Identification of cheese species origin by pattern recognition processing of elemental data

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Summary
Pattern recognition techniques - cluster, principal component, factor and canonical discriminant analysis, were used for differentiation of cheese species using such elemental markers as Ba, Cr, Cu, Hg, Mg, Mn, Ni and V. Cluster analyses showed some preliminary results intimated partial species differentiation of cheeses. Principal component and factor analysis appeared to be effective methods not only for data visualisation but for efficient differentiation of cow and sheep cheese species as well. Canonical discriminant analysis demonstrated a 95% potential to distinguish differences and correct classification of cheese species according to the used specific elemental data.

Keywords
species origin; cow cheese; sheep cheese; elemental markers; pattern recognition

In order to guarantee authenticity of cheeses, suitable analytical traceability methods are required that may be used by official authorities to control the correctness of the information appearing on the label. The ability to determine the species origin of milk products is important in order to protect consumer interests and producing companies from fraud caused by the use of other than the declared milk in the cheese production. Currently, extensive literature on research dealing with the detection of milk from different species in raw and processed milk products is available. The EC reference method, based on the isoelectric focusing of γ-caseins, has proved to be the most reliable for the detection of cow milk in cheeses made from mixtures of different milk [1]. Isoelectric focusing is able to detect cow milk in Roquefort cheese [2], Mozarella cheese [3], or in ovine, caprine and buffalo cheese [4].

A number of enzyme-linked immunosorbent assays (ELISA) procedures has been developed for the detection of milk adulteration in dairy products. ELISA has been successfully applied to the detection of cow or goat milk in sheep cheese [5-7]. A number of methods proposed for the detection of cow, sheep and goat milk in mixtures of milk and cheese has been reviewed by RAMOS and JUAREZ [8]. The detection limit of immunological and electrophoretic methods is about 0.5% [9]. It is recommended that ELISA should be used in combination with PCR to ensure compliance with current legislation [10].

Polymerase chain reaction (PCR) is one of the most used molecular biology tools and has been used by many authors for milk species identification in milk products. The PCR technique was proposed to identify cow and water buffalo DNA in milk and mozzarella cheese [11] and for the qualitative detection of cow milk in mixed cheese [12].

PCR was performed to detect the corresponding DNA in milk and cheese with a detection limit of about 0.5% cow milk in sheep or goat cheese [13], to detect cow milk in goat cheese with a detection limit of less than 0.1% [14], to detect cow milk in "buffalo" Mozzarella cheese [15] and also for differentiation of feta cheeses made from cow, sheep and goat milk [16].

In recent years, computerized pattern recognition analysis has become an effective tool to categorize different food samples by considering many variables that can be measured, often in a single analytical determination. Pattern recognition involves subdisciplines such as discriminant analysis, principal component and cluster analysis. Cluster analysis is a classification method that is used to arrange a set of cases into clusters. Principal component analysis (PCA) is a widely used multi-

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variate analytical statistical technique to reduce dimensionality of the data (but retaining most of the original variability in the data set) by linear combinations of original dependent variables to a smaller set of new uncorrelated variables (called principal components), or in the case of factor analysis to a new set of variables (called factors) based on patterns of correlation among the original variables. Canonical discriminant analysis generates canonical variables, which are linear combinations of the original variables that describe the variation between pre-specified classes in a manner analogous to the way in which PCA summarizes the variation among individual samples. Discriminant function analysis here refers to a group of pattern recognition classification methods that use known data to determine a discriminant function, which can then be used to classify unknown samples into predetermined classes.

Multivariate analysis of chromatographic, electrophoretic or elemental data has been shown to represent a powerful method for discrimination between cheeses of different geographical origin, varieties, quality or for judging cheese maturation. The concentration ranges of some elements in milk and cheese are closely dependent upon animal species and feeding, time of year, environmental conditions and manufacturing processes. Results of a study of selected trace elements (Al, Ba, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Pt, Sr and Zn) showed considerable differences between the sheep and goat milk as well as related products [17]. Rincon et al. used Cu, Fe, Zn, Mn, Ca, Mg, Na and K determined by atomic absorption spectroscopy (AAS) as markers for milk species [18]. These elements clearly distinguished cow milk from sheep and goat milk. A statistical study of correlation, factorial and discriminant analysis on the metal composition (Se, Fe, Cu, Zn, Na, K, Ca, Mg) of different types of milk (human, cow, goat, pasteurized, and powdered infant formula) was carried out to differentiate the samples. The discriminant functions were able to classify correctly 98% of the samples within each type of milk [19]. The contents of nine mineral elements (P, Ca, Na, K, Mg, Zn, Fe, Cu and Mn) were determined in cheeses belonging to nine Spanish varieties. Using stepwise discriminant analysis, 76% of samples were classified correctly according to milk species from which they were produced. When the cheeses were differentiated according to the variety, 92% of samples of unripened cow milk cheeses, 85% of samples of ripened cow milk cheeses and 93% of samples of ripened goat milk cheeses were correctly classified [20]. The mineral composition of sheep, cow and goat milk and samples of different types of pure-milk cheeses made from the milks of these species was analysed by Martin-Hernandéz et al. [21]. Stepwise discriminant analysis of the milk samples demonstrated that variables K/Mg, Na/Ca, Zn, Cu/Zn and Cu/Na were the most useful in differentiating the samples, achieving correct classification in 98.2% of samples. The most useful variables for cheese were Fe/K, Na/Ca, Zn/Cu, Na/Mg and Zn, which facilitated the correct classification in 97.1% of samples. Three goat milk cheeses were successfully distinguished using variables K/Zn, Fe/Cu and P. Using classification functions obtained by discriminant analysis, 94.1% of Mahon cheese samples were correctly classified into traditional or industrial groups, and 89.7% of samples into fresh, half-ripened, ripened and old-ripened groups [22]. Some promising results were obtained from identification of specific sensory patterns for several cheese varieties with special attention to sheep, goat and cow cheeses [23]. Linear discriminant analysis showed that sheep milk cheese varieties with unique sensory characteristics were very different from each other.

From the highlighted review we can see that authentication of cheeses requires special analytical tools and that the elemental data are not frequently used for identification of cheese species. This paper summarizes results obtained from species identification of cow and sheep cheeses on the Slovakian market using selective elemental data and pattern recognition analysis.

MATERIALS AND METHODS

Samples

Forty Slovakian cow hard cheeses of emmental and edam type from 11 different producers were obtained from the retail network in Bratislava. One-hundred-sixteen sheep cheeses (40 sheep hard cheeses and 76 bryndza cheeses) were obtained directly from 9 Slovakian regional producers. Cheese samples were vacuum- or hand-packaged and stored at 4 °C until the analysis. All samples were analysed for the contents of elemental markers Ba, Cr, Cu, Hg, Mg, Mn, Ni and V, which were selected on the basis of geochemical characterization of Slovakian regions using the distribution data pupblished in the Slovakian geochemical atlas of soils, according to their variability in the producing areas [24, 25]. All samples were analysed in triplicate.

Cheese sample preparation for atomic absorption spectrometry

Each sample of cheese (0.5 g) was digested

using the microwave digestion system MLS 1200 MEGA (Milestone, Sorisole, Italy) with 4 ml of 65% HNO_3 and 0.5 ml H_2O_2. The following microwave digestion programme was applied: 250 W (1 min), 0 W (1 min), 250 W (5 min), 400 W (5 min) and 650 W (5 min). The digested sample was adjusted to the volume of 10 ml with ultrapure water.

Reagents

All chemicals were of analytical grade. Stock solutions of each metal (Ba, Cr, Cu, Hg, Mg, Mn, Ni, and V at concentration of 1.00 $g.l^{-1}$) were from Merck (Darmstadt, Germany). Working standard solutions were prepared by suitable dilution of stock solutions. Nitric acid of suprapure quality was purchased from Merck. Lanthanum chloride 5% as ionic suppressor was obtained from Slovak Institute of Metrology (Bratislava, Slovakia). Ultrapure water from Milli-Q system (Analyst HP, Wolf, United Kingdom) with conductivity of 18 MΩ was used throughout.

Atomic absorption spectrometry

A Perkin Elmer 4100 atomic absorption spectrometer (Norwalk, Connecticut, USA) equipped with a deuterium lamp background-correction system and HGA 700 graphite tube atomizer with pyrolytically coated graphite tubes and flame was used for metal determination. Metals Mg, Mn were determined using an air/acetylene flame. Metals Ba, Cr, Cu, Ni and V were measured on graphite tube atomizer. All results were expressed as the average of triplicate measurements. The instrumental conditions for the determination are shown in Tab. 1 and analytical characteristics for the determination of elements in samples by AAS in Tab. 2. The accuracy of the results was verified by standard addition method, because there were no available certified reference materials for determination of elements in cheese. Recovery of the method was assessed in the matrix by analysis of fortified samples. Fortification of samples was performed before microwave digestion using solutions of selected elements. The mean recoveries of elements are presented in Table 2. The accuracy of method was tested by determination of the concentration of Ba, Cr, Cu, Hg, Mg, Mn, Ni and V in the reference material NCS ZC 73008 (rice) and NCS ZC 73013 (spinach; both from China National Analysis Center for Iron and Steel, Beijing, China); results are shown in Tab. 3. The quantification limit was defined as the elements concentration of the reagent blanks corresponding to the ten-fold standard deviation of the reagent blanks ($n = 10$). The uncertainty of A-type (U_A) was a standard deviation of measurement of matrix. The uncertainty of B-type (U_B) included sample mass, volume,

Tab. 1. Instrumental conditions for determination of individual elements by AAS.

Element	Wavelength [nm]	Lamp current [mA]	Technique by AAS	Gas	Signal type	Suppressor modifier
Ba	553.6	25	GF	Argon	AA	No
Cu	324.8	15	GF	Argon	AA-BG	No
Cr	357.9	25	GF	Argon	AA-BG	No
Mg	285.2	15	F	C_2H_2 - air	AA-BG	0.1% $LaCl_3$
Mn	279.5	20	F	C_2H_2 - air	AA	No
Ni	232.0	25	GF	Argon	AA-BG	No
V	318.4	25	GF	Argon	AA-BG	No

AAS - atomic absorption spectrometry, GF - graphite furnace, AA - atomic absorption, AA-BG - atomic absorption with background correction, F - flame AAS.

Tab. 2. Analytical characteristics for the AAS determination of elements in the cheese matrix.

Element	*LOQ* [$mg.kg^{-1}$]	U_A [%]	U_B [%]	U_C [%]	*R* [%]
Ba	0.005	8.5	10.2	13.3	103
Cu	0.010	0.8	6.9	6.9	109
Cr	0.004	3.2	4.5	5.3	102
Ni	0.010	1.0	14.4	14	103
Mg	0.090	1.2	3.3	3.5	96
Mn	0.060	4.3	5.1	6.6	101
V	0.006	1.2	6.2	6.3	102

AAS - atomic absorption spectrometry, *LOQ* - limit of quantification, U_A - uncertainty of A-type, U_B - uncertainty of B-type, U_C - combined uncertainty, *R* - recovery.

Tab. 3. Certified and found contents of markers in CRM determined by AAS.

Element	Values	NCS ZC 73008 rice	NCS ZC 73013 spinach
Ba	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	0.40 ± 0.09 0.42 ± 0.02 105.0	–
Cr	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	–	1.4 ± 0.2 1.6 ± 0.02 114.2
Cu	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	4.9 ± 0.3 4.8 ± 0.2 98.0	8.9 ± 0.4 8.8 ± 0.3 98.5
Hg	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	0.0053 ± 0.0005 0.0056 ± 0.0001 105.6	0.020 ± 0.003 0.025 ± 0.001 125.0
Ni	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	0.27 ± 0.02 0.28 ± 0.01 103.7	0.92 ± 0.12 0.95 ± 0.05 103.4
Mg	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	410 ± 60 440 ± 32 107	–
Mn	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	17 ± 1 17.2 ± 0.2 100.9	41 ± 3 41.5 ± 0.8 101.2
V	$X_{cert} \pm s_x$ [mg.kg^{-1}] $X_{found} \pm s_x$ [mg.kg^{-1}] R [%]	–	0.87 ± 0.23 0.86 ± 0.17 98.8

AAS - atomic absorption spectrometry, X_{cert} - certified content of element, X_{found} - found content of element, R - recovery, s_x - standard deviation.

slope of the calibration curve and the signal measured. The combined standard uncertainty (U_C) was calculated as $\sqrt{(u_A^2 + u_B^2)}$.

Determination of Hg

For determination of mercury in cheeses, atomic absorption spectrometer AMA 254 (Altech, Prague, Czech Republic) was used. This is a single-purpose mercury analyser, in which mercury vapour is generated after thermal oxidation treatment of the sample in a stream of oxygen. Absorbance of Hg is then measured using AAS at a wavelength of 254 nm. The accuracy of the method was established by the determination of mercury content in rice and spinach certified reference materials (CRM, Tab. 3), and in skim milk powder CRM BCR-150 (Community Bureau of Reference, Brussels, Belgium). The values found were: 0.0091 mg.kg^{-1}, s_x = 0.0004 mg.kg^{-1}, certified value: 0.0094 mg.kg^{-1}, s_x = 0.0017 mg.kg^{-1} with an accuracy of 96.8%.

Statistics

The following pattern recognition techniques were used: cluster analysis (CA), principal component analysis (PCA), factor analysis (FA) and canonical discriminant analysis (CDA). Statistical programme Unistat® (Unistat, London, United Kingdom) was used.

RESULTS AND DISCUSSION

Results from AAS determination of selected elements as markers of cheese species originating from Slovakia are displayed in a box and dot plot, Fig. 1. This figure demonstrates the high variability of selected elements and some significant differences in markers contents between cow and sheep cheeses, namely in the case of Cr, Cu, Mn and Ni. Obtained elemental data are also well visualized by pattern recognition techniques (Fig. 2-5).

Fig. 2 shows preliminary predisposition to natural groupings among the samples of cow and sheep cheeses obtained by hierarchical cluster analysis of the elemental data. Cluster analysis was performed on the basis of the block (Manhattan) distance and the method of complete linkage, which select the largest distance between pairs of elements in each cluster. As can be seen on the dendrogram (Fig. 2), at a dissimilarity distance of about 400, cheeses are grouped into two major clusters. Sheep cheeses

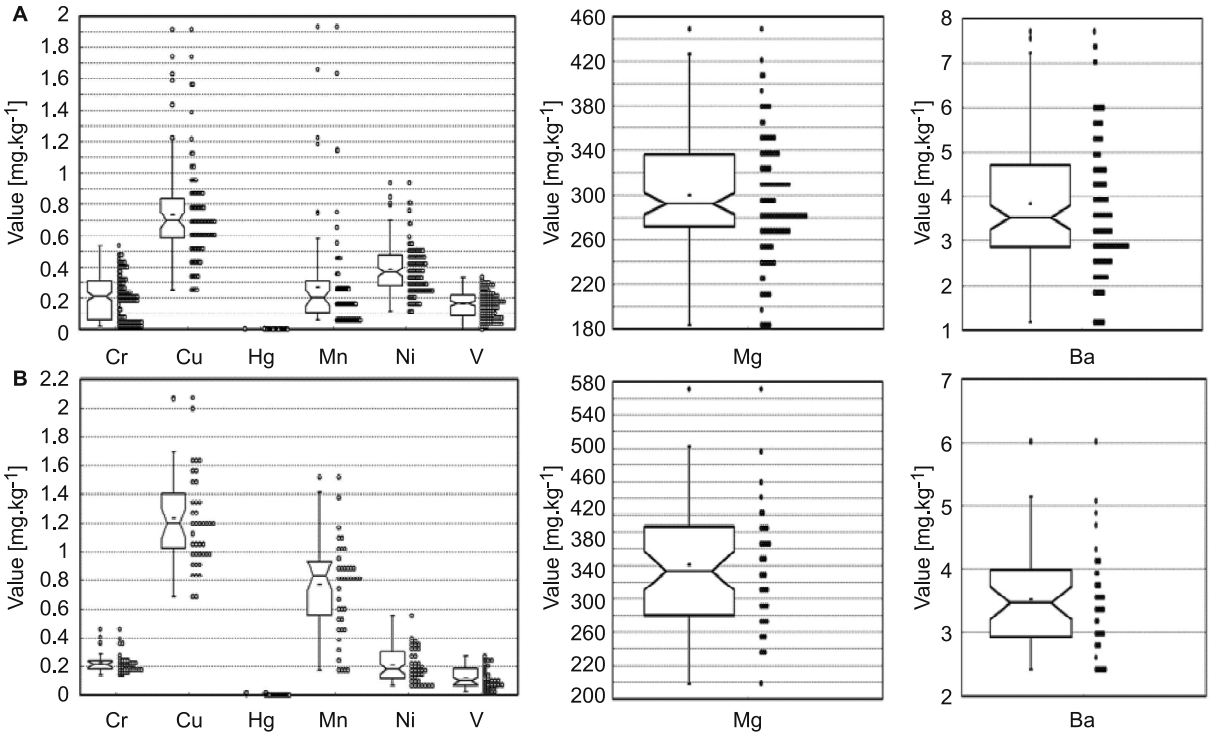

Fig. 1. Box-whisker and dot plot of data from AAS determination of elemental markers of Slovakian sheep and cow cheeses.
A - sheep cheeses, B - cow cheeses.

predominate in the first cluster, and cow cheeses predominate in the second. Both groups consist of many incorrectly classified samples, but tendency for differentiation is visible.

In order to achieve better differentiation of cow and sheep cheeses, a principal component analysis (PCA) was performed with the data to describe the main variations between the cheese species. Scores of the first three principal components, cumulatively accounting for about 60% of the variance, were normalized and plotted, as shown in Fig. 3. PCA effectively visualized all data and correctly arranged cheese samples into two isolated groups.

While the aim of PCA is simply to transform the original variables into a new set of variables, factor analysis attempts to construct a mathematical model explaining the correlation between a large set of variables. Factor analysis with the varimax-rotation of the whole set of elemental

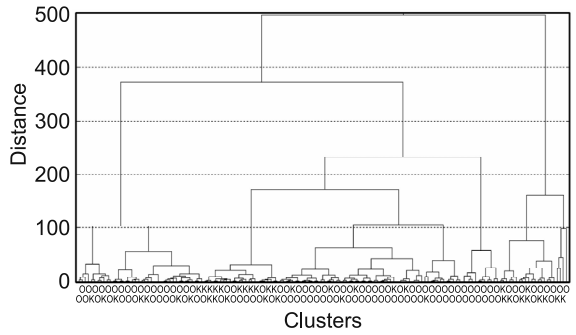

Fig. 2. Dendrogram of the Slovakian cow (K) and sheep (O) cheeses clustering.
Measure: Block, Method: Average within groups, Markers: Ba, Cr, Cu, Hg, Mg, Mn, Ni, and V.

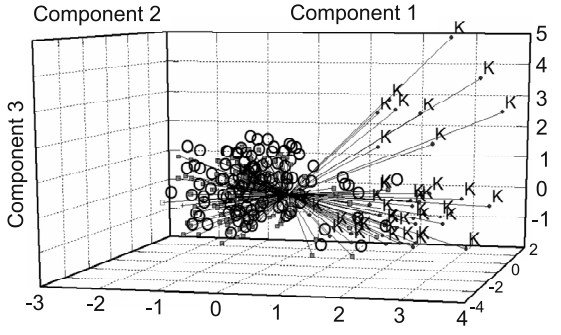

Fig. 3. Principal component analysis of Slovakian cow (K) and sheep (O) cheeses.
Markers: Ba, Cr, Cu, Hg, Mg, Mn, Ni, and V (Components 1–3: linear combinations of original dependent variables).

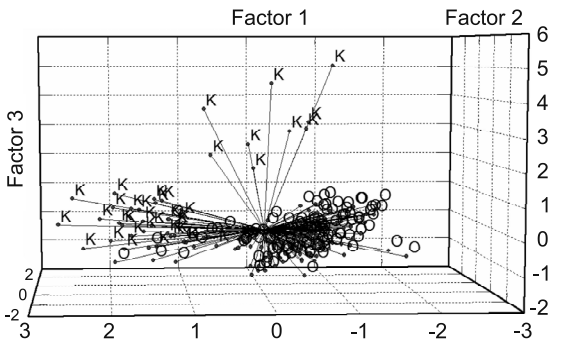

Fig. 4. Plot of factor scores of Slovakian cow (K) and sheep (O) cheeses.
Varimax rotation, Markers: Ba, Cr, Cu, Hg, Mg, Mn, Ni, and V (Factors 1–3: new set of variables based on correlation among the original variables).

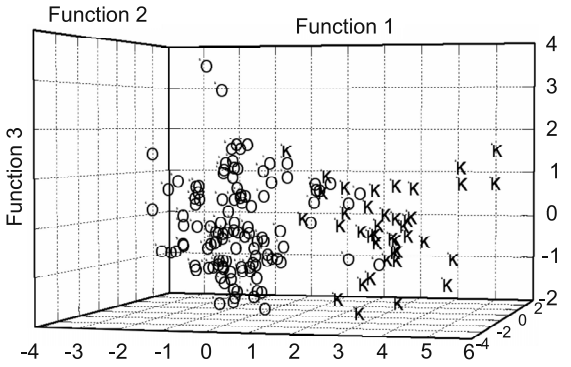

Fig. 5. Canonical discriminant analysis of Slovakian cow (K) and sheep (O) cheeses.
Markers: Ba, Cr, Cu, Hg, Mg, Mn, Ni, and V (Function 1–3: canonical variables, which are linear combinations of the original variables).

data showed the similar results as were found in the case of PCA and clearly differentiated cheese species (Fig. 4). The first three factors explained more than 73% of the variation in the elemental data. Variability of data for cow cheeses can be mainly explained by means of Mg, Hg and Cu, and variability of sheep cheeses by means of Cr, Ni, and V as marker elements.

Results from canonical discriminant analysis demonstrated very high potential to distinguish differences between cow and sheep cheeses. Discriminant function correctly classified 95% of the samples according to their species origin (Fig. 5).

Good results obtained from pattern recognition processing of elemental data set of cow and sheep cheeses, with an exception of cluster analysis, are in a very good relation with results achieved by HERNANDÉZ et al. [21], RINCON et al. [18], FRESNO et al. [20], CONI et al. [17], and RODRÍGEZ [19]. Excellent efficiency of the statistical pattern recognition techniques used in this work relates very probably with certain analogous elemental markers of cheese species origin (mainly Cu, Mg, and Mn). Because the markers were selected on the basis of variability of the elements in pasture soils in Slovakia, the strong segregation of cheese species may be explained by means of geochemical differences of cow and sheep pasture soils. Sheep pastures are predominantly located in the mid-mountain regions, while the cow ones in lowland agricultural areas. For this reason, the contents of minerals and other trace elements varied greatly in the feeding diet and enhanced the statistical differentiation of cheese species as well. Very high correlations of the marker elements were found for bryndza sheep cheese between their contents in pasture soil, grass and bryndza cheese (0.9414–0.9999). By-products from bryndza production (sheep milk, lumpy cheese, whey, boiled sheep whey) significantly correlated with the soil elemental markers as well (0.8246–0.9996).

CONCLUSION

Our results show that specific elemental data and pattern recognition techniques may be used to discriminate Slovakian commercial cow and sheep cheeses to identify their species origin. Principal component, factor and discriminant analysis demonstrated a very significant and effective segregation of sheep and cow cheeses according to their species origin. Further research is being undertaken to extend the method to other cheese species and to determine the detection limit of this procedure with cheese mixtures. This may be the basis for the development of simple and rapid protocols for cheese classification.

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