

Geographical verification of Slovenian milk using stable isotope ratio, multi-element and multivariate modelling approaches

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Abstract

This work examines the use of stable isotopes and elemental composition for determining geographical origin and authenticity of cow milk from four geographical regions of Slovenian. Samples (277) were collected during summer and winter (2012 to 2014). It was possible to discriminate milk samples according to the year, season and production region using discriminant analysis (DA). The overall temporal prediction variability was 84.6% and 56.4% for regional differences. It was also possible to discriminate milk from three geographic regions, although Alpine samples overlap with Dinaric and Pannonian ones. Prediction ability was the highest for the Pannonian (82.1%) and lowest (26.9%) for the Alpine region. Pairwise comparison using OPLS-DA also displaying good regional predictability (≥ 0.77) with $\delta^{13}\text{C}_{\text{cas}}$ values and Br content carrying the most variance. A model based on DD-SIMCA was also developed and applied to the control of Slovenian milk. The results revealed the mislabeling of three Slovenian milk products.

Keywords: cow milk, IRMS, elemental composition, stable isotope ratios, multivariate analysis

1. INTRODUCTION

Milk and dairy products are in considerable demand, making them attractive targets for fraudulent practices, such as mislabelling or economic adulteration (illegal substitution of inferior or cheaper ingredients for profit). These practices affect both consumers and producers.

The EC regulation protects consumers through a system of effective and impartial controls that define, within the Common Market, the safeguard of the ‘Protected Designation of Origin’ (PDO) (EC regulation n° 510/2006). The typical characteristics of a PDO dairy products demand that food is produced or grown in the specific geographical region with required technology that is unique for this region. Such identification is also of economic importance to many stakeholders allowing them recognition and a premium price.

This increase in awareness by consumers, regulators and producers over the importance of correctly declared food products has, in turn, led to an increase in the number of studies aimed at developing methods and techniques for verifying the origin of food (Drivelos & Georgiou, 2012). In particular stable isotope fingerprinting of the most abundant bioelements (i.e., $^1\text{H}/^2\text{H}$, $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$, $^{18}\text{O}/^{16}\text{O}$, and $^{34}\text{S}/^{32}\text{S}$) combined with various chemometric tools has been successfully applied in different food commodities to discriminate the geographical origin including vegetables (Mahne Opatić, Nečemer, Lojen & Vidrih, 2017;), beverages (juices, coffee, tea; Bizjak Bat et al., 2016; Cengiz, Turan, Ozdemir, Albayrak, Perincek & Kocabas, 2017) and cereal crops (rice, wheat; Li, Keely, Chan, Baxter, Rees & Kelly, 2015; Rashmi, Shree & Singh, 2017). One of the most recent significant advances in analytical chemistry has been the development of compound-specific stable isotope analysis CSIA of organic compounds using gas and/or liquid chromatography coupled to isotope ratio mass spectrometry. These techniques enable identification of relevant biomarkers (fatty acids, sugars) that can improve geographical discrimination (Paolini, Bontempo & Camin, 2017; She et al., 2019). In the case of dairy, the studies demonstrated that stable isotope ratios of H, C, N and O of milk and cheese were linked to the territory, particularly the type of vegetation and the environment.

The $\delta^{18}\text{O}$ content in milk reflects the isotope composition of the groundwater drunk by animals, which is influenced by geographical factors such as altitude, latitude, and distance from the sea (Crittenden, Andrew, LeFournour, Young, Middletom & Stockmann, 2007; Chesson, Valenzuela, O'Grady, Cerling & Ehleringer, 2010). Seasonal variations in isotopic composition are also apparent; for example, in summer, milk water has higher $\delta^{18}\text{O}$ values as dairy animals graze plants containing ^{18}O -enriched water (Garbaras, Skipityte, Šapolaite, Ežerinskis & Remeikis, 2019). This enrichment occurs because transpiration from leaves favours the lighter isotope of ^{16}O .

The $\delta^{13}\text{C}$ values in milk are mainly dependent on the animal's diet, especially if they are fed on maize, which is a C_4 cycle plant (Camin, Perini, Colombari, Bontempo & Versini, 2008). The main factor influencing the distribution of C isotopes in plants and their products is linked to the process of photosynthetic atmospheric CO_2 fixation. Plants using C_4 metabolic pathway such as maize, exhibit a $^{13}\text{C}/^{12}\text{C}$ ratio (expressed as $\delta^{13}\text{C}$ value) that differs significantly from plants using C_3 metabolic pathway (-10‰ to -20‰ for C_4 plants, and -22‰ to -33‰ for C_3 plants). Factors other than CO_2 -fixation pathway may also affect the isotopic composition of plants. These include local atmospheric CO_2 concentrations, plant variety, plant physiology (nutritional status of cells, enzyme levels, growth rate), water-use efficiency and cultivation practice. Further it was found that especially $\delta^{13}\text{C}$ values in milk in combination with other isotopes ($\delta^{15}\text{N}$ values) or other analytical parametrs (fatty acids, alfa-linolenic acids, vitamin E) can be used to discriminate between organically and conventionally produced milk (Kaffarnik, Schroder, Lehnert, Baars & Vetter, 2014; Chung et al., 2018; Chung et al., 2020). The $\delta^{15}\text{N}$ values in milk are generally influenced by the intensity of agricultural use, since the $\delta^{15}\text{N}$ values

in dairy products reflect – by way of the plants consumed by animals – the isotope composition of the original soil. Whereas the presence of nitrogen-fixing plants in the animal's diet can lead to lower $\delta^{15}\text{N}$ values because these plants utilize both atmospheric and soil nitrogen (Camin, Bontempo, Perini & Piasentier, 2016) .

In the case of sulphur, assimilation in plant material is mostly a unidirectional process, where isotope fractionation is related more to physical than biological processes and results in a 1 – 2‰ depletion in ^{34}S in the bulk plant, relative to its primary material, soils (Tanz & Schmidt, 2010). The geology of a region, fertilizer application (organic or artificial), anthropogenic atmospheric sulphur dioxide deposition and the distance from the coast (due to atmospheric seawater fallout), can all affect the natural variability of sulphur in the soils. The $\delta^{34}\text{S}$ values generally vary locally and are typical for defined cultivation areas (Crittenden et al., 2007). However, higher $\delta^{34}\text{S}$ values are found in arid regions, especially near the sea (high amounts of marine aerosols), or near salt deposits in bedrock (Krivachy (Tanz), Rossmann & Schmidt, 2015).

The multi-element stable isotope application was successfully applied to differentiate the milk according to the geographical origin. It was found that milk from different Italian origins could be discriminated based on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (Scampicchio et al. 2012), while Luo, Dong, Luo, Xian, Guo & Wu (2016) showed a good separation of milk originating from different countries (Australia, New Zealand, Germany, France, USA and China) using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in casein and $\delta^2\text{H}$ and $\delta^{18}\text{O}$ in milk water. Further $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{18}\text{O}$, and $\delta^{34}\text{S}$ values were the main parameters important to discriminate organic milk from different regions in Korea (Chung et

al., 2020). The differentiation according to the geographical origin could be even more effective when combined with elemental analysis. The multi-element composition of animal tissues to some extent also reflects the composition of elements in the vegetation that animals eat. It has been proven that the regional-specific distribution of elements is transmitted from the soil into the plant and passed on to the animals fed by locally or regionally grown pasture. The nutrient and element bioavailability depend on pH of the soil, clay mineralogy and humic complexes and thus give us direct information about geographical origin (Wang, Takematsu & Ambe, 2000). However, animals brought up on commercial animal feed lose the regional context.

To date, several studies have used both isotopes and elements jointly in milk and dairy products (Camin et al., 2016 and references within). Sacco et al. (2009) analysed milk samples from different geographical areas, including Italy and six other EU countries, to determine whether their chemical composition could be used to identify their geographical origin. Elemental concentrations (Ba, Mn, Zn, Fe and Cu) were determined together with C and N isotopic ratios and chemical analysis such as fatty acids, sugars, aminoacids. Beside fatty acids composition stable carbon isotopes were the most important parameters for origin discrimination. Pillonel et al., (2003) achieved separation of Emmental cheese from Finland, Bretagne and Savoie, while Bontempo et al., (2011) successfully distinguished between traditional alpine and pre-alpine Italian cheese. Furthermore, Camin (2012) and co-workers present two models with excellent classification success rates (> 98%), one for predicting the geographic origin of the 7 European hard cheese production, and another for discrimination of PDO Parmigiano Reggiano cheese from 9 European and 2 non-European producers. The last two papers deal with the combined use of elemental and stable isotope data to differentiate milk from different regions in Argentina

(Griboff, Baroni, Horacek, Wunderlin & Monferran, 2019) and to distinguish PDO and non-PDO Mozzarella di Bufala Campana (Bontempo et al., 2019). The results indicated that stable isotope ratio and elemental composition could enable detection of the regional provenance of dairy products. Furthermore, the isotopic fingerprinting can be combined with other methods such as, MIRS, NIRS, NMR, HPLC and GC to improve the determination of the origin of milk (Table S1). Table S1 summarizes publications dealing with the geographical origin of milk using different analytical methods combined with appropriate statistical analysis.

In Slovenia, Nečemer, Potočnik & Ogrinc, (2016) systematically defined authentic Slovenian cow, sheep and goat milk and cheese and determined the geographical origin, which contributed towards supporting the existence of a Slovenian PDO cheese and protect consumers and producers alike from mislabelling. However, this research was restricted only to two years of investigation (2012, 2013) and the main focus was the differentiation according to the milk species and cheese production. In the present study we included additional year of milk production (2014) and more stable isotope parameters ($\delta^2\text{H}_{\text{cas}}$, $\delta^{18}\text{O}_{\text{cas}}$, $\delta^{34}\text{S}_{\text{cas}}$) to make the approach more robust and developed the model for verification of the origin of commercial milk samples. It is important for Slovenia to promote and to raise awareness about the importance and characteristics of locally produced and processed food. For example, most milk and dairy products, produced and processed in Slovenia, now use the “Selected Quality - Slovenia” mark that helps the consumers to easily recognize local quality products. This is because the Slovenian dairy industry is small and can compete globally only by maintaining the high quality of their products including milk. Thus, the aim of this study was to establish an isotopic, and elemental database of Slovenian cow milk to (i) study the effect of year and season

on milk composition in relation to diet composition; (ii) differentiate milk according to geographical origin using appropriate statistical methods; and (iii) develop a model based on existing data to verify if milk on the Slovenian market really corresponds to its declaration.

2. MATERIAL AND METHODS

2.1 Samples

During a 3-years study, from 2012 to 2014, 277 raw cow milk samples were obtained two times per year (in winter and summer) from four geographical regions: Mediterranean, Pannonian, Dinaric and Alpine in Slovenia (Figure 1). This regionalization of Slovenia is primarily based on the analysis of geology, surface relief, climate, vegetation, and land use. The geographical characteristics of the sampling locations with latitude, longitude, distance from the sea and altitude are presented in Table S2. Mean temperature and mean amount of precipitation for different seasons (winter, summer) and different years (2012, 2013 and 2014) are also included. In addition, eleven commercial milk samples declared as Slovenian milk, and 13 samples of milk from four other European countries (Croatia, Germany, Austria and Italy) were collected to verify their authenticity and for evaluating and demonstrating model robustness. All samples were kept in plastic containers and stored at -20°C in plastic containers.

2.2 Stable isotope measurements

2.2.1 Isolation of casein in milk

Milk fat was removed by centrifugation (Type Centric 322 A, TEHTNICA, Slovenia, 10 min at 3200 rpm) and casein precipitated from the skimmed milk by acidification at pH of 4.3 with 2M HCl (Carlo Erba, Val de Reuil, Italy) followed by centrifugation for 10 min at 3200 rpm. At pH = 4.3, which is the isoelectric point for casein, only casein was precipitated. The precipitate was rinsed twice with Milli-Q water (Millipore, USA), followed by acetone and petrolether (Carlo Erba, Val de Reuil, Italy) and freeze-dried.

2.2.2 Isotope Ratio Mass Spectrometry (IRMS) measurements

Stable isotope ratio measurements were performed using isotope ratio mass spectrometry (IRMS) and reported using the δ – notation in ‰ with the following equation 1 (Brand, Coplen, Vogl, Rosner & Prohaska, 2014):

$$\delta(^i/^jE) = \delta(^i/^jE) = \frac{(^i/^jR_P - ^i/^jR_{Ref})}{^i/^jR_{Ref}} \quad (1)$$

Where superscripts i and j denote the highest and the lowest atomic mass number of element E, and R_P and R_{Ref} indicate the ratio between the heavier and the lighter isotope ($^2\text{H}/^1\text{H}$, $^{13}\text{C}/^{12}\text{C}$, $^{18}\text{O}/^{16}\text{O}$, $^{15}\text{N}/^{14}\text{N}$, $^{34}\text{S}/^{32}\text{S}$) in the sample (P -product) and reference material (Ref), respectively. The $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values are reported relative to the V-SMOW (Vienna-Standard Mean Ocean Water) standard, $\delta^{13}\text{C}$ values to the V-PDB (Vienna-Pee Dee Belemnite) standard, and the $\delta^{34}\text{S}$

187 sulphur values relative to the V-CDT (Vienna Cañon Diablo Troilite) standard. The $\delta^{15}\text{N}$ values
188 are reported relative to AIR.

189 The $^{18}\text{O}/^{16}\text{O}$ ratio in milk water ($\delta^{18}\text{O}_w$) was determined directly in milk using the equilibration
190 method where the sample was purged with a reference CO_2/He gas (5% CO_2 , 95% of He) at
191 40°C for 3 h. Measurements were performed using a Multiflow system (IsoPrime, Cheadle
192 Hulme, UK) connected to a continuous flow IRMS (GV Instruments). Each sample was
193 measured twice and the mean value was considered. Analyses were normalised against two
194 internal laboratory reference materials: snow water ($\delta^{18}\text{O} = -19.73 \pm 0.02\text{‰}$) and seawater
195 ($\delta^{18}\text{O} = -0.34 \pm 0.02\text{‰}$). To ensure accuracy of the results, independent laboratory reference
196 material MilliQ water ($\delta^{18}\text{O} = -9.12 \pm 0.04\text{‰}$) was analysed periodically through the sequence
197 and used as control material. The internal laboratory and independent laboratory reference
198 materials were calibrated against international reference materials: V-SLAP2 (Standard Light
199 Antarctic Precipitation, $\delta^{18}\text{O} = -55.5 \pm 0.02\text{‰}$) and V-SMOW (Vienna-Standard Mean Ocean
200 Water 2, $\delta^{18}\text{O} = 0 \pm 0.02 \text{‰}$). The analytical precision of $\delta^{18}\text{O}$ measurements was $\leq 0.10\text{‰}$ in
201 the form of $\pm$ standard deviation relative to the laboratory reference material MilliQ water.

202 Freeze-dry casein sample from 2012 was weighed to approximately 1 mg in a tin capsule,
203 closed with tweezers and placed into the autosampler of the elemental analyser. Each sample
204 was measured twice and the mean value was considered. All analyses for $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$
205 determination were performed separately on a Europa Scientific 20-20 continuous flow IRMS
206 with an ANCA-SL solid-liquid preparation module (Sercone, Crewe, UK). The internal
207 laboratory reference material UreaC (Europa), ($\delta^{13}\text{C}$ value of $-28.3 \pm 0.2\text{‰}$; $\delta^{15}\text{N}$ value of

208 $-0.36 \pm 0.2\text{‰}$) was used to normalise the results. The analytical precision, expressed as standard
209 deviation from laboratory reference material, was $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.3\text{‰}$ for $\delta^{15}\text{N}$.

210 For $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ and $^{34}\text{S}/^{32}\text{S}$ determination in samples from 2013 and 2014, 10 mg of dry
211 casein samples were analysed simultaneously using the IsoPrime 100 – Vario PYRO Cube
212 (OH/CNS Pyrolyser/Elemental Analyser) (IsoPrime, Cheadle, Hulme, UK). Each sample was
213 measured at least twice and the mean values was considered. The results were normalised
214 against the international standards: IAEA-CH-7 ($\delta^{13}\text{C} = -32.15 \pm 0.03\text{‰}$), IAEA-CH-6 ($\delta^{13}\text{C}$
215 $= -10.45 \pm 0.03\text{‰}$), IAEA-CH-3 ($\delta^{13}\text{C} = -24.72 \pm 0.04\text{‰}$), IAEA-N-1 ($\delta^{15}\text{N} = -0.4 \pm 0.0\text{‰}$),
216 IAEA-N-2 ($\delta^{15}\text{N} = +20.3 \pm 0.2\text{‰}$), IAEA-S-1 ($\delta^{34}\text{S} = -0.3\text{‰}$), IAEA-S-2 ($\delta^{34}\text{S} = +22.49 \pm$
217 0.16‰). To ensure accuracy of the results other reference materials included CRP-IAEA casein
218 ($\delta^{13}\text{C} = -20.3 \pm 0.09\text{‰}$, $\delta^{15}\text{N} = +5.62 \pm 0.19\text{‰}$, $\delta^{34}\text{S} = +4.18 \pm 0.74\text{‰}$), and Casein, B2155
219 Sercon ($\delta^{13}\text{C} = -26.98 \pm 0.13\text{‰}$, $\delta^{15}\text{N} = +5.94 \pm 0.08\text{‰}$, $\delta^{34}\text{S} = +6.32 \pm 0.8\text{‰}$) were analysed
220 periodically through the sequence and used as control materials. The analytical precision of
221 measurements, expressed as standard deviation relative to control material, was $\pm 0.2\text{‰}$ for
222 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ and 0.3‰ for $\delta^{34}\text{S}$.

223 The procedure for determining the ratios $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ in casein ($\delta^{18}\text{O}_{\text{cas}}$, $\delta^2\text{H}_{\text{cas}}$) involved
224 transferring 0.2 mg of casein to a silver capsule and analysing the sample simultaneously on
225 DELTA XP IRMS (Thermo Scientific) coupled with a TC/EA pyrolyser (Thermo Finnigan).
226 For normalisation of the results, two internal laboratory reference materials were used: CBS
227 (Caribou Hoof Standard) with $\delta^2\text{H}$ values of $-157 \pm 0.9\text{‰}$ and $\delta^{18}\text{O}$ values of $+3.8 \pm 0.3\text{‰}$, and
228 KHS (Kudu Horn Standard) with $\delta^2\text{H}$ value of $-35.3 \pm 1.1\text{‰}$ and $\delta^{18}\text{O}$ value of $+20.3 \pm 0.3\text{‰}$.

These two standards were used for normalization of the data, since at the moment there are no recognized international standards available.

2.3 Multi-elemental analysis

The multi-elemental composition was determined using two different methods: Energy Dispersive X-ray Fluorescence Spectrometry (EDXRF) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The EDXRF was applied to analyse elemental fingerprint of P, S, Cl, K, Ca, Br, Rb and Sr, while ICP-MS was used to determine Mn, Fe, Cu and Se and toxic elements such as As, Cd and Pb present at low concentration ranges. These elements could not be determined by EDXRF due to its poor sensitivity, but are important for geographical discrimination.

2.3.1 Elemental analysis performed by Energy Dispersive X-ray Fluorescence Spectrometry (EDXRF)

Multi-elemental composition including P, S, Cl, K, Ca, Zn, Br, Rb, Sr content in milk samples were determined with Energy Dispersive X-ray Fluorescence Spectrometry (EDXRF). Each freeze-dried sample (0.5 – 1.0 g) was pressed into a pellet using a hydraulic press. The disc radioisotope excitation sources of Fe-55 (25 mCi) and Cd-109 (20 mCi) (Eckert & Ziegler, Berlin, Germany) were used as the primary excitation sources. The emitted fluorescence radiation was measured using a Si(Li) detector (Canberra, Industries, Meriden, U.S.A.). The

spectrometer was equipped with a vacuum chamber (Fe-55) for measurement of elements of P, S and Cl. The energy resolution of the spectrometer was 175 eV at 5.9 keV. The analysis of complex X-ray spectra was performed by AXIL (IAEA, Vienna, Austria) spectral analysis program (Nečemer, Kump & Vogel-Mikuš, 2011). An in-house developed QAES (Quantitative Analysis of Environmental Samples) software was used for quantification (Nečemer et al., 2011). The method's estimated uncertainty was 5% to 10%. The accuracy was checked against reference material BCR 151 (Skimmed milk powder) (Potočnik, Nečemer, Mazej, Jaćimović & Ogrinc, 2016).

2.3.2 Multi-elemental analysis using inductively coupled plasma mass spectrometry (ICP-MS)

Multi-elemental analysis in freeze-dried milk samples from 2012 and 2013 were further determined using inductively coupled plasma mass spectrometry (ICP-MS) using two-sample preparation procedures: microwave digestion and dilution. Both are described in full by Potočnik et al., (2016). The 2014 milk samples were prepared using only the microwave digestion procedure.

Microwave digestion: An aliquot of dry raw milk (1 ml) was weighed into a quartz tube. To this was added 1 mL of 65 % HNO₃, (Merck, Germany, suprapur) and 1 mL of 30 % H₂O₂, (Merck, Germany, suprapur). The sample was then subjected to closed vessel microwave digestion (Microwave system ETHOS 1, MILESTONE SN 130471) at 1500 W: ramp to 130°C 10 min, ramp to 200°C 10 min, hold 20 min, cool 20 min. The samples were then left to

equilibrate to room temperature. The digest was then quantitatively transferred to a 10 mL polyethylene graduated tube and filled to the mark with Milli-Q water (Millipore, USA). Each sample was diluted five times before analysis. External calibration was performed using a CP multi-element standard solution XVI (CertiPUR®, Merck).

Dilution: In brief, each lyophilized Milk sample (0.15 g) was diluted five times with a solution containing 25% ammonia, 0.5 g l⁻¹ Triton X-100, and 0.5 g l⁻¹ EDTA in Millipore water.

All samples were measured using an Octapole Reaction System (ORS) ICP-MS (7500ce, Agilent) equipped with an ASX-510 Autosampler (Cetac). To ensure quality of the results/data, blank samples, control samples and certified reference materials were run together with the samples on a daily basis. The accuracy of the results was checked using the following certified reference materials: a whole milk powder (NIST 8435, National Institute of Standard and Technology), a non-fat milk powder (NIST 1549, National Institute of Standard and Technology), and three skim milk powders (BCR 150, ERM-BD 150, ERM-BD 151; EC-JRC-IRMM). The results obtained for the multielement analysis of certified reference materials are shown in Table S3. Since there are no reference materials for As, the competency of the laboratory was verified by participating in the FAPAS (Food and Environmental Research Agency, Sand Hutton, York, VB) interlaboratory comparison scheme (Potočník et al., 2016).

The limits of detection (LOD) for Cd, Pb, As, Se, Mn, Cu, Zn and Fe – calculated as three times the standard deviation of the blank sample – were 0.1, 0.2, 0.05, 2, 1, 6, 35 and 30 ng/g of milk samples, respectively, and the limit of quantification (LOQ), calculated as ten times the standard deviation of the blank samples for Cd, Pb, As, Se, Mn, Cu, Zn and Fe was 0.3, 0.7, 0.16, 7, 3,

20, 116 ad 100 ng/g of milk samples, respectively Estimated combined uncertainty was 5% for Cu, Zn, Fe, 10% for Se, Mn and 15% for As, Cd, and Pb. More detailed information about methodology are describet in [Potočnik et al., \(2016\)](#).

2.4 Statistical evaluation

Statistical calculations were carried out using the XL-STAT software package ([Addinsoft, Long Island, NY, USA, 2019](#)). Basic statistics included mean values (median and arithmetic mean), standard deviation (S.D.), minimum, maximum, analysis of variance ANOVA and Kruskal-Wallis test. Normally distributed data were treated with ANOVA using Duncan's test to compare the means, while the Kruskal–Wallis test was used for not-normally distributed data with a *post-hoc* Dunn test to compare the medians. Both the one-way ANOVA and the two-way ANOVA were performed on ranked data. One-way ANOVA was performed to determine the significant temporal (season, year) and spatial difference of variables. As different factors act simultaneously only two-way ANOVA, with different fixed parameters: year and season; tear and location; season and location was applied to the data. In the statistical test probability (p) values of less than 0.05 were used to indicate a significance level. If the significance was noted in a response factor, the calculation was followed by post-hoc testing using the Tukey's Honestly Significant Difference (HSD) test. For not normally distributed data, one-way analysis of variance by ranks (Kruskal-Wallis test) was performed.

Further, clustering - an unsupervised data mining approach, was applied to all milk samples with regard to the temporal variability and geographical discrimination. Tha basic idea of

clustering is to split the dataset from all samples, in our case all milk samples, into clusters (i.e. groups) such that milk samples from the same cluster share similar characteristics and are more similar than milk samples from other clusters. By applying clustering, we actually split all milk samples based on the basis of similarity and dissimilarity between them. For further usage of results obtained in this study, when a new milk sample comes, first we should describe it with the same variables that are used to describe the milk samples in this study, and then by applying the similarity between it and all milk samples that are involved in the study, we can find where it belongs and with which milk samples share the most common characteristics. Additionally, if for some milk sample some of the measurements are missing, we can calculate or borrow its values, based on the most similar samples that are obtained from the clustering result. In our case, to calculate the similarity between the milk samples, we used the Gower's distance from the R package 'cluster' since we have a combination of numerical (i.e. measurements) and categorical variables (i.e. year, etc). Next, the 'partitioning around medoids' (PAM) algorithm was applied to divide the milk samples into different clusters ([Kaufman and Rousseeuw, 1990](#)), so that those that belong to the same cluster all share similar characteristics.

To determine the key factors responsible for differentiating between production year and season, and for determining the geographical origin, the study applied discriminant analysis (DA). Moreover, orthogonal projection to latent structure-discriminant analysis (OPLS-DA) was performed using all data to assess the geographical indication of milk samples, while data driven soft independent modelling of class analogy (DD-SIMCA) were used to verify the declaration of Slovenian milk samples from the market.

3 RESULTS AND DISCUSSION

3.1 Elemental and isotope composition of cow milk

Table 1 presents the elemental composition (content in Ca, K, Cl, S, P, Zn, Br, Rb, Sr, Mn, Fe, Cu, Se, As, Cd, Pb) and stable isotope values of $\delta^{18}\text{O}_w$ in the cow milk samples as well as stable isotope values ($\delta^2\text{H}_{\text{cas}}$, $\delta^{13}\text{C}_{\text{cas}}$, $\delta^{15}\text{N}_{\text{cas}}$, $\delta^{18}\text{O}_{\text{cas}}$, $\delta^{34}\text{S}_{\text{cas}}$) in casein isolated from cow milk samples. The given values are the mean and standard deviation (SD) of obtained results and literature values. These data represent the database of authentic Slovenian milk sample.

Statistical analysis of results are presented in Table S4. The elemental composition of the milk samples is similar to that reported in the literature regarding the high levels of calcium, potassium, manganese, zinc and selenium, but differ in the lower amounts of sodium, iron, copper and bromine. It is assumed that the high levels of zinc and selenium in cow's milk are related to feeding practices and the use of mineral supplements. The levels of the toxic elements: As, Pd and Cd, are lower in Slovenian milk compare to the literature data and pose no health risk (Dobrazanski, Kolacz, Gorecka, Chojnacka & Bartkowiak, 2005). Significant differences in relation to the year of production was observed for all elements except for Se and Pb, while season of production was significant only for Mn, Ca, Zn, Rb and Pb with higher concentrations observed for Ca in Mn in winter and lower concentrations of Zn, A, Pb and Rb in summer. Significant was also interaction between year and season of production except for Se. Between

the 15 elements determined, nine (Mn, Cu, Se, Cl, Zn, Br, Rb and Sr) show significant regional variation. Mediterranean region was characterized by lower concentrations of Zn comparing to other regions (Table 1). The only observable difference in the levels of Cl is between the Alpine region (84 ± 22 mg/100 g in 2012; 90 ± 9 mg/100 g in 2013 and 95 ± 9 mg/ 100 g in 2014) and the Mediterranean region (66 ± 18 mg/100 g in 2012; 80 ± 14 mg/100 g in 2013 and 92 ± 18 mg/ 100 g in 2014). On the other hand, the lowest concentrations of Cu were determined in Alpine region (3.11 ± 0.67 μ g/100 g) and the highest in Mediterranean region (5.83 ± 2.24 μ g/100 g), which can be distinguished from each other with the other two regions falling in between. Significant differences between regions were also apparent with regards to the levels of Mn, Se, Br, Rb and Sr. In the case of Se, the content in milk from the Dinaric region was higher compared to the Mediterranean and Alpine regions, whilst the Br content of milk from the Panonnian region is the lowest and statistically different from the others. With mixed-effects ANOVA (year x region; season x region), statistical differences in all elements except for Pb, Fe, Cu, K and P were also confirmed. The literature data (references in Table 1) show that the elemental contents in cow milk vary considerably especially for Zn and their composition appears to be affected by genetics, physical and environmental factors.

The $\delta^{18}\text{O}_w$ values in raw milk varied over the sampling period from -8.0‰ to 0.3‰ , the $\delta^{18}\text{O}_{\text{cas}}$ values were from 9.0‰ to 14.6‰ , and the $\delta^2\text{H}_{\text{cas}}$ values were from -149‰ to -93‰ . Statistically significant difference was observed in $\delta^{18}\text{O}_w$ values according to the year, season, region and interactions therein. The $\delta^{18}\text{O}_w$ values were higher in 2012 comparing to 2013 and 2014 and were higher during the summer. Turkey contrasts tests show that the $\delta^{18}\text{O}_w$ values were the highest in Mediterranean region with the mildest climate in Slovenia, differentiating it

from the Pannonian region. Our findings are consistent with those of previous reports showing that $\delta^{18}\text{O}$ values in milk water show a clear seasonal variation, displaying a greater ^{18}O content in the summer in milk water relative to ground water (Crittenden et al., 2007; Chung et al., 2020), related to the effect of a high evapotranspiration rate in fresh plant feed and animals during summer. The $\delta^{18}\text{O}_{\text{cas}}$ values are in agreement with literature values, whereas the $\delta^2\text{H}_{\text{cas}}$ values are lower than literature values. No correlation was observed between $\delta^{18}\text{O}_{\text{cas}}$ and $\delta^2\text{H}_{\text{cas}}$ values, which supports the finding from previous studies where 30% of the H and 70% of the O in milk protein derives from local water (Ehleringer, Bowen, Chesson, West, Podlesak & Cerling, 2008). The remaining fraction originates from the diet. No statistically significant difference was observed in $\delta^{18}\text{O}_{\text{cas}}$ according to the season and region, however in 2013 statistically lower values were observed compared to 2014. For $\delta^2\text{H}_{\text{cas}}$ values the only statistical difference was observed according to the season ($p < 0.05$) with the highest values observed in summer and interaction between season and region.

A broad range of $\delta^{13}\text{C}_{\text{cas}}$ values was observed (Table 1) from -28.2 to -17.8‰ . These differences are related to the cows fed – which depends, to a large extent, on the location of the herd. The $\delta^{13}\text{C}$ values higher than -23.5‰ indicate the presence of maize (C_4 plant) in the diet (Camin et al., 2008). The $\delta^{15}\text{N}_{\text{cas}}$ values are also broadly distributed (2.5 to 9.6‰) during the sampling period. The presence of leguminous plants and the absence of intensive agricultural practices can explain the low $\delta^{15}\text{N}_{\text{cas}}$ values. Alternatively, the highest $\delta^{15}\text{N}_{\text{cas}}$ values occur in the samples from the Alpine (9.5 ‰ in 2012 and 8.3‰ in 2013) and Dinaric (9.6‰ in 2012) regions. The high $\delta^{15}\text{N}_{\text{cas}}$ are supported by the high $\delta^{13}\text{C}_{\text{cas}}$ values and indicate an increasing content of maize silage in the diet, which typically grows on intensively fertilized fields. In

Slovenia, more than 80% of the milk samples indicate the presence of maize in the diet and mainly originates from the Pannonian region with intensive milk production by stabled cows. Statistically significant difference was observed in $\delta^{13}\text{C}_{\text{cas}}$ and $\delta^{15}\text{N}_{\text{cas}}$ values according to the year, season, region and interactions therein. Both higher $\delta^{13}\text{C}_{\text{cas}}$ and $\delta^{15}\text{N}_{\text{cas}}$ values were observed in 2013, while during the winter lower $\delta^{13}\text{C}_{\text{cas}}$ and higher $\delta^{15}\text{N}_{\text{cas}}$ values were determined. Further, it is also possible to distinguish Mediterranean region from Pannonian, whilst differentiation between the Dinaric and Alpine regions based on $\delta^{13}\text{C}_{\text{cas}}$ and $\delta^{15}\text{N}_{\text{cas}}$ values was not possible.

The $\delta^{34}\text{S}_{\text{cas}}$ values range from 0.5 to 7.7‰ and fall within the range (3 to 8‰) found in European dairy products (Camin et al., 2016), but are significantly lower than $\delta^{34}\text{S}$ values in New Zealand milk that ranged from 7.7‰ to 14.8‰, reflecting seaspray effect (Crittenden et al., 2007). This study found no correlation between $\delta^{15}\text{N}_{\text{cas}}$ and $\delta^{34}\text{S}_{\text{cas}}$ values, even though similar factors influence their isotopic fractionation, which suggests that $\delta^{34}\text{S}_{\text{cas}}$ values depend more on geology than on feeding regime. The estimated sulphur trophic shift between animals and their diet ranged between 0‰ and +1‰ (Harrison et al., 2011) and small differences could be found among the various animal organs. For $\delta^{34}\text{S}_{\text{cas}}$ values the only statistical difference was observed according to the region and interactions between season and region. Tukey contrasts indicate that based on $\delta^{34}\text{S}_{\text{cas}}$ values the Mediterranean region is distinguishable from the Alpine and Pannonian regions, and that the Dinaric region is also distinct from the Pannonian region, while differentiating between the Dinaric and Mediterranean regions was not possible.

3.2 Temporal variability

Clustering was performed using 24 variables to describe the milk samples. The analysis included the measured values, geographical origin, year of production (2012, 2013 and 2014) and sampling season (summer, winter). For several samples, the $\delta^{18}\text{O}_{\text{cas}}$, $\delta^2\text{H}_{\text{cas}}$, and $\delta^{34}\text{S}_{\text{cas}}$ were missing, and for this reason, these three variables were excluded from further analysis. Geographical origin was also omitted to test if the other variables can determine the origin of the milk samples. Before performing the clustering, we used the data to estimate the optimal number of clusters. Several methods to perform this analysis exist. In our case, the number of clusters was estimated using the average silhouette width, which defines how well each milk sample lies within its cluster. To perform this, the average silhouette width is calculated for different numbers of clusters (i.e. in our case starting from two till 15), from which the optimal number of clusters is the one that corresponds to the maximum value of all possible tested values. The optimal number of clusters is six and the most influence parameters are the categorical variables: season, year and feeding parameter. Although cluster analysis produced six clusters, they did not sufficiently differentiate geographical origin, while season and year most influenced clustering (Figure 2). We should mention again that the geographical origin was not used as a parameter in the clustering since we would like to make sure that this will come as a result. However, the insufficient discrimination by this method can be related to the number of milk samples obtained from some regions.

The results were further evaluated using multivariate analysis. In this case, 176 samples consisting of authentic Slovenian milk samples from summer and winter 2012 (12s,w), 2013

(13s,w) and 2014 (14s,w) were statistically evaluated. Parameters included stable isotope measurements ($\delta^{13}\text{C}_{\text{cas}}$, $\delta^{15}\text{N}_{\text{cas}}$, $\delta^{18}\text{O}_w$) and elemental concentrations (P, S, Cl, K, Ca, Mn, Fe, Cu, Se, Zn, Br, Rb, Sr). Figure 3a,b presents the results of the DA as a discriminant function score plot (a) and a discriminant loadings plot (b). In the functional score plot, each group (centroid) are represented by a scatter plot, while in the loadings plot they appear as a set of vectors indicating the degree of association of the corresponding initial variables with the first two discriminant functions. In the latter, the degree of distribution of each parameter in the classes is revealed. Except the 2012 and 2013 winter samples (12w, 13w) that overlap, good separation between year and season of production was achieved. The most influential parameter for the summer 2012 (12s) class (see top left of biplot a) is $\delta^{18}\text{O}_w$ (direction and length of its vector is towards the left part of the biplot). The $\delta^{18}\text{O}_w$ values in summer 2012 were higher compared to other seasons, which is probably a result of the hot summer and drought conditions that year affecting the metabolism of the cows (Critteenden et al., 2007). Winter and summer samples 2014 (the right lower part of biplot (a) stand out from the other classes on account of the high levels of P and S. Separation between the classes 13s and 13w (12w) is likely a result of the higher values of $\delta^{13}\text{C}_{\text{cas}}$ and $\delta^{15}\text{N}_{\text{cas}}$ in the winter group indicating higher proportion of maize in the diet, while from the element a contribution of Ca, Zn and Mn should be mentioned. A leave-one-out cross-validation (LOOCV) classified 84.6% of the 169 samples correctly.

3.3 Geographical discrimination

Discriminant analysis (DA) was further tested if milk samples can discriminate between the Alpine, Mediterranean, Pannonia and Dinaric regions (Table 1). This calculation was first performed on the samples from the same production year in 2012, 2013 and 2014. The best separation was obtained for 2013 and 2014 as a result of including the $\delta^2\text{H}_{\text{cas}}$, $\delta^{18}\text{O}_{\text{w}}$ and $\delta^{34}\text{S}_{\text{cas}}$ values. The results are presented in Figure 3c,d, showing three separated groups, while Alpine group is overlapping with Dinaric and Panonnian ones. The group from Mediterranean region is well separated from the others due to the higher $\delta^{18}\text{O}_{\text{w}}$ and $\delta^{34}\text{S}_{\text{cas}}$ values and higher concentrations of Cu. For the Dinaric group (lower left part in the graph), it is the Cl and Br concentrations together with Rb and Se that has the most separation power. The variables that contribute to the separation of Pannonian group were $\delta^2\text{H}_{\text{cas}}$, Mn, $\delta^{18}\text{O}_{\text{cas}}$, Sr and $\delta^{13}\text{C}_{\text{cas}}$ values. The prediction ability was the highest for Pannonian region (82.1%) and the lowest for Alpine region (26.9%) with the overall prediction of 56.4%.

Further, OPLS-DA tests for pairwise comparisons among all four regions (Figure 4) was calculated similar than in the study performed by [Chung et al. \(2020\)](#). These models had an explanatory power of 80%–100% (F1) for variation in the X variables and displayed high quality and goodness of fit and predictability (≥ 0.77) to differentiate among the geographical regions in Slovenia. The lowest prediction was obtained between Alpine and Dinaric region and Alpine and Pannonian region, which supports our DA model. It was found, that the prominent factor influencing OPLS-DA models was $\delta^{13}\text{C}_{\text{cas}}$ (VIP value: 1.33–1.97), except for the discriminative model between Dinaric with Panonian and Alpine regions where

concentrations of Br are more important (VIP: 1.39-2.36) (Fig. 4a,g). The findings of the study show the importance of combining both elemental and isotopic values and support other similar studies on by other authors (Bontempo et al., 2011; Camin et al., 2012; and Nečemer et al., 2016).

3.4 Evaluation of authenticity of Slovenian milk in the market

The data set of milk samples was then used to check the authenticity of commercial samples available on the market. In this case, the data set contained 76 samples of authentic Slovenian milk samples (SI, N=52), samples of milk available on the Slovene market (Si-m, N=10), Austrian (AT, N=3), Italian (IT, N=3), German (DE, N=3) and Croatian (HR, N=5) milk samples. The measured parameters included stable isotope data ($\delta^2\text{H}_{\text{cas}}$, $\delta^{13}\text{C}_{\text{cas}}$, $\delta^{15}\text{N}_{\text{cas}}$, $\delta^{18}\text{O}_{\text{w}}$, $\delta^{18}\text{O}_{\text{cas}}$, $\delta^{34}\text{S}_{\text{cas}}$) and thirteen individual elements (P, S, Cl, K, Ca, Mn, Fe, Cu, Se, Zn, Br, Rb, Sr). For statistical evaluation one-class classifier method DD-SIMCA that was found to be one of the most suitable methods for authentication studies was used (Rodionova, Titova & Pomerantsev, 2016). This method is similar to non-target analysis of adulterants as the acceptance boundaries are developed on the authentic samples purely without the necessity of known adulterant training samples. Authentic milk samples were used as the target class, while foreign samples and samples from Slovenian market declared as Slovenian were used in the test set. Acceptance areas defined for the give α -values are shown in Figure 5. The object located inside the area are considered as member of the target class. The dotted line corresponds to $\alpha = 0.05$. Five objects were classified as extremes. Finally, acceptance line that correspond

to $\alpha = 0.01$ (solid line) was used and two training sets were tested. Figure 5 shows that all foreign samples are classified out of the acceptance criteria, while seven of Slovenian market samples correspond to the target group. Three Slovenian market samples fall out of the acceptance criteria of $\alpha = 0.01$, indicating possible mislabeling. These results indicate that DD-SIMCA classification using a combination of stable isotope values and elemental composition as parameters, can be a suitable approach to support the verification of the declaration of the geographical origin of milk, and thus provide information related to authenticity.

4 CONCLUSION

Characterization of Slovenian milk was performed using stable isotopes of light elements in combination with elemental composition. The aim was to create, implement, update and maintain a database of authentic Slovenian milk samples, for the years 2012 to 2014, which could then be used to classify samples according to the year, season and production origin using multi-variate analysis. The obtained data were also used to verify the declaration of Slovenian milk in a real-world application using DD-SIMCA. The results show that three Slovenian market samples fall out of the acceptance criteria, indicating possible mislabeling. The information available through this study can be used to increase transparency in the milk and dairy product supply chain. Further, the established system is applicable to other countries and commodities.

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534

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664

665

666 FIGURE CAPTIONS

667 **Fig 1:** Sampling location of raw cow milk covering different geographical regions in Slovenia

668 **Fig 2:** Cluster analysis in milk samples. Results indicate that year and season parameters are
669 the most influence variables for the clustering

670 **Fig 3:** Stable isotope and elemental composition analysis (a): discriminant function score plot
671 of the 176 authentic Slovenian milk samples from summer/winter in 2012 (12s, N = 18, 12w,
672 N = 33), 2013 (13s, N = 31, 13w, N = 37), 2014 (14s, N = 24, 14w, N = 33). Stable isotope and
673 elemental analysis; (b): discriminant loading plot of the correlations between the initial
674 variables and the discriminant functions; (c) score plot of DA modelling of the data set of 101
675 raw cow milk samples produced from Alpine, Dinaric, Mediterranean and Pannonian
676 geographical regions in 2013 and 2014; (d) discriminant loading plot of the correlations
677 between the initial variables and the discriminant function.

678 **Fig 4:** OPLS-DA score plots and VIP values in the pairwise comparison between different
679 region derived from all isotopic and elemental composition data of milk samples. The ellipse
680 on the score plot represents the 95% confidence interval. Red-dotted line indicates a criteria to
681 identify the variable important to the model developed.

682 **Fig 5:** Application of DD-SIMCA for milk authentication. AUTHENTIC-SLO is the target
683 validation set, while FOREIGN-MARKET and SLO-MARKET are two full classes for
684 verification of authenticity. PCA model with 2 PCs. Dotted line is the threshold for $\alpha = 0.05$,
685 for $\alpha = 0.01$.

686 TABLES

687 **Table 1**

688 Mean and standard deviation (SD) of the elemental and stable isotope composition in cow milk
689 in our study from the year 2012, 2013, 2014 in comparison to the min and max range of the
690 literature data (Dobrazanski, Kolacz, Gorecka, Chojnacka & Bartkowiak, 2005, Knobbe et al.,
691 2006, Crittenden et al., 2007, Camin et al. 2008, Sola-Larranaga & Navarro-Blasco, 2009,
692 Chesson et al., 2010, Bontempo, Lombardi, Paolleti, Ziller & Camin., 2012, Zamberlin,
693 Antunac, Havranek & Samaržija, 2012, Chung, Park, Yoon, Yang & Kim, 2014, Erich et al.,
694 2015, Griboff et al., 2019).

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