CHEMOMETRIC ANALYSIS OF MINERALS AND TRACE ELEMENTS IN RAW COW MILK FROM THE COMMUNITY OF NAVARRA (SPAIN)

Sola-Larrañaga, Cristina; Navarro-Blasco, Iñigo*

University of Navarra, School of Sciences, Department of Chemistry, Irunlarrea 1, Pamplona (Navarra), E-31.080, Spain

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 interprets reasonably well the correlations of these studied variables. These variable associations may be attributed to the 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 as a complementary information to PCA. This work shows that PCA and LDA are useful chemometric tools for the multivariate characterisation of raw cow milk.

Keywords: Cow milk; Atomic spectrometry; Pattern recognition; Multivariate analysis; Principal component analysis; Linear discriminant analysis

1. Introduction

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

Mineral and trace element concentrations in raw cow milk are not constant but mainly vary in according to two kinds of factors, those related with secretion from the mammary gland, such as the lactation state, animal species and healthy status, and extrinsic factors: season, dairy cattle ration (nutrtitional status of cow), environment (nature of soil and localization of the animal farm). In this respect, several studies has been carried out to assess mineral content of cow milk from different areas (Lante, Lomolino, Cagnin, & Spettoli, 2004, Dobrzarnski, Kolacz, Górecka, Chojnacka & Bartkowiak, 2005; Hermansen, Badsnerg, Kristensen & Gundersen, 2005; Muñiz-Naveiro, Domínguez-González, Bermejo-Barrera, Cocho de Juan, Fraga Bermúdez, Goris Pereiras, et al. 2005; Licata, Trombetta, Cristani, Giofre, Martino, Calo et al., 2004; Rodríguez, Sanz & Díaz, 2001; Orak, Yanardag & Hugul, 2000; Simsek, Gültekin, ÖKsüz & Kurultay, 2000; O'Brien, Mehra, Connolly & Harrington, 1999), as well as to evaluate preliminary correlations between animal feeding, environmental manufacturing process and elemental profile in cow milk and dairy products (Coni, Bocca, Coppolelli, Caroli, Cavallucci & Trabalza Marinucci, 1996; Coni, Bocca, Ianni & Caroli, 1995; Coni, Caroli, Ianni & Bocca, 1994).

However, multivariate data analysis may be used to obtain more additional information. Pattern recognition techniques has been applied to estimate food quality in view of its quantitative characteristic of mineral and trace element composition. The chemometric procedures appear to be usefully assessing authentification and in the identification of several factors affecting the quality of different foods, such as meat, seafood, milk and dairy products, vegetables, honey, coffee, tea, fruit juices and alcoholic beverages (Szefer, 2007). Thus, chemometric techniques applied to the data of macro and micronutrients in raw cow milk might provide an interesting and promising approach to perform the classification and to identify possible sources of influence onto elemental profiles.

The present study has applied principal component analysis (PCA) for processing data concerning 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 cow milk in order to distinguish the group belonging to different seasons and area of milk collection and to study any association existing among these elements which are reflected on the main influence factors above mentioned. Linear discriminant analysis was also applied, as supervised pattern recognition method, with the objective to describe the discriminant functions and therefore, data distributions might be identified as criteria of seasonal or area classification for raw cow milk samples studied.

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 determination. 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, the focal length is 1 m. The main argon flow was 12 l min^{-1}, and the cooling flow 0.45 l min^{-1}. The nebulizer 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); phosphorous, 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 atomizers and Zeeman background correction was used. 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.

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)- (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 -20º C. Bottles were opened under flow laminar bench, using vinyl talc-free gloves (Rotiprotect® Carl Roth, Karlsruhe, Germany). Special care was devoted to minimize the risk of external contamination.

2.3. Reagents and solutions

All the chemicals used were of the highest purity available and all the 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 (Darmstad, Germany) was further purified by sub-boiling distillation in 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 $mg \cdot ml^{-1}$ Rh, prepared by dissolving 0.5 g citric acid monohydrate (pro analysis, Merck) in 5 ml of $Rh(NO_3)_2$ (1000 $mg \cdot l^{-1}$ certipur, Merck), a solution of magnesium nitrate (0.150 g $Mg(NO_3)_2 \cdot 6H_2O$, suprapur, Merk) 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) were diluted in 100 ml with ultrapure water) were used as matrix modifier for selenium, chromium, cadmium and lead, respectively.

2.4. Analytical procedures

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

In order to determine the concentration of minerals and trace elements, 5 ml of the raw cow milk were placed into 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 next temperature stages: 25-140 ºC for 10 min, 140-150 for 4 min, 150 ºC for 7 min, 150-180 ºC for 10 min, and 180 ºC for 20 min, all of them at 1000 W, followed immediately by ventilation at room temperature (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 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 summarized 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. Also Table 2 contains the LOD of 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 control were run to satisfy the criteria established in the quality program and to provide on-going quality control information.

2.6. Statistical data processing

Statistical data processing univariate characterization was carried out previously to check and describe the distribution data of each element analyse.

A data matrix, whose rows are the different raw cow milk samples analysed (cases) and whose columns are descriptors corresponding 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 representation allows the finding of patterns hidden in the dataset.

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

3. Results and discussion

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

Descriptive statistics of all data distribution of 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 (righ-handside 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.

3.2. Principal component analysis

3.2.1. Global approximation

The data set of parameters analyzed was subjected to a PCA in order to decrease the number of descriptors retaining the maximum amount of variability present in experimental data. Prior to performing PCA, the suitability of data for factor analysis was checked. Inspection of correlation matrix between variables revealed a great number of coefficients higher than 0.250 (data not shown). The determinant value (0.00325) of 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 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 characterizes those parameters (protein, fat, calcium, magnesium, phosphorous, sodium) associated with lactogenesis process at the mammary gland, homeostatically controlled and regulated by the secretor cell through a mechanism fully known (Jensen, 1995).

Iron, manganese and lead are the dominating variables in the second factor, although zinc, selenium, cadmium, aluminium and copper are also correlated with a lower representation. 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 mineral (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 wet period, contribute its secretion in synthesized cow 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, selenium cow milk concentration was gradually declining during autumn, winter and spring months. It reflects a negative loading (-0.360) in the second rotated factor.

Third factor mainly reflects the complex factor of the geographical situation. The found correlation between parameters studied indicates that arise 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 characterize the high quality of cow milk from north zone against south region. Additionally, selenium-poor soil localized in north zone has a marked effect on lower levels of selenium available to plants and consequently to ruminants that feed on them. This finding is evidenced by the negative loading (-0.396). On the other hand, the marine influence is related to the lowest and highest potassium values determined in north and south cow milk, respectively. The potassium exchangeable character could explain the high negative correlation found in this factor.

Fourth factor is not intuitive. It could be related with food habits modification between climatic seasons with incorporation of fodder to diet on those favourable seasons. A high corporal storage of cadmium when a vegetarian diet with high phytate contain is supplied has been demonstrated in animals studies. Moreover, this kind of diet is natural source of copper and manganese which have 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 implies a small increase in fat content of cow milk (coefficient value: 0.301), and a shorter need to drink water by cattle. In such a way that the cows cover the 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 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. It is well known the essential role of selenium in cattle health (Nutrition Research Council, 2001). Selenium principally as selenium yeast is incorporated in a mineral pre-mix for addition of the fodder. In the same way, several studies have demonstrated the benefit of chromium supplementation (Escobosa & Ávila, 2001) under inorganic or organic form, as chromium (III) picolinate and enrichment yeast (Hegoczki, Suhajda, Janzso & Vereczkey, 1997); being significantly stimulated during the last decade.

Principal component analysis allows visualizing the information of the data in graphical representations, making easy the observation and interpretation of the information for the simple understanding. Scatterplot of loadings for studied macro and micronutrients (Fig. 2) shows the associations obtained between elements after the Varimax orthogonal rotation in order to visualize the discriminating efficiency of the principal components.

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

In short, it is necessary to emphasize that this chemiometric methodology provides a useful tool, by means of the employment of a statistical package widely used by the scientific community, to approach a complex problem, difficult to interpret with the traditional logic.

3.2.2. Factor analysis by seasons

The separately season factor analysis might provide a detailed information particularly about the longitudinal 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),.

Nevertheless, prior to performing a principal component analysis in order to decrease the number of descriptors responsible for the highest percentage of a total variance of the experimental data from different season, the suitability of data was assessed.

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 observation of obtained factors describes globally the longitudinal changes in the mineral composition of cow milk during the course of year (Lante et al., 2004; Orak et al., 2000; Rodríguez et al., 2001; Ricón, Moreno, Zurera & Amaro, 1994; Moreno-Rojas, Amaro & Zurera, 1993). Seasonal variations in the composition of fat and casein have been found in cow's milk. Thereby, calcium, phosphorous and magnesium are grouped on 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 (first and fourth factors in autumn and winter, 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 (Silva, Lopes, Nóbrega, Souza & Nogueira, 2001; Hazell, 1985).

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

Feeding and regional factors are showed separately in the factors 2 and 3, and 4 and 5, during summer and spring, respectively. On the contrary, 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 emphasized. It is recognized 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 the typical commercial dairy concentrate is also reflected on 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, 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 or environmental pollution (Dobrzarnski et al., 2005; Licata et al., 2004; Simsek et al., 2000). This aspect is clearly observed on 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 non-toxic trivalent chemical form supplied by 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 to 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 autumn matrix) or the regional area (e.g. lead, third factor in summer matrix).

Just as expected, the results obtained here permit to complete and interpret as far as possible 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 cow milk classification attending to those studied variables which are 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 cow milk samples.

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

In the light of aforementioned, many factors are involved in seasonal variation of contents in cow milk. Therefore, it is not surprising that the variables used in extracted discriminant functions are numerous. Exclusively protein, fat, sodium, iron and cadmium variables have been excluded from factors by stepwise method, taking into account their lower variability observed through the different seasons. Three discriminant functions, linear combinations of quantization variable selected, have been extracted to represent the different cow milk samples in the established space. The following three eigenvalues and canonic correlations (given in parenthesis) 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 cow milk samples collected from different seasons is very satisfactory reaching a 97.1% of cases correctly grouped, with a very high specificy and sensibility. Three-dimensional plot of discriminant functions derived from the eleven selected variables is represented in Fig. 4. As can be observed, cow milk samples have an excellent resolution and nearly compete separation rate.

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

Four variables (protein, fat, manganese and lead) were selected to represent the cow 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 to feeding practices; and lead is linked to contamination from external sources.

However, the differentiation and classification of cow milk samples with respect to origin zone was insufficiently satisfactory and poorly characterised. A total of 47.8% of the cow milk samples was correctly classified, with 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; albeit all of these variables could be reflected on cow milk mineral content.

4. Conclusions

According to the results obtained, multivariate techniques are able to differentiate and classify raw cow 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 recognized in order to relate with intrinsic and extrinsic factors. Their application was helpful for deeper understanding of the changes in the composition of cow milk through the different seasons. Consequently, the discrimination of cow milk samples collected from 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 paediatrics agencies and European Economic Community legislation in force.

Acknowledgement

The authors are grateful to the Fundación Universitaria de Navarra (FUNA) for financial support.

References

Coni, E., Bocca, A., Coppolelli, P., Caroli, S., Cavallucci, C., & Trabalza Marinucci, M. (1996), Minor and trace element content in sheep and goat milk and dairy products. *Food Chemistry*, 57, 253-260.

Coni, E., Bocca, A., Ianni D., & Caroli, S. (1995). Preliminary evaluation of the factors influencing the trace element content of milk and dairy products. *Food Chemistry*, 52, 123-130.

Coni, E., Caroli, S., Ianni D., & Bocca, A. (1994). A methodological approach to the assessment of trace elements in milk and dairy products. *Food Chemistry*, 50, 203-210.

Dobrzarnski, Z., Kolacz, R., Górecka, H., Chojnacka, K., & Bartkowiak, A. (2005). The content of microelements and trace elements in raw milk from cows un the Silesian region. *Polish Journal of Environmental Studies*, 14(5), 685-689.

Escobosa, A., & Ávila, S. (2001). Chapter 9: Alimentación. In: S. Avila, & A. Gutierrez, *Producción de leche con ganado bovino*. Mexico: Digital Book FMVZ-UNAM. http://www.fmvz.unam.mx/biblivir/BvS1Lb/BvS1Pdf/Avila/cap9.pdf.

Hazell, T. (1985). Minerals in foods: dietary sources, chemical forms, interactions, bioavailability. *World Review of Nutrition and Dietetics*, 46, 1-123.

Hegoczki, J., Suhajda, A., Janzso, B., & Vereczkey, G. (1997). Preparation of chromium enriched yeasts. *Acta Alimentaria*, 26, 345-358.

Hermansen, J.E., Badsnerg, J.H., Kristensen, T., & Gundersen, V. (2005). Major and trace elements in organically or conventionally produced milk. *Journal of Dairy Research*, 72, 362-368.

International Dairy Federation. (2000). International Standard 141C. Whole milk - Determination of milk fat, protein and lactose content - Guidance on the operation of mid-infrared instruments. Brussels: International Dairy Federation.

Jensen, R.G. (1995). *Handbook of milk composition*. New York: Academic Press.

Lante, A., Lomolino, G., Cagnin, M., & Spettoli, P. (2004). Content and characterisation of minerals in milk and in Crescenza and Squacquerone Italian fresh cheeses by ICP-OES. *Food Control*, 17, 229-233.

Licata, P., Trombetta, D., Cristani, M., Giofre, F., Martino, D., Calo, M., & Naccari F. (2004). Levels of "toxic" and "essential" metals in samples of bovine milk from various dairy farms in Calabria, Italy. *Environmental International*, 30, 1-6.

Marengo, E., & Aceto, M. (2003). Statistical investigation of the differences in the distribution of metals in Nebbiolo-based wines. *Food Chemistry*, 81, 621-630.

Mata, L., Sánchez, L., & Calvo, M. (1996). Cadmium uptake by Caco-2 cells. Effect of some milk components. *Chemico-Biological Interactions*,100(3), 277-288.

Moreno-Rojas, R., Amaro, M., & Zurera, G. (1993). Concentration and seasonal variation of calcium, magnesium, sodium and potasium in raw, ewe and goat milk. *International Journal of Food Sciences and Nutrition*, 45, 99-105.

Muñiz-Naveiro, O., Domínguez-González, R., Bermejo-Barrera, A., Cocho de Juan, J.A., Fraga Bermúdez, J.M., Goris Pereiras, A., López Santamariña, A., Martínez Lede, I., Valledor Puente, J., Fernández-Couto Gómez, L., & Bermejo-Barrera, P. (2005). Selenium content and distribution in cow's milk supplemented with two dietary selenium sources. *Journal of Agricultural and Food Chemistry*, 53, 9817-9822.

Nutrition Research Council. (2001). *Nutrient requirements of dairy cattle, 7th revised ed*. Washington: National Academia Press.

O'Brien, B., Mehra, R., Connolly, J.F., & Harrington, D. (1999). Seasonal variation in the composition of Irish manufacturing and retail milks. 4. Minerals and trace elements. *Irish Journal of Agricultural and Food Research*, 38, 97-99.

Orak, H., Yanardag, R., & Hugul, M. (2000). The levels of sodium, potassium, magnesium and calcium in various milk samples of Turkey. *Nahrung*, 44, 285-287.

Pallant, J. (2003). *SPSS Survival Manual: A Step by Step Guide to Data Analysis Using SPSS for Windows (version 10 and 11)*. Maidenhead: Open University Press.

Ricón, F., Moreno, R, Zurera, G, & Amaro, M. (1994). Mineral composition as a characteristic for the identification of animal origin of milk. *Journal of Dairy Research*. 61, 151-154.

Rodríguez, E.M., Sanz, M., & Díaz, C. (2001). Mineral concentrations in cow's milk from the Canary Island. *Journal of Food Composition and Analysis*, 14, 419-430.

Silva, F.V., Lopes, G.S., Nóbrega, J.A., Souza G.B., & Nogueira A.R.A. (2001). Study of the protein-bound fraction of calcium, iron, magnesium and zinc in bovine milk. *Spectrochimica Acta Part B*, 56 1909-1916.

Simsek, O., Gültekin, R., ÖKsüz O., & Kurultay, S. (2000). The effect of environmental pollution on the heavy metal content of raw milk. *Nahrung*, 44 (5), 360-363.

Szefer, P. (2007). Chemometric techniques in analytical evaluation of food quality. In: P. Szefer, & J.O. Nriagu, *Mineral components in foods (pp. 69-121)*. Boca Raton: CRC Press Taylor & Francis Group.

Vegarud, G.E., Langsrud, T., & Svenning, C. (2000). Mineral-binding milk proteins and peptides; occurrence, biochemical and technological characteristics. *British Journal of Nutrition*, 84(suppl. 1), S91-S98.

Legends of figures

Figure 1. Sampling localization site.

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

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

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

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

Instrumental parameters				
	Chromium	***Selenium***	***Cadmium***	***Lead***
Wavelength *(nm)*	*357.9*	196.0	228.8	283.3
Slit width *(nm)*	*0.7*	2.0	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

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

Table 2. Quality control parameters: Detection limits expressed in raw cow milk, recovery assays and analysis of the certified reference material SRM 1549 non-fat milk powder (mg or μg g^{-1})

	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]

[a] mg/L
[b] μg/L
[c] ng/L
[d] μg/g

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

		Range		***Mean***	***s.d.***	***Skewness***	***Kurtosis***
	n	*min.*	*max.*				
ein (% w/w)	347	2.66	3.69	3.19	0.16	-0.094	0.154
(% w/w)	347	1.93	4.59	3.81	0.31	-0.758	3.964
mg l^{-1})	347	760	1380	970	98	0.613	0.372
g l^{-1})	347	550	1123	785	98	0.571	0.133
(mg l^{-1})	347	69.4	126.4	91.8	9.4	0.473	0.125
mg l^{-1})	347	234	502	372	40	0.435	1.232
g l^{-1})	347	1143	1554	1344	65	0.003	0.229
µg l^{-1})	347	2532	7737	4631	855	0.490	-0.014
µg l^{-1})	347	57.6	759.3	290.0	104.1	0.794	1.306
(µg l^{-1})	347	7.1	54.8	29.1	9.2	0.377	-0.365
µg l^{-1})	347	7.2	357.8	51.8	36.1	3.834	22.910
ug l^{-1})	347	< LOD	24.35	4.03	3.43	1.948	5.406
ug l^{-1})	347	< LOD	40.60	9.77	7.61	1.137	1.364
ug l^{-1})	347	47	1598	369	209	1.921	7.457
µg l^{-1})	347	0.55	18.70	5.23	2.85	1.091	2.208
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**

Table 5. Varimax rotated factor matrix by seasons (Summer and Autumn).

Summer	***Component***						
	1	2	3	4	5	6	7
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	***Component***						
	1	2	3	4	5	6	7
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**

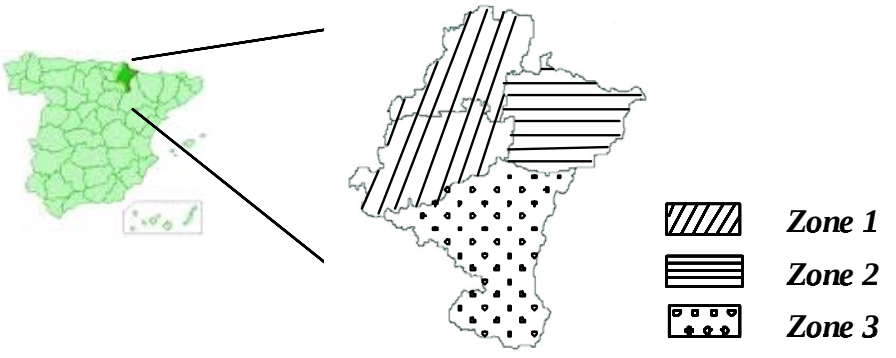
Table 5 cont. Varimax rotated factor matrix by seasons (Winter and Spring)

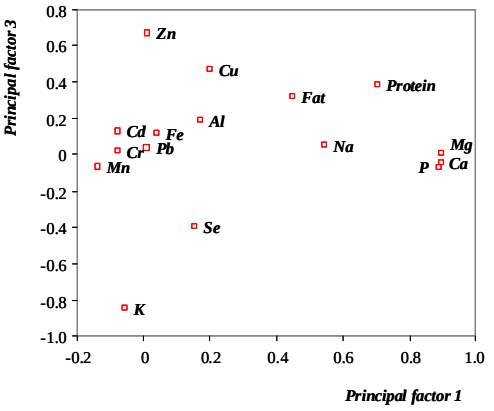
Winter	***Component***					
	1	2	3	4	5	6
P	**0.872**	-0.064	-0.132	-0.114	-0.021	0.208
Protein	**0.843**	-0.058	-0.049	0.218	0.172	-0.004
Zn	**0.579**	**0.401**	0.097	0.096	0.111	0.135
Mn	-0.203	**0.775**	0.139	0.248	0.142	0.132
Cu	0.200	**0.740**	-0.050	-0.029	-0.102	-0.142
Fe	-0.003	**0.663**	0.052	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**

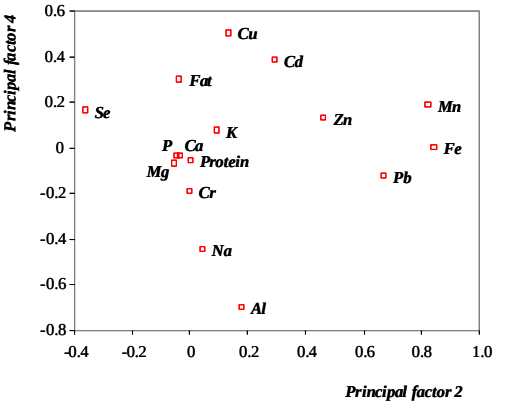
Spring	***Component***					
	1	2	3	4	5	6
Ca	**0.802**	-0.052	0.067	-0.131	0.006	-0.276
P	**0.764**	-0.132	-0.308	0.045	0.141	0.317
Zn	**0.749**	0.071	0.130	0.133	-0.038	0.200
Protein	**0.527**	0.315	0.051	0.160	0.062	-0.049
K	0.162	**-0.766**	-0.144	0.096	0.022	-0.303
Fat	0.104	**0.671**	0.129	-0.090	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**

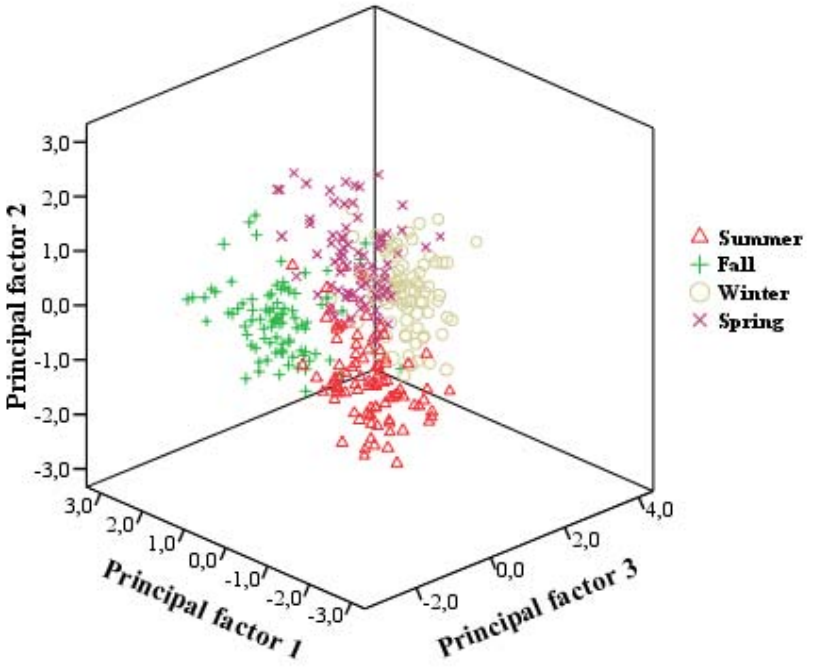
Table 6. Stepwise discriminant analysis and coefficients of discriminant functions according to seasonal criteria

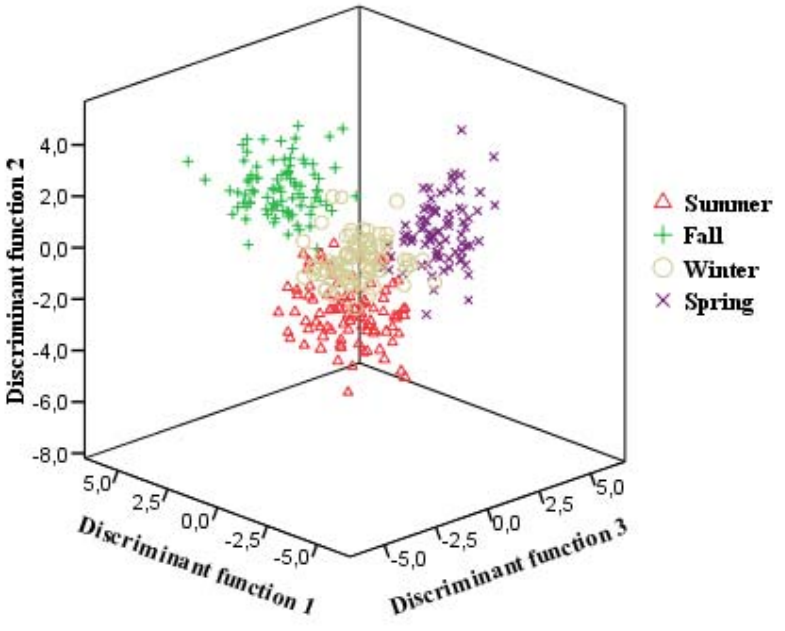
Step	***Variable***	***Wilks' Lambda***	***p***		***Coefficients***		
					1	*2*	*3*
1	[Zn]	0.351	<0.001	[Ca]	0.006	0.005	0.002
2	[P]	0.109	<0.001	[P]	0.007	0.006	0.001
3	[Pb]	0.065	<0.001	[Mg]	0.059	0.029	-0.016
4	[Mn]	0.045	<0.001	[K]	0.000	-0.004	0.007
5	[Ca]	0.034	<0.001	[Zn]	-0.002	0.000	-0.001
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


Zone 1
Zone 2
Zone 3


Principal factor 3
Principal factor 1
Zn
Cu
Fat
Protein
Al
Cd
Fe
Na
Mg
Cr
Pb
Mn
P
Ca
Se
K


Principal factor 4
Principal factor 2
Cu
Cd
Fat
Se
Mn
Zn
K
P
Ca
Fe
Protein
Mg
Pb
Cr
Na
Al


Principal factor 2
3,0
2,0
1,0
0,0
-1,0
-2,0
-3,0
Principal factor 1
3,0
2,0
1,0
0,0
-1,0
-2,0
-3,0
Principal factor 3
-2,0
0,0
2,0
4,0
Summer
Fall
Winter
Spring


Discriminant function 2
4,0
2,0
0,0
-2,0
-4,0
-6,0
-8,0
5,0
2,5
0,0
-2,5
-5,0
Discriminant function 1
-5,0
-2,5
0,0
2,5
5,0
Discriminant function 3
Summer
Fall
Winter
Spring