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Chemometric analysis of minerals and trace elements in raw cow milk from the community of Navarra, Spain

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ABSTRACT

The concentrations of protein, fat, five minerals (Na, K, P, Ca and Mg) and nine trace elements (Fe, Zn, Cu, Mn, Se, Al, Cd, Cr and Pb) have been determined in 347 samples of raw cow milk from the community of Navarra, north Spain, using infrared analysis, atomic absorption spectrometry (flame and electrothermal atomisation) and inductively coupled plasma atomic emission spectroscopy. A preliminary chemometric study with the use of pattern recognition methods was carried out in order to characterise, classify and distinguish the different collected samples on the basis of their contents. Principal component analysis (PCA) has permitted the reduction of 16 variables to five principal components which interpret reasonably well the correlations of these studied variables. These variable associations may be attributed to intrinsic (lactogenesis) and other extrinsic factors, such as seasonal variation, animal feeding or geographical situation. Changes in these contents during different seasons were also assessed and consistently interpreted. Linear discriminant analysis (LDA) was used to explore cow milk samples, classifying according to season or geographical location, providing complementary information to PCA. This work shows that PCA and LDA are useful chemometric tools for the multivariate characterisation of raw cows' milk.

1. Introduction

Many constituents of cows' milk can be broadly categorised according to their physical properties or/and physiological functions. In this complex biological fluid, minerals occur in chemical equilibrium between the free ions and complexes with various components, such as protein, lipids, carbohydrates and low molecular weight ligands like citrate and amino acids (Vegarud, Langsrud, & Svenning, 2000).

Mineral and trace element concentrations in raw cows' milk are not constant but mainly vary according to two kinds of factors, those related with secretion from the mammary gland, such as the lactation state, animal species and health status, and extrinsic factors, such as season, dairy cattle ration (nutritional status of cow), environment (nature of soil and locality of the farm). In this respect, several studies have been carried out to assess mineral content of cows' milk from different areas (Dobrzarnski, Kolacz, Górecka, Chojnacka, & Bartkowiak, 2005; Hermansen, Badsnerg, Kristensen, & Gundersen, 2005; Lante, Lomolino, Cagnin, & Spettoli, 2004; Licata et al., 2004; Muñiz-Naveiro et al., 2005; O'Brien, Mehra, Connolly & Harrington, 1999; Orak, Yanardag, & Hugul, 2000; Rodríguez, Sanz, & Díaz, 2001; Simsek, Gültekin, ÖKsüz, & Kurultay, 2000), as well as to evaluate preliminary correlations between animal feeding, manufacturing process and elemental profile in cows' milk and dairy products (Coni et al., 1996; Coni, Bocca, Ianni, & Caroli, 1995; Coni, Caroli, Ianni, & Bocca, 1994).

Multivariate data analysis may be used to obtain additional information. Pattern recognition techniques have been applied to estimate food quality, based on its mineral and trace element composition (Szefer, 2007). Chemometric techniques applied to the data of macro- and micronutrients in raw cow milk might provide an interesting and promising approach to classification and to identify possible sources of influence on elemental profiles.

The present study has applied principal component analysis (PCA) for processing data concerning the concentration of protein, fat, minerals (Na, K, P, Ca and Mg) and trace elements (Fe, Zn, Cu, Mn, Se, Cr, Al, Cd and Pb) in raw cows' milk, in order to distinguish groups belonging to different seasons and area of milk collection, and to study any association existing among these elements which reflect the main influence factors mentioned above. Linear discriminant analysis was also applied, as a supervised pattern recognition method, with the objective of describing the discriminant functions and, therefore, data distributions might be identified as criteria of seasonal or area classification for raw cows' milk samples studied.

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2. Experimental

2.1. Apparatus

A Milkoscan apparatus (MilkoScan FT6000, Foss Electric, Hillerød, Denmark) was used to evaluate the protein and fat contents in raw milk samples. Ethos Plus microwave labstation with computer-controlled easywave software (Milestone, Sorisole, Italy) was used to digest the milk samples. A Jobin-Yvon JY38S Plus Sequential (Horiba Jobin Yvon S.A.S., Longjumeau, France) ICP-AES spectrometer powered by a 40.68 MHz radiofrequency generator at 1100 W was used for Ca, Mg, P and Al determinations. This instrument operates in the sequential measurement mode (radial measurements) and has a Czerny-Turner mounting with a 2400 grooves mm^{-1} holographic plane grating, with focal length of 1 m. The main argon flow was 12 l min^{-1}, and the cooling flow 0.45 l min^{-1}. The nebuliser was a Meinhard type with Scott concentric nebulisation chamber, operated at 34 psi, with argon aerosol gas, and a 0.6 l min^{-1} flow rate. Sample aspiration was forced by means of a Spetec Perimax 12 peristaltic pump with a 1.4 l min^{-1} sample delivery rate. The analytical lines (and the integration times) used for the different elements were: calcium, 717.933 nm (0.5 s); magnesium, 483.231 nm (0.5 s); phosphorus, 213.618 nm (1.0 s) and aluminium, 396.152 nm (0.5 s). The signals were obtained 15 mm above the load coil and background corrected.

Nebuliser clogging was avoided by using suitable uptake and rinsing cycles. A 30 s pre-observation aspiration time and a 30 s rinse time with ultrapure water were applied. Warm-up time (plasma on) was 30 min.

A Perkin-Elmer Model AAnalyst 800 atomic absorption spectrometer equipped with flame and graphite furnace atomisers and Zeeman background correction was used (Perkin Elmer, Waltham, MA). Sodium and potassium were determined by atomic emission at 589.0 and 766.5 nm, using an air/acetylene flame with an oxidant fuel flow of 17.0 and 2.0 ml min^{-1}, respectively. Zinc, iron, copper and manganese measurements were performed by atomic absorption at 213.9, 248.3, 324.8 and 279.5 nm, using hollow cathode lamps operated at 15, 30, 15 and 20 mA and bandwidths of 0.7, 0.2, 0.2 and 0.2 nm, respectively. A high-sensitivity nebuliser was used.

Transversely-heated graphite tubes with end caps supplied by Perkin-Elmer, were used for chromium, selenium, cadmium and lead determinations. Measurements (integrated absorbance peak areas) were carried out by using single element hollow lamps (chromium, cadmium and lead) and electrodeless discharge lamp exclusively for selenium. The instrumental setting and optimising temperature program of the spectrometer are summarised in Table 1. Argon was used as the inert gas, the flow rate being 250 ml min^{-1} during all stages except atomisation, when the flow was stopped.

Table 1
Instrumental parameters and graphite furnace program (temperature and time) for Cr, Se, Cd and Pb determination in raw cow milk digested samples

	Chromium	Selenium	Cadmium	Lead
Instrumental parameters				
Wavelength (nm)	357.9	196	228.8	283.3
Slit width (nm)	0.7	2	0.7	0.7
Lamp current (mA)	25	280	4	10
Sample – mod. vol. (μl)	10–10	10–20	20–10	15–5
Measurement mode	Peak area	Peak area	Peak area	Peak area
Background correction	Zeeman	Zeeman	Zeeman	Zeeman

Step	Temperature (°C)	Ramp (s)	Hold (s)	Argon flow (ml min^{-1})	Read on
Temperature programs					
	Cr, Se, Cd, Pb	Cr, Se, Cd, Pb	Cr, Se, Cd, Pb		
Drying	130	15, 20, 15, 15	40, 60, 45, 40	250	–
Charring	1400, 1700, 1600, 1350	10	20, 10, 20, 20	250	–
Atomisation	2300, 2600, 2400, 2450	0	5	0	Yes
Cleaning	2500, 2600, 2400, 2450	1	5, 4, 3, 3	250	–
Cooling	20	–	–	250	–

2.2. Collection and handling of raw cow milk samples

A total of 347 samples were collected following a strict protocol during the four seasons from different farms in the Community of Navarra (Spain): Zone 1 – Northwest region: Ultzama (n = 76), Norte Aralar (n = 32), Alto Bidasoa (n = 63), and Baztán (n = 122); Zone 2 – Pirenaica region: Auñamendi (n = 32), and Zone 3 – Media-Ribera region: Tafalla (n = 22). The region studied is shown in Fig. 1.

Raw cow milk was collected in acid-washed 100 ml low-density polyethylene bottles (Plastibrand®, Brand, Wertheim, Germany). In the laboratory, raw cow milk was stored at −20 °C. Bottles were opened on a laminar flow bench, using vinyl talc-free gloves (Rotiprotect® Carl Roth, Karlsruhe, Germany). Special care was devoted to minimise the risk of external contamination.

2.3. Reagents and solutions

All the chemicals used were of the highest purity available and all materials were nitric acid-washed and rinsed with ultrapure water.

High quality water, obtained using a Milli-Q system (Millipore Iberica S.A., Madrid, Spain) with a resistivity of 18.1 MΩ cm was used exclusively.

Concentrated 65% nitric acid from Merck (Darmstadt, Germany) was further purified by sub-boiling distillation in a quartz still (Hans Kürner, Rossenheim, Germany).

Ca, PO_4^{3-}, Mg, Na, K, Fe, Cu, Zn, Al, Pb, Cd, Se, Cr and Mn standard solutions (1000 mg l^{-1}) were supplied by Merck. The calibration standard solutions used were made by appropriate dilution of the stock standards.

A solution of rhodium in citric acid, (containing 1 mg ml^{-1} Rh, prepared by dissolving 0.5 g citric acid monohydrate (Pro analysis, Merck) in 5 ml of $Rh(NO_3)_2$ (1000 mg l^{-1} Certipur, Merck), a solution of magnesium nitrate (0.150 g $Mg(NO_3)_2 \cdot 6H_2O$; Suprapur, Merck) diluted in 100 ml ultrapure water, and a solution of magnesium nitrate–ammonium dihydrogenphosphate (0.6 g $Mg(NO_3)_2 \cdot 6H_2O$ (Suprapur, Merck) and 1 g $NH_4H_2PO_4$ (Suprapur, Merck), both diluted in 100 ml ultrapure water) were used as matrix modifiers for selenium, chromium, cadmium and lead, respectively.

2.4. Analytical procedures

Fat and protein contents in raw milk samples were obtained by infrared analysis in accordance with the standard method (IDF 141C:2000) described by the International Dairy Federation. (2000).

In order to determine the concentration of minerals and trace elements, 5 ml of the raw cows' milk were placed into a high-pressure Teflon bomb and digested with 15 ml of sub-boiling nitric acid on the Ethos Plus microwave workstation. The microwave digestion program applied included the following temperature stages: 25–140 °C for 10 min, 140–150 °C for 4 min, 150 °C for 7 min, 150–180 °C for 10 min, and 180 °C for 20 min, all of them at 1000 W, followed immediately by ventilation at room temperature

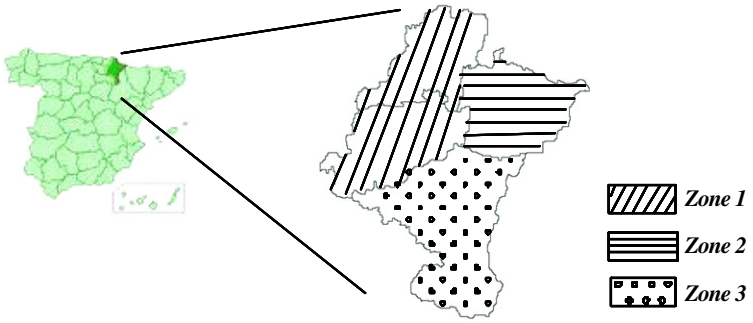

Fig. 1. Sampling location site.

(20 min). The acid-digested sample solution was diluted in a 25 ml volumetric flask with ultrapure water. Triplicate digestions were conducted for each sample.

Calibrations were accomplished using direct calibration against aqueous standards. Working standard solutions were made up each day by dilution from stock standard solution in enough sub-boiling nitric acid to give a final acid concentration similar to digest samples.

2.5. Quality control

The reliability of the method was tested with the certified reference material SRM 1549. All the results for the measurements of minerals and trace elements in reference material are summarised in Table 2. Recoveries of the elements analysed performed by spiking the SRM 1549 samples before digestion and an internal aqueous quality control, were satisfactory, ranging from 96.9% to 103.2%.

Detection limit (LOD) was calculated according to the definition and criteria established by IUPAC ($x_b + 3\sigma_b$), as the average of three times the standard deviation of the reagent blank. Table 2 contains the LOD values for the mineral and trace elements analysed, expressed in terms of raw cow milk ($n = 12$). Throughout the course of the study, both SRM 1549 digestion sample in-house controls were run, to satisfy the criteria established in the quality program and to provide on-going quality control information.

Table 2
Quality control parameters

	Recovery (%)		Reference material		LOD
	SRM 1549 ($n = 10$)	Int. standard ($n = 10$)	Certified	Experimental	
Na	100.2 ± 1.9	101.6 ± 2.2	4.97 ± 0.10	4.92 ± 0.02	0.023[a]
K	100.6 ± 1.9	101.5 ± 2.7	16.9 ± 0.3	16.74 ± 0.24	0.028[a]
Ca	100.4 ± 1.3	101.0 ± 2.8	13.0 ± 0.5	12.73 ± 0.35	0.111[a]
P	98.8 ± 1.0	100.4 ± 1.5	10.6 ± 0.2	10.47 ± 0.16	0.183[a]
Mg	103.2 ± 0.9	99.8 ± 0.5	1.20 ± 0.03	1.21 ± 0.09	0.017[a]
Zn	98.1 ± 0.9	98.6 ± 1.3	46.1 ± 2.2	48.00 ± 0.67	0.020[a]
Fe	98.8 ± 1.2	99.8 ± 1.3	1.78 ± 0.10	1.80 ± 0.03	0.010[a]
Cu	99.1 ± 1.8	99.6 ± 1.4	0.7 ± 0.1	0.6 ± 0.1	0.921[b]
Mn	99.7 ± 1.1	98.3 ± 0.6	260 ± 60[d]	236 ± 28[d]	0.280[b]
Cr	102.0 ± 0.8	101.8 ± 1.2	2.6 ± 0.7[d]	1.9 ± 0.1[d]	0.088[b]
Se	98.8 ± 2.5	99.1 ± 1.3	110 ± 10[d]	108 ± 3[d]	0.468[b]
Al	96.9 ± 1.3	99.4 ± 1.6	(2)[d]	1.60 ± 0.36[d]	1.167[b]
Cd	101.5 ± 2.6	101.2 ± 4.6	0.5 ± 0.2[d]	0.4 ± 0.2[d]	0.087[c]
Pb	99.1 ± 2.7	99.2 ± 2.3	19 ± 3[d]	17 ± 2[d]	0.111[c]

Detection limits expressed in raw cow milk, recovery assays and analysis of the certified reference material SRM 1549 non-fat milk powder (mg or $\mu g\, g^{-1}$).
[a] $mg\, l^{-1}$.
[b] $\mu g\, l^{-1}$.
[c] $ng\, l^{-1}$.
[d] $\mu g\, g^{-1}$.

2.6. Statistical data processing

Statistical data processing univariate characterisation was carried out to check and describe the distribution data of each element analysed.

A data matrix, whose rows are the different raw cow milk samples analysed (cases) and whose columns are descriptors corresponding to protein, fat, minerals and trace elements content determined (variables) was built for further multivariate analysis.

The 347 cases are divided into four seasons and three areas, and 16 variables (protein, fat, Na, K, Ca, P, Mg, Fe, Zn, Cu, Mn, Se, Cr, Al, Cd, Pb) were taken into consideration, as described above. The data were autoscaled before PCA, in order to achieve independence on the different scale factors of the element concentration, whereas LDA was performed on the original data since its results are not affected by the scale factors of the different variables (Marengo & Aceto, 2003).

PCA reduces the dimensionality of the original data matrix retaining the maximum amount of variability. It provides a new set of variables (principal components, PCs) which facilitates the discovery of patterns hidden in the dataset.

LDA, as a supervised technique, provides a discriminant model with respect to the descriptors previously defined (seasons or areas). Both, univariate and multivariate analyses were performed by means of the statistical package SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Macronutrients, minerals and trace elements analysis in raw cow milk

Descriptive statistics of all data for protein, fat, minerals and trace elements analysed in raw cow milk samples are presented in Table 3. The concentration distributions are characterised by the arithmetic mean value, standard deviation at 95% confidence interval and range. Nearly all element distributions show a remarkable symmetry, proved by means of the low values expressed by skewness statistics. Exclusively, certain elements such as copper, chromium, aluminium and cadmium appear to be positively skewed with scores clustered to the left at the low values, and at the same time, high positive Kurtosis value, indicating a rather peaked distribution (clustered in the centre). On the other hand, protein and fat data present negative skewness values that indicate a clustering of scores at the end (right-hand side of distribution graph).

Despite the test results of normality (Kolmogorov–Smirnov statistic), suggesting a violation of the assumption of normality, a fact quite common in larger samples (Pallant, 2003), the inspection of the normal probability plots studied (Normal *Q*–*Q* plot) reveals a Gaussian model for all data distribution.

Table 3
Descriptive statistics of data in raw cow milk samples.

	Range			Mean	s.d.	Skewness	Kurtosis
	n	Min.	Max.				
Protein (% w/w)	347	2.66	3.69	3.19	0.16	−0.094	0.154
Fat (% w/w)	347	1.93	4.59	3.81	0.31	−0.758	3.964
Ca (mg l^{-1})	347	760	1380	970	98	0.613	0.372
P (mg l^{-1})	347	550	1123	785	98	0.571	0.133
Mg (mg l^{-1})	347	69.4	126.4	91.8	9.4	0.473	0.125
Na (mg l^{-1})	347	234	502	372	40	0.435	1.232
K (mg l^{-1})	347	1143	1554	1344	65	0.003	0.229
Zn (μg l^{-1})	347	2532	7737	4631	855	0.490	−0.014
Fe (μg l^{-1})	347	57.6	759.3	290.0	104.1	0.794	1.306
Mn (μg l^{-1})	347	7.1	54.8	29.1	9.2	0.377	−0.365
Cu (μg l^{-1})	347	7.2	357.8	51.8	36.1	3.834	22.910
Cr (μg l^{-1})	347	<LOD	24.35	4.03	3.43	1.948	5.406
Se (μg l^{-1})	347	<LOD	40.60	9.77	7.61	1.137	1.364
Al (μg l^{-1})	347	47	1598	369	209	1.921	7.457
Pb (μg l^{-1})	347	0.55	18.70	5.23	2.85	1.091	2.208
Cd (μg l^{-1})	347	<LOD	1.73	0.40	0.28	1.822	4.246

Table 4
Component in Varimax rotated space

	Component				
	1	2	3	4	5
Mg	**0.898**	−0.052	0.011	−0.068	0.008
P	**0.897**	−0.046	−0.041	−0.032	0.018
Ca	**0.890**	−0.034	−0.069	−0.033	−0.036
Protein	**0.704**	0.002	0.385	−0.052	0.064
Na	**0.546**	0.004	0.052	**−0.442**	−0.257
Fat	**0.449**	−0.034	0.323	0.301	0.139
Fe	0.040	**0.845**	0.115	0.005	−0.003
Mn	−0.138	**0.824**	−0.063	0.187	−0.080
Pb	0.010	**0.672**	0.038	−0.123	−0.016
K	−0.052	0.093	**−0.846**	0.080	0.095
Zn	0.015	**0.463**	**0.666**	0.131	−0.060
Al	0.174	0.179	0.188	**−0.699**	0.261
Cu	0.200	0.135	**0.472**	**0.504**	0.120
Cd	−0.078	0.293	0.126	0.386	−0.088
Cr	−0.078	−0.003	0.018	−0.191	**0.821**
Se	0.153	−0.360	−0.396	0.167	**0.551**

3.2. Principal component analysis

3.2.1. Global approximation

The data set of parameters analysed was subjected to a PCA, in order to decrease the number of descriptors retaining the maximum amount of variability present in the experimental data. Prior to performing PCA, the suitability of the data for factor analysis was checked. Inspection of the correlation matrix between variables revealed a great number of coefficients higher than 0.250 (data not shown). The determinant value (0.00325) of the correlation matrix was low. All variables show a significant correlation with at least one other variable. The Kaiser–Meyer–Olkin measure of sampling adequacy was 0.738, exceeding the recommended value of 0.6, and the Barlett's test of sphericity (value 935.30) reached statistical significance ($p < 0.001$), supporting the factorability of the correlation matrix. The matrix is, therefore, appropriate for principal component analysis.

When PCA was applied to the autoscaled data matrix, five principal components with eigenvalues exceeding one were extracted, according a Kaiser Criterion which explains up to 64% of the total variance (23.0%, 17.6%, 9.1%, 7.4% and 6.9%, respectively). In order to get a more explicit assignment of experimental variables, the PC extracted correlation matrix was subjected to the Varimax rotation. The rotated factor matrix is shown in Table 4. After orthogonal rotation, easier interpretation of the factors was possible. This five-factor model interprets reasonably well the correlations of these studied variables.

The obtained variable associations may be attributed to the intrinsic and extrinsic factors. The first factor characterises those parameters (protein, fat, calcium, magnesium, phosphorous, sodium) associated with the lactogenesis process at the mammary gland, homeostatically controlled and regulated by the secretor cell through a fully known mechanism (Jensen, 1995).

Iron, manganese and lead are the dominating variables in the second factor, although zinc, selenium, cadmium, aluminium and copper are also correlated to a lower extent. The variability of these elements contents is a result of seasonal variation. Thus, it was found, except for selenium, a significant progress of the concentration values from summer to spring throughout the period of study. This fact can be explained by two different factors: feeding and metabolic adaptation during the climatic season. The use of enriched fodder, including essential minerals (iron, manganese, copper, zinc) and potential toxic elements (aluminium, lead and cadmium) incorporated in the industrial handling process as contaminant from ingredients; and the higher bioavailability of these elements in the wet period, contribute to their secretion in cows' milk. In the case of selenium, seasonal conditions influence the amount of this element in alfalfa and other plants, therefore pasture selenium levels tend to be lowest in the spring when the plant is growing the fastest. As a consequence, the selenium concentration in cows' milk was gradually declining during autumn, winter and spring months. This is reflected by a negative loading (−0.360) in the second rotated factor.

The third factor mainly reflects the complexity of the geographical situation. The found correlation between parameters studied indicates that it arises from some additional sources, involving geomorphological environment and weather conditions in the study zone. In this sense, the representative minerals, zinc and copper, have significant positive loadings (0.666 and 0.472, respectively), together with the lower coefficients found for the macronutrients, protein and fat, which characterise the high quality of cows' milk from the north zone against the south region. Additionally, selenium-poor soil located in the north zone has a marked effect on levels of selenium available to plants and consequently to ruminants that feed on them. This finding is proved by the negative loading (−0.396). On the other hand, a marine influence is related to the lowest and highest potassium values determined in north and south cows' milk, respectively.

The fourth factor is not intuitive. It could be related to modification in food habits between climatic seasons with incorporation of fodder into the diet. A high corporeal storage of cadmium when a vegetarian diet with high phytate contain is supplied has been demonstrated in animal studies. Moreover, this kind of diet is a natural source of copper and manganese, which have a synergic effect on dietary intake of this heavy metal (Mata, Sánchez, & Calvo, 1996). These findings are in agreement with the coefficients found in this factor (0.504, 0.386 and 0.187 for copper, cadmium and manganese, respectively). In addition, the usual practice of a rich intake of alfalfa, fodder and cereals causes a small increase in fat content of cow milk (coefficient value: 0.301), and a reduced need to drink water by cattle. Hence, cows meet their salt requirement not by a salt lick but rather by means of vegetarian diet, intrinsically linked with a lower intake of tap water, treated in the purifying plant with aluminium salts to remove organic matter. Both dietary intake facts are indicative of negative coefficients (−0.442 and −0.699, for sodium and aluminium, respectively) associated in this fourth factor.

Finally, the fifth factor can be interpreted *a priori* by a simple argument. The additives and dietetic supplements used traditionally in animal feeding are mainly reflected by selenium and chromium

with highly positive loadings (0.551 and 0.821, respectively). The essential role of selenium in cattle health is well known (Nutrition Research Council, 2001). Selenium, principally as selenium yeast, is incorporated in a mineral pre-mix for addition to fodder. In the same way, several studies have demonstrated the benefit of chromium supplementation (Escobosa & Ávila, 2001) in inorganic or organic form, as chromium (III) picolinate and enrichment yeast (Hegoczki, Suhajda, Janzso, & Vereczkey, 1997).

Principal component analysis allows visualisation of data in graphical representations, simplifying the observation and interpretation of information. A scatter plot of loadings for studied macro- and micronutrients (Fig. 2) shows the associations obtained between elements after the Varimax orthogonal rotation, in order to visualise the discriminating efficiency of the principal components.

The application of a factor analysis model to data from multielemental analysis of cows' milk samples in the space defined by the first, second and third principal factors, is noteworthy (Fig. 3). As can be seen in the three-dimensional plot, four clusters are clearly distinguished according to the season in which the cow milk samples were collected, setting a differentiation criterion.

In short, it is necessary to emphasise that this chemometric methodology provides a useful tool, using a statistical package widely used by the scientific community, to approach a complex problem, difficult to interpret using traditional logic.

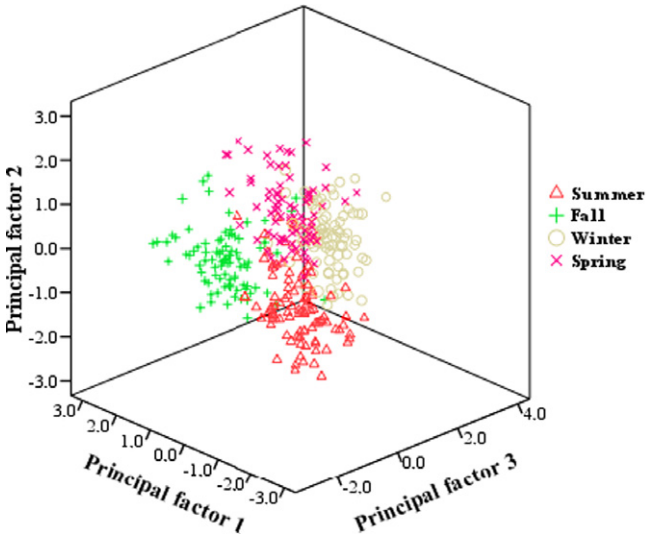

Fig. 3. Three-dimensional representation of the principal component scores for raw cow milk samples differentiated according to seasonal criteria.

3.2.2. Factor analysis by seasons

Factor analysis might provide detailed information on the changes and associations existing among the different elements studied, in view of the large number of samples collected in each season (summer: 93, autumn: 87, winter: 84 and spring: 81).

Table 5 shows the rotated component matrix obtained from the principal component correlation matrix, in order to facilitate the interpretation of the results.

The thorough analysis of the obtained factors describes globally the changes in the mineral composition of cows' milk during the course of the year (Lante et al., 2004; Moreno-Rojas, Amaro, & Zurera, 1993; Orak et al., 2000; Ricón, Moreno, Zurera, & Amaro, 1994; Rodríguez et al., 2001). Seasonal variations in the composition of fat and casein have been found in cows' milk. Thereby, calcium, phosphorus and magnesium are grouped in the first factor together with the protein fraction during the summer and spring whereas these macrominerals are separated on different factors and strongly associated to fat in autumn and winter (first and fourth factors, respectively). This fact is in agreement with the physiological basis of mineral secretion in milk synthesis by the lactating mammary gland. Macrominerals occur under the chemical forms of calcium phosphate, calcium phosphocaseinate and free magnesium, associated with the colloidal suspension of casein micelles (Hazell, 1985; Silva, Lopes, Nóbrega, Souza, & Nogueira, 2001).

Sodium appears generally in a single factor (summer: fourth factor; autumn: seventh factor; winter and spring: fifth factor) together with other ionic elements, positively associated with magnesium or negatively with potassium. It denotes an exclusive saline source, used routinely in many diets for cattle feeding.

Feeding and regional factors are showed separately in factors 2 and 3 during summer, and 4 and 5 during spring. On the contrary, during cool seasons, typical diets based on high concentrates, fodders or ingredients mix are represented in a single component factor, fourth and second in autumn and winter, respectively.

The occurrence of selenium and chromium in the summer rotated matrix (seventh factor) must be emphasised. It recognises the use of mineral supplements, commonly selenium and chromium, with a broad range of benefits to animal health during the dry period.

Selenium supplementation administered in a typical commercial dairy concentrate is also reflected in the fifth, sixth and second factors in the autumn, winter and spring matrices, coming together with various minerals and trace elements frequently used in formulating rations, such as the essentials elements magnesium, iron,

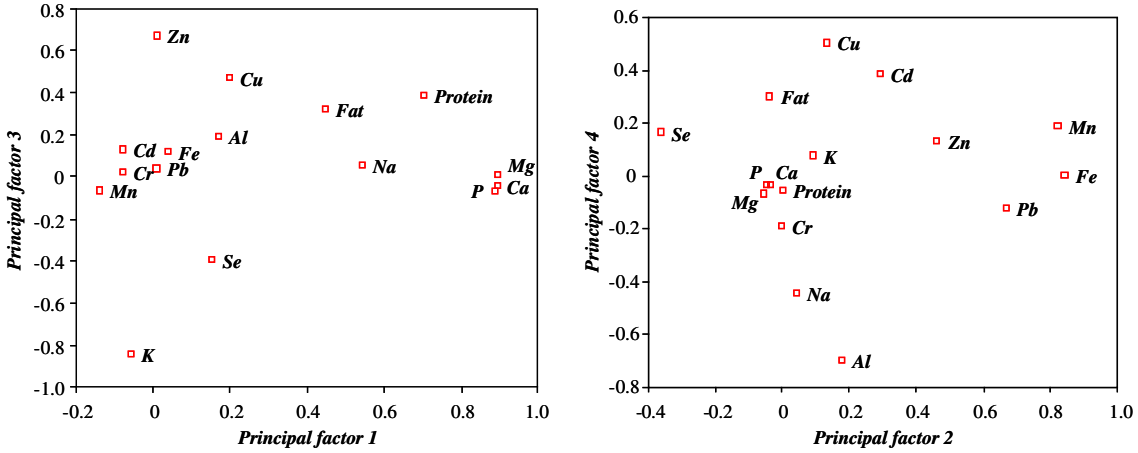

Fig. 2. Principal component analysis plot (two dimensional) for macronutrients, minerals and trace elements analysed (PF1 versus PF3 and PF2 versus PF4).

Table 5
Varimax rotated factor matrix by seasons (Summer, Autumn, Winter and Spring)

	Component						
	1	2	3	4	5	6	7
Summer							
Ca	**0.857**	−0.023	−0.051	0.110	−0.021	0.098	−0.054
P	**0.797**	0.120	0.243	−0.157	0.164	−0.170	0.080
Mg	**0.729**	0.046	0.081	0.101	−0.149	−0.090	0.108
Mn	0.036	**0.931**	0.031	0.075	0.040	0.035	0.046
Fe	0.099	**0.894**	0.033	0.103	0.087	0.091	0.124
Cu	−0.015	**0.521**	0.358	−0.273	−0.164	−0.160	−0.150
Zn	0.326	0.186	**0.678**	−0.244	−0.183	−0.051	−0.152
Protein	0.344	0.160	**0.677**	0.061	−0.036	−0.002	−0.036
Pb	−0.317	−0.241	**0.621**	0.091	0.075	0.168	0.239
Al	−0.010	−0.029	0.062	**0.804**	0.028	−0.173	0.210
Na	0.106	0.202	−0.205	**0.712**	−0.043	0.234	−0.312
Fat	0.054	0.044	−0.044	0.168	**0.859**	0.061	−0.091
K	−0.177	0.017	−0.137	−0.451	**0.677**	−0.012	0.049
Cd	−0.183	0.112	0.115	−0.034	0.072	**0.855**	0.169
Se	−0.168	0.142	**0.529**	−0.011	0.032	**−0.593**	0.330
Cr	0.124	0.101	−0.029	0.028	−0.070	0.077	**0.911**
Autumn							
Ca	**0.844**	0.009	0.116	−0.181	0.059	0.065	0.186
Mg	**0.751**	0.098	**0.443**	−0.032	0.049	−0.233	0.037
Cu	**0.577**	−0.228	−0.157	0.292	0.113	0.188	−0.204
Cr	0.011	**0.780**	−0.026	−0.167	−0.092	0.050	0.019
Al	−0.085	**0.698**	−0.153	0.298	0.152	0.193	0.238
Pb	0.086	**0.498**	−0.373	−0.031	−0.254	0.055	**−0.461**
P	0.034	−0.075	**0.822**	0.072	0.100	0.013	−0.040
Zn	0.292	−0.225	**0.635**	0.213	0.047	0.050	−0.117
Fe	−0.092	−0.020	0.178	**0.865**	0.051	−0.042	0.075
Mn	0.175	**−0.527**	0.067	**0.535**	−0.166	−0.012	0.166
Cd	−0.020	0.047	0.016	**0.520**	−0.494	−0.169	−0.242
Protein	−0.005	0.220	**0.403**	−0.089	**0.702**	−0.106	0.099
Fat	**0.428**	−0.174	−0.025	0.056	**0.660**	0.006	−0.178
Se	−0.044	0.095	−0.070	−0.029	0.066	**0.882**	−0.067
K	0.199	0.188	0.358	−0.155	**−0.425**	**0.612**	0.075
Na	0.079	0.089	−0.134	0.044	−0.030	−0.036	**0.870**

	Component					
	1	2	3	4	5	6
Winter						
P	**0.872**	−0.064	−0.132	−0.114	−0.021	0.208
Protein	**0.843**	−0.058	−0.049	0.218	0.172	−0.004
Zn	**0.579**	**0.401**	0.097	0.096	0.111	0.135
Mn	−0.203	**0.775**	0.139	0.248	0.142	0.132
Cu	0.200	**0.740**	−0.050	−0.029	−0.102	−0.142
Fe	−0.003	**0.663**	0.052	0.123	−0.083	0.106
Al	0.131	−0.140	**−0.767**	0.027	−0.026	−0.020
Cd	0.127	0.171	**0.747**	−0.053	−0.019	−0.344
Pb	−0.071	−0.152	**0.690**	0.282	0.009	0.284
Cr	−0.158	0.041	0.202	**0.697**	0.151	0.059
Fat	0.271	0.189	0.052	**0.692**	−0.227	0.210
Ca	0.274	0.279	−0.285	**0.577**	0.081	−0.007
Na	0.099	−0.095	−0.001	−0.180	**0.856**	0.080
K	−0.093	−0.024	−0.018	−0.222	**−0.743**	0.057
Se	0.050	0.043	0.030	0.175	−0.117	**0.809**
Mg	0.394	0.107	−0.070	−0.017	0.230	**0.701**
Spring						
Ca	**0.802**	−0.052	0.067	−0.131	0.006	−0.276
P	**0.764**	−0.132	−0.308	0.045	0.141	0.317
Zn	**0.749**	0.071	0.130	0.133	−0.038	0.200
Protein	**0.527**	0.315	0.051	0.160	0.062	−0.049
K	0.162	**−0.766**	−0.144	0.096	0.022	−0.303
Fat	0.104	**0.671**	0.129	−0.090	0.184	−0.112
Se	0.186	**0.622**	−0.025	0.214	−0.032	−0.234
Cd	−0.166	0.166	**0.685**	0.085	−0.074	−0.140
Cu	0.140	0.023	**0.658**	−0.147	−0.165	0.201
Fe	0.207	0.011	0.283	**0.809**	0.249	−0.062
Al	−0.062	−0.014	**−0.450**	**0.750**	−0.138	0.155
Na	−0.041	0.025	−0.205	0.011	**0.807**	0.059
Mg	**0.512**	0.303	−0.083	0.005	**0.542**	0.142
Mn	0.211	0.103	**0.490**	0.300	**0.532**	0.054
Pb	0.120	0.057	0.036	−0.061	−0.024	**0.711**
Cr	−0.051	−0.243	−0.027	0.218	0.291	**0.596**

manganese or copper and the potentially toxic impurities aluminium and lead.

The controversial origin of chromium in cow milk is worth mentioning. Several researchers have related the metallic element to a source of environmental pollution (Dobrzarnski et al., 2005; Licata et al., 2004; Simsek et al., 2000). This aspect is clearly observed in the second, third and sixth principal factors in the outstanding seasons of maximum industrial pollution, autumn, winter and spring, respectively; where chromium appears associated to other heavy metals. However, during the summer, chromium is connected with a non-toxic trivalent chemical form, supplied in commercial supplements recommended for dairy cattle nutrition.

The higher need of drinking water by cows during the hot season is explained by positive (aluminium and sodium) coefficients found in the fourth factor of summer-rotated matrix. Additionally, the incorporation of alfalfa or forage in the diet through spring is related to a lower water need. This finding is indicated by the aluminium and sodium negative coefficients showed in the third component.

Finally, with regard to the adventitious contamination by potentially toxic trace elements, mainly expressed by those factors related to the feeding pattern (e.g., cadmium, fourth factor in the autumn matrix) or the regional area (e.g., lead, third factor in the summer matrix).

Just as expected, the results obtained here allow further interpretation of the initial information provided by the global matrix principal component analysis.

3.3. Discriminant analysis

Linear discriminant analysis is a useful complement to PCA. Its application in this study was to assess the adequacy of cows' milk classification, focusing on those studied variables which were previously related with the season or geographical location.

Variable selection in stepwise LDA was selected by means of Wilks' lambda statistic. Stepwise discriminant analysis is a method for seeking out subsets of variables most used to discriminate between the cows' milk samples.

The result of the applied discriminant analysis, according to seasonal criteria for each step is summarised in Table 6.

Many factors are involved in the seasonal variation of the contents in cows' milk. Therefore, it is not surprising that the variables used in extracted discriminant functions are numerous. Protein, fat, sodium, iron and cadmium variables have been excluded from the factors by a stepwise method, taking into account their lower variability observed through the different seasons. Three discriminant functions, linear combinations of quantitation variables

Table 6
Stepwise discriminant analysis and coefficients of discriminant functions according to seasonal criteria

Step	Variable	Wilks' lambda	*p*		Coefficients		
					1	2	3
1	[Zn]	0.351	<0.001	[Ca]	0.006	0.005	0.002
2	[P]	0.109	<0.001	[P]	0.007	0.006	0.001
3	[Pb]	0.065	<0.001	[Mg]	0.059	0.029	−0.016
4	[Mn]	0.045	<0.001	[K]	0.000	−0.004	0.007
5	[Ca]	0.034	<0.001	[Zn]	−0.002	0.000	−0.001
6	[Se]	0.026	<0.001	[Mn]	−0.034	0.012	0.107
7	[K]	0.021	<0.001	[Cu]	0.000	0.002	−0.011
8	[Mg]	0.020	<0.001	[Cr]	−0.008	−0.077	−0.069
9	[Cu]	0.018	<0.001	[Se]	0.070	−0.064	−0.003
10	[Al]	0.017	<0.001	[Al]	−0.001	0.002	0.000
11	[Cr]	0.016	<0.001	[Pb]	−0.092	0.259	0.213
				Constant	−8.318	−9.773	−9.530

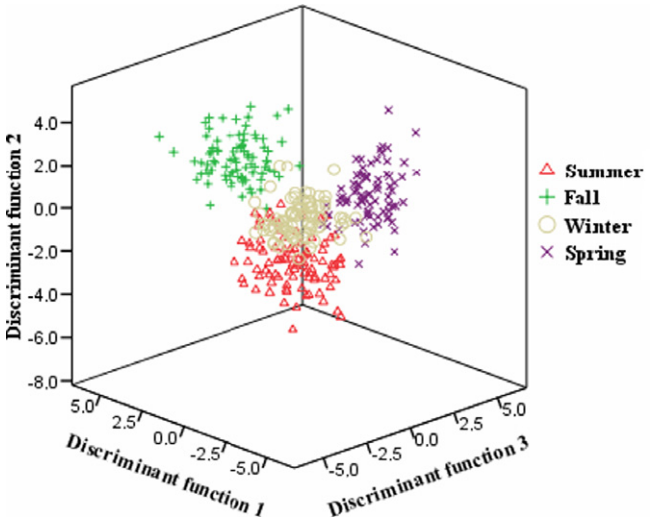

Fig. 4. Projections of raw cow milk samples analysed according to seasonal criteria in the space formed by the three discriminant functions after LDA.

selected, have been extracted to represent the different cows' milk samples in the established space. The following three eigenvalues and canonic correlations (given in parentheses) were calculated: 5.997 (0.926); 2.509 (0.846) and 1.567 (0.781), explaining about 59.5%, 24.9% and 15.6% of the total variance, respectively. Canonical discriminant function coefficients obtained are also reported in Table 6.

The classification results of the cows' milk samples collected from different seasons are very satisfactory, allowing 97.1% of cases to be correctly grouped, with a very high specificity and sensitivity. A three-dimensional plot of discriminant functions derived from the eleven selected variables is represented in Fig. 4. As can be observed, cows' milk samples have an excellent resolution and nearly complete separation rate.

On the other hand, a similar statistical study of discriminant analysis was realised considering other factors such as geographical location, proximity of pollution points, type of feeding or drinking water. The complexity of these variables has invalidated the achieved results, except for the geographical zone variable in which two discriminant functions were obtained.

Four variables (protein, fat, manganese and lead) were selected to represent the cows' milk samples. This variables selection seems to be a proper illustration of the content variability: the macronutrients, protein and fat, are directly related to dairy farm region; manganese is associated with feeding practices; and lead is linked to contamination from external sources.

However, the differentiation and classification of cow milk samples with respect to origin was unsatisfactory and poorly characterised. A total of 47.8% of the cow milk samples was correctly classified, with a high number of wrong assignments. These results are in part due to the influence of other different factors involved: types and habits of feeding, nutritional additives and supplements, contamination from external sources, stress factors such as climate, disease or lactation; all of these variables could be reflected in cows' milk mineral content.

4. Conclusions

According to the results obtained, multivariate techniques are able to differentiate and classify raw cows' milk using the profile of mineral and trace elements. By application of PCA and LDA, correlations between studied variables were highlighted and some patterns were recognised, in order to relate with intrinsic and extrinsic factors. Their application was helpful for deeper understanding of the changes in the composition of cows' milk through the different seasons. Consequently, the discrimination of cows' milk samples collected in different seasons was excellent.

These findings might be of special relevance for infant formula manufacturing, due to the variability in raw material used, in order to assure an adequate nutrient content of adapted and follow-up formulas, subjected to guidelines proposed by different paediatric agencies and European Economic Community legislation.

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