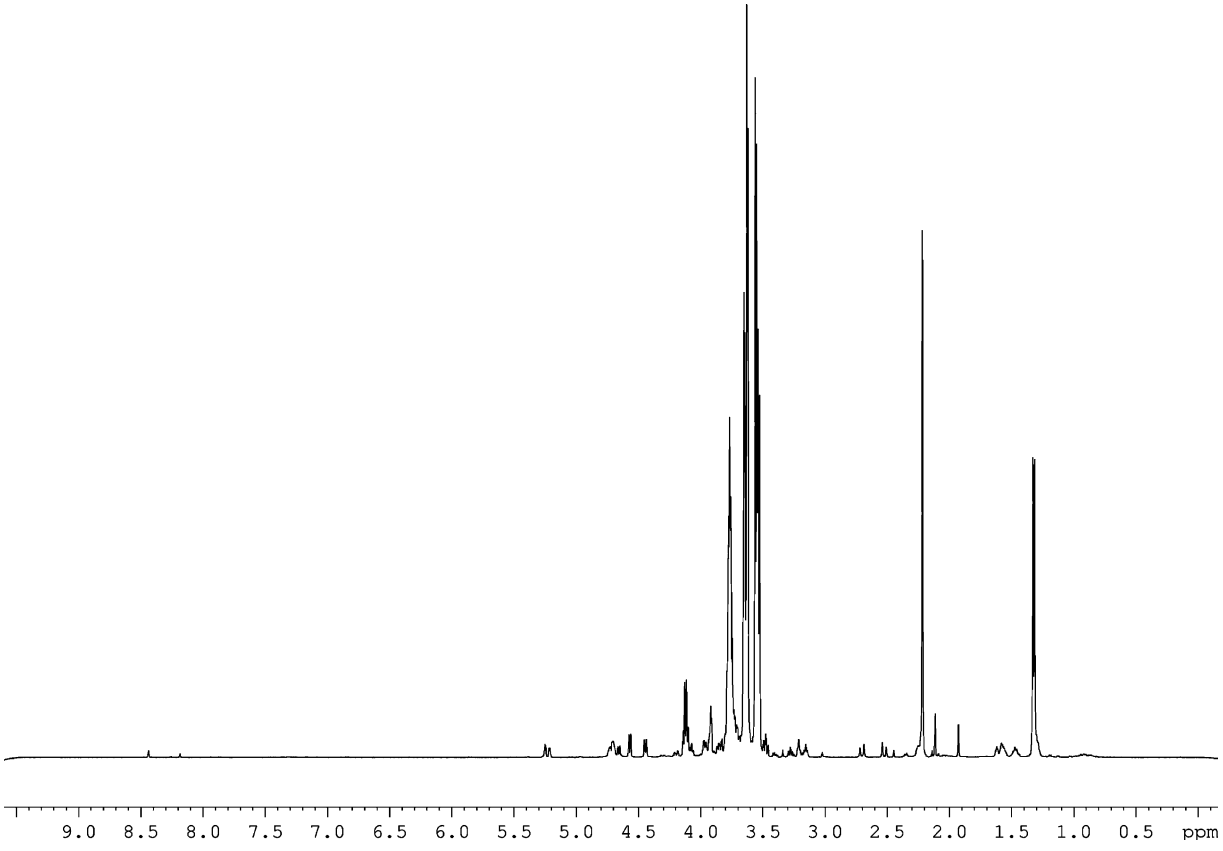

Fig. 6. ^{1}H NMR spectrum of the aqueous extract of a buffalo milk mozzarella.

important variables. PC1 was highly correlated with Na, Li, K, and protein content. These variables contain information useful for distinguishing the milk samples according to their geographical locations.

The results obtained by HCA confirm a separation of samples according to geographical origin. The dendrogram shown in Fig. 3 was obtained by applying a complete linkage procedure to Euclidean distance. At a linkage distance of 6, two clusters (containing, respectively, samples of the same origin) and an outlier can be distinguished. The outlier was a sample from Apulia, which had lower contents of fat and protein and the higher contents of dry extract and potassium.

Discriminant analysis was afterwards applied to the first four PCs in order to classify milk samples in two separate groups according to geographical origin. The samples were divided into a training set, to develop a rule for the classification of the unknown samples, and a test set on which the model could be tested. The percentage of the members of the learning set and the test set correctly classified by the model (classification ability and the prediction ability, respectively) were both of

Table 1
Assignment of resonances in 500 MHz ^{1}H NMR spectrum of the aqueous extract of a buffalo milk mozzarella

δ ^{1}H (ppm)	Multiplicity	COSY	δ ^{13}C	Assignment
0.91		1.72		CH_3 leucine
0.92		1.25		CH_3 isoleucine
0.95	d			CH_3 leucine
0.99	d	2.28		CH_3 valine
1.02	d	2.28		CH_3 valine
1.32	d	4.11	20.82	CH_3 lactic acid
1.36	d	4.40		CH_3 threonine
1.46	m	3.77		CH_3 alanine
1.69	m	3.02		CH lysine
1.72	m	3.03		CH_2 leucine
1.88	m	3.75		CH_2 lysine
1.92	s		23.67	CH_3 acetic acid
2.05	m	2.35		CH glutammate
2.11	s	2.35		CH glutammate
2.34	m	2.12		CH_2 glutammate
2.44	s		33.64	CH_2 succinate
2.52	d	2.70	45.45	CH_2 citric acid
2.70	d	2.52	45.45	CH_2 citric acid
3.02	m	1.69		CH_2 lysine
3.04	m	3.15		CH tyrosine
3.15	m	3.04		CH tyrosine
3.27	t	4.65	74.65	CH β-glucose
3.39	dd			CH β-glucose
3.47	dd		72.53	CH β-galactose
3.54	dd		63.26	CH_2 glycerol
3.58	m		75.58	CH α-glucose
3.63	dd		63.26	CH_2 glycerol
3.65	m	3.77	73.51	CH β-galactose
3.70	m		61.87	CH α-galactose
3.75	m	1.88		CH lysine
3.75	m	2.05–2.11		CH glutammate
3.76	m		72.8	CH glycerol
3.77	m	3.65	63.26	CH β-galactose
3.84	m		72.09	CH α-glucose
3.85	m		60.70	CH β-glucose
3.91	m			CH tyrosine
3.91	m	3.65	69.36	CH β-galactose
3.95	m	3.80	70.88	CH α- galactose
4.07	t	3.71–3.95	71.22	CH α-galactose
4.11	q	1.32	69.31	CH lactic acid
4.56	d	3.47	97.22	CH anomeric β-galactose
4.65	d	3.27	96.65	CH anomeric β-glucose
5.21	d	3.58	92.51	CH anomeric α-glucose
5.24	d	3.70–3.95–4.07	93.09	CH anomeric α-galactose
6.85	d	7.02		C3,C5 ring tyrosine
7.02	d	6.85		C2,C6 ring tyrosine
8.43	s			CH formic acid

100%. The prediction ability was estimated, either on a test set containing 30% of samples randomly extracted, or by the "leave one out" method that repeats the model calculations n times, each time leaving out a different observation and predicting it from a model fitted to the other $n-1$ observations.

For milk samples, the isotopic parameters alone were evaluated. Fig. 4 shows a scatter plot of $^{13}C/^{12}C$ vs. $^{15}N/^{14}N$. From this plot, the importance of the isotopic parameters can be deduced; milk samples are separated according to geographical origin. The higher ^{13}C content in Campania milk could be due to a greater amount of maize in the buffalo diet (Rossmann et al., 1998).

3.2. Mozzarella samples

3.2.1. 1st data set

The moisture, ash, Zn and Ba contents were excluded from the statistical analyses since they showed correlations with other variables. Ni, Pb and Se content were also excluded because they were below the detection limit of the ICP-AES. Therefore, PCA was performed on a matrix of 13 analytical parameters for 14 samples. The first five PCs explain 82% of the total variance. The scatter plot of PC2 vs. PC1, explaining 47% of the total variance (Fig. 5), indicates that samples from Apulia and samples from Campania are not as well separated as the respective milk samples. A little separation according to geographical origin was achieved out on PC2. The loadings of variables showed that some parameters, which were important for the discrimination of milk samples, did not contribute to the discrimination of mozzarella samples. For example, Na content has a high loading on PC1, as for milk samples, but for mozzarella samples, the discrimination is on PC2, which is more correlated with Li, Mg, K and Ca contents. Indeed, Na content is affected by the salting process. Therefore, it is possible that the cheese manufacturing processes undergone by the milk tend to reduce the remarkable differences existing between unprocessed milk samples.

3.2.2. 2nd data set

A typical ^{1}H NMR spectrum of the aqueous extract of a mozzarella sample is showed in Fig. 6. In this spectrum peaks of some organic acids, sugars and amino acids can be distinguished and their assignments are listed in Table 1.

The heights of those signals showing neither overlapping nor correlation with other signals were considered and normalized to the sum of heights of the same

Table 2
Loadings of the original set of variables associated with the first four PCs obtained from NMR data of the aqueous extracts of mozzarella samples

Variable	PC1	PC2	PC3	PC4
8.18 ppm	−0.36	−0.08	0.44	0.01
4.55 ppm	−0.41	−0.09	−0.32	−0.07
4.43 ppm	−0.41	−0.13	−0.30	0.11
3.37 ppm	−0.38	0.10	0.27	0.18
3.33 ppm	−0.26	0.11	0.06	−0.83
3.21 ppm	−0.45	0.17	−0.02	0.17
3.04 ppm	−0.13	0.47	−0.57	−0.04
1.91 ppm	0.08	0.66	0.05	0.33
$^{15}N/^{14}N$	0.25	−0.21	−0.41	−0.07
$^{13}C/^{12}C$	0.19	0.46	0.18	−0.34

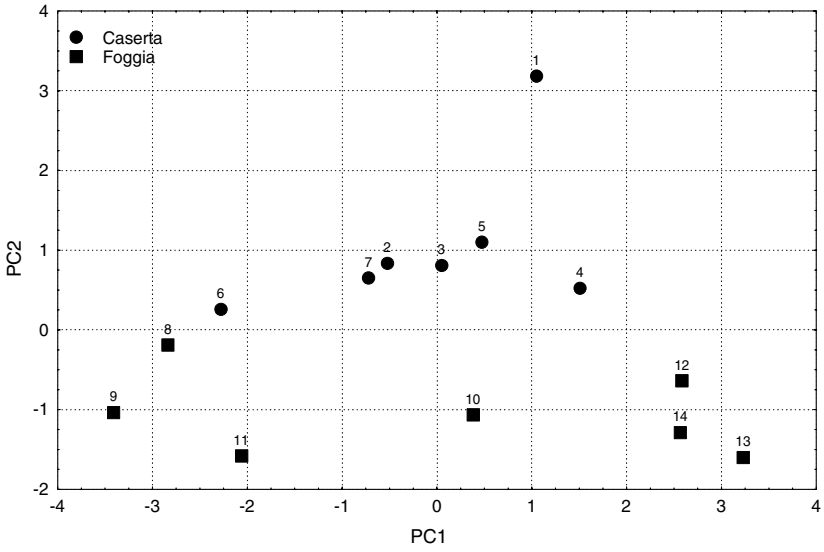

Fig. 7. Scatter plot of the scores of the mozzarella samples from the first two principal components PC1 and PC2 obtained using NMR and IRMS data.

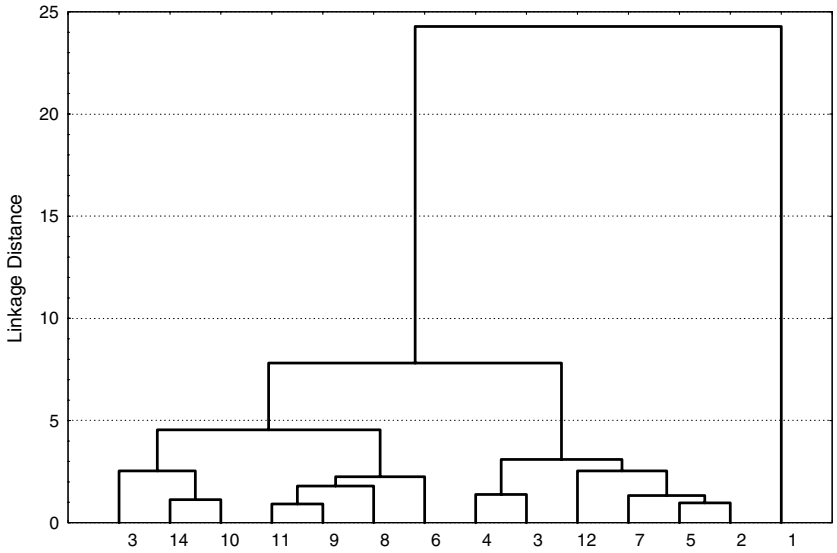

Fig. 8. Dendrogram of the mozzarella samples obtained using NMR and IRMS data.

peaks. These signals were submitted to analysis of variance to select the most discriminating peaks to be used as variables, together with isotope ratios, for the statistical analysis.

The first four PCs, obtained by the PCA, explain 85% of the total variance. From the scatter plot (Fig. 7), it can be seen that the separation of samples according to geographical origin was realized on PC2: samples from Campania have positive scores whereas samples from Apulia have negative scores. From the loadings in Table 2, it can be seen that the ratio $^{13}C/^{12}C$ and peaks at 1.91 and 3.04 ppm are the variables with the higher loading for the geographical discrimination. The last two signals are due to the acetate and tyrosine, respectively. The acetate is a marker of heterofermentative metabolism with respect to the osmofermentative metabolism (Bottazzi, 1993), whereas tyrosine content is a marker of proteolytic activity (Coppola et al., 1988).

From the dendrogram obtained with HCA (Fig. 8), seven clusters are visible: three of them contain samples from Campania and the other four contain samples from Apulia. These clusters are visible at a similarity level of 53% on the dendrogram. The latter was obtained by scaling the same variables used for the PCA and applying the average linkage to Euclidean distance between samples.

With discriminant analysis, applied on the first four PCs, the classification ability and the prediction ability were 100% and 93%, respectively.

4. Conclusion

The data collected on milk samples were useful for their geographical discrimination. A noteworthy result was obtained with isotopic ratios that could be used alone for this differentiation, mainly reflecting a different type of feeding. The most interesting aspect of this result consists of the rapidity of IRMS analysis in comparison with routine analyses.

As far as buffalo milk mozzarella samples are concerned, only the coupling of the isotopic parameters with NMR data determined on the aqueous mozzarella extracts allowed good results concerning geographical origin discrimination. This was due to compounds deriving from metabolic activities, which occur during the processes of mozzarella preparation. This measure should be repeated in following years and on a higher number of samples in order to analyse the annual variability. The future development of this work will consist of extending this method to the characterization of other typical cheeses.

References

Addeo, F., Moio, L., Chianese, L., Stingo, C., Resmini, P., Berner, I., Krause, I., Di Luccia, A., & Bocca, A. (1995). Use of plasmin to increase the sensitivity of the detection of bovine milk in ovine cheese by gel isoelectric focusing of γ 2-caseins. *Milchwissenschaft, 50*, 83–85.

Bottazzi, V. (1993). *Microbiologia lattiero-casearia*. Bologna: Edagricole.

Bréas, O., Reniero, F., & Serrini, G. (1994). Isotope ratio mass spectrometry: Analysis of wines from different European countries. *Rapid Communication in Mass Spectrometry, 8*, 967–970.

Brescia, M. A., Di Martino, G., Fares, C., Di Fonzo, N., Platani, C., Ghelli, S., Reniero, F., & Sacco, A. (2002a). Characterization of italian durum wheat semolina by means of chemical analytical and spectroscopic determinations. *Cereal Chemistry, 79*(2), 238–242.

Brescia, M. A., Caldarola, V., de Giglio, A., Benedetti, D., Fanizzi, F. P., & Sacco, A. (2002b). Characterization of the geographical origin of Italian red wines based on traditional and nuclear

magnetic resonance spectrometric determinations. *Analytica Chimica Acta, 458*, 177–186.

Brescia, M. A., Caldarola, V., Buccolieri, G., Dell'Atti, A., & Sacco, A. (2003). Chemometric determination of the geographical origin of cow milk using ICP-OES data and isotopic ratios. *Italian Journal of Food Science, 3*, 329–336.

Coppola, S., Parente, E., Dumontet, S., & La Peccerella, A. (1988). The microflora of natural whey cultures utilized as starters in the manufacture of Mozzarella cheese from water–buffalo milk. *Le Lait, 68*, 295–310.

European Communities (1992). Regulation 2081/92, Official Journal of European Communities, L208.

European Communities (1996). Regulation 1107/96, Official Journal of European Communities, L148.

Kornexl, B. E., Werner, T., Rossmann, A., & Schmidt, H. L. (1997). Measurement of stable isotope abundances in milk and milk ingredients – a possible tool for origin assignment and quality control. *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung A – Food Research and Technology, 205*, 19–24.

Manca, G., Camin, F., Coloru, G. C., Del Caro, A., Depentori, D., Franco, M. A., & Versini, G. (2001). Characterization of the geographical origin of Pecorino Sardo cheese by casein stable isotope (C-13/C-12 and N-15/N-14) ratios and free amino acid ratios. *Journal of Agricultural and Food Chemistry, 49*, 1404–1409.

Mauriello, G., Moio, L., Genovese, A., & Ercolini, D. (2003). Relationships between flavoring capabilities, bacterial composition, and geographical origin of natural whey cultures used for traditional water–buffalo Mozzarella cheese manufacture. *Journal of Dairy Science, 86*, 486–497.

Pillonel, L., Badertscher, R., Bütikofer, U., Casey, M., Dalla Torre, M., Lavanchy, P., Meyer, J., Tabacchi, R., & Bosset, J. O. (2002). Analytical methods for the determination of the geographical origin of Emmentaler cheese. Main framework of the project; chemical, biochemical, microbiological, colour and sensory analyses. *European Food Research and Technology, 215*, 260–267.

Romano, P., Ricciardi, A., Salzano, G., & Suzzi, G. (2001). Yeast from Water Buffalo Mozzarella, a traditional cheese of the Mediterranean area. *International Journal of Food Microbiology, 69*, 45–51.

Rossmann, A., Kornexl, B., Versini, G., Pichlmayer, F., & Lamprecht, G. (1998). Origin assignment of milk from alpine regions by multielement stable isotope ratio analysis (Sira). *La Rivista di Scienza dell'Alimentazione, 1*, 9–21.

Rossmann, A., Haberhauer, G., Holzl, S., Horn, P., Pichlmayer, F., & Voerkelius, S. (2000). The potential of multielement stable isotope analysis for regional origin assignement of butter. *European Food Research and Technology, 211*, 32–40.