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Discrimination markers for the geographical and species origin of raw milk within Romania

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ABSTRACT

Isotopic and elemental compositions of raw bovine and ovine milk samples from three farms situated in Transylvania were investigated to see how differences in water composition and feeding regime were reflected in the milk samples. By applying chemometric analysis, a marker association suitable for the origin and species differentiation of raw milk samples is proposed in this pilot work. The most powerful combination of markers involved isotope ratios of oxygen ($^{18}O/^{16}O$) and carbon ($^{13}C/^{12}C$) combined with Sr and Mn concentrations. Also, the species differentiation between bovine and ovine milk products was made by the corroboration of Sr concentration with isotope ratio of hydrogen. The results obtained from cross-validation showed that resulting models of discriminant analysis allowed the correct classification of 90.9% of samples related to geographical origin, while for raw milk speciation the correct classification percentage was 96.4%.

1. Introduction

Stable isotope ratios of bio-elements ($^{2}H/^{1}H$; $^{18}O/^{16}O$; $^{13}C/^{12}C$; $^{15}N/^{14}N$), combined with elemental profiles has often been used for the determination of the authenticity of different food products such as wines, fruit juices, milk and dairy products (Kelly, Heaton, & Hoogewerff, 2005; Zhao et al., 2014). Besides milk authentication with respect to geographical origin, another important issue relates to the detection of milk produced by different animal species; many people are allergic to cows' milk and therefore they consume milk from other animals, e.g., sheep or goats. Previous studies have shown that multi-element profiles could be a useful tool for distinguishing both geographical origin and animal species (Camin, Perini, Colombari, Bontempo, & Versini, 2008; Crittenden et al., 2007; Ritz et al., 2005).

Stable isotope ratios of H, O, C and N in animals and animal products such as milk and cheese are directly influenced by the animals' diet and drinking water (Kornexl, Werner, Rossmann, & Schmidt, 1997; Molkentin & Giesemann, 2007). Isotope ratios of hydrogen and oxygen from milk reflect the isotopic composition of feed and water consumed by animals. Meanwhile, relative carbon stable isotope abundances in bulk milk mainly reflect the isotopic composition of the animals' feed.

Mineral and trace element concentrations in raw milk are not constant; they vary based on intrinsic and extrinsic factors. The intrinsic factors are those related to secretion from the mammary gland, such as the lactation state, animal species and health status, while extrinsic factors include season, dairy cattle ration (nutritional status of cow) and environment (nature of soil and locality of the farm) (Sola-Larrañaga & Navarro-Blasco, 2009).

Chemometric techniques applied to analytical data might provide an interesting and promising approach for classifying and identifying possible sources of influence upon elemental profiles (Sola-Larrañaga & Navarro-Blasco, 2009). The use of multi-element analysis has proved to be a good tool for origin authentication purposes of milk: for instance, cow versus buffalo (Benincasa, Lewis, Sindona, & Tagarelli, 2008). This association between analytical techniques and statistical tools was applied for differentiating milk or cheese samples according to their origin (Bontempo et al., 2011; Sacco et al., 2009).

In this study, ovine and bovine milk samples, along with the feed and water used for animal breeding, were tested for stable

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isotope ratios ($^1H/^2H$; $^{18}O/^{16}O$; $^{13}C/^{12}C$) and multi-elemental content. Milk was collected from three Transylvanian farms, located at different altitudes, but at distances of about 100 km from each other. Sheep milk was produced and collected only from two farms. The feeding regimes were similar for sheep and cows breeding in the same farm. The results obtained were processed by chemometric tools to find the best markers that could individualise both milk geographical provenance and also animal species.

2. Materials and methods

2.1. Sample collection

A total number of 77 samples, consisting of 28 raw milk (22 bovine milk and 6 ovine milk), 21 dry forages, 16 grass and 12 water samples from three Transylvanian farms in Romania were collected during one year, to determine which markers were most suitable to differentiation of milk origin and species type. The chosen farms are representative for Transylvania, being situated at different altitudes. Thus, Farm 1 is at an altitude of 1000 m, being characterised by a mean temperature of 6 °C, and an annual precipitation quantity of around 650–800 mm. The second farm, Farm 2, is characterised by a climate with an average temperature between 6 and 8 °C, is located at 570 m altitude and has an average annual precipitation of 800–950 mm. For Farm 3, situated at 400 m of altitude, the mean annual temperature is about 9.6 °C, while the mean precipitation is around 866 mm per year.

From all these farms, bovine milk was sampled from August 2014 to August 2015, while ovine milk was collected only from Farm 1 and Farm 3 during May–July, 2015.

2.2. Isotope ratio mass spectrometry measurements

The procedure of isotope ratio mass spectrometry (IRMS) consists of measuring the isotope ratio of an analyte converted into a simple gas, isotopically representative of the original sample, before entering into the ion source of an IRMS. The values were expressed in δ‰, i.e., $\delta(‰) = (R_X/R_S - 1) \times 1000$, where R_X is the $^{18}O/^{16}O$, $^{13}C/^{12}C$ or $^2H/^1H$ isotopic ratio sample and R_S is the $^{18}O/^{16}O$, $^{13}C/^{12}C$ or $^2H/^1H$ isotopic ratio of the standard. The isotopic values are expressed against international standards Vienna – Standard Mean Ocean Water (V-SMOW) for $\delta^{18}O$ and δ^2H and Vienna Pee Dee Belemnite (V-PDB) for $\delta^{13}C$.

For $\delta^{18}O$ and δ^2H analyses, a cryogenic distillation system under static vacuum was used to extract the water contained in milk and grass samples, always with quantitative recovery of water (Magdas & Puscas, 2011). The measurement of $\delta^{18}O$ and δ^2H values were made using a liquid-water isotope analyser (DLT-100, Los Gatos Research, CA, USA) and were calibrated against laboratory standards (Std): Std. 1, $\delta^{18}O = -11.54‰$; $\delta^2H = -79.0‰$; Std. 2, $\delta^{18}O = -7.14‰$; $\delta^2H = -43.6‰$; Std. 3, $\delta^{18}O = -2.96‰$; $\delta^2H = -9.8‰$. The standardisation of $\delta^{18}O$ and δ^2H values against international V-SMOW scale was done by calibration of the working standards with IAEA V-SMOW-2, Standard Light Antarctic Precipitation (SLAP-2, $\delta^{18}O = -55.5‰$, $\delta^2H = -427.5‰$ versus V-SMOW-2), and Greenland Ice Sheet Precipitation (GISP, $\delta^{18}O = -24.76‰$, $\delta^2H = -189.5‰$ versus V-SMOW-2) standards. The limit of uncertainty of the isotopic analysis was ±0.2‰ for $\delta^{18}O$ and ±0.6‰ for δ^2H. Each sample was analysed six times, but only the last three injections were taken into consideration for the calculations.

The determination of $\delta^{13}C$ from bulk milk samples (freeze-dried before analysis) were carried out on an Elemental Analyser (Flash EA1112 HT, Thermo Scientific, Waltham, MA, USA), coupled with an isotope ratio mass-spectrometer (Delta V Advantage, Thermo Scientific). For the quality control of analysis, three replicates of NBS-22 oil standard were analysed at the beginning of each sequence, then three replicates from each sample were measured. NBS-22 oil with a certified value of −30.031‰ versus V-PDB (IAEA, Vienna, Austria) was used as standard. The uncertainty was: ±0.3 for $\delta^{13}C$, ±0.4 for $\delta^{18}O$ and ±0.3 for δ^2H measurements.

2.3. Inductively coupled plasma-mass spectrometry measurements

The majority of inductively coupled plasma mass spectrometry (ICP-MS) applications involve the analysis of aqueous samples, directly or following sample pre-treatment, due to advantages of working with samples in solution. To determine the concentration of minerals and trace elements, 0.1 g of sample was placed into a Teflon bomb and digested with 5 mL of nitric acid in an oven at 200 °C for 12 h. The acid-digested sample solution was diluted in a 50 mL volumetric flask with ultrapure water. For each sample analysis, three replicates were measured to assure the control quality of measurements. All determinations were carried out by a Perkin Elmer ELAN DRC (e) mass spectrometer. Calibration standard solutions and internal standards were prepared by successive dilution of a high purity ICP, Multi-Element Calibration Standard 3 (10 mg L^{-1} Al, As, Ba, Be, Bi, Ca, Cd, Co, CR, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Ni, Pb, Rb, Se, Na, Ag, Sr, Tl, V, U, Zn standard, Matrix: 5% HNO_3, PerkinElmer Life and Analytical Sciences).

Accuracy was assured by analysing certified materials: SRM 1548a (for Al, As, Cd, Cs, Ni, Pb, Se, and Zn), SRM 2387 (for Ca, Cu, Fe, Mg, Mn, P, K, Na and Zn), and SRM 1547 (for V, Rb, Sr and Ba). For other elements, two samples spiked with a known amount of certified standard solution, were applied. Repeatability was verified by analysing five samples (from each analysed matrix: milk, water, grass and dried forages) in duplicate, obtaining an average standard deviation percentage below 4% (for Cr, Mn, Ni, Zn, As, Rb, Sr and Ba), below 7% (for P, Mg, K, Na, Ca, Li, V, Al, Cu, Se, Ga, Pb and Bi), and below 10% (for Fe, Co, Cd and Cs).

2.4. Statistical analysis

Chemometric data processing was performed using specific software. The experimental results were subjected to analysis of variance (ANOVA) and discriminant analysis (DA). ANOVA is used when the means of two or more groups (previously defined) need to be compared. The parameters that differentiate the groups are statistically significant if $p < 0.05$ (Field, 2009). ANOVA analysis was performed to reveal the parameters that could distinguish different types of milk sample, with respect to their geographical provenience (Farm 1, Farm 2 and Farm 3) and animal species (cow or sheep). The differences were considered significant at the level of $p < 0.05$ for a 95% confidence interval.

DA provides a classification model characterised by a linear dependence of the classification scores with respect to the descriptors (groups defined previously), which maximise the ratio between-class variance and minimise the ratio of within-class variance. DA assumes a priori knowledge of the group membership of each sample in a training set. In DA, groups are supposed to follow a multivariate normal distribution and to be linearly independent. The sample data minus one observation are used for the estimation of the discriminant functions, and then the omitted variable is classified from them; the procedure was repeated for all observations and so each sample was classified by discriminant functions which were estimated without its contribution ("leave-one-out" cross validation) (Dias, Peres, Veloso, & Machado, 2009). The success of the discrimination model was measured by the proportion of cases correctly classified using this cross-validation (Osorio, Koidis, & Papademas, 2015).