CHEMICAL AND ISOTOPIC METHODS FOR DETERMINING AUTHENTICITY AND GEOGRAPHICAL ORIGIN OF MILK AND DAIRY PRODUCTS

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Doctoral Dissertation
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UPORABA KEMIJSKIH IN IZOTOPSKIH METOD ZA UGOTAVLJANJE PRISTNOSTI IN GEOGRAFSKEGA POREKLA MLEKA IN MLEČNIH IZDELKOV

Doktorska disertacija

Supervisor: Prof. Dr. Nives Ogrinc

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To Uroš and Zoja because they believed in me and understood!

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Abstract

Increasing public awareness of food quality and safety has led to an increase in the demand for sensitive and reliable methods for the detection of food adulteration. Also, despite the numerous analytical techniques developed for the verification of food authenticity and provenance, it seems that stable isotope and elemental fingerprinting as well as DNA-based genetic methods are becoming increasingly important.

This doctoral thesis deals with milk and dairy products as an example commodity due to its high nutrient content and importance in the human diet, a high level of trade, growing competition in the market and the increasing complexity of supply chains. The main objective is the introduction and validation of specific methods in which a combination of different stable isotopic ratios of light bio-elements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$), multi-elemental analyses, fatty acid profiles and real-time polymerase chain reaction (PCR) are exploited to determine the authenticity and provenance of Slovenian milk and dairy products. These parameters were used to establish the first database of authentic Slovenian cow, sheep and goat's milk and cheese and to develop appropriate statistical models to differentiate milk according to geographical origin and to verify if the characteristics of milk or a dairy product on the Slovenian market corresponds to the label declaration.

The thesis consists of seven distinct, but closely interrelated, studies. In the first three studies, the main issues were to ensure the quality and comparability of elemental and isotopic composition data. The focus is on establishing appropriate protocols for the harmonization of methods and describes my role in an inter-laboratory assessment (CCQM-K140) of stable carbon isotope ratios in bulk honey and the development of food matrix reference materials, namely USGS82 to USGS91. The fourth study deals with the first systematic characterization of authentic Slovenian cow, sheep and goat's milk and cheese based on stable isotope analysis and elemental composition to trace the origin of premium dairy products, which contribute to supporting the existence of Slovenian PDO cheeses. Regional discrimination of sheep's milk was possible when taking into consideration the following elements P, S, K, Cl, Ca, and Zn content and the $\delta^{13}C$ and $\delta^{15}N$ values in casein. Further species differentiation based on discriminant analysis revealed that sheep and goat's milk and cheese is distinguishable from cow's milk with a predictive ability of 95.2%. The fifth investigation examines the use of stable isotopes and multi-elemental composition for the determination of the geographical origin of authentic cow's milk from four geographical regions in Slovenia, namely Alpine, Dinaric, Mediterranean and Pannonian. This study revealed that it was possible to discriminate milk samples according to the production year, season and region based on discriminant analysis and that three Slovenian market samples fell outside of the acceptance criteria, i.e., they were mislabelled. The sixth investigation looks at using the fatty acid profile and the stable isotope composition ($\delta^{13}C$) of individual fatty acids for determining the authenticity (geographical origin) of Slovenian milk. The study revealed that differences in the fatty acid profile are more dependent on the year and season than on production region. It also shows that $\delta^{13}C$ values in individual fatty acids are better biomarkers for studying

metabolic transformation processes in ruminants than for discriminating between different production origins. The final part of this dissertation was dedicated to the verification of the authenticity of commercial dairy products from Slovenian market where molecular methods (real-time PCR) and fatty acid composition were used to determine the adulteration of dairy products. The results showed that real-time PCR could identify milk from different species if the content of cow's milk is >0.1% and provide a semi-quantitatively analysis when the content of cow's milk is >0.5%. Real-time PCR also provided a rapid, robust and sensitive tool for the identification of adulterated sheep's and goat's milk cheese with cow's milk. Moreover, differences in fatty acid composition meant that the adulteration of raw cow's milk with <5% vegetable palm tallow was easily detected. On the Slovenian market, the most often mislabelled products were goat's and sheep's cheese with >20% not consistent with the declaration on the label.

In summary, this thesis shows the important role that stable isotope ratio analysis of light elements, multi-elemental composition as well as molecular methods (real-time PCR) and chromatographic analysis of fatty acids have on verifying the authenticity and geographical origin of not just milk and dairy products. The knowledge gained from this work is broadly applicable and as such, can be readily transferred to other commodities and production regions.

Povzetek

Vse večja ozaveščenost javnosti o kakovosti in varnosti hrane je privedla do vse večjega povpraševanja po občutljivih in zanesljivih metodah za ugotavljanje potvorb. Kljub temu, da obstajajo že številne metode in pristopi za preverjanje avtentičnosti živil, postajajo stabilni izotopi lahkih elementov, elementna sestava ter genetske metode, ki temeljijo na analizah DNA, pomembne tehnike pri ugotavljanju pristnosti in geografskega porekla v živilih.

Doktorsko delo se osredotoča na mleko in mlečne izdelke, saj so le-ti pomemben vir hranil v prehrani, s tržnega vidika pa so visoko povpraševan izdelek, ki izkazuje hitro tržno rast, poleg tega pa je konkurenčnost dobaviteljev pogosto odvisna od vedno bolj kompleksnih dobavnih verig. Glavni cilj disertacije je vpeljava in validacija specifičnih metod, kot so uporaba stabilnih izotopov lahkih bio-elementov ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$), elementna sestava, maščobno kislinska sestava ter PCR v realnem času, s pomočjo katerih bomo lahko določili avtentičnost in geografsko poreklo slovenskega mleka in mlečnih izdelkov. To delo je bilo uporabljeno za vzpostavitev in izdelavo prve baze podatkov pristnih vzorcev slovenskega kravjega, ovčjega in kozjega mleka in sirov ter pri razvoju ustreznih statističnih modelov za razlikovanje mleka glede na geografsko območje in preverjanju skladnosti deklaraciji mleka in mlečnih izdelkov.

Doktorsko delo je sestavljeno iz sedmih ločenih, vendar tesno povezanih študij. V prvih treh študijah je glavni poudarek na zagotavljanju visokokakovostnih in primerljivih podatkov v elementni in izotopski sestavi mleka. Osredotočili smo se na vzpostavitev ustreznih protokolov, ki zagotovijo kakovostne in verodostojne podatke. Sodelovali smo v medlaboratorijski primerjavi (CCQM-K140) za določanje razmerja stabilnega izotopa ogljika v medu in pri razvoju referenčnih materialov (USGS82 do USGS91) za meritve stabilnih izotopov lahkih elementov v matrici živil. Četrta študija obravnava prvo sistematično karakterizacijo pristnega kravjega, ovčjega in kozjega mleka in sira na podlagi stabilnih izotopov in elementne sestave, z namenom da bi izsledili poreklo višje kakovostnih slovenskih mlečnih izdelkov, kar je pripomoglo k uvedbi slovenskega sira z oznako – zaščitena označba porekla (ZOP). Regionalno razvrščanje ovčjega mleka je bilo mogoče ob upoštevanju naslednjih parametrov; vsebnosti elementov P, S, K, Cl, Ca, Zn in $\delta^{13}C$ in $\delta^{15}N$ vrednosti v kazeinu. Nadaljnje razlikovanje mleka glede na vrsto je pokazalo, da diskriminantna analiza omogoči razlikovanje med ovčjim, kozjim in kravjim sirom, saj je bilo 95,2 % vzorcev pravilno uvrščenih. V peti študiji smo preučili uporabo stabilnih izotopov in večelementno sestavo za določanje geografskega izvora pristnega kravjega mleka iz štirih geografskih regij v Sloveniji; alpska, dinarska, panonska in mediteranska regija. Raziskava je pokazala, da je mogoče vzorce mleka razlikovati z diskriminantno analizo glede na leto, letni čas in območje proizvodnje ter da trije vzorci iz slovenskega tržišča ne ustrezajo postavljenim merilom ustreznosti, kar lahko kaže na napačno označevanje deklaracij izdelkov. Šesta raziskava poda odgovor na vprašanje o pomembnosti maščobnih kislin in izotopski sestavi ogljika ($\delta^{13}C$) v posameznih maščobnih kislinah pri določanju geografskega porekla pristnega slovenskega mleka. Študija je pokazala, da na maščobno kislinsko sestavo mleka bolj vpliva leto in letni čas proizvodnje kot geografsko

območje ter da se $\delta^{13}C$ posameznih maščobnih kislin lahko bolje uporablja kot bio-marker za preučevanje procesov presnove v prežvekovalcih kakor pa za razlikovanje po geografskem poreklu. V zadnjem delu disertacije sem se osredotočila na preverjanje pristnosti komercialno dostopnih mlečnih izdelkov s slovenskega trga, kjer se je za določitev možne potvorbe uporabilo molekularno metodo PCR v realnem času in maščobno kislinsko sestavo. Rezultati so pokazali, da se omenjena metoda lahko uporabi za kvalitativno identifikacijo kravjega mleka, če je vsebnost kravjega mleka > 0,1 %, in semi-kvantitativno identifikacijo, če je vsebnost kravjega mleka >0,5 %. PCR v realnem času tako predstavlja hitro, robustno in senzitivno metodo za določanje potvorb v ovčjih in kozjih sirih s primesjo kravjega mleka. Prav tako se je izkazalo, da je vsebnost maščobnih kislin v mleku in mlečnih izdelkih pomembna metoda za določanje potvorb s primesjo rastlinskih maščob, saj lahko določimo dodatek rastlinskih maščob kravjemu mleku že v vsebnostih, manjših od 5 %. Rezultati raziskav, izvedenih na vzorcih sirov, dostopnih na slovenskem tržišču, razkrivajo, da so največje nepravilnosti prisotne pri označbah ovčjih in kozjih sirov, saj več kot 20 % komercialno dostopnih sirov ne ustreza deklaraciji.

Če povzamemo, rezultati te študije kažejo, da so analize stabilnih izotopov lahkih elementov in večelementne sestave, molekularne metode (PCR v realnem času) in kromatografske metode za določanje maščobnih kislin uporabne metode za preverjanje pristnosti in geografskega porekla živil, vključno z mlekom in mlečnimi izdelki. Vzpostavljen sistem ima lahko tudi širšo uporabo, saj ga je mogoče prenesti tako na drugo državo kakor tudi na druga živila.

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Abbreviations

%	... percent
‰	... permille
C14:0	... myristic acid
C16:0	... palmitic acid
C18	... fatty acids with 18 carbon atoms
C18:3ω-3	... α-linolenic acid
C20:5ω-3	... eicosapentaenoic acid (EPA)
C22:6ω-3	... docosahexaenoic acid (DHA)
C_3	... C_3 plants, Calvin photosynthetic pathway
C_4	... C_4 plants, Hatch-Slack photosynthetic cycle
C4:0	... butyric acid
CAM	... CAM plants, Crassulacean Acid Metabolism
CCQM	.. Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CIAAW	... Commission on Isotopic Abundances Atomic Weights
cis-9, *trans*-11 C18:2	... the isomer of conjugated linoleic acid (CLA)
CLA	... conjugated linoleic acid
CO_2	.. carbon dioxide
CSIA	... compound specific isotope analysis
DHA	... docosahexaenoic acid
DMS	... dimethyl sulphide
DNA	... deoxyribonucleic acids
ED-XRF	... dispersion X-ray fluorescence spectroscopy
ELISA	... enzyme-linked immunosorbent assay
EPA	... eicosapentaenoic acid
FA	... fatty acid
FID	... flame ionization detector
FIRMS	.. Forensic Isotope Ratio Mass Spectrometry
GC	... gas chromatography
GC-MS	... gas chromatography-mass spectrometry
GCO	... gas chromatography olfactometry
GMO	... genetically modified organisms
H_2S	... hydrogen sulphide
HPLC	... high-pressure liquid chromatography
i	... the mass number of the heavier isotope of an element(E)
IAEA	... International Atomic Energy Agency
ICP-MS	... inductively coupled plasma mass spectrometry
IR	... infrared spectroscopy
IRMM	... Institute of Reference Materials and Measurements
IRMS	... isotope ratio mass spectrometry
IUPAC	... International Union of Pure and Applied Chemistry

j	... mass number of the lighter isotope of an element (E)
JSI	... Jožef Stefan Institute
km	... kilometre
LC	... liquid chromatography
LC-TOF	... liquid chromatography Time-of-Flight (TOF)
MALDI-TOF	... matrix-assisted laser desorption/ionization time-of-flight
MC-ICP-MS	... multi-collector-inductively coupled plasma – mass spectrometry
MIR	... Mid-infrared spectroscopy
MS	... mass spectrometry
N_2	.. nitrogen
NIR	... Near-infrared spectroscopy
NIST	... National Institute of Standard and Technology
NMR	... nuclear magnetic resonance
PCR	... polymerase chain reaction
PDO	... Protected Designation of Origin
PGI	... Protected Geographical Indication
PTR-MS	... proton transfer reaction–mass spectrometry
PUFA	... polyunsaturated fatty acid
RM	... reference material
RNA	... ribonucleic acid
R_p	... the ratio between the heavier and the lighter isotope in the sample
R_{Ref}	... the ratio between the heavier and the lighter isotope in the reference material
SC	... somatic cell
SFA	... saturated fatty acid
SNIF	... site-specific natural isotopic fractionation
SO_2	.. sulphur dioxide
TIMS	... thermal ionization mass spectrometry
TOF	... Time-of Flight
TSG	... Traditional Speciality Guaranteed
USGS	... United States Geological Survey
UV	... ultraviolet
VCDT	... Vienna Cañon Diablo Troilite
VPDB	... Vienna Pee Dee Belemnite
VSMOW	... Vienna Standard Mean Ocean Water
α	... alpha
δ	... delta, relative isotopic composition, value given in part per thousand or "permille"
$\delta^{13}C$	... isotopic composition of carbon
$\delta^{15}N$	... isotopic composition of nitrogen
$\delta^{18}O$	... isotopic composition of oxygen
$\delta^{2}H$	... isotopic composition of hydrogen
$\delta^{34}S$	... isotopic composition of sulphur
ω	... omega
mUr	.. micro urey

Chapter 1

Theoretical Background

Food scandals and incidents, concern for health and improving socioeconomic circumstances have led to an increase in consumer demand for higher quality and safer food. As a result, there has been an upsurge in research and development in the food sciences and a demand for food supply chain transparency-enabling technologies.

The main reasons for the development and application of methods and techniques in food science are compliance with legal and labelling requirements, quality assessment, determination of nutrient values, and the detection of adulterations (Picó, 2012). The concept of food "authenticity" refers to its genuineness and intactness, and that a food product complies with its label description. It also encompasses characteristics, such as origin (specific, geographic or genetic), production management system (conventional, organic, traditional practices, free-range) and processing technology. Consumer expectation, legislative needs and developments in instrumental analysis, should also be kept in mind when considering the term "food quality" as well as obtaining healthy and safe food products (Aung & Chang, 2014; Danezis et al., 2016).

From the consumer's perspective, healthy food products are those that have the proper relationship between macronutrients such as proteins, carbohydrates, fats, and fibber and minor nutrients such as vitamins and trace elements (Müller & Steinhart, 2007), which are not only a source of energy but also an affordable way to prevent disease. The guaranteeing of correct labelling of food products also increases awareness about the importance of labelling not just from the consumer's perspective but also among regulatory authorities, food processors and retailers, and others (Karoui, 2012).

In 2002, the European Parliament and the Council established *Regulation (EC) No 178/2002* for protecting the rights of consumers to receive truthful information about the origin, content and use of the aliments through labelling. However, for economic and production reasons, many food product manufacturers act deceitfully by falsely declaring a product's contents or through unlawful adulteration, which can destabilize the market and disrupt the regional economy and even the national economy (Cordella et al., 2002). Globalization and the international movement of food products and related raw materials, especially luxury products or those subject to natural fluctuations, and a large number of intermediaries between the primary producers and end consumers (merchants, distributors, and importers), provides plenty of opportunities for adulterated products to enter the market (Karoui, 2012; Kelly et al., 2005). Intentionally mislabelled or adulterated products can represent significant savings for food producers (Dennis, 1998). Typical frauds include:

- addition of undeclared supplements/ingredients
- addition of cheap substitutes (dilution)
- discordance with the technological process
- incorrect indication of geographical origin

- mixing different food products of different origin (addition of cow's milk to sheep or goat's milk)
- obtaining food from a diseased animal

Such practices are becoming increasingly sophisticated and harder to detect, and staying one step ahead of the criminals has meant developing cost-effective analytical strategies capable of offering qualitative and quantitative results while increasing the sensitivity, precision, specificity, and speed of analysis (Karoui, 2012). The need to monitor the authenticity and quality of food has also led to an increase in demand for sensitive and reliable methods to determine whether adulteration has taken place.

A variety of analytical techniques, for the verification of food authenticity and provenance, have been developed and tested. All of them have strengths and weaknesses; however, it seems that stable isotope and elemental fingerprinting as well as DNA-based genetic methods have become increasingly important in establishing authenticity and geographical origin. To date, scientists have successfully applied stable isotope analysis of bio-elements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) combined with multielement analysis for the authentication of various food commodities including vegetables, beverages and cereal crops (Bizjak Bat et al., 2016; Carter et al., 2015; Cengiz et al., 2017; Gopi et al., 2019; Li et al., 2015; Mahne Opatić et al., 2017, 2018; Park et al., 2019; Rashmi et al., 2017). One recent advance has been the development of compound-specific stable isotope analysis (CSIA) of organic compounds using gas or liquid chromatography coupled to isotope ratio mass spectrometry and the identification of relevant biomarkers such as fatty acids (FAs), sugars, amino acids, and citric acid (Guyon et al., 2014; Paolini et al., 2017; She et al., 2019).

A common requirement in food authenticity and traceability studies is a suitable product reference database. Creating such a database, however, represents a major investment in terms of both time and cost, since it requires samples from a broad and representative range of geographical, seasonal, dietary and production conditions. In order to evaluate the authenticity of commercial products, they must be first characterized and then compared with reference samples in the databank and evaluated in terms of their fit within defined statistical boundaries. Only a few databases of food products covering wide regional distributions currently exist of which the wine database is the most developed in the EU. The two other official comprehensive databanks that are updated annually and had been created by product-specific association or consortiums are the PDO Grana Padano and Parmigiano Reggiano Databank for cheese and the AIJN European Fruit Juice Association databank for fruit juice. The second group of databanks were created either within the framework of smaller projects or specifically to detect fraud like the Italian PDO databank for olive oil, butter circular trade or Beluga caviar (Camin et al., 2017).

This PhD thesis deals with milk and dairy products as an example commodity due to its high nutrient content and importance in the human diet, a high level of trade, growing competition in the market and increasing complexity of food supply chains. The main focus was to establish the first database of authentic Slovenian cow's, sheep's and goat's milk and cheese that includes isotope parameters, elemental composition and the content and stable isotope composition of individual FAs. This thesis also covers the development of appropriate statistical models to differentiate milk according to geographical origin and to verify if milk on the Slovenian market corresponds to its stated declaration.

The thesis is organized in five core chapters with the content of each chapter summarized as follows:

Chapter 1 presents the theoretical background, description of milk and dairy products, different methods to determine the authenticity of milk and dairy products with an

emphasis on isotopic and elemental composition. Special attention is given to overcoming metrological issues in determining stable isotope ratios.

Chapter 2 presents the aims and objectives of the thesis. It also presents the work done and includes six relevant publications. The first three publications deal with the establishment of appropriate protocols to ensure the harmonization of methods and the quality and comparability of elemental and isotopic data. The fourth publication deals with the implementation of the methods to analyse authentic Slovenian cow's, sheep's and goat's milk and cheese and to trace the origin of Slovenian dairy premium products. The fifth publication investigates the use of stable isotopes and multi-elemental compositions for determining the geographical origin of authentic cow's milk from four geographical regions in Slovenia. This work includes establishing a model for verifying if milk on the Slovenian market corresponds to that declared on the label. The sixth publication investigates whether the FA composition and the stable carbon isotope ration of individual FAs in milk are useful biomarkers for determining its provenance.

Chapter 4 describes the application of molecular methods (real-time PCR) and FA analysis for verifying the authenticity of commercial dairy products, focusing on low and high-value cheese products on the Slovenian market.

Chapter 5 presents the conclusions of this work and identifies possible future research directions.

1.1 Milk and Dairy Products

Milk is a secretion from the mammary glands of female mammals and typically comprises 87% of water, 4.6% of lactose (sugar), 3.3% of protein and around 4% of fat – the most valuable component (Haug et al., 2007). Ruminant milk fat is an essential component in dairy and many food products and is composed of lipids (99%), especially triacylglycerides (95 – 96%) that consist of different amounts of FAs of different length and saturation (Renou et al., 2004a). A large portion of milk fat is composed of a limited number of FAs containing 4 to 18 carbon atoms, where FAs with an even number of carbon atoms predominate. This diversity in FAs composition in milk arises from the effects of ruminal biohydrogenation on dietary FAs, and the range of FAs synthesized *de novo* in the mammary gland. Thus, many factors affect the FA composition of milk, including genetic (breed), stage of lactation, milking operation, season, geographical origin, feeding (grazing, silage type, oil supplementation). Long-chain (predominantly C_{18}) FAs and some C16:0 FAs arise from the plasma lipids depended on the amount of fat in the diet, while short-chain and medium-chain FAs (C4:0 to C14:0) and C16:0 FAs are synthesized *de novo*. Milk FAs of dietary origin accounts approximately 55% of total FAs in milk fat, while *de novo* FA synthesis accounts for the rest (Palmquist, 2006).

Milk and dairy products are an important source of nutrients in the human diet, including vitamins, minerals, protein, and fats that provide the body with energy. The human body, however, does not have the enzymes that can insert a double bond in the ω-3 (omega-3) position and cannot synthesise ω-3 FAs, such as α-linolenic acid, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA). Thus, most animals and humans can only get these fats from food sources. In the human diet, milk and dairy products contribute between 18% – 24% total fat, 30% – 40% total saturated fatty acids (SFAs) and 20% – 25% total *trans* fatty acids. Bovine milk contains a large number of FAs, such as polyunsaturated fatty acids (PUFAs) in the ω-3 FA group, and the conjugated linoleic acid (CLA) isomer *cis*-9, *trans*-11 C18:2. The main ω-3 FA in milk is α-linolenic acid (C18:3ω-3). In milk, a small amount of docosahexaenoic acid (C20:5ω-3) and

eicosapentaenoic acid (C22:6ω-3) is also present. Therefore, it is recommended that the optimum ratio between ω-6 and ω-3 FA in the human diet should be 1:1 (Ellis et al., 2006).

The most common form of milk traded globally is the dehydrated (spray-dried) form, due to a wide range of use in different milk-based products (for example, infant formula, yoghurt and chocolate). Food producers also use milk by-products, such as whey proteins, lactose from cheese manufacturing, casein and milk fat from milk powder manufacturing as a major or minor ingredient in food products to improve their nutritional and physical functionality.

Food adulteration and fraud for profit have been practised since ancient times, but nowadays, such practices have become increasingly more sophisticated. The fact that milk and dairy products are used extensively makes them vulnerable to economic adulteration, and, there is a high risk of intentionally mislabelled products entering the market, especially when the price is a factor. In fact, according to Moore and co-workers, milk is the second most adulterated foodstuff (Moore et al., 2012).

There are several categories of fraud in milk such as (1) false declaration of milk's geographical or species origin, (2) dilution of milk with water or the addition of low-cost adulterant mixtures to increase the value of lower quality of milk and (3) abstraction of fat from milk (Ehtesham et al., 2017). Thus, selecting the appropriate analytical method or procedure is crucial when ensuring the safety of milk and dairy products. For this, different analytical procedures have to be established for different production steps to enhance quality control management systems. The three critical stages of production require fast process analytics with simple and inexpensive methods, elaborated analysis methods for qualification and quantification of residues and contaminants – so-called surveillance methods as well as sophisticated methods for research (Müller & Steinhart, 2007).

1.2 Different Methods for Determining Authenticity of Food: Specific Example – Dairy Product

Compliance with label description in food products is of particular interest for both consumer and regulatory authorities since food products are often the target of fraudulent labelling. The development and application of methods and techniques in food science have grown in parallel to consumer concern over food safety and quality. For most Europeans, food origin is one of the main authenticity issues, e.g., French wine and Italian cheese have a long tradition of geographical indication, and European Union protects food and beverages of a particular quality through Council Regulation EEC No 2081/92 and Regulation EEC No 1151/2012. The legislation makes sure that products with clear geographical origin such as PDO (Protected Designation of Origin), PGI (Protected Geographical Indication) and TSG (Traditional Specialties Guaranteed) are officially protected. The typical characteristic of PDO products is their unified geographical location of production, processing and preparation, while PGI is related to a geographical area where at minimum one production step occurs (Danezis et al., 2016).

However, determining food authenticity has become an issue in quality control and food safety, and there is a pressing need for accurate, standardized food authentication methods and techniques. To date, various analytical techniques have been used for food authentication including molecular (genomics – proteomics), immunological, isotopic, elemental and chromatographic techniques, vibrational and fluorescence spectroscopy, NMR (nuclear magnetic resonance), sensory analysis, non-chromatographic mass spectrometry (MS) and chemometric and bioinformatics tools (Danezis et al., 2016).

1.2.1 Molecular techniques

Molecular methods are based on the characterization, isolation and manipulation of molecular components of cells and organisms such as nucleic acids (DNA) and ribonucleic acid (RNA) (Singh et al., 2012). In food authentication, molecular methods are mostly used for species detection and identification. DNA is identical in all somatic cells of a given organism and is highly stable, which allows the analysis of processed food products. The majority of the DNA-based methods focus on using Polymerase Chain Reaction (PCR) to amplify the specific part of DNA of interest. The specific lengths of DNA can be amplified a sufficient number of times to allow the DNA to be analysed; thus, the amount of the required material can be as small as a few cells. However, traditional techniques have been replaced with new techniques such as genomics and proteomics, which are usually applied to the identification of false description and mislabelling of foods, for example, the detection of genetically modified organisms (GMOs), seafood authentication, authentication of kosher and halal meat, detection of horse meat and pork in food labelled as beef, game meat authentication, botanical origin of foods (olive oil, wine, tomato products, tea, and cocoa), and species origin (meat, milk, and fish) authentication (Danezis et al., 2016). Although they are the most specific and sensitive methods for food authentication, they also require expensive laboratory equipment and a high degree of expertise (Asensio et al., 2008).

1.2.2 Chromatographic techniques

Chromatography is a technique for separating chemically similar compounds in complex matrices based on the distribution of components between a mobile and a stationary phase. It can be used for quantitative and quantitative analysis of numerous components such as peptides, lipids, carbohydrates, amino acids, FAs, organic acids, nucleic acids, additives (e.g., colorants, aromas and preservatives) since these compounds have a wide range of polarity. For instance, Gas Chromatography-Mass Spectrometry (GC-MS/MS), Liquid Chromatography-Mass Spectrometry (LC-MS/MS) and Liquid Chromatography-Time-of-Flight Mass Spectrometry (LC-TOF-MS) are the most frequently used analytical techniques for food authentication. Liquid chromatography separation is based on polarity, electrical charge and molecular weight. It is, therefore, suitable for proteins, amino acids, carbohydrates, vitamins, phenolic compounds, triglycerides, chiral compounds, and pigments. At the same time, gas chromatography is more suited to the analysis of volatile or semi-volatile molecules/compounds such as FAs, triglycerides, waxes, sterols, hydrocarbons, alcohols, tocopherols. Although they are of considerable value, these methods are not convenient for routine analysis, since they are relatively costly, time-consuming and complex to perform (Danezis et al., 2016).

1.2.3 Vibrational and fluorescence spectroscopy

Infrared spectroscopy (IR) measures the vibrations of molecules and is based on measurements of the wavelength and intensity of the absorption of infrared light by a sample positioned in the path of an IR beam. The infrared portion of the electromagnetic spectrum is divided into three regions: the near (NIR), mid (MIR) and far-infrared, named for their relation to the visible spectrum. Both the NIR and MIR spectrum reveals information about the molecular bonds and can give details of the type of molecules present in food. Raman spectroscopy is another technology based on fundamental vibrations that provide chemical and structural information to specific chemical functional group (C=C, C≡C, C≡N) (Luykx & van Ruth, 2008; Reid et al., 2006). Both IR and Raman spectroscopy

are rapid and non-destructive techniques and easily applied in food quality and authenticity research. These methods cannot eliminate the need for more detailed analyses since special care is required concerning possible interference between components possessing similar IR spectra. Fluorescence spectroscopy is also a rapid and non-destructive technique and can be used to identify and analyse fluorescent compounds at trace levels and provide information about structure, formulation and stability. Molecules such as polyaromatic hydrocarbons and heterocycles with rigid molecular skeletons can be detected by fluorescence spectroscopy. The emission spectrum is highly dependent on the excitation wavelength; thus, both an excitation and an emission spectrum can be obtained. The advantage of fluorescence spectroscopy is the absence of interferences and that higher-order data can be obtained. At the same time, a major disadvantage is that the technique is susceptible to light scattering. At present, it is not possible to correct for this effect mathematically since information on the amount of scattering is not contained in the spectrum (Karoui & De Baerdemaeker, 2007). Since many foods contain proteins, peptides and free amino acids, which include tryptophan, tyrosine and phenylalanine residues, fluorescence spectroscopy can provide information on the presence of these aromatic compounds. Fluorescence spectroscopy has been used to obtain information about protein structure and protein interactions in dairy products as well as for determining the geographical origin of cheese, milk and olive oil (Luykx & van Ruth, 2008).

1.2.4 Nuclear magnetic resonance

Nuclear Magnetic resonance (NMR) is a suitable method for obtaining structural information of molecular compounds with high analytical precision. The major disadvantage of NMR is that it is one of the most expensive analytical technique considering initial capital investment and running costs. The combination of ^{1}H NMR and ^{13}C NMR with advanced chemometric methods was successfully applied to study the profile of a food product in relation to its geographical origin (Luykx & van Ruth, 2008). Site-Specific Natural Isotopic Fractionation NMR (SNIF-NMR) is applicable for the analysis of small molecules such as ethanol. This technique is the official method (Regulation No 2676/90) for determining the geographical origin of wines. NMR has also been used to successfully identify the geographical origin of coffee, olive oils, honey, fish, spirits, vinegar, saffron (Danezis et al., 2016), and milk and cheese (De Angelis Curtis et al., 2000; Hinrichs et al., 2007; Renou et al., 2004)

1.2.5 Sensory analysis

Products with high organoleptic qualities are becoming increasingly popular among consumers, which has led to the development of new sensory analysis methods. Appearance, aroma, flavour and texture properties are essential characteristics that determine the quality of products. Traditional sensory techniques require a well-trained panel of human assessors, who have a reasonable level of sensory perception, commitment and motivation, and are trained in the use of standardized and systematic sensory methods to obtain reliable results. However, for an objective, specific, and rapid sensory analysis, the development of instrumental techniques is needed. Gas Chromatography Olfactometry (GCO), biomimetic sensors: electronic tongue (e-tongue), electronic nose (e-nose), electronic eye (e-eye), are all techniques that could objectively recognize and quickly characterize a sample in the same way as an expert tasting panel. The advantages include minimum sample preparation, simplicity, rapidity and low-cost (Danezis et al., 2016).

1.2.6 Non-chromatographic mass spectrometry

Mass spectrometry (MS) is a technique that separates ionized atoms or molecules according to their mass to charge ratio (m/z). As a stand-alone technique, mass spectrometric methods such as Proton transfer reaction mass spectrometry (PTR-MS), Matrix-assisted laser desorption/ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) and Ambient Mass Spectrometry techniques such as Direct Analysis in Real-Time (DART-MS) can be used. PTR-MS is a method for rapid and non-invasive online monitoring of volatile organic compounds, such as alkenes, alcohols, aldehydes, aromatics, ketones, nitriles and sulphides, in complex matrices, based on soft ionization methods (Danezis et al., 2016; Luykx & van Ruth, 2008). MALDI-MS is used in a variety of applications such as proteomics, DNA/RNA, lipids, oligosaccharides, phosphopeptides, bacteria, small molecules, and synthetic polymers and require a low amount of sample. DART-MS is a variant of ambient mass spectrometry, capable of rapidly analysing a large variety of samples, with minimum sample preparation and high salt tolerance. These techniques have been used to prevent food fraud by uncovering incorrect description and mislabelling of foods, for example, the adulteration of sheep's and goat's milk with less expensive cow's milk or fresh cow's milk with powdered milk (Danezis et al., 2016).

1.2.7 Immunological techniques

Immunological techniques have been applied successfully to the food authentication and control because they are sensitive, fast, cost-effective and have high throughput. Immunoassays are analytical tools that can provide information about small molecules (pesticides or antibiotics), macromolecular species (proteins) and microorganisms (bacteria, virus) based on the specific interaction between antibodies and their cognate antigens. The most used immunological technique is the Enzyme-linked immunosorbent assay (ELISA). ELISA involves the cultivation of an enzyme (a protein that catalyses a biochemical reaction) to detect the presence of an antibody or an antigen in a sample (Karoui, 2012; Reid et al., 2006). ELISA has been successfully used in the detection of the presence of meat from different species in food products, the presence of vegetable proteins in milk powder, and for differentiating milk from different ruminant species and the addition of low levels (0.1%) of cow's milk to sheep's and goat's milk (Reid et al., 2006). Until recently, the main limitation of the ELISA technique is that the target proteins sometimes are denatured during food processing, and therefore the target protein epitope may not be present in the condition detectable by the antibodies (Asensio et al., 2008).

1.2.8 Elemental techniques

The composition of major and trace elements in plants, to some extent, reflects that of the soil; thus, vegetation is a reflection of the bio-available and mobilized nutrients present in the soil, which is characteristic for a specific geographical location. Variation of elemental concentration is a consequence of different fertilization regime, harvesting, botanical origin, soil type, pollution, and production year. However, the elemental composition of foods of animal origin depends on the animal's diet (fodder and vegetation), drinking water, pollution and soil composition (Kelly et al., 2005).

There are several techniques available for performing simultaneous multi-element chemical analysis. Among them, energy dispersion X-ray fluorescence spectroscopy (EDXRF) and various forms of inductively coupled plasma mass spectrometry (ICP-MS) are the most commonly used (Khan et al., 2014). Each of these techniques provides a chemical profile (fingerprint), which can be used to characterize the material in question

and to compare it with other samples or reference materials. Inductively coupled plasma mass spectrometry (ICP-MS) has been widely used for the analysis of minor and trace elements in food since it provides excellent results and allows the simultaneous and sequential determination of multiple elements. The advantages of this technique are the short analysis times low limits of detection (Birghila et al., 2008; Kelly et al., 2005). Alternatively, EDXRF is a highly sensitive, fast and relatively cheap technique that offers the possibility of performing direct multi-element analysis of solid samples over a wide dynamic range. The method is based on the detection of fluorescence generated when bombarding the sample with high energy X-rays. The technique is especially useful in food authentication and traceability studies where there is a requirement for a database of genuine samples to which a sample can be compared to establish its authenticity (Drivelos & Georgiou, 2012; Kelly et al., 2005; Kropf et al., 2010).

1.2.9 Isotopic techniques

Isotopic techniques can distinguish chemically identical compounds based on their isotopic composition. Isotopes are atoms of the same element that have different numbers of neutrons, resulting in a different atomic mass. There are two main groups of stable isotopes, 1) light or bio-elements, where the most commonly used isotopic ratios are $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$ and 2) heavy isotopes, where the most commonly used isotopic ratios are $^{87}Sr/^{86}Sr$, $^{206}Pd/^{204}Pb$, $^{207}Pb/^{204}Pb$, $^{208}Pb/^{204}Pb$, and $^{143}Nd/^{144}Nd$. Stable isotopes are measured using an instrument called an isotope ratio mass spectrometer (IRMS) coupled with different preparation systems such as an Elemental Analyser, Pyroliser, Equilibration device, or either to a gas chromatograph (GC) or a high-pressure liquid chromatograph (HPLC). Due to the ability of the IRMS to measure isotope distribution at natural abundance levels with great accuracy and high precision it can be used for detecting adulteration of any raw material or any food (Meier-Augenstein, 1999). Further, when gas chromatography (GC) is coupled to an IRMS through a combustion furnace (C), it is possible to perform highly precise compound-specific isotope analysis (CSIA), especially at natural isotopic abundances as used in tracer studies and allows the determination of the origin of certain organic compounds such as FAs at the molecular level (Meier-Augenstein, 2002). Heavier isotope ratio measurements are usually made using Multi Collector – Inductively Coupled Plasma – Mass Spectrometry (MC-ICP-MS) and Thermal Ionization Mass Spectrometry (TIMS) (Danezis et al., 2016).

Isotopic techniques are appropriate in food authentication since the isotopic composition of an agricultural product depends on various factors related to geography (climatic condition, altitude, and pedology), the geology of the region in which food ingredients are produced, and factors such as fertilizers and the specific feed present in the diet of the farm animals. However, as a first approximation, natural abundance measurements provide information on plant 'type' or diet (mostly carbon and nitrogen isotopes) and geographical origin (mostly hydrogen and oxygen isotopes) and can differentiate between production methods (organic and conventional). Such methods also allow the extent and content of adulteration, provenance and the year of production to be determined (Meier-Augenstein, 2002; Ogrinc et al., 2014).

1.2.10 Chemometric and bioinformatics

Chemometrics is a mathematical and statistical method applied to analytical data in order to obtained relevant chemical information. Food authentication studies produce large complex datasets, which require sophisticated statistical chemometric and bioinformatic tools for effective interpretation. At present, there are no standard guidelines for describing

workflow, which is evidenced by the diversity in experimental design and the reporting of results. Thus, the ability to compare the results from similar studies is challenging. Nevertheless, both methods and results must follow specific rules such as (i) provide an accurate data, which is usually achieved using standard certified reference materials; (ii) providing correct storage and handling of the samples, and (iii) comparable data across different studies (Danezis et al., 2016).

Since this thesis deals mainly with the use of stable isotope ratios of light elements in combination with elemental composition for food authenticity, the following sections describe both methods in detail.

1.3 Stable Isotopes of Light Elements – Definition and Calculation

The following chapter of the thesis provides the theoretical background of stable isotopes of light elements, including definitions and the metrological approach needed to ensure accurate results.

The most common light elements used in food authenticity studies are H, C, N, O and S. These elements have different stable isotopes and occur in different natural abundances, where the lighter isotope is dominant, and one or two of the heavier isotopes have a natural abundance of a few percent or less (Table 1.1).

Table 1.1: Mean natural abundance of stable isotopes used in ecological research and their corresponding international reference standards (adapted from Kelly, 2003).

Element	**Isotope**	**Abundance (%)**	**International reference standard**
Hydrogen	1H	99.985	VSMOW (Vienna – Standard Mean Ocean Water)
	2H	0.015	
Carbon	12C	98.89	VPDB (Vienna – Pee Dee Belemnite)
	13C	1.11	
Nitrogen	14N	99.63	Air (Atmospheric nitrogen)
	15N	0.37	
Oxygen	16O	99.759	VSMOW (Vienna – Standard Mean Ocean Water)
	17O	0.037	
	18O	0.204	
Sulphur	32S	95.00	CDT (Vienna – Canyon Diablo Troilite)
	33S	0.76	
	34S	4.22	

Natural isotopic variations are small, typically with differences as little as 0.00001 being informative; moreover, the absolute isotopic abundances are less important than the changes in isotopic abundances that have occurred. For this reason, it is more convenient to express isotope data in the δ – notation in per mill (‰). The δ value can be expressed also in the unit name "urey" (Ur) in order to comply with the International System of Units (SI). A δ value in traditional form of -20‰ can be expressed as -20 mUr.

Thus, δ notation is defined as "the difference between the ratios of the number of heavier and the number of lighter stable isotopes in a given material versus the ratio in a reference material, normalised by the ratio of the reference material relative to an international reference standard" (Brand and Coplen 2012) according to the equation:

$$\delta(^{i/j}E) = \delta^{i/j}E = \frac{^{i/j}R_P - ^{i/j}R_{Ref}}{^{i/j}R_{Ref}} \quad (1.1)$$

Here, i and j denote the highest and the lowest atomic mass number of element E, respectively, R_P and R_{Ref} indicate the ratio between the heavier and the lighter isotope (^{2}H/^{1}H, ^{13}C/^{12}C, ^{15}N/^{14}N, ^{18}O/^{16}O, ^{34}S/^{32}S) in the sample (P) and reference material (Ref), respectively. δ^2H and δ^{18}O values are typically reported relative to the VSMOW (Vienna-Standard Mean Ocean Water) standard, δ^{13}C values are reported relative to the VPDB (Vienna-Pee Dee Belemnite) standard. In contrast, sulphur and nitrogen are reported relative to VCDT (for Vienna Cañon Diablo Troilite) and AIR, respectively (Brand et al., 2014).

1.3.1 Determination/reporting of stable isotope composition

In the stable isotope approach, isotopic abundances are reported relative to reference material (RM). These RMs are used to make sure that measurements are both repeatable and reproducible, which makes the selection, calibration and proper use of an international and in-house RM indispensable. Measurements must also be consistent and repeatable over long periods as well as reproducible by other laboratories. For this reason, comparability between laboratories is essential for the development of an international database for supporting the scientific and criminal investigations and facilitate the globalization of scientific networks. The best way to achieve reliable data is for all reported measurements to be traceable to internationally agreed-upon references (see Table 1.1) as well as the fundamental analytical principle of identical treatment by which samples and RMs are processed in an identical manner. Accurate measurements also require normalization using at least two isotopic RMs with contrasting isotopic compositions in order to anchor delta values to a conventional delta scale and compensate for differences in responses of analytical instrumentation (Carter & Fry, 2013; Meier-Augenstein & Schimmelmann, 2019).

1.3.1.1 Internationally accepted reference materials

Calibration or primary reference materials described the physical materials, which currently define the international δ – scales (see Table 1.2). These materials have exact isotope δ-values by consensus with no uncertainty (Carter & Fry, 2013). When a primary RM has been exhausted, the replacement RM must be calibrated against the original primary RM, with the minimum uncertainty possible. For example, VSMOW2 and SLAP2 were replacements for VSMOW and SLAP, while IAEA-603 replaced NBS 19 (Dunn & Carter, 2018). In routine stable isotope ratio measurements, primary RMs (and their replacements) are rarely used due to their incompatibility with the sample introduction system and limited stock, which restricts supply (Carter & Fry, 2013; Meier-Augenstein & Schimmelmann, 2019).

Table 1.2: Internationally agreed zero-points, their primary reference materials and currently available replacement reference materials at the highest metrological level (Brand et al., 2014; Dunn & Carter, 2018; Meier-Augenstein, 2017).

Reference material	**Compound name**	**Zero-point material**	**δ^{2}H (‰)**	**δ^{13}C (‰)**	**δ^{15}N (‰)**	**δ^{18}O (‰)**	**δ^{34}S (‰)**
VSMOW	Water	VSMOV	0			0	
SLAP	Water	VSMOW	0			-55.5	
VSMOW2[a]	Water	VSMOW	0 ± 0.3			0 ± 0.02	
SLAP2[a]	Water	VSMOW	-427.5 ± 0.3			-55.50 ± 0.02	
NBS 19[b]	Calcium carbonate	VPDB		+1.95		-2.2	
IAEA-603		VPDB		+2.46 ± 0.01		-2.37 ± 0.04	
Air N_2[c]	Atmospheric nitrogen	Atmospheric nitrogen			0.0		
IAEA-N-1	Ammonium sulfate	Atmospheric nitrogen			+0.43 ± 0.07		
USGS32	Potassium nitrate	Atmospheric nitrogen			+180		
IAEA-S-1[b]	Silver sulfide	VCDT					-0.3

[a] Replacement for the original VSMOW and SLAP.
[b] Limited supply – 0.5 g available for purchase every three years.
[c] While atmospheric nitrogen could be considered to be a primary RM; it is difficult to define a reproducible isotope ratio and is considered the zero-point of the scale.

Most primary RMs are inorganic compounds or materials and are not suited for the stable isotope analysis of organic compounds. Thus, secondary reference materials have been designed and developed to represent a variety of typical organic compounds or substances that are analysed and are traceable to the scale defining the primary RMs. These materials have been distributed by international recognized organizations, namely the International Atomic Energy Agency (IAEA), United States Geological Survey (USGS), Institute for Reference Materials and Measurements (IRMM), and the National Institute of Standards and Technology (NIST). The values of secondary RMs are often evaluated and submitted based on international inter-laboratory exercises organized by different organizations such as the IAEA, USGS, and IRMM. However, unlike primary RMs, the specified δ values of secondary RMs are associated with an uncertainty envelope (Meier-Augenstein & Schimmelmann, 2019).

1.3.1.2 In-house (lower-level) reference materials

Most laboratories prepare so-called in-house RMs, which they use for day-to-day quality control, and their preparation and use should follow the same guidelines as any international RM. They also need to be chemically similar to the samples analysed routinely, i.e., have typical δ-values, and must be calibrated against international RMs and the overall uncertainty calculated (Carter, 2017)

1.3.1.3 Appropriate choice of reference materials for normalization

Two-point normalization based on linear regression, where the intercept and the slope is used for recalculating raw data, is becoming increasingly common and follows on from the implementation of the two-point anchoring concept by the International Union of Pure and Applied Chemistry (IUPAC) (Brand et al., 2014; Skrzypek, 2013). There are several international available distributors for reference materials for stable isotope ratio analysis, although several do not have up-to-date isotope data, inventory or offer insufficient documentation of isotopic characterization (Meier-Augenstein & Schimmelmann, 2019).

Most international RMs are listed on the website of the International Union of Pure and Applied Chemistry (IUPAC) Commission on Isotopic Abundances Atomic Weights (CIAAW). In 2014, Brand et al. published a report on international isotopic reference materials. At the time of writing, a complete, up-to-date listing of available internationally RMs was not available on the CIAAW webpage[1]. Nevertheless, available internationally accepted RMs for stable isotope ratio analysis are listed in Table 1.3.

[1] https://www.ciaaw.org/carbon-references.htm

Table 1.3: Internationally accepted reference materials for stable isotope ratio analysis (modified by Camin et al., 2017).

Reference material (ID)	**Material**	$\delta^{2}H_{VSMOW}$ (‰)	$\delta^{13}C_{VPDB}$ (‰)	$\delta^{15}N_{Air}$ (‰)	$\delta^{18}O_{VSMOW}$ (‰)	$\delta^{34}S_{VCDT}$ (‰)
VSMOW2[a]	water	0 ± 0.3			0 ± 0.02	
GRESP[a]	water	-257.8 ± 0.4			-33.39 ± 0.04	
SLAP2[a]	water	-427.5 ± 0.3			-55.50 ± 0.02	
IAEA-604[a]	Enriched water	+799.9 ± 0.4			-5.86 ± 0.04	
IAEA-605[a]	Enriched water	+5997.9 ± 0.5			-3.02 ± 0.04	
IAEA-606[a]	Enriched water	+15993.6 ± 1.0			+2.43 ± 0.04	
IAEA-607[a]	Enriched water	+802.4 ± 0.4			+99.02 ± 0.13	
IAEA-608[a]	Enriched water	+6014. 3 ± 0.5			+736.4 ± 0.9	
IAEA-609[a]	Enriched water	+16036 ± 1			+1963.7 ± 2.2	
NBS 22[a,b]	Oil	-117.2[b] ± 0.6	-30.02[b] ±0.04			
NBS 28[a]	Quartz Sand				+9.58[c] ± 0.09	
USGS24[a]	Graphite		-16.049 ± 0.035			
IAEA-600[a,b,d]	Caffeine	-156.1 ± 1.3[b]	-27.73[b] ± 0.04	+1.02[b] ± 0.05	-3.48[d] ± 0.53	
IAEA-601[a,d]	Benzoic acids		-28.81[d] ± 0.04		+23.14[d] ± 0.19	
IAEA-602[a]	Benzoic acids		-28.85[d] ± 0.04		+71.28[d] ± 0.36	
IAEA-CH-3[a]	Cellulose		-24.724 ± 0.041			
IAEA-CH-7[a,b]	Polyethylene	-99.2[b] ± 1.2	-32.14[b] ± 0.05			
IAEA-303[a]	^{13}C Labelled Sodium-Bicarbonate		(Amp. A) +93.3 (Amp. B) +466			

Reference material (ID)	**Material**	$\delta^{2}H_{VSMOW}$ (‰)	$\delta^{13}C_{VPDB}$ (‰)	$\delta^{15}N_{Air}$ (‰)	$\delta^{18}O_{VSMOW}$ (‰)	$\delta^{34}S_{VCDT}$ (‰)
IAEA-603[a]	Calcite		+2.46 ± 0.01		-2.37 ± 0.04	
NBS 18[a,d]	Calcite		-5.01 ± 0.03		+7.20 ± 0.03[d]	
IAEA-CO-8[a]	Calcite		-5.764 ± 0.032		-22.7 ± 0.2	
IAEA-N-1[a,d]	Ammonium sulphate			+0.43[d] ± 0.07		
IAEA-N-2[a,d]	Ammonium sulphate			+20.41[d] ± 0.07		
USGS32[a]	Potassium nitrate			+180 ± 1	+25.7 ± 0.4	
USGS25[a,e]	Ammonium sulphate			-30.41[e] ± 0.27		
USGS26[a,e]	Ammonium sulphate			+53.75[e] ± 0.24		
NSVEC[a,c]	Nitrogen Gas-N_2			-2.78[c] ± 0.03		
IAEA-305[a]	^{15}N labelled ammonium sulphate			(Vial A) +39.8 (Vial B) +375. 3		
IAEA-310[a]	^{15}N labelled urea			Vial A) +47.2 (Vial B) +244.6		
IAEA-S-1[a]	Silver sulphide					-0.30
IAEA-S-2[a,d]	Silver sulphide					+22.62[d] ± 0.16
IAEA-S-3[a,d]	Silver sulphide					-32.49[d] ± 0.16
IAEA-S-4[a,c]	Sulphur					+16.86[c] ± 0.03
IAEA-SO-5[a,d]	Barium sulphate					+0.49[d] ± 0.11
IAES-SO-6[a,d]	Barium sulphate					-34.05[d] ± 0.08
USGS34[e]	Potassium nitrate			-1.8 ± 0.1	-27.78 ± 0.37	
USGS35[e]	Sodium nitrate			+2.7 ± 0.1	+56.81 ± 0.31	
USGS37[e]	Potassium perchlorate				-17.00 ± 0.1	

Reference material (ID)	**Material**	$\delta^{2}H_{VSMOW}$ (‰)	$\delta^{13}C_{VPDB}$ (‰)	$\delta^{15}N_{Air}$ (‰)	$\delta^{18}O_{VSMOW}$ (‰)	$\delta^{34}S_{VCDT}$ (‰)
USGS38[e]	Potassium perchlorate				+52.5 ± 0.3	
USGS39[e]	Potassium perchlorate				+22.3 ± 0.6	
USGS40[e]	L-glutamic acids		-26.39 ± 0.04	-4.52 ± 0.06		
USGS41a[e]	L-glutamic acids enriched in ^{13}C and ^{15}N		+36.55 ± 0.08	+47.55 ± 0.15		
USGS42[e]	Tibetan human hair powder (< 100 mesh)	-72.9 ± 2.2	-21.09 ± 0.1	+8.05 ± 0.1	+8.56 ± 0.1	+7.84 ± 0.25
USGS43[e]	Indian human hair powder (< 60 mesh)	-44.4 ± 2.0	-21.28 ± 0.1	+8.44 ± 0.10	+14.11 ± 0.1	+10.46 ± 0.22
CBS[e]	Caribou Hoff Standard					
KHS[e]	Kudu Horn Standard					
USGS44[e]	Calcium carbonate		-42.1 ± 0.05			
USGS45[e]	Biscayne Aquifer Drinking Water	-10.3 ± 0.4			-2.238 ± 0.011	
USGS46[e]	Ice Core Water	-235.8 ± 0.7			-29.80 ± 0.03	
USGS47[e]	Lake Louise Drinking Water	-150.2 ± 0.5			-19.80 ± 0.02	
USGS48[e]	Puerto Rico Precipitation	-2.0 ± 0.4			-2.224 ± 0.012	
USGS49[e]	Antarctic Ice-core Water	-394.7 ± 0.4			-50.55 ± 0.04	
USGS50[e]	Lake Kyoga Water	+32.8 ± 0.4			+4.95 ± 0.02	
USGS51[e]	Nitrous Oxide			+1.32 ± 0.04	+41.23 ± 0.04	
USGS52[e]	Nitrous Oxide			+0.44 ± 0.02	+40.64 ± 0.03	
USGS53[e]	Lake Shala Distilled Water	+40.2 ± 0.4			+5.47 ± 0.03	
USGS54[e]	Canadian lodgepole pine wood powder	-150.4 ± 1.1	-24.43 ± 0.02	-2.42 ± 0.32	+17.79 ± 0.15	

Reference material (ID)	Material	$\delta^{2}H_{VSMOW}$ (‰)	$\delta^{13}C_{VPDB}$ (‰)	$\delta^{15}N_{Air}$ (‰)	$\delta^{18}O_{VSMOW}$ (‰)	$\delta^{34}S_{VCDT}$ (‰)
USGS55[e]	Mexican ziricote wood powder	-28.2 ± 1.7	-27.13 ± 0.02	-0.3 ± 0.4	+19.12 ± 0.07	
USGS56[e]	South African red ivorywood powder	-44.0 ± 1.8	-24.34 ± 0.01	+1.8 ± 0.4	+27.23 ± 0.03	
USGS57[e]	Biotite	-91.5 ± 2.4				
USGS58[e]	Muscovite	-28.4 ± 1.6				
USGS61[e]	Caffeine	+96.9 ± 0.9	-35.05 ± 0.04	-2.87 ± 0.04		
USGS62[d]	Caffeine	-156.1 ± 2.1	-14.79 ± 0.04	+20.17 ± 0.06		
USGS63[e]	Caffeine	+174.5 ± 0.9	-1.17 ± 0.04	+37.83 ± 0.06		
USGS64[e]	Glycine		-40.81 ± 0.04	+1.76 ± 0.06		
USGS65[e]	Glycine		-20.29 ± 0.04	+20.68 ± 0.06		
USGS66[e]	Glycine		-0.67 ± 0.04	+40.83 ± 0.06		
USGS67[e]	n-hexadecane	-166.2 ± 1.0	-34.50 ± 0.05			
USGS68[e]	n-hexadecane	-10.2 ± 0.9	-10.55 ± 0.04			
USGS69[d]	n-hexadecane	+381.4 ± 3.5	-0.57 ± 0.04			
USGS70[e]	Icosanoic acid methyl ester (C20 FAME)	-183.9 ± 1.4	-30.53 ± 0.04			
USGS71[e]	Icosanoic acid methyl ester (C20 FAME)	-4.9 ± 1.0	-10.50 ± 0.03			
USGS72[e]	Icosanoic acid methyl ester (C20 FAME)	+348.3 ± 1.5	-1.54 ± 0.03			
USGS73[e]	L-valine		-24.03 ± 0.04	-5.21 ± 0.05		
USGS74[e]	L-valine		-9.30 ± 0.04	+30.19 ± 0.07		
USGS75[e]	L-valine		+0.49 ± 0.07	+61.53 ± 0.14		

Reference material (ID)	Material	$\delta^{2}H_{VSMOW}$ (‰)	$\delta^{13}C_{VPDB}$ (‰)	$\delta^{15}N_{Air}$ (‰)	$\delta^{18}O_{VSMOW}$ (‰)	$\delta^{34}S_{VCDT}$ (‰)
USGS76[e]	Methylheptadecanoate	-210.8 ± 0.9	-31.36 ± 0.04			
USGS77[e]	Polyethylene powder	-75.9 ± 0.6	-30.71 ± 0.04			
NBS 22a[e]	Vacuum oil, regular	-120.4 ± 1.0	-29.72 ± 0.04			
USGS78[e]	Vacuum oil, ^{2}H-enriched	+397.0 ± 2.2	-27.72 ± 0.04			
VSMOV	Water (original reference)	0			0	
GFLES-1[e]	Water enriched in ^{2}H	+80.1 ± 0.4			-6.25 ± 0.04	
GFLES-2[e]	Water enriched in ^{2}H	+159.9 ± 0.4			-6.21 ± 0.04	
GFLES-3[e]	Water enriched in ^{2}H	+280.2 ± 0.4			-6.14 ± 0.04	
GFLES-4[e]	Water enriched in ^{2}H	+399.8 ± 0.4			-6.08 ± 0.04	
NBS 123[c]	Sphalerite					+17.09 ± 0.19
NBS 127[c]	Barium sulphate				+8.59[d]± 0.26	+21.12 ± 0.22
IRMM-BCR-656[f]	Ethanol (wine origin)		-26.91 ± 0.07			
IRMM-BCR-657[f]	Glucose		-10.76 ± 0.04			
IRMM-BCR-660[f]	Ethanol (wine origin)		-26.72 ± 0.09			

[a]Reference material ID and their δ values are taken from https://nucleus.iaea.org/rpst/ReferenceProducts/ReferenceMaterials/Stable_Isotopes/index.htm.
[b]See also reference Schimmelmann et al., 2016
[c]Reference material ID and their δ values are taken from https://www-s.nist.gov/srmors/viewTableV.cfm?tableid=42
[d]See also reference Brand et al., 2014
[e]Reference material ID and their δ values are taken from https://isotopes.usgs.gov/lab/referencematerials.html.
[f]Reference material ID and their δ values are taken from https://ec.europa.eu/jrc/en/reference-materials/catalogue.

Since scale normalization requires at least two RMs acting as scale anchors, it is essential to choose the right pair of reference materials to use as end-points. This choice also depends on the assumed isotope values in the samples. Selected RMs should, therefore, cover a wide δ – value range or bracket, which covers all δ – values of the unknown samples and thus provide more accurate result normalization of the measured δ – values. Furthermore, physical properties and chemical elemental composition of selected RMs should be considered (Meier-Augenstein & Schimmelmann, 2019). For example, organic materials that contain hydrogen in an exchangeable position such as hydroxyl group in proteinaceous material will readily exchange hydrogen with atmospheric moisture leading to potentially erroneous δ^2H results. Before analysis, it is advantageous to dry the sample to eliminate any absorbed moisture and monitor this process while treating RMs identically. In this way, RMs and samples are equilibrated simultaneously with regards to atmospheric moisture. Further, the measured RM values can be used to normalize the raw δ-values of the samples; thus the RM matrix must resemble that of the samples as closely as possible or at least in terms of the fraction of exchangeable hydrogen. However, only a few RMs are available for the determination of δ^2H values such as USGS42 – Tibetan human hair and USGS43 – Indian human hair, CBS – Caribou Hoof Standard and KHS – Kudu Horn Standard (Camin et al., 2017).

In the case of amine salts or nitrogen-bearing organic compounds, it is also important to choose appropriate RMs in terms of matrix matching. In ammonium salts or organic amines or amides, nitrogen is present in an oxidation state ranging from -IV to -I and can be oxidized to states of oxidation of 0 (N_2) or possibly +II (NO). While in nitrates, nitrogen is present in its higher state of oxidation +V and cannot be oxidized further, but must be reduced to +IV, NO_2 or +II, NO. Thus, if we want to ensure identical treatment of nitrogen-bearing organic compound (amino acids or hetero-aromatic compounds), organic RMs (e.g., L-valine or caffeine) should be used for normalization. In the case of nitrate samples, it is also important to used nitrate RMs as scale anchors (Meier-Augenstein & Schimmelmann, 2019).

1.3.2 Natural variation in stable isotope composition

Natural variations in isotope abundance (or fractionation) occur for elements of low atomic numbers due to different chemical and physical properties and is a consequence of the differences in isotope mass. The main processes that produce isotope fractionation are kinetic ones, which depend primarily on differences in reaction rates of isotopic molecules and isotope exchange reactions (thermodynamic process) and relate to the energy state of the system, for which the isotope distribution changes between different chemical substances (reactant vs product), phases (vapor vs liquid) or individual molecules.

Molecules that contain heavy isotopes are more stable since they have higher dissociation energies, which leads to the preferential accumulation of the heavy isotope in the compound. Also, because the bonds between the lighter isotopes are easier to break, they react faster and become concentrated in the products, while the residual reactants become enriched with heavy isotopes. Some biological reactions (enzymatic) and many other phenomena such as evaporation, diffusion, condensation, dissociation are all associated with the kinetic isotope effect (Benson et al., 2006; Hoefs & Stable, 2009; Kendall & Mcdonnell, 1998). The thermodynamic isotope effect is related to physicochemical properties such as melting point, boiling point, vapor pressure, and molar volume all of which are related to vibration energy levels (Benson et al., 2006). The main factors affecting the variability of the five isotopic ratios: $^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$, in food authenticity studies are discussed in further detail.

1.3.2.1 Carbon

Different isotopic compositions of carbon in natural substances are associated with isotopic fractionation in the carbon cycle, and the primary carbon source is atmospheric CO_2 ($\delta^{13}C$ = -7.9‰ to -8.2‰). The variations in $^{13}C/^{12}C$ ratio mainly arise due to exchange processes between the atmosphere and the hydrosphere, which results in the enrichment in ^{13}C, while the intake of biospheric carbon by biological processes leads to depletion in ^{13}C. Plants fix carbon in their cells by assimilating carbon dioxide through photosynthesis, which actively discriminates against ^{13}C due to enzymatic and physical processes related to different photosynthetic pathways. Photosynthetic carbon fixation is also dependent on environmental conditions such as humidity, temperature, ambient CO_2 levels (land and ocean), light intensity, and fertilization. Metabolic reactions can create a kinetic isotope effect leading to further fractionation. For example, the products formed during the reaction of carbohydrates to protein and lipids have 2‰ and 5‰ lower $\delta^{13}C$ values, respectively (van Leeuwen et al., 2014).

Plants have evolved three photosynthetic pathways, namely the C_3 or Calvin cycle, C_4 or Hatch-Slack cycle, and through CAM (Crassulacean Acid Metabolism) metabolism. Each cycle has a different capacity to fixate atmosphere CO_2 due to carboxylase enzymes, resulting in photosynthesis products that have different $\delta^{13}C$ values compare to atmospheric CO_2 (Michener & Lajtha, 2007).

1.3.2.1.1 C_3 plants and Calvin cycle

Plants that only use the Calvin cycle are C_3 plants and thrive in areas of moderate temperatures and light intensity. These plants (C_3 plants) include, among others, cereal grains; wheat, rice, barley, oats, most trees, shrubs and cool-season grasses as well as peanuts, cotton, tobacco, tomatoes, apples, soybean, and spinach. The principal of the Calvin cycle is the fixation of atmospheric CO_2 with 5-carbon sugar, ribulose 1,5-biphosphate (RuBR) by the enzyme ribulose-1,5-diphosphate carboxylase/oxygenase to produce two 3-carbon molecules in 3-phosphoglyceric acid (3-PGA). The enzymatic reaction discriminates against the heavier carbon isotope ^{13}C (from CO_2), and plants become enriched in ^{12}C (O'Leary, 1988). Nevertheless, the final $\delta^{13}C$ values are affected not only by the enzymatic reaction but also by fertilization, plant organs (leaves or seeds), temperature, salinity, CO_2 concentration, and photorespiration (O'Leary, 1981). The $\delta^{13}C$ values of C_3 plants vary from -35 to -22‰ (Figure 1.1).

1.3.2.1.2 C_4 plants and the Hatch-Slack cycle

Hatch-Slack or C_4 plants, which include warm-season grasses, maize, sugar cane, often arise in tropical regions and thrive under conditions of high temperature, intense light, low CO_2 and high O_2 concentrations. In the Hatch-Slack cycle, CO_2 initially enters the leaf through the stoma and is taken up via the carboxylation of phosphoenolpyruvate, by the enzyme phosphoenolpyruvate carboxylase in the mesophyll cells. The intermediate of this carboxylation is oxaloacetic acid (OOA), a 4-carbon compound, which gets converted to malic (MAL) or aspartic (ASP) acid. The substrate for the enzyme is dissolved CO_2 which it converts to HCO_3^-. Diffusion limits the incorporation of CO_2 into the leaf and its dissolution in the water. In this pathway, the carboxylation is more limiting than the diffusion, which discriminates against the lighter carbon isotope and C_4 plants become enriched in ^{13}C, ($\delta^{13}C$ = -17 and -9‰; Figure 1.1) (O'Leary, 1988).

1.3.2.1.3 CAM plants and crassulacean acid metabolism

The third group of plants are those that use Crassulacean Acid Metabolism (CAM) to fixate CO_2. Examples include desert plants (cacti and orchids) and other succulents (pineapple and agave). These plants are characteristic of hot and dry environments and survive by closing their stomata during the day and opening them at night when they absorb CO_2 and fix it into malic acid by enzyme phosphoenolpyruvate carboxylase in a process similar to that in C_4 plants. In contrast, during the daytime, the stomata are tightly closed to prevent water loss, and the 4C acids (Malic acid) are decarboxylated to release CO_2, which then enter the Calvin cycle (O'Leary, 1988). Since the CAM plants used both C_3 and C_4 pathways, $\delta^{13}C$ values vary between -27 and -12‰ (see Figure 1.1).

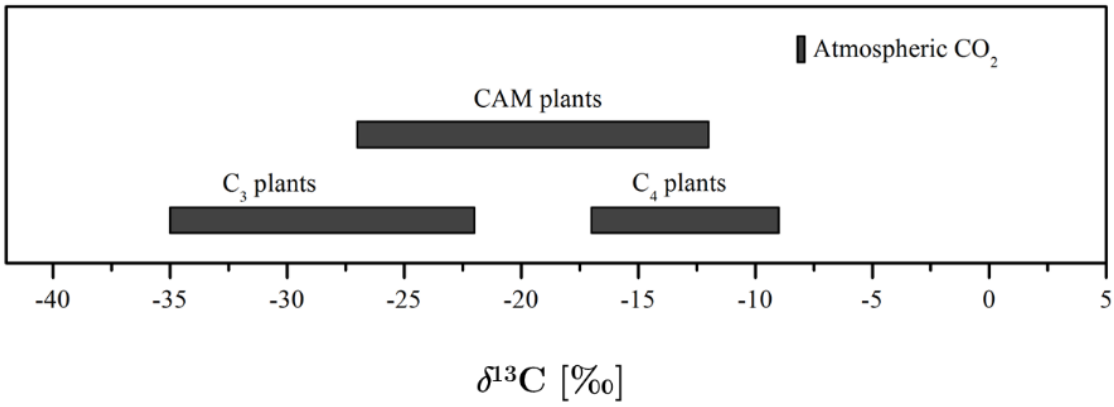


Figure 1.1: Examples of natural $\delta^{13}C$ values grouped according to the origin along the scale of ^{13}C natural abundance (modified from van Leeuwen et al., 2014).

Since a farm animal's diet usually consists of various plant species, it is possible to observe variations in $\delta^{13}C$ values in different animal tissues (lipid fraction and muscles). However, as Bahar and co-workers (2005) report shifting cattle from a C_3 diet to C_4 diet (maize), increases the $\delta^{13}C$ values of defatted dry muscle and its lipid fraction. Camin et al., (2008) also found that a 10% increase in maize in the animal's diet corresponded to a 0.7 to 1.0‰ shift in the $\delta^{13}C$ values of casein isolated from milk and dairy products.

1.3.2.2 Nitrogen

Nitrogen is also a crucially important component for all life from single-cell organisms to invertebrates. It can affect the rate of decomposition of plant and animal material and is a component of amino acids, the building blocks for proteins. Different forms of nitrogen circulate among the atmosphere, plants and microbes in different oxidation states from -IV to +V resulting in different isotopic compositions. The largest pool of available nitrogen is atmospheric molecular nitrogen (N_2) an inert compound, which is unavailable for use by most organisms (Sutton et al., 2011).

1.3.2.2.1 The nitrogen cycle and isotopic fractionation

The conversion of nitrogen can be carried out through both biological and physical processes, whereby atmospheric nitrogen (N_2) is transformed into other forms of nitrogen, such as inorganic (ammonium, nitrates and nitrites) and organic compounds (amines, amino acids and proteins) and further stored in the soil (Figure 1.2). Fixation is a process

that converts atmospheric nitrogen ($\delta^{15}N = 0‰$) into other forms by bacteria found inside the root nodules of legumes by nitrogenase enzymes. Typical $\delta^{15}N$ values are -3 and +1‰. Nitrogen can also be fixed abiotically by lightning and by human activities such as the production of energy or fertilizers and crop cultivation. Assimilation is related to the incorporation or uptake of ammonium, nitrate or nitrite. First, assimilated nitrogen oxides are reduced to ammonium by nitrate or nitrite reductase and further combined into organic matter by microorganisms. Assimilation discriminates against ^{15}N, resulting in lower $\delta^{15}N$ values compare to substrates and are in the range -1.6 to +1‰.

Mineralization or ammonification is a process in which organic nitrogen in the soil is transformed into ammonia. The fractionation between soil organic N and soil ammonium is ±1‰. Nitrification is the biological oxidation of ammonia to nitrite performed by soil bacteria *Nitrosomonas*, which is then oxidized to nitrate by *Nitrobacter*. In soils, $\delta^{15}N$ ranges from -29 and -19‰. Volatilization losses of ammonia from the soil to the atmosphere depend on many factors such as soil pH, wind speed, moisture, and temperature. Ammonia in the atmosphere has lower $\delta^{15}N$ values compared to the residual gas, due to fractionation between ammonium and ammonia in solution and between aqueous and gaseous ammonia and the diffusive loss of ^{15}N-depleted ammonia. Denitrification is the biologically mediated reduction of nitrate to nitrogen (N_2). Fractionation during denitrification causes the substrate to become enriched in ^{15}N, increasing the $\delta^{15}N$ values of the residual nitrates and a decrease in the concentration of nitrates (Kendall & Mcdonnell, 1998). The natural isotope abundance ^{15}N of soil is affected by many processes including mineralization, nitrification, volatilization, and denitrification and factors such as soil depth, vegetation, climate, particle size, salinity, and cultural history.

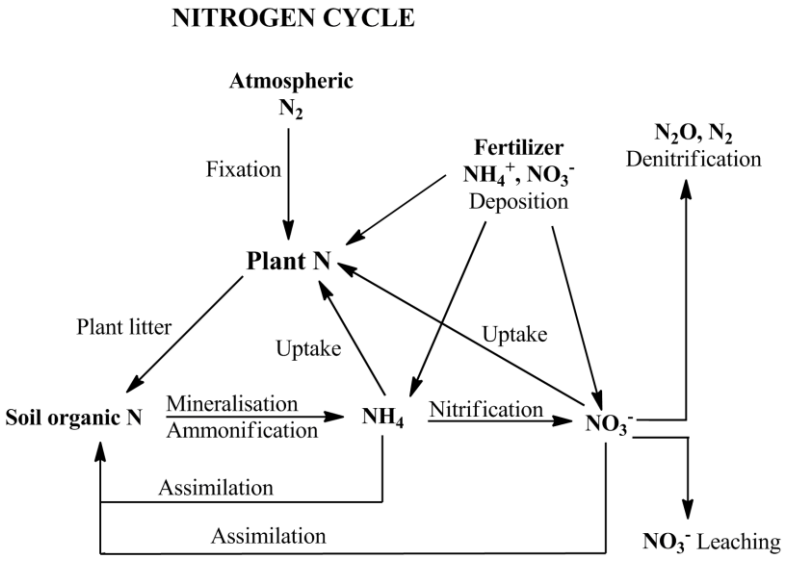


Figure 1.2: Processes in nitrogen cycle that affecting $\delta^{15}N$ values in ecosystem (modified from Kendall & Mcdonnell, 1998).

However, the main factor that affects crop soil is fertilizer practice, particularly the use of high concentrations of nitrates. Inorganic fertilizers produced from atmospheric N_2 by the Haber-Bosch process, which include urea, ammonium nitrate and potassium nitrate have $\delta^{15}N$ values of between -6 and +6‰. In contrast, $\delta^{15}N$ values for organic fertilizers (cover crops, plants composts, and animal manure) are much higher and range between +0.6 and +36.7‰, The $\delta^{15}N$ values of animal manure range between +10 and +25‰. This extensive range of $\delta^{15}N$ values in manure can be attributed to nitrogen originating from

different trophic levels. Leguminous and nitrogen-fixation plants can fix nitrogen directly from the atmospheric air and consequently have $\delta^{15}N$ values around 0‰ (Kendall & Mcdonnell, 1998; van Leeuwen et al., 2014).

1.3.2.3 Sulphur

In the environment, sulphur can be present as a non-metal in ore deposits or as sulphates in evaporites. It is also a minor component in igneous and metamorphic rocks, organic substances, marine waters and sediments as sulphide and sulphate. Sulphur occurs in different oxidation states (−II to +VI) and forms deposits of sulphates, which can be further reduced and deposited as sedimentary sulphides or in organic matter.

However, isotopic discrimination during sulphur transformation and isotopic composition of sources result in natural abundances of sulphur isotopes with $\delta^{34}S$ values ranging from -40 to +40‰. Soil sulphates and atmospheric SO_2, as well as seawater, are a major S source in plants (Krivachy Tanz et al., 2015). Oceanic sources affect the isotopic composition of atmospheric sulphur in coastal areas up to 100 km from the ocean due to sulphate aerosols derived from sea spray and result in the airborne deposition of marine sulphur particles from the ocean to the coast, which have a $\delta^{34}S$ value close to that of the ocean (+21‰). Anthropogenic emission and sulphide ores also affect $\delta^{34}S$ values of atmospheric sulphur through the precipitation, due to the different $\delta^{34}S$ values of sources ($\delta^{34}S$ coal ranging from -30 to +30‰, $\delta^{34}S$ gas and oil are from -5 to +30‰, while $\delta^{34}S$ values in sulphide ores range from -25 to +25‰. Another factor that influences the isotopic composition of atmospheric sulphur are biogenic emissions such as hydrogen sulphide (H_2S) and dimethyl sulphide (DMS), which are depleted in ^{34}S due to the kinetic isotope effect that occurs during dissimilatory sulphate reduction, and can be further incorporated into soil and vegetation. Dissimilatory sulphate reduction, which discriminates in favour of ^{32}S, produces isotopically light H_2S and results in differences in $\delta^{34}S$ values of mineral sources. In marine sediments, sulphides have lower $\delta^{34}S$ values (-49.6 to +5.2‰) compare to sulphates (average $\delta^{34}S$ of +20‰) (Kendall & Mcdonnell, 1998). In contrast, assimilatory sulphate reduction, which is the transformation of sulphur from sulphate into organic sulphur (proteins and descendants, vitamins, coenzymes and redox centers), results in far smaller sulphur isotope fractionation, and the isotopic composition of bulk plant sulphur is lower by 1 to 2‰ relative to its primary source (soils, sea spray sulphates and SO_2 from the atmosphere). The transferred $\delta^{34}S$ signal from plants material to animals does not alter the $\delta^{34}S$ values in the food chain, which can then be used in nutritional research (Tanz & Schmidt, 2010). The $\delta^{34}S$ values vary locally and are typical for defined cultivation areas, i.e., higher $\delta^{34}S$ values are found in arid regions, especially near the sea or near salt deposits (Krivachy Tanz et al., 2015).

1.3.2.4 Oxygen

Oxygen atoms in plant and animal tissues predominantly come from meteoric water, which originates from the ocean through evaporation and subsequent condensation in precipitation. The $\delta^{18}O$ values of ocean water range from -1 to +0.7‰ and are close to that of international reference standard – the Vienna Standard Mean Ocean (V-SMOW, $\delta^{18}O = 0$‰). Isotopic exchange reactions, which occur between different phases (equilibrium fractionation) and diffusion processes of the water vapor (kinetic fractionation) result in isotopic variations (Figure 1.3), which affects the $\delta^{18}O$ values in plants (Krivachy Tanz et al., 2015; Marshall et al., 2008).

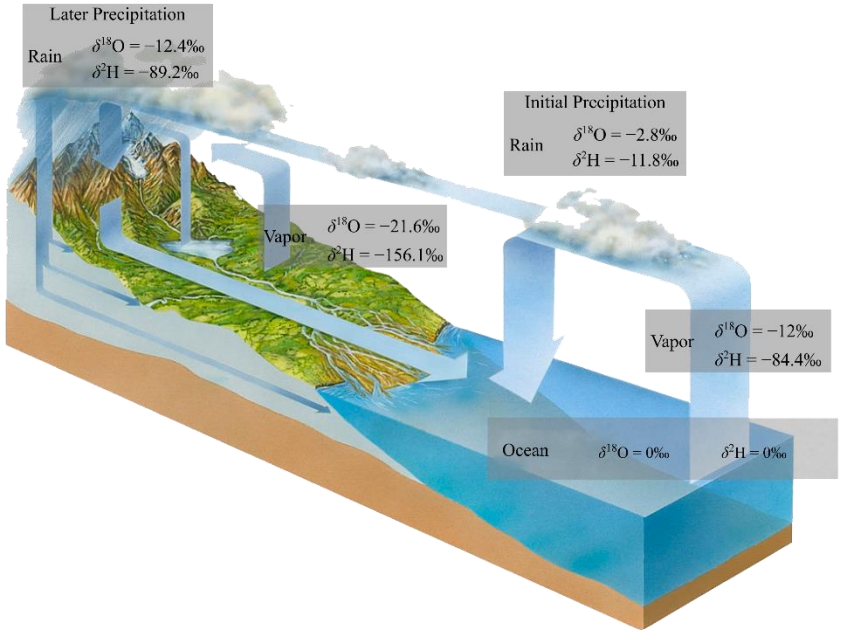


Figure 1.3: A schematic diagram showing isotopic fractionation in the water cycle (adapted from Meier-Augenstein, 2017).

The two main factors affecting the isotopic composition of precipitation are temperature, and the proportion of the original vapor that remains after the precipitation has begun. Increasing temperature causes enrichment of ^{18}O and fractionation at a rate of approximately 0.5‰ for every degree °C. Since fractionation is temperature-dependent, fractionation it is higher at lower temperatures, which means that the isotope ratio of water vapor at 25°C is 9.2‰ more negative than that of liquid water, while at 0°C the vapour would be 11.0‰ more negative (Marshall et al., 2008). A similar effect is also observed with increasing elevation, corresponding to lower temperatures, since the cooling of air masses at higher altitudes produces higher precipitation, which over time cause isotopically lighter rain clouds. Consequently, regions of higher-elevation receive ^{18}O depleted precipitation. Isotopic composition of precipitation varies from approximately -0.15 to -0.5‰ per 100 m increase in elevation. Increasing distance from the equatorial regions causes a decrease in condensation temperature and consequently removes water vapor from the air mass resulting in precipitation that has more negative isotopic values over higher latitude for approximately -0.5‰ per degree latitude. However, since the precipitation becomes enriched in ^{16}O as it moves towards the cold polar regions, polar ice is a reservoir of ^{16}O enriched water compare to seawater. Liquid phases in initial precipitation are enriched in ^{18}O compared to subsequent ones. The result is that precipitation gets isotopically lighter as it continues to rain, because, during long periods of rainfall, evaporation is reduced by the higher relative humidity – a phenomenon known as “rainout effect” or “amount effect.” Distance from the sea also an effect, since rainout reduces the heavy isotope composition of an air mass as it moves inland. Precipitation over inland areas tends to be characterized by temperature variations resulting from so-called seasonal trends. All of these factors are responsible for the localized individual oxygen isotope characteristics of meteoric and groundwater (Krivachy Tanz et al., 2015; Marshall et al., 2008; McGuire & McDonnell, 2008).

To conclude, isotopic variation in plant water is mainly due to the isotopic variations in precipitation or groundwater inputs, and enrichment of the ^{18}O in water due to

evaporation from the soil surface, and evaporation from the leaf surface during transpiration, which depends on assimilation type (C_3, C_4, and CAM plant), plant species and organ, and local conditions and climate (water availability). Incorporation of different reactions between plant water, atmospheric CO_2 ($\delta^{18}O$ = +40.3‰ to +42.5‰) and O_2 ($\delta^{18}O$ = +23.5 to +23.8‰) implies different $\delta^{18}O$ values in plant products (Schmidt et al., 2001). In animal originating food drinking water, intrinsic water in food, water originating from organically bound oxygen and atmospheric dioxygen converted in the respiratory chain cause differences in $\delta^{18}O$ values. For example, carbonyl compounds (aldehydes and ketones) can exchange oxygen atoms with plant water and cause fractionation of approximately 27‰. Esters and amides can have slightly lower values (23‰), due to the intramolecular isotope effect on the oxygen elimination from the carboxyl moiety. Carboxyl group can also exchange oxygen atoms via hydrates resulting fractionation of approximately 19‰ relative to the water (Krivachy Tanz et al., 2015; Schmidt et al., 2001).

1.3.2.5 Hydrogen

The primary source of hydrogen in nature is the hydrosphere also called the “water sphere”, as it includes all of the Earth’s water found in streams, lakes, oceans, ice, groundwater and air. The ocean holds 97 percent of the Earth's water, making it the main reservoir for hydrogen in nature. Clouds, formed from evaporating ocean water transport water from the ocean to the continents. The movement of the water vapor leads variability in δ^2H values, which depends on geo-location, due to the evaporation from oceans and subsequent condensation in terms of precipitation (Figure 1.3) (Meier-Augenstein, 2018).

Several factors such as latitude, altitude, temperature and distance to the open seas, “control” the isotopic composition of meteoric water and hence freshwater globally (δ^2H = +20 to -270‰), with less negative δ^2H values being typical of warmer coastal/near-equatorial regions and low-elevation/low-latitude and more negative δ^2H values being typical of cooler inland and high-altitude/high-latitude regions (Meier-Augenstein, 2018). Generally, more negative δ^2H values of precipitation are found during the winter and more positive values during the summer.

The δ^2H values of plant tissues are related to the isotopic composition of the source water available to the plant during growth since hydrogen for land plants originates from the water taken up by the roots from the soil, which derives mainly from local precipitation. The soil water taken up by the roots is then transported through the plant's xylem system, a process that does not result in significant isotopic fractionation. However, when xylem water leaves the stem and enters the evaporating tissues (leaf, fruits), the plant tissue water is further modified as a result of evaporation and is isotopically enriched in the heavy isotopes (Chesson et al., 2018).

Isotopic fractionation of hydrogen also takes place during the biosynthesis of carbohydrates, lipids and proteins. The biosynthesis of lipids is associated with considerable isotopic fractionation of around -200‰ versus δ^2H values of the source water. However, the biosynthesis of carbohydrates such as sugars and cellulose seems to be mostly unaffected, since the δ^2H values strongly correlate with those of source water (Meier-Augenstein, 2018).

In contrast to plants, the source for hydrogen in animal tissues, particularly for land-living animals, can be divided into three main pools: hydrogen derived from drinking water, hydrogen derived from dietary sources and exchangeable hydrogen (ambient water hydrogen). The δ^2H values in animal tissues are primarily controlled by diet, which in turns is strongly correlated with the δ^2H values of source water (soil or irrigation) (Meier-Augenstein, 2018).

1.4 Application of Stable Isotopes and Elemental Composition in Food: Milk and Dairy Products

Increased awareness of consumers, regulatory authorities and food processors of the importance of correctly declared food products has led to the development and application of food authenticity and traceability-enabling techniques. Stable isotope profiling of various bio-elements has been used to determine the authenticity of plant and animal products with some success. However, the stable isotope approach is even more effective when combined with elemental analysis. The following discussion provides an overview of both methods as applied to milk and dairy products.

Milk and dairy products constitute a major commodity group that has been the subject of many studies attempting to differentiate milk products from different production regions (geographical origin) as well as for guaranteeing the unadulterated status and quality of the dairy products. This increase is reflective of the fact that provenance has become a significant issue in the context of food safety and quality as well as in consumer and brand protection. For example, the EU recently introduced the quality labels Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialities Guaranteed (TSG) trademarks, which have been assigned to numerous products based strictly on their geographic and production origin. The techniques employed for differentiation of PDO and PGI dairy products involve stable isotope analysis either on its own or in combination with elemental analysis, since stable isotopes mostly depend on climatic and geographical condition, while the elemental composition is affected by geology and the pedological characteristics of the soil, excluding "contamination" processes (Bontempo et al., 2012; Drivelos & Georgiou, 2012; Griboff et al., 2019; Nečemer et al., 2016).

1.4.1 Stable isotope composition of dairy products

The first application of isotope ratio mass spectrometry was made in 1997 by Kornexl and co-workers, who found that $\delta^{13}C$ and $\delta^{15}N$ values reflect the diet fed to dairy cows and that only carbon and nitrogen isotopes were not useful in assigning the origin of the milk. The authors did find that the most useful tool for determining milk's geographical origin was the $\delta^{18}O$ composition of milk water. Crittenden et al. (2007) confirmed the potential of stable isotope ratios of C, N, O, S and Sr for distinguishing between milk products produced in different regions in Australia and that oxygen and carbon isotope ratios confirmed predicted isotope fractionation patterns based on the latitude and climate of each region. The authors also determined that milk samples from Australasia were enriched in ^{18}O and ^{34}S compared to European dairy products. Stevenson and co-workers (2015) looked at a similar set of isotopes (H, O, N, C and Sr) of cheese, milk, animal feed and soil from six artisanal cheese production sites in Quebec, Canada and demonstrated the potential of isotope geochemistry in linking land to agricultural produce. This differentiation is possible because the $\delta^{18}O$ and $\delta^{2}H$ content in milk water reflect the isotope composition of the animals drinking water, which is influenced by geographical factors such as altitude, latitude, and distance from the sea (Chesson et al., 2010; Crittenden et al., 2007). Studies have also documented that in milk water, the ^{18}O content between 2 and 6‰ higher compared to the cows drinking water (Camin et al., 2008; Chesson et al., 2010; Kornexl et al., 1997). Seasonal variations in isotopic composition are also apparent. In summer, milk water contains higher $\delta^{18}O$ values since dairy animal's graze on plants containing ^{18}O enriched water due to evapotranspiration from the leaves (Kornexl et al., 1997; Rossmann, 2001).

In other studies, Luo et al. (2016) were able to identify the geographical origin of milk, using $\delta^{13}C$ and $\delta^{15}N$ values in extracted protein and $\delta^{18}O$ and $\delta^{2}H$ values of milk water and differentiate milk from Australia and New Zealand, Germany and French, the USA and China. Further, Chung et al. (2020) found that $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values were the main parameters important in discriminating organic milk from different regions in Korea. Stable isotope ratios of carbon and nitrogen in the casein fraction were successfully used to distinguished Pecorino Sardo Cheese from Sardinia from that produced in Sicily and Apulia (Manca et al., 2001) and buffalo mozzarella originating from two areas in southern Italy (Brescia et al., 2005). When combined with ^{2}H, ^{34}S and $\delta^{13}C$ and $\delta^{18}O$ values in glycerol, it was also possible to classify the geographical provenance of a high-value cheese from Spain, Italy and French (Camin et al., 2004) and Sardinian Peretta cheese from that of competitors cheese produced in Northern Europe (Manca et al., 2006). The successful classification of these products was possible because the $\delta^{13}C$ value of the milk is dependent on the proportion of C_3 (grass) and C_4 (maize) plants in animals' diet since grass-feeding regime in comparison with a regime where a higher amount of maize in the diet leads to more negative $\delta^{13}C$ values in the protein fraction (Camin et al., 2008). The isotopic composition of nitrogen is not only dependent on dietary habits but also on factors such as agricultural practice, aridity, salinity, and distance from the sea. The $\delta^{15}N$ value of milk is mainly influenced by the intensity of agricultural use, because the $\delta^{15}N$ values in dairy products reflect, through the plants consumed by animals, the isotope composition of the soil (Kornexl et al., 1997; Manca et al., 2001). Higher amounts of leguminous (nitrogen fixation plants) result in low $\delta^{15}N$ values in the feed and subsequently in dairy products because these plants use both atmospheric and soil nitrogen as a nitrogen source. The result is lower ^{15}N content than in plants that just rely on soil nitrogen since ^{15}N in legumes is enriched relative to atmospheric nitrogen (Camin et al., 2008; Rossmann, 2001).

Regarding sulphur, the $\delta^{34}S$ values vary locally and are typical for defined areas (Crittenden et al., 2007), since the source of the sulfuric in animal tissues is from plants. Many factors such as local geology, fertilizer application (organic or artificial), anthropogenic atmospheric sulfuric dioxide deposition and distance from the coast (due to atmospheric seawater fallout), influence the $\delta^{34}S$ values in terrestrial plants. The estimated sulfuric trophic shift between animals and their diet range between 0‰ and 1‰ and small differences can be observed among the various animal (Camin et al. 2016). However, higher $\delta^{34}S$ values occur in arid regions, especially near the sea where there are high amounts of marine aerosols, or near salt deposits in bedrock (Krivachy Tanz et al., 2015).

Stable isotope ratio analysis of carbon and nitrogen were also used to discriminate between organic and conventional milk from Korea (Chung et al., 2014). In organic milk, the mean $\delta^{13}C$ value was higher, while the mean $\delta^{15}N$ value was lower compared to conventional milk. Similarly, Capici and co-workers (2015) also verified that it was possible to distinguish between Stelvio cheese produced from raw milk and those prepared from pasteurized milk based on the $\delta^{13}C$ and $\delta^{15}N$ values in fat and resulting de-fatted cheese fraction. The authors found that they could distinguish samples prepared with raw milk from those prepared with pasteurized milk. Furthermore, they also demonstrated that in cheese, it is possible to detect the fraudulent addition of extraneous matter based on isotope values such as those of reconstructed milk obtained from milk powder.

A correlation between precipitation and geographical location and the $\delta^{2}H$ values of specific FA (butyric, myristic, palmitic and oleic acid) in milk and bulk milk powder allowed Ehtesham et al. (2013a) to discriminate between producing regions in New Zealand with a 91% correct classification rate. Furthermore, by using principal component analysis (PCA), the authors were able to that $\delta^{2}H$ and $\delta^{13}C$ values of four FAs (butyric, myristic, palmitic and oleic acid) and bulk milk powder could distinguish between milk produce in New Zealand North from milk produced in the South Islands (Ehtesham et al., 2013b), and

that the four selected FAs make suitable biomarkers for investigating provenance. Ehtesham et al. (2015) also focused on the relationship between δ^2H of FAs in milk, milk water, and both feed and farm water simultaneously. The δ^2H values of FAs in feed and δ^2H values of farm water are related to the δ^2H values of milk FAs, milk solids, and milk water and could be a biogeochemical fingerprint of the environment. This finding should be taken into consideration for dried milk products when water is removed or the product altered and can help determine the dietary regime and geographical origin of dry and processed products (cheese, butter, and milk powder).

In the case of other dairy products, butter has been subjected to numerous food scandals, which include its substitution with animal fats or synthetic products as well as mislabelling with incorrect geographical origin. A combination of δ^{13}C, δ^{15}N δ^{18}O δ^{34}S, and ^{87}Sr/^{86}Sr data is one way to enable the regional provenance of butter from several European and non-European countries (Rossmann et al., 2000).

1.4.2 Elemental composition of dairy products

The content of major and trace elements in milk and dairy products are not constant and reflect the content of the elemental composition of the soil. The genetics of individual animal species also affect the elemental composition of milk, as does the stage of lactation, farm management practices (nutritional regime), fertilizer application and industrial activities (Sola-Larrañaga & Navarro-Blasco, 2009), which explains the observed variations in the elemental profile of milk between and within countries. Some of the first studies on this subject showed that elemental composition in milk and dairy products could be used to identify the species of ruminant (Benincasa et al., 2008; Fresno et al., 1995) or by determining specific elements including toxic ones, to estimate food quality (Coni et al., 1996; Esposito et al., 2017; González-Martín et al., 2011; Rey-Crespo et al., 2013). More recently, the trace element composition was applied to the characterization of milk and cheese since they can provide sufficient information to distinguish between different producing regions. For instance, Del Signore and co-workers (2004) proved that the elemental profiles Li, K, Mn, Se, Rb, and Cs could be used to discriminate between cheese from three Italian regions, regardless of the type of milk (cow or sheep). Suhaj and Koreňovská (2008) also successfully classified typical Slovak national Bryndza sheep's cheese from nine different producing regions in Slovakia as well as from other Central European countries, while Bresciaand co-workers (2005) used K and Li as a useful marker for distinguishing milk and mozzarella cheese from two areas in Southern Italy. Further, Zain et al. (2016) studied the elemental composition of milk from a tropical region where there are no dramatic changes in season. The authors observed that Malaysian milk differs significantly from the milk from farms in northern and southern regions of Iran as well as from factory milk samples consistent with milk from New Zealand, Australia, Azerbaijan, Belgium, Canada, and the USA purchased from supermarkets in Malaysia and Turkey.

1.4.3 Stable isotope and elemental approach in food authenticity

To date, several studies have used both isotopic and elemental profiles for the determination of the origin of milk and dairy products. Pillonel et al., (2003) were able to distinguish between Emmental cheese produced in Finland, Bretagne and Savoie based on the content of Ca, Mg, Na, K, Mn, Mo and I; the stable isotopes δ^2H, δ^{13}C, δ^{15}N, δ^{34}S and ^{87}Sr/^{86}Sr), and on levels of the radioactive elements ^{90}Sr, ^{234}U, and ^{238}U. Similarly, Sacco et al., (2009) could distinguish cow's milk from Southern Italy from that of Central Europe (Austria, Croatia, Hungary, Germany, and Slovenia), and found that δ^{13}C values had the most discriminating power. Bontempo et al., (2011), successfully distinguished between

traditional alpine and pre-alpine Italian PDO cheese according to origin and type, since the $\delta^{13}C$ in casein and glycerol provided an estimate of the maize uptake in the animal's diet. Camin and co-workers (2012) presented two models with excellent classification success rates (>98%), one for predicting the geographic origin of seven kinds of European hard cheese, and one for discriminating PDO Parmigiano Reggiano cheese from nine European and two non-European producers. Nečemer and co-workers (2016) were also able to define the geographical origin of authentic Slovenian cow's, sheep's and goat's milk and cheese, work that contributed towards supporting the existence of a Slovenian PDO cheese. However, it was only in 2015, that an international collaborative study was performed to validate the isotopic and elemental methods used to analysis cheese, in order to support official recognition of their use in determining the authenticity of cheese products (Camin et al., 2015).

A combination of stable isotope and multi-elemental analysis can also be applied to support the authenticity of whole milk powder or powdered dairy product, in some cases, at the manufacturer or factory level. McLeod and others (2016) determined $\delta^{13}C$ and $\delta^{15}N$ and 13 elements in goat's milk powder. The authors showed that it is possible to use a chemical fingerprint of production batches of goat whole milk powder produced by different manufacturers to verify the source of the product at the manufacturer or factory level, and potentially at the individual batch level.

Among more recent studies, Griboff and co-workers (2019) verified milk from different regions in Argentina, linking the composition of the milk with that of the soil, drinking water and forage. In addition, using chemometrics tools, the authors were able to differentiate milk from four production regions in Argentina. Further Bontempo et al., (2019) investigated the Mozzarella di Bufala Campana PDO production chain from milk to cheese, in order to determine the suitability of using elemental and isotopic compositions for cheese traceability. Despite elemental and isotopic differences occurring during processing, and those relating to seasonal differences and production region, the group were able to create a model capable of distinguishing PDO and non-PDO mozzarella products, even when non-PDO cheeses were produced outside of the Mozzarella di Bufala Campana reference area.

Chapter 2

Hypothesis and Aims

The need to monitor the authenticity and quality of milk and dairy products has created a demand for sensitive and reliable methods to determine whether or not food fraud has taken place. It is known that the isotopic composition of dairy products reflects the dietary regime and the metabolism of milk-producing animals, and based on this knowledge, stable isotope ratios (expressed as δ in ‰) have been used to authenticate milk and dairy products from different regions with different dietary regimes and production systems. Geographical origin of milk and dairy products can also be determined using a combination of elemental and stable isotopes analysis (Camin et al., 2016). Sacco et al. (2009) analysed milk samples from different geographical areas, including Italy and some EU countries to determine whether their chemical composition could be used to identify their geographical origin. In their study, elemental concentrations (Ba, Mn, Zn, Fe and Cu) were determined together with C and N isotopic ratios and chemical analyses of fatty acids, sugars, and amino acids. The results suggested that fatty acid (FA) composition could be a determinant of provenance and adulteration of milk and dairy products. It is known that the FA composition, as well as the isotopic composition of the FAs, is influenced by the dairy cow's diet (Karoui & De Baerdemaeker, 2007; Meier-Augenstein, 2002; Molkentin & Giesemann, 2007; Richter et al., 2012).

A common type of adulteration of dairy products appearing on the market is the addition of tallow to raw cow's milk. The detection of this kind of fraud is based on analysing the FA composition non-milk fat, and use this to determine the amount of tallow in raw cow's milk (Ulberth, 1995). The addition of very small amounts of tallow (1.2%) can be detected using this method. The FA composition could also be used to check the authenticity of goat's and sheep's milk. Although the lipids of cow, goat, and sheep are somewhat similar, there are significant differences in the content of short-chain FAs. These differences are particularly noticeable in the case of caproic (C6:0), caprylic (C8:0) and capric (C10:0) acids, which are more abundant in goat's and sheep's milk compared to cow's milk. Thus, the addition of cow's milk to goat's and sheep's milk or cheese could be detected based on the FA profile (MacGibbon & Taylor, 2006).

Slovenia is a small country in terms of surface area, but is pedologically and climatically diverse, which makes it an ideal location for studying the natural factors which govern the isotopic distribution in milk and its products. The primary objective of this dissertation was to determine the authenticity and geographical origin of Slovenian milk and dairy products. Two approaches were followed to realise this objective: 1) the combined use of elemental and stable isotope analysis of H, C, N, O and S; and 2) the determination of the FA content and stable isotope composition of individual fatty acids. The aim was also to examine the effect of season and geographical location on proportional changes in milk fat content in relation to the animal's diet.

One of the main issues in food authenticity is to ensure the high-quality and comparability of elemental and isotopic data. The initial focus of this thesis was on establishing appropriate protocols to ensure harmonization of methodologies and data quality. As a designated metrology institute, the JSI participated in an inter-laboratory assessment (CCQM-K140) of stable carbon isotope ratio determination of bulk honey. Further, the aim was to characterize the FA composition of milk adulterated with varying amounts of vegetable tallow and to verify the authenticity of cheese from cow's, sheep's and goat's milk available on the Slovenian market and to use the developed methods to qualitatively and quantitatively analyse dairy products.

This thesis tests the following hypotheses:

- The geographical origin of milk can be determined based on its elemental and isotopic composition.
- The stable isotope composition of fatty acids is a better parameter for discriminating the geographical origin of milk than using elemental and bulk isotopic composition.
- By knowing the FA composition of milk, the addition of cow's milk to sheep's and goat's milk can be determined.
- Most types of cheese (cow, goat, sheep) on the Slovenian market are correctly labelled.

Chapter 3

Publications

3.1 Scientific Paper: "Multi - Elemental Composition of Slovenian Milk: Analytical Approach and Geographical Origin Determination"

In this chapter, the paper entitled "*Multi-elemental composition of Slovenian milk: analytical approach and geographical origin determination*" by Doris Potočnik, Marijan Nečemer, Darja Mazej, Radojko Jaćimović and Nives Ogrinc is presented. The paper was published in *Acta IMEKO*, 2016 and compares three different techniques: energy dispersive X-ray fluorescence spectrometry (EDXRF), k_0-instrumental nuclear activation analysis (k_0-INAA) and inductively coupled plasma mass spectrometry (ICP-MS) for determining the multi-elemental composition of milk samples. It also investigated whether it is possible to discriminate milk according to its geographic production region in Slovenia based on its elemental composition.

In order to ensure consistent, reliable and accurate data, it is necessary to validate analytical methods. In this work, quality assurance of the results was checked by analyzing certified reference materials: Whole milk powder NIST 8435 and Non-fat Milk Powder NIST 1549 both from the National Institute of Standard and Technology; Skimmed Milk Powder BCR 150, ERM DB-150 and ERM DB-151 obtained from the EC-JCR-IRMM. The results obtained using the validated EDXFR, k_0-INAA and ICP-MS methods were in good agreement with the stated certified reference material values. Except for Se, As, Cd and Pb, method precision was satisfactory, and the relative standard deviation for each element was below 10%. For the other elements, their measured concentrations were close to their limit of detection, and the precision was >10%. The results obtained by independent k_0-INAA and EDXRF for selected macro- and micro-elements in milk samples are also in good agreement. Only two samples exceeded the 95% confidence interval for Rb and Zn, where k_0-INAA gave lower values than EDXRF.

The results also revealed that among the applied analytical techniques, EDXRF was the most practical, greenest and cost-effective for determining levels of P, S, Cl, K, Ca, Zn, Br, Rb ad Sr in milk. However, for analysing Mn, Fe, Cu, and Se and toxic elements such as As, Cd and Pb, ICP-MS was the method of choice on account of its superior sensitivity and accuracy.

In this study, both methods were used to determine the multi-elemental composition of Slovenian raw cow's milk samples collected in December 2013 from four distinct geographical regions: Mediterranean, Pannonian, Alpine and Dinaric, in Slovenia. A statistical analysis of the data using linear discriminant analysis (LDA) was also performed in order to classify milk samples according to the production region. The results revealed

only partial separation of the milk samples, and the overall prediction ability was 66.7%. The elements Ca, S, P, K and Cl had the most discrimination power.

In this paper, I was responsible for the collection and storage of milk samples provided by different Slovenian dairy producers. I also prepared all the samples and standards for analysis, including the microwave digestion of milk samples and certified reference materials. The k_0-INAA was performed with assistance from the Radiochemistry group at the Department of Environmental Science, Jožef Stefan Institute, and the EDXRF analysis with the assistance of the Department of Low and Medium Energy Physics also at the Jožef Stefan Institute. I also collected and in part, analysed the data and jointly prepared the manuscript.

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Multi-elemental composition of Slovenian milk: analytical approach and geographical origin determination

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ABSTRACT
The main objective in multi-elemental analysis in food has traditionally been and still is, to ensure food quality and safety. Three different methods were investigated in the present study to obtain the elemental content in milk samples: energy dispersive X-ray fluorescence spectrometry (EDXRF), k_0-instrumental nuclear activation analysis (k_0-INAA) and the inductively coupled plasma mass spectrometry (ICP-MS). Results indicate that the obtained data with different methods are in good agreement with certified reference materials. Precision was found to be satisfactory with relative standard deviations always between 1 and 10 %, except for Se, As, Cd and Pb. The concentrations of these elements were close to the detection limit and thus, precision higher than 10 %. An intercomparison exercise between EDXRF and k_0-INAA showed satisfactory agreement and only two samples exceeded 95 % confidence interval for Rb and Zn with lower data obtained by k_0-INAA. It was found that EDXRF was the cheapest, simplest and most environmental friendly method for analysis of multi-elemental composition (P, S, Cl, K, Ca, Zn, Br, Rb, Sr) in milk samples, while for the determination of Mn, Fe, Cu, Se content and possible identification of pollutants such as As, Cd and Pb ICP-MS it was a method of choice due to its excellent sensitivity and accuracy. These two methods were also used to determine the multi-elemental composition in Slovenian raw cow milk from different geographical regions: Alpine, Mediterranean, Dinaric and Pannonian in December 2013. Linear discriminant analysis (LDA) was used to explore multi-elemental analysis of milk samples to obtain classification according to geographical regions. Regional discrimination was most successful taking into account Ca, S, P, K, and Cl with prediction ability of 66.7 %.

Section: RESEARCH PAPER

Keywords: milk; EDXRF; k_0-INAA; ICP-MS; LDA; geographical origin

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1. INTRODUCTION

Milk is an important everyday nutrition, since it is a source for many key nutrients including proteins, energy, many essential minerals and vitamins, factors that all contributed to an increased consumption in recent years. Milk contains more than 20 different micro and macro elements. Among them are elements such as Cu, Co, Se, Zn, Mn, Mo, I and Fe that are essential for human health in certain concentrations. These elements have a beneficial effect on animals and humans, since they are important for normal functioning of metabolism, growth and development and any concentration discrepancies of these elements may cause disturbance in organisms [1], [2].

Concentration of micro and macro elements in milk is not constant, but is influenced by genetics of individual animal species, stage of lactation, farm management (nutritional regime) and environment factors such as season, locality of the farm, nature of soil, fertilizer application and industrial activities [3]. Industrial and other anthropogenic activities could also influence the distribution and levels of elements, mainly toxic elements such as Cd, Ni, Co, Pb, Cr, Hg in milk, since they are

accumulated through the food chain [1], [4], [5]. They can easily accumulate in plants grown on polluted soils and thus cause toxic effects in cattle and consequently also in humans who consume "contaminated" milk [4], [6]. For this reason, it is necessary to control the concentration of micro and macro elements/toxic elements in consumed food.

Further, multi-element analysis has been successfully used for the determination of the geographical origin of different foodstuff such as wine [7], [8] onion [9]-[11], tea [12] and tomatoes [13]. In relation to milk and dairy products, most of the studies have been performed on cheese for descriptive purposes [14] and for origin authentication purposes [15]. Sacco et al. [16] used multi-elemental analysis in combination with stable isotope analysis for differentiation between Southern Italy and foreign milk. Trace elements in milk and dairy product have been used to investigate and to provide information about correlation between animals fodder, time of year, elemental conditions. It was found that season has more influence on mineral concentration than regions [15].

Several different techniques are available for simultaneous multi-element chemical analysis including: energy dispersion X-ray fluorescence spectrometry (EDXRF), neutron activation (k_0-instrumental nuclear activation analysis; k_0-INAA), inductively coupled plasma atomic emission spectroscopy (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) [1]. Each of these techniques has the capacity to provide a chemical profile or chemical fingerprinting, which can be used to characterize the material in question and to compare it with other samples or reference materials. Among inductively coupled plasma systems ICP-AES and ICP-MS have been widely used for the analysis of minor and trace elements in food, since they provide satisfactory result and allow performing simultaneous-sequential determination of multiple elements. The advantages of these techniques are short time of analysis and low limit of detection [4], [17]. On the other hand, EDXRF as a highly sensitive, fast and cheap technique offers the possibility of performing direct multi-element analysis of solid samples over a wide dynamic range. This technique is especially useful in food authentication and traceability studies where there is a requirement for a database of genuine samples to which the sample can be compared to establish its authenticity. For effective data processing, the large number of independently measured parameters is needed including multi-elemental composition [18]-[20]. Another nondestructive multi-element technique is k_0-INAA. The main disadvantages of this method are long turn-around time, is labour intensive and a nuclear reactor or other source of activating particles is required. Nevertheless, providing a different approach, this method is often an important asset in studies regarding the analysis of standard reference materials [21]-[23] and as an additional technique in evaluation of the accuracy of analytical results [24].

Thus, the main objectives of this paper are: (1) to verify the methods for elemental composition in milk samples including EDXRF, k_0-INAA and ICP-MS; and (2) to differentiate the milk according to geographical region in Slovenia based on elemental composition. Sample preparation and the analysis procedure for each of the above mentioned analytical techniques are described and a comparison of analytical and other parameters such as uncertainty, accuracy, limits of detection (LOD), cost of analysis per sample, instrumental cost etc. is critically evaluated.

2. MATERIALS AND METHODS

2.1. Sampling

Samples of the whole milk were provided by four Slovenian diary producers: Ljubljanske mlekarne d.d., Pomurske mlekarne d.d., Mlekarna Planika d.o.o. and Mlekarna Celeia d.o.o. in December 2013 covering different geographical regions (Mediterranean, Pannonia, Dinaric and Alpine) in Slovenia. The samples were stored at -20°C before analysis. All together 40 samples were obtained for multi-elemental analysis.

2.2. Energy Dispersive X-ray Fluorescence Spectrometry (EDXRF)

Multielement determination of macro (P, S, Cl, K, Ca) and micro elemental (Zn, Br, Rb, Sr) content was nondestructively performed by Energy Dispersive X-ray Fluorescence Spectrometry (EDXRF). The analysis was performed on freeze-dried samples. The pellets were prepared from 0.5 to 1.0 g of powder sample material by a pellet die and hydraulic press. For excitation, the disc radioisotope excitation source of Fe-55 (25 mCi) and Cd-109 (20 mCi) from Eckert and Ziegler were used. The emitted fluorescence radiation was measured by en energy dispersive X-ray spectrometer constituted of a Si(Li) detector (Canaberra), a spectroscopy amplifier (Canberra M2024), ADC (Canberra M8075) and PC based MCA (S-100 Canberra). The spectrometer was equipped with a vacuum chamber (Fe-55) for the measurement of light elements P-Cl, while the energy resolution of the spectrometer was 175 eV at 5.9 keV.

The analysis of complex X-ray spectra was performed by the AXIL spectral analysis program [24], [25]. For the evaluated uncertainty of this procedure, it is required to include the statistical uncertainty of measured intensities and the uncertainty of the mathematical fitting procedure. For this purpose, quantification was then performed utilizing QAES (Quantitative Analysis of Environmental Samples) software, developed in our laboratory [24], [25]. The estimated uncertainty of the analysis was around 5 % to 10 %. Rather high total estimated uncertainty is mainly due to contributions of matrix correction and geometry calibration procedures, which include errors of tabulated fundamental parameters, and contributions of spectrum acquisition and analysis. In the case of EDXRF, the limits of detection calculated from the signal to background ratio [26] on the realistic sample NIST 1549 for P, S, Cl, K, Ca, Zn, Br, Rb and Sr were 400, 200, 100, 20, 15, 4, 2, 1, 1 μg/g of dry milk sample, respectively.

2.3. k_0-INAA measurements

For k_0-INAA measurements aliquot of the freeze-dry sample (0.10 to 0.20 g) was weighed into a polyethylene ampoule (SPRONK System, The Netherlands). For the determination of long-lived radionuclides the sample was irradiated together with the standard Al-Au (0.1 %) for 12 hours. Irradiation was carried out in a reactor TRIGA Mark II. After irradiation the sample was kept in a polyethylene ampoule and gamma activity of radionuclides induced in the sample after 4, 9-15, and 24-33 days of cooling was measured. All measurements were performed on HPGe detector (40 and 45 % yield). For the evaluation of gamma spectra we used the program HyperLab 2002, and element content was calculated with the program Kayzero for Windows. Limits of detection for Br, Ca, K, Rb, Zn calculated as three times the standard deviations of the blank sample, are 1, 500, 500, 5, 5 μg/g milk sample, respectively.

The uncertainty level for elemental determination by k_0-INAA was 6–10 %, in the case of lower elemental content such as Se and Mn the uncertainty is higher, around 20 %.

2.4. ICP-MS measurements

For multi-elemental analysis of milk samples with ICP-MS two procedures of sample preparation were used:

a) Microwave digestion: about 1 mL of milk sample (0.15 g of lyophilized sample) was weighed into quartz tubes. 1 mL 65 % HNO3, (Merck, Germany, suprapur) and 1 mL 30 % H_2O_2, (Merck, Germany, suprapur) were added and samples were subjected to closed vessel microwave digestion (Microwave system ETHOS 1, MILESTONE SN 130471) at max. power of 1500 W: ramp to 130°C 10 min, ramp to 200 °C 10 min, hold 20 min, cooling 20 min. Then the samples were equilibrated to room temperature. The solution was quantitatively transferred into 10 mL polyethylene graduated tubes and filled to the mark with Mili-Q water. Before determination by ICP-MS, samples were diluted five times. External calibration was made with ICP Multi Element Standard solution XVI CertiPUR (Merck).

b) Dilution (adapted from Barany [27]): An aliquot of 1 mL of milk sample (0.15 g of lyophilized sample) was diluted five times with alkaline (Merck, suprapur) solution containing Triton X-100 (Sigma Aldrich, sigmaultra) and ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, Fisher Scientific, analytical reagent grade). For calibration the standard addition procedure was utilised.

Measurements of prepared solutions were made by an Octapole Reaction System Inductively Coupled Plasma Mass Spectrometer (7500ce, Agilent) equipped with an ASX-510 Autosampler (Cetac). Instrumental conditions: Babington nebulizer, Scott-type spray chamber, spray chamber temperature 5 °C, plasma gas flow rate 15 L/min, carrier gas flow rate 0.8 L/min, make-up gas flow rate 0.1 L/min, sample solution uptake flow rate 1 mL/min, RF power 1500 W, reaction cell gas helium 4 mL/min, isotopes monitored ^{55}Mn, ^{56}Fe, ^{63}Cu, ^{66}Zn, ^{75}As, ^{78}Se, ^{111}Cd, ^{114}Cd, ^{206}Pb, ^{207}Pb, ^{208}Pb, isotopes monitored as internal standard added to all solutions: ^{45}Sc, ^{69}Ga, ^{89}Y, ^{157}Gd. Tuning of the instrument was made daily using a solution containing Li, Mg, Y, Ce, Tl and Co.

Concentrations of Cd, Pb and As in milk samples are usually very low <0.5 ng/g and thus, insufficient detection limits were obtained with microwave digestion. Therefore preparation with simple dilution of milk samples was introduced. Limits of detection for Cd, Pb, As, Se, Mn, Cu, Zn and Fe, calculated as three times the standard deviations of the blank sample, were 0.1, 0.2, 0.05, 2, 1, 6, 35 and 30 ng/g milk sample, respectively. Estimated combined uncertainty of results was 5 % for Cu, Zn, Fe, 10 % for Se, Mn and 15 % for As, Cd, Pb.

2.5. Quality control

The accuracy of results was checked as follows:

a) analysis of the certified reference materials: Whole milk powder NIST 8435, Non-fat Milk Powder NIST 1549 (both National Institute of Standard and Technology) and Skim Milk Powder BCR 150 (EC-JRC-IRMM), ERM-BD150 and ERM-BD151 (EC-JRC-IRMM);
b) comparison with independent methods: EDXRF and k_0-INAA;
c) for As there are no reference materials, therefore, comparison was made by an independent method radiochemical neutron activation analysis [28], [29]: result for internal quality control milk sample by k_0-RNAA 0.28 ± 0.02 ng/g and by ICP-MS 0.36 ± 0.05 ng/g;
d) participation in interlaboratory comparison scheme FAPAS (Food and Environmental Research Agency, Sand Hutton,York, VB).

2.6. Statistical evaluation

Statistical calculations and multivariate analysis were carried out using XLSTAT software package (Addinsoft, New York, USA) Basic statistics included mean values (median and arithmetic mean), standard deviation (S.D.), minimum and maximum. Multivariate analysis involved linear discriminant analysis (LDA).

3. RESULTS AND DISCUSSION

3.1. Results on certified reference materials

Results of the validation of the accuracy of multi-elemental analysis performed by ICP-MS with certified reference materials (NIST 1549, NIST 843) are collected in Table 1, while results of the validation of the accuracy with certified reference material ERM-BD 150 and ERM-BD 151 with all three methods are collected in Table 2.

Results indicate that the obtained data with different methods are in good agreement with certified reference materials. Precision was also found to be satisfactory with relative standard deviations (RSDs) always between 1 and 10 %. Only in four cases (of Se, As, Cd and Pb), for which the

Table 1. Validation of the accuracy of ICP-MS multi-elemental procedures proposed (NIST 1549, NIST 8435 and BCR 150).

	NIST 1549		NIST 8435		BCR 150	
Element	Certified value	Found Value	Certified value	Found value	Certified value	Found value
			dry weight mg/kg			
Mn	0.26 ± 0.06	0.25 ± 0.03	0.17 ± 0.05	0.19 ± 0.02	-	-
Fe	1.78 ± 0.10	1.71 ± 0.09	1.80 ± 1.10	2.0 ± 0.1	11.8 ± 0.6	9.79 ± 0.48
Cu	0.70 ± 0.10	0.67 ± 0.03	0.46 ± 0.10	0.40 ± 0.02	2.23 ± 0.08	1.95 ± 0.1
Zn	46.1 ± 2.2	39.3 ± 2.0	28.0 ± 3.1	23.8 ± 1.2	-	-
Se	0.11 ± 0.1	0.11 ± 0.1	0.131 ± 0.14	0.128 ± 0.01	-	-
Cd	0.0005 ± 0.0002	0.0005 ± 00001	-	-	0.0218 ± 0.0014	0.0203 ± 0.0031
Pb	0.019 ± 0.003	0.023 ± 0.004	-	-	1.00 ± 0.04	0.84 ± 0.13

Table 2. Determination of element concentrations in mg/kg in reference materials by ICP-MS, EDXRF and k_0-INAA (ERM-BD 150 and ERM-BD 151).

	ERM-BD 150				ERM-BD 151			
Element	Certified value	Found value by EDXRF	Found value by k_0-INAA	Found value by ICP-MS	Certified value	Found value by EDXRF	Found value by k_0-INAA	Found value by ICP-MS
Ca	13900 ± 800	13000 ± 1000	13487 ± 1013	-	13900 ± 700	13200 ± 1100	13309 ± 1018	-
Mg	1260 ± 100	-	1266 ± 117	-	1260 ± 70	-	1268 ± 112	-
Mn	0.289 ± 0.018	-	0.313 ± 0.101	-	0.29 ± 0.03	-	0.306 ± 0.072	-
P	11000 ± 600	9100 ± 900	-	-	11000 ± 600	9600 ± 900	-	-
K	17000 ± 700	16500 ± 1300	17665 ± 1263	-	17000 ± 800	16200 ± 1300	17447 ± 1246	-
Se	0.188 ± 0.014	-	0.201 ± 0.053	0.227 ± 0.022	0.19 ± 0.04	-	0.188 ± 0.046	0.224± 0.022
Na	4180 ± 190	-	4397 ± 313	-	4190 ± 230	-	4348 ± 308	-
Sr	-	3.71 ± 0.3	-	-	-	4.10 ± 0.3	-	-
Zn	44.8 ± 2.0	44.3 ± 3.5	46.7 ± 3.7	42.2 ± 2.1	44.9 ± 2.3	43.0 ± 3.4	46.1 ± 4.0	44.3 ± 3.2
Cu	1.08 ± 0.06	-	-	1.05 ± 0.05	5.00 ± 0.23	-	-	5.24 ±0.33
Cl	9700 ± 2000	9600 ± 900	10143 ± 743	-	9800 ± 1200	9000 ± 900	10077 ± 737	-
Cd	0.0114 ± 0.0029	-	-	0.0122 ± 0.002	0.106 ± 0.013	-	-	0.106 ±0.008
Pb	0.019 ± 0.004	-	-	0.019 ± 0.003*	0.207 ± 0.014	-	-	0.206 ± 0.02
S	-	3300 ± 300	-	-	-	3300 ± 300	-	-
Br	-	13.2 ± 1.0	-	-	-	13.6 ± 1.0	-	-
Rb	-	17.7 ± 1.4	-	-	-	17.0 ± 1.4	-	-

measured concentrations were very close to the detection limit, precision was worse and higher than 10 %.

3.2. Comparison of elemental composition between EDXRF and k_0-INAA methods

Comparison of results of milk samples obtained by EDXRF and k_0-INAA is presented in Table 3. It was found that only two samples exceeded 95 % confidence interval for Rb and Zn with lower data obtained by k_0-INAA (bold values in Table 3).

The possible reason for this discrepancy could be in the inhomogeneity of distribution of these two elements in the two aliquotes of the same sample analysed by EDXRF and k_0-INAA.

3.3. Results of the interlaboratory comparison scheme FAPAS

We participated in the interlaboratory comparison scheme FAPAS in 2012, 2013 and 2014 for milk samples in powdered form with all analytes present at low natural levels. Results are collected in Table 4. The results indicated good agreement between measured and assigned values. With these independent assessment competence of our laboratory to analyse low levels of As, Cd and Pb was demonstrated.

Common characteristic of all three analytical techniques applied EDXRF, k_0-INAA and ICP-MS is their multi-element capability. Preparation of samples was simple and nondestructive in the case of EDXRF and k_0-INAA, meanwhile ICP-MS required decomposition of samples. It is obvious that the most sensitive method applied in this study was ICP-MS with LODs in the range of few tens of ng/g.

The sensitivity of EDXRF and k_0-INAA is comparable to each other, according to obtained estimated uncertainty (5-10 %) and LODs for the analysed elements in the range from hundred to few µg/g. This means that LODs of ICP-MS were approximately two orders of magnitude lower compared to EDXRF and k_0-INAA. On the other hand, the determination of elements such as P, S, Cl by ICP-MS was impossible due to the fact that the ions formed by the ICP discharge are typically positive ions, $M+$ or M^{+2}, therefore, elements that prefer to form negative ions (such as Cl, I, F, etc.) are very difficult or impossible to determine by ICP-MS.

On the other hand, EDXRF enables analysis of very important macro elements (P, S, and Cl) in milk samples, meanwhile k_0-INAA as an alternative analytical tool allows the determination of only Cl. Determination of P was impossible due to the production of pure negatron emitter 32P after activation. In the case of S, poor activation of ^{37}S (short half life $t_{1/2}$ = 5.05 min) resulted in high LODs (3000-5000 µg/g). Since the S content found in milk was in the concentration range of 2000-3000 µg/g, it was evident that determination of S by k_0-INAA was omitted due to its insufficient sensitivity. The advantage of k0-INAA in relation to EDXRF is the determination of other important macronutrients Mg and Na. The determination of Mg and Na by EDXRF was impossible due to insufficient instrumental sensitivity in concentration range from one to few thousand µg/g found in real milk samples. Another disadvantage of the k_0-INAA and EDXRF techniques was their inability to perform the analysis of toxic elements such as As, Pb and Cd in the concentration range found in milk (Table 2).

Further, considering the cost per sample, its multi-elemental capability, simple non-destructive sample preparation, the minimum number of steps in the measurement and quantification procedure, EDXRF was undoubtedly the cheapest, simplest and most environmental friendly method among the applied analytical techniques and the most suitable for analysis of multi-elemental composition (P, S, Cl, K, Ca, Zn, Br, Rb, Sr) in milk samples. However, for the analysis of elemental content of Mn, Fe, Cu, Se and possible identification of pollutants such as As, Cd and Pb the ICP-MS method was found as a method of choice due to its excellent sensitivity and accuracy. These two methods were also used for the determination of multi-elemental content in authentic Slovenian raw cow milk.

3.4. Multi-elemental composition of Slovenian milk

The elemental content of Slovenian raw cow milk from different geographical regions collected in December 2013 is presented in Table 5. The elemental composition is as follows K > Ca > P, Cl > S > Zn > Rb > Br > Fe > Sr > Cu> Mn >

Table 3. Comparison between k_0-INAA and EDXRF methods for selected macro- and micro-elements in milk samples. Samples exceeding 95 % Confidence interval of comparison are marked bold.

Sample no.	k_0-INAA					EDXRF				
	Br	Ca	K	Rb	Zn	Br	Ca	K	Rb	Zn
	dry weight mg/kg									
1	14.0	7970	10969	12.1	28.8	13.9	8710	11200	13.2	30.4
2	11.4	8210	10595	22.1	18.9	11.7	9530	10600	23.3	21.5
3	9.04	7520	8706	6.85	23.3	8.87	7430	8210	7.98	25.2
4	7.71	8680	11800	12.6	31.7	7.85	9210	11600	14.0	33.3
5	7.77	8200	11110	10.3	29.2	8.38	8720	11600	8.85	30.9
6	8.32	8350	11300	12.9	28.5	6.97	8760	11200	14.2	29.9
7	8.10	7670	10390	11.9	27.3	8.38	8740	10600	12.5	28.7
8	7.59	8610	10880	11.5	26.7	8.27	10000	12100	12.9	29.8
9	8.34	8590	11140	16.6	26.6	7.74	9000	11300	19.3	29.6
10	11.4	8120	10980	9.33	29.2	11.4	8810	11300	8.3	30.6
11	8.22	8550	11332	14.0	29.0	9.74	8950	11900	15.0	31.8
12	14.6	8410	11170	12.5	28.6	13.2	9680	12600	11.9	29.6
13	9.12	8420	11230	20.3	27.7	7.93	9660	12400	20.3	29.8
14	16.0	6040	8595	11.1	23.0	15.9	7150	8610	11.4	23.0
15	10.9	7020	9898	**22.3**	**23.6**	14.3	6150	8070	**28.1**	**30.9**
16	14.1	9470	12251	14.1	29.8	14.0	9930	12600	13.3	30.1
17	16.6	9000	12635	22.8	31.2	17.4	9630	12900	23.6	29.6
18	9.44	6070	8113	**9.72**	**20.7**	12.5	6360	8320	**15.4**	**26.0**
19	13.7	7340	10140	15.7	25.6	13.8	8700	10700	16.5	26.1
20	27.5	7660	10332	14.8	23.2	28.2	8510	10900	14.9	26.1

Se > Pb > As > Cd and is consistent with literature data. The content of the elements, such as Cd, Cu, Fe, Mn, Ni, Pb, Sr and Zn is influenced by feed and environmental conditions [30]. The concentrations of toxic elements such as As, Cd and Pb, are low and do not represent a threat to human health.

Multi-elemental composition of milk was used for possible discrimination among four Slovenian geographical regions. Results of statistical evaluation by LDA are presented in Figure 1. Partial separation between groups was obtained, where Alpine and Pannonian groups were well separated, while Dinaric and Mediterranean groups were overlapping. The later two groups are close to each other and thus insufficiently separation was obtained; however their discrimination tendency is promising. The most important factors affecting the geographical origin were Ca, S, P, K, and Cl. In a cross validation test 66.7 % of the samples are classified correctly. The highest rate of classification was for the Alpine and Dinaric samples (78.6 % and 71.4 %, respectively). It is expected that more precise and efficient separation between four geographical regions could be obtained by the involvement of more analysed parameters such as stable isotope values of milk samples.

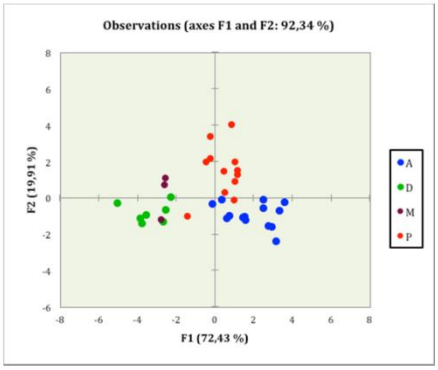


Figure 1. Discrimination between geographical regions using significant elemental parameters. Function 1 represents 72.43 % of variability, while function 2 represents 19.91 % of variability.
Label: A-Alpine, D-Dinaric, M- Mediterranean, P – Pannonian.

Table 4. Results of the metallic contaminants in milk powder in interlaboratory schemes FAPAS in 2012, 2013 and 2014.

Element	2012: FAPAS 07172		2013: FAPAS 07190		2014: FAPAS	
	Assigned value	Found value	Assigned value	Found value	Assigned value	Found value
	µg/kg					
As	56.4 ± 12.4	45.2 ± 6.1	49.1 ± 10.8	53.5 ± 6.0	58.1 ± 12.8	53.8 ± 1.6
Cd	18.59 ± 4.09	19.9 ± 5.8	16.2 ± 3.56	15.8 ± 1.0	12.2 ± 2.69	12.5 ± 0.8
Pb	66.17 ± 14.6	51.8 ± 3.3	50.8 ± 11.2	47.8 ± 3.4	47.5 ± 10.5	49.3 ± 3.0

Table 5. The summary of elemental data in Slovenian raw cow milk collected in December 2013.

Elements	Mediterranean			Pannonia			Alpine			Dinaric		
Ca (mg/100 g)	110	±	14	113	±	11	113	±	15	111	±	14
K (mg/100 g)	128	±	20	145	±	14	146	±	20	144	±	18
Cl (mg/100 g)	74	±	17	84	±	9	89	±	10	91	±	13
S (mg/100 g)	24	±	3	27	±	4	26	±	5	28	±	5
P (mg/100 g)	62	±	12	77	±	10	78	±	13	76	±	12
Na (mg/100 g)	33	±	4	35	±	2	35	±	3	33	±	7
Zn (mg/100 g)	329	±	57	392	±	28	374	±	34	368	±	34
Br (mg/100 g)	147	±	32	106	±	14	139	±	40	197	±	71
Rb (mg/100 g)	190	±	100	196	±	67	210	±	63	215	±	40
Sr (mg/100 g)	27	±	6	29	±	5	30	±	15	20	±	7
Fe (mg/100 g)	33	±	3	25	±	6	28	±	5	29	±	5
Ni (mg/100 g)	4.5	±	0.7	4.6	±	1.2	4.5	±	1.0	5.6	±	1.1
Mo (mg/100 g)	7.4	±	1.2	7.5	±	2.5	8.7	±	1.8	8.8	±	2.2
Mn (mg/100 g)	3.9	±	0.9	2.8	±	1.1	2.4	±	0.8	2.9	±	0.8
Cu (mg/100 g)	7.0	±	3.4	4.6	±	1.2	4.4	±	1.4	5.3	±	1.5
Se (mg/100 g)	2.0	±	0.2	1.6	±	0.4	1.6	±	0.5	2.0	±	0.8
As (mg/100 g)	0.043	±	0.004	0.042	±	0.017	0.043	±	0.015	0.057	±	23
Cd (mg/100 g)	0.006	±	0.004	0.005	±	0.003	0.004	±	0.002	0.007	±	0.004
Pb mg/100 g)	0.076	±	0.051	0.055	±	0.035	0.076	±	0.057	0.064	±	0.027

4. CONCLUSIONS

There is a need for reliable milk element concentration data to provide information about nutritional uptake and at the same time to provide information about the excess amounts of trace and toxic elements. In addition, multi-elemental composition provides important information on the authenticity and geographical origin of food including milk and dairy products. This paper provides some interesting comparisons between three different techniques (EDXRF, k_0-INAA and ICP-MS) in determination of multi-elemental composition in milk samples. Quality assurance proved entirely satisfactory for all involved measurements and an intercomparison exercise between EDXRF and k_0-INAA showed satisfactory agreement.

The simple, fast, and inexpensive EDXRF method in combination with ICP-MS, which was found the most appropriate technique for the analysis of elemental content of Mn, Fe, Cu, Se and toxic elements such as As, Cd and Pb, were further used for multi-elemental analysis of Slovenian raw cow milk samples. Multi-elemental content in milk samples was combined further in a linear discriminant model to discriminate milk according to geographical origin. Only partial separation was possible using elemental content with overall predicting ability of 66.7 %. It is expected that if elemental data are used in conjunction with other characteristic chemical indices such as isotope analysis, a more holistic and accurate picture in relation to geographical region separation could be created. These data represent a basis for a database of authentic samples of milk in Slovenia, which could be incorporated into a traceability system.

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3.2 Scientific Paper: "Lessons Learned from Inter-Laboratory Studies of Carbon Isotope Analysis of Honey"

This chapter describes the work published in the paper entitled "*Lessons learned from inter-laboratory studies of carbon isotope analysis of honey*" by Philip J.H. Dunn, Sarah Hill, Simon Cowen, Heidi Goenaga-Infante, Mike Sargent, Ahmet Ceyhan Gören, Mine Bilsel, Adnan Şimşek, Nives Ogrinc, Doris Potočnik, Paul Armishaw, Lu Hai, Leonid Konopelko, Yan Chubchenko, Lesley A. Chesson, Gerard van der Peijl, Cornelia Blaga, Robert Posey, Federica Camin, Anatoly Chernyshev, and Sadia A. Chowdhury. The paper was published in *Science & Justice*, 2019.

Stable carbon isotope ratio measurements are used to differentiate plants utilizing different photosynthetic pathways, knowledge of which can be used to develop methods capable of detecting the adulteration of honey with high fructose corn syrup and other C_4 plant sugars. Such methods, however, must meet specific performance criteria, particularly regarding measurement uncertainty and traceability. Inter-laboratory exercises are, therefore, an invaluable tool for assessing the measurement capabilities of laboratories. Comparability of results between laboratories at the highest metrological level is one of the fundamental aims of an interlaboratory comparison. In the present study, national and designated metrology institutes from five different countries participated in an inter-laboratory comparison study (CCQM-K140) to determine stable carbon isotope ratios in bulk honey. The same samples were sent to forensic isotope analysis laboratories, contacted via the Forensic Isotope Ratio Mass Spectrometry (FIRMS) Network that could not participate directly in the metrological comparison. This comparison, among others, includes a discussion of the application of various performance metrics, measurement and data handling methods employed by all of the participants and the analysis of the data. The results from this study demonstrated that the majority of participants ensured the traceability of their results, and obtained measurement uncertainties were acceptable. Several forensic laboratories only reported precision rather than full estimates of measurement uncertainty, which is also a common practice outside metrology institutes. However, there was good agreement between metrology institutes and forensic laboratories in term of carbon isotope ratio data. The maximum difference in $\delta^{13}C_{\text{VPDB-LSVEC}}$ value for the bulk honey reported was 0.37‰, while the expanded measurement uncertainty (k = 2) reported was 0.28‰, which was >2.5 times the average standard deviation reported by the FIRMS participants.

In this paper, I performed stable carbon isotope measurements of honey and estimated the measurement uncertainty for bulk honey. The measurement and data handling experience and analysis acquired while performing the EA-IRMS analyses, during this exercise was replicated during my PhD work.

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Lessons learned from inter-laboratory studies of carbon isotope analysis of honey

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ABSTRACT

Forensic application of carbon isotope ratio measurements of honey and honey protein to investigate the degree of adulteration with high fructose corn syrup or other C_4 plant sugars is well established. These measurements must use methods that exhibit suitable performance criteria, particularly with regard to measurement uncertainty and traceability – low levels of adulteration can only be detected by methods that result in suitably small measurement uncertainties such that differences of 1‰ or less can be reliably detected. Inter-laboratory exercises are invaluable to assess the state-of-the art of measurement capabilities of laboratories necessary to achieve such performance criteria. National and designated metrology institutes from a number of countries recently participated in an inter-laboratory assessment (CCQM-K140) of stable carbon isotope ratio determination of bulk honey. The same sample material was distributed to a number of forensic isotope analysis laboratories that could not participate directly in the metrological comparison. The results from these studies have demonstrated that the majority of participants provided isotope delta values with acceptable performance metrics; that all participants ensured traceability of their results; and that where measurement uncertainties were reported; these were fit-for-purpose. A number of the forensic laboratories only reported precision rather than full estimates of measurement uncertainty and this was the major cause of the few instances of questionable performance metrics. Reporting of standard deviations in place of measurement uncertainties is common practice outside metrology institutes and the implications for interpretations of small differences in isotopic compositions are discussed. The results have also highlighted a number of considerations that are useful for organisers of similar inter-laboratory studies in the future.

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1. Introduction

The determination of stable carbon isotope ratios as isotope delta values ($\delta^{13}C$ values) has many applications within forensic sciences including source inference (e.g. [1]), detection of food adulteration and authenticity (e.g. [2]) and distinguishing materials that are otherwise chemically and physically identical (e.g. [3]). Forensic applications of isotope delta values require analyses that meet the needs of stakeholders such as the police, courts and regulators and it is therefore essential that measurement results are compatible regardless of which laboratory provides them. Carbon isotope ratio testing of honey is usually performed to detect the addition of high-fructose corn syrup or other C_4 plant sugars within C_3 plant-based honey. AOAC has a standard method for this test which depends on the determination of the carbon isotope ratio of the bulk honey as well as of the extracted honey protein to estimate the amount of adulteration using the following equation [4]:

$$\%\ C_4\ Sugar = \frac{\delta^{13}C_{Protein} - \delta^{13}C_{Honey}}{\delta^{13}C_{Protein} - (-9.7)} \times 100 \quad (1)$$

Most unadulterated honeys will yield ≤7% C_4 sugars [4] and as the $\delta^{13}C_{VPDB\text{-}LSVEC}$ difference between the honey and protein increases, so too does the calculated degree of adulteration. The application of this method requires adherence to the common method protocol, isotope ratio analyses that afford traceability to the internationally agreed carbon isotope delta scale to ensure compatibility of results and that provide adequate measurement uncertainty to detect low levels of adulteration.

Achieving compatibility of results between laboratories at the highest metrological level is one of the fundamental aims of the interlaboratory comparisons (ILCs) organised by the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM). The Inorganic Analysis Working Group (IAWG) of the CCQM has previously organised a number of ILCs of isotope ratio measurements for the national and designated metrology institutes (NMIs and DIs) which participate in its activities. The aim was to demonstrate compatibility of their results, including investigating light element isotope delta values of methionine, strontium isotope ratios in wine and lead isotope ratios in bronze [5].

Under most circumstances metrology institutes aim to obtain measurement data traceable to the SI. However, the requirement in forensic and other applications to distinguish very small variations in isotope ratios of elements such as carbon is presently achievable only using isotope delta values. The IAWG participants were, therefore, granted an exception to use delta values with traceability to the reference materials that define the position of the zero point until a scale with SI-traceability can be established at the required level of uncertainty [6]. There were a number of incentives for conducting a new comparison on isotope delta values: firstly, the need for IAWG members to publicly demonstrate their measurement capabilities; secondly, to assess the compatibility of metrology institute results reported using an isotope delta scale; and finally it had been almost a decade since light element isotope ratio measurements were the subject of a CCQM comparison. Two metrology institutes, LGC in the UK and Scientific and the Technological Research Council of Turkey's National Metrology Institute (TÜBİTAK UME), therefore recently coordinated an ILC of the stable carbon isotope analysis of bulk honey (CCQM-K140) [7]. This CCQM comparison highlighted that there was good agreement in obtained $\delta^{13}C$ values between the participants despite the significant differences in methods applied, particularly in regard to the data reduction and measurement uncertainty estimation approaches [7].

CCQM comparisons demonstrating compatibility between participants underpin the measurement services offered by metrology institutes (e.g. production and certification of reference materials and provision of reference values). On the other hand, field laboratories applying isotope ratio analysis to forensic investigations/questions generally only provide a commercial analytical service and simply report analytical results to their stakeholders. As forensic laboratories address different needs and use different approaches, the comparison of data obtained by metrological institutes to the forensic stable isotope community is useful. The results of such comparisons demonstrate the extent to which metrological principles are adopted outside of metrology institutes and more importantly should encourage the forensic laboratories to appreciate the benefits of a metrological approach. There are also implications from measurement uncertainty for forensic application of carbon isotope ratio measurements of honey as well as isotope ratios in other materials.

In this work, we briefly review the results from the CCQM comparison study and compare them to those obtained by a number of forensic stable isotope laboratories contacted via the Forensic Isotope Ratio Mass Spectrometry (FIRMS) Network that analysed the same honey material. This comparison includes discussions of the relative merits of the application of various performance metrics, measurement and data handling methods employed by all participants, comparison of the reported results, discussion of the performance of the forensic laboratories and implications for forensic analysis of honey.

2. Materials and methods

2.1. Participation

The participants within the studies described in this work are those listed under the author affiliations. No indication is given as to which participants participated in which of the two aspects (the CCQM comparison and the FIRMS study) to preserve anonymity. Note that Analytica Laboratories reported two separate results due to having used two different measurement approaches and these two results are listed separately in results tables, figures and within the text (hence the total number of results is thirteen while there are only twelve institutions). Also, for administrative reasons, one of the CCQM laboratories (CCQM Laboratory A) participated in a separate study conducted in parallel with CCQM-K140 and its data were not included in the CCQM-K140 report (but are described herein). Studies were coordinated by LGC and TÜBİTAK UME.

2.2. Honey material

Each participant within these studies (CCQM and FIRMS) received at least one amber borosilicate vial containing 2 g of honey. This was the same material that is now commercially available from the TÜBİTAK UME as "UME CRM 1312 – Honey (Unadulterated)." Prior to use in these studies, TÜBİTAK UME had investigated the homogeneity of the units of honey as well as performing experiments to determine the short and long-term stability of the honey material. The results of these preliminary studies can be found in the published final report for the CCQM study [7] as well as on the certificate for UME CRM 1312 [8].

2.3. Participants' methods

For CCQM participants, other than CCQM Laboratory A, full details of participant sample handling, instrumental and data handling methods can be found in the published final report [7]; however a brief summary is presented in Table 1. CCQM Laboratory A and participants within the FIRMS parallel study were also free to use whatever analytical measurement method they chose, with the proviso that full method details including source of traceability to the VPDB-LSVEC scale were reported. This differs from the AOAC method where the protocol is specified. Participants were required to report the $\delta^{13}C_{VPDB\text{-}LSVEC}$ of the honey together with an indication of measurement uncertainty. For the FIRMS laboratories where measurement uncertainty estimation might not be a common practice, the standard deviation of

Table 1
Sample preparation, instrumental and data handling information for the twelve laboratories participating in this study (one participant used two different techniques and therefore reported two results).

Laboratory	Principle	Mass of honey analysed	Corrections						Normalisation		Quality control material(s)
			^{17}O	Blank	Linearity	Drift	Memory	Other	Type	RMs Used	
		µg									
FIRMS 1	EA-IRMS	120 ± 50	SSH	n	y (Sharp)	n	n	n	Two point	IAEA-CH-7 USGS24	In-house tape
FIRMS 2	EA-IRMS	1100 to 1200	SSH	n (blank below detection limit)	n (mass controlled)	y (using QC material)	n	n	Two point	USGS40 USGS41	Cane sugar
FIRMS 3	CM-CRDS	1100 to 1200	n/a	n (blank below detection limit)	n (mass controlled)	y (using QC material)	n	n	Two point	USGS40 USGS41	Cane sugar
FIRMS 4	EA-IRMS	1200 ± 120	SSH	n (none observed)	n (mass controlled)	n (none observed)	n (none observed)	n	Two point	USGS40 USGS41	In-house glutamic acid
FIRMS 5	EA-IRMS	500 to 1000	Craig	n	y (no detail)	n	n	n	Multiple point	NBS-22 IAEA-CH-6 LSVEC	None reported
FIRMS 6	EA-IRMS	1500 to 2500	SSH	n	y (no detail)	n	n	n	Two point	Two in-house glucoses	IAEA-CH-7
FIRMS 7	EA-IRMS	3000 ± 300	Craig	y (automatic)	n (none observed)	y (spl/std. bracketing)	n	n	Two point	In-house flour and sucrose	NBS-22 IAEA-CH-6
CCQM 1	EA-IRMS	103 ± 66[a]	IUPAC	y (mass balance)	n (mass controlled)	n (none observed)	n (none observed)	n	Multiple point	NBS-22 USGS40 IAEA-CH-6 USGS41	FIRMS Olive Oil FIRMS Chitin In-house Glycine USGS 42
CCQM 2	EA-IRMS	200 ± 10	SSH	y (mass balance)	n (mass controlled)	n (none observed)	n (none observed)	n	Two point	IAEA-CH-6 NBS-22	None reported
CCQM 3	EA-IRMS	2000 to 2500	Craig	n	n	y (using QC material)	n	n	Two point	IAEA-CH-6 IAEA-CH-7	None reported
CCQM 4	EA-IRMS	900 ± 100	SSH	n (none observed)	n (mass controlled)	y (spl/std. bracketing)	n (avoided)[c]	n	Two point	NBS-22 USGS24	USGS40
CCQM 5	EA-IRMS	600 to 1000	IUPAC	y (mass balance)	n (none observed)	y (using QC material)	n (none observed)	n	Multiple point	IAEA-CH-7 IAEA-CH-6 USGS40 IAEA-600	Two in-house honey materials
CCQM A	CM-CRDS	90,000 ± 9000[b]	n/a	n	n	y	n	n	Two point	IAEA-CO-8 IAEA-CH-7	None reported

[a] Standard deviation of masses analysed.
[b] Custom offline combustion used significantly larger amount of honey than the online processes used by other participants.
[c] Carryover from graphite into subsequent analyses was observed; however the affected analyses were discarded rather than a memory correction being applied.

the independent replicates was requested as an indication of method precision if the participants were inexperienced with estimation of measurement uncertainty. A brief description of the instrumental and data handling methods applied by these participants can also be found within Table 1.

2.4. Performance metrics

Within the CCQM study, the reference value was determined according to the method described within the published final report, i.e. the arithmetic mean of participant results (excluding CCQM Laboratory A), which was $\delta^{13}C_{VPDB\text{-}LSVEC} = -24.095 \pm 0.107‰$ (expanded uncertainty, $k = 2.776$) [7]. CCQM inter-comparison studies also report a degree of equivalence (DoE) which is a measure of metrological compatibility and it is expressed quantitatively by two terms: the deviation from the reference value and the uncertainty of this deviation (at a 95% level of confidence). The method used for calculation of DoEs will depend on the statistical method used to determine the reference value [9] and for CCQM-K140 details can be found in the final report [7].

Z-scores for participants were calculated as follows:

$$z = \frac{(x - ref)}{U_{ref}} \tag{2}$$

where x is the value reported by the participant in this study and *ref* and its associated expanded uncertainty, U_{ref}, (with $k = 2.776$) were the reference value taken from the CCQM study [7].

The participant results and reference values were also used to calculate ζ-scores which provide a means to examine the plausibility of participants' measurement uncertainty estimate. The ζ-scores were calculated as follows:

$$\zeta = \frac{(x - ref)}{\sqrt{(u_x)^2 + (u_{ref})^2}} \tag{3}$$

where x is the value reported by the participant, u_x is the standard uncertainty in the value as reported by the participant (or the reported standard deviation where the measurement uncertainty was not reported) and *ref* and its associated standard uncertainty, u_{ref}, (with $k = 1$) were the reference value taken from the CCQM study [7].

E_n numbers for the participant results were also calculated. These are very similar to the ζ-scores but use expanded rather than standard uncertainties for both the participant result and reference value:

$$E_n = \frac{(x - ref)}{\sqrt{(U_x)^2 + (U_{ref})^2}} \tag{4}$$

where x is the value reported by the participant, Ux is the expanded uncertainty in the value as reported by the participant (here a k factor of 2 has been applied to the standard deviations reported in place of measurement uncertainties by some participants) and ref and its associated expanded uncertainty, Uref, (with k = 2.776) were the reference value taken from the CCQM study [7].

Unless specifically stated, all measurement uncertainties within this manuscript are expanded uncertainties (U). These are standard uncertainties (u) multiplied by a k-factor (usually $k = 2$ and so $U = 2u$) so that the resulting uncertainty range has a 95% confidence level of including the true value.

3. Results

3.1. Instrumental and data handling methods

3.1.1. Instrumental approach

The majority of participants across all three studies employed elemental analyser-isotope ratio mass spectrometry (EA-IRMS) to determine the $\delta^{13}C_{VPDB\text{-}LSVEC}$ value of the bulk honey. Only two participants, FIRMS Laboratory 3 and CCQM Laboratory A, did not use EA-IRMS – both used a combustion module either directly coupled to cavity ring down spectroscopy instrumentation (CRDS, a form of isotope ratio infrared spectroscopy) or using an offline sample preparation and transfer.

3.1.2. ^{17}O correction

For EA-IRMS measurements of carbon isotope ratios of CO_2 gas, the isotopic composition of the oxygen must be taken into account by application of a so-called ^{17}O correction. These corrections use one of three different sets of algorithms and are typically carried out automatically within instrumental software packages but can also be applied offline. The Craig correction [10] is the simplest to apply but makes assumptions regarding oxygen isotopic fractionation which are not supported by experimental evidence. CCQM Laboratory 3 and FIRMS Laboratories 5 and 7 used the Craig correction approach for ^{17}O. The improved algorithm suggested by Santrock, Studley and Hayes (SSH) [11] is available within some IRMS software packages. This is considered an exact approach but requires an iteration procedure to determine the $^{18}O/^{16}O$ ratio in the sample CO_2. Six participants, CCQM Laboratories 2 and 4 as well as FIRMS Laboratories 1, 2, 4 and 6, applied the SSH algorithm to correct for ^{17}O. The final approach, which is endorsed by the Commission on Isotopic Abundances and Atomic Weights (CIAAW) of the International Union of Pure and Applied Chemistry (IUPAC), is a linear approximation rather than an exact solution and also uses more up-to-date values for the absolute isotope ratios of VPDB than the SSH approach [12]. Only two participants, CCQM Laboratories 1 and 5 used the CIAAW-recommended approach.

Provided that raw $\delta^{13}C$ values of samples and the reference materials (RMs) used for scale normalisation are measured against the same working reference, and that the same ^{17}O correction is applied to all materials within an analytical sequence, then the bias in scale-calibrated $\delta^{13}C_{VPDB\text{-}LSVEC}$ values introduced via the choice of ^{17}O correction approach will be $< 0.001‰$.

3.1.3. Blank correction

Three of the CCQM participants (CCQM Laboratories 1, 2 and 5) carried out a blank correction using the mass balance approach described in the FIRMS Good Practice Guide for IRMS [13]. The other two did not report the need to carry out a correction for the blank contribution. CCQM Laboratory A did not carry out a blank correction as a result of using an offline combustion process and a significantly larger amount of honey material. Only one FIRMS laboratory, FIRMS Laboratory 7, found a blank level that required correction and therefore carried out a blank correction of their raw δ values using an automated procedure within the instrumental software. The remaining FIRMS laboratories found negligible blank contributions and therefore deemed a blank correction unnecessary.

The effect of blank correction on measured isotope delta values will depend on the difference in peak size between sample and blank signals and also upon the difference in isotopic composition. Where the blank is significantly smaller than the same signal (i.e. by a factor of 100 or more), then the difference between the measured isotope delta value before and after application of a blank correction will be minimal, as too will be the contribution of blank to the measurement uncertainty.

3.1.4. Linearity correction

All participating laboratories controlled the mass of honey analysed to some degree, with some having very low tolerances while others having much wider ranges of acceptable weight (Table 1). Within the CCQM study, no participants reported application of a correction to measured data to account for variation in sample mass analysed. FIRMS Laboratories 2, 3, 4 and 7 deemed a linearity correction was unnecessary within the ranges of mass of honey analysed. FIRMS Laboratory 1 applied a linearity correction as described by Sharp [14] while FIRMS Laboratories 5 and 6 applied a linearity correction based on the change in delta value of repeated injections of working gas with

Table 2
Reported $\delta^{13}C_{VPDB\text{-}LSVEC}$ values, standard uncertainties or standard deviations, expanded uncertainties and performance metrics for each participating laboratory together with the reference value from CCQM-K140 [7]. Questionable performance metric scores are highlighted in bold text; there were no unsatisfactory performance metric scores in these studies.

Laboratory	n	δ13CVPDB-LSVEC	u	U (k = 2)	z-score	ζ-score	En Number
		‰	‰	‰			
FIRMS 1	5	−24.25	0.03*		−1.45	**−3.17**	**−1.26**
FIRMS 2	5	−24.137	0.038*		−0.39	−0.78	−0.32
FIRMS 3	5	−24.117	0.153*		−0.21	−0.14	−0.07
FIRMS 4	5	−24.089	0.021*		0.06	0.14	0.05
FIRMS 5	11	−24.08	0.15	0.30	0.14	0.10	0.05
FIRMS 6	5	−23.93	0.2*		1.54	0.81	0.40
FIRMS 7	7	−23.88	0.08*		**2.01**	**2.42**	**1.12**
CCQM 1		−24.20	0.09	0.18	−0.98	−1.07	−0.50
CCQM 2		−24.146	0.139	0.278	−0.48	−0.35	−0.17
CCQM 3		−24.09	0.05	0.11	−0.05	0.08	0.03
CCQM 4		−24.03	0.05	0.11	0.61	1.03	0.42
CCQM 5		−23.993	0.042	0.084	0.95	1.79	0.75
CCQM A		−24.20	0.45	0.90	−0.98	−0.23	−0.12
Reference value		−24.095 ± 0.107‰ (expanded uncertainty, $k = 2$)					

* This is a standard deviation (Section 4.3).

varying intensity.

IRMS instrument manufacturers typically specify control limits for change in isotope delta with sample signal. These might be for example 0.05‰ nA^{-1} and therefore if variation in signal amplitude is > 1 nA, there will be > 0.05‰ variability in measured isotope delta value resulting from the linearity effect, which will be reflected in the standard deviation of replicate analyses. The degree to which a correction for linearity contributes to measurement uncertainty will depend on the magnitude of the effect being corrected as well as the mathematical approach employed.

3.1.5. Drift correction

CCQM Laboratories 1 and 2 as well as FIRMS Laboratories 1, 4, 5 and 6 did not find any evidence of drift within their analytical sequences and therefore did not apply a drift correction. CCQM Laboratories 3 and 5 as well as FIRMS Laboratories 2 and 3 applied a drift correction based upon the analysis of QC materials dispersed throughout their analytical sequences while CCQM Laboratories 4 and 6 and FIRMS Laboratory 7 employed a sample-standard bracketing technique within each sequence to account for instrumental drift.

The use of QC materials to correct for drift within a sequence of analyses will result in additional contributions to measurement uncertainty for each sample result from the QC results. If the drift correction also shifts results such that the QC result post-correction matches the expected result (e.g. after [15]), then there will also be an uncertainty contribution from the expected value for the QC material. The magnitude of these contributions will depend on the extent of observed drift and the correction algorithm applied.

3.1.6. Normalisation

All participants applied normalisation using at least two RMs as recommended by the CIAAW [16] thereby providing adequate traceability to the reporting scale. While all participants within the CCQM study used secondary reference materials directly for normalisation, this was only the case with five of the seven FIRMS laboratories. These secondary materials included USGS24, USGS40, USGS41, NBS 22, IAEA-CH-6, LSVEC, IAEA-CH-7, IAEA-600 and IAEA-CO-8 and the assigned values were taken from the relevant IUPAC Technical Report [17]. The remaining participants (FIRMS Laboratories 6 and 7) used in-house RMs that had been previously calibrated against primary and/or secondary reference materials, resulting in a slightly longer traceability chain. Both of these laboratories did include secondary reference materials amongst their quality control materials. Only one participant across both studies used exactly matrix-matched QC materials, i.e. honey (CCQM Laboratory 5).

The contribution to measurement uncertainty resulting from normalisation of results to the reporting scale will include not only the uncertainty of the known values of the RMs (for those listed above the standard uncertainties are 0.05‰ or less), but also the uncertainty related to the analysis of those RMs as well as the sample. The choice of RMs, both in terms of calibration range that they afford but also in their associated measurement uncertainty, will impact the uncertainty in normalized isotope delta values obtained. These issues have been discussed in previous publications [e.g. 18–21]. It is also important to consider the nature of the RMs used for normalisation of results. When analysing organic materials it is preferable to use organic RMs, however provided that quantitative conversion of the RM to the analyte gas can be demonstrated and that the same is also true for samples analysed using the same instrumental method, then any RM can be used. For many materials, proving that conversion to the analyte gas is complete is difficult and therefore the use of matrix-matched RMs is critical.

3.1.7. Estimation of measurement uncertainty

Details of the approaches used by CCQM Laboratories 1–5 to estimate their measurement uncertainties can be found in the final report for CCQM-K140 [7]. CCQM Laboratory A combined the uncertainty components arising from measurements (type A), from sample preparation (type B), from the expected $\delta^{13}C_{VPDB\text{-}LSVEC}$ value of one RM and from their normalisation approach. CCQM Laboratory A was relatively lacking in experience with the instrumentation used and hence why they participated in a parallel study (results from which were excluded during calculation of the reference value) and it is unsurprising that their estimation of measurement uncertainty turned out to be very conservative. FIRMS Laboratory 5 combined an estimate of within-laboratory reproducibility obtained from QC data with the uncertainty component arising from method and laboratory bias, which was estimated from proficiency testing (PT) data. Both of these participants combined the individual uncertainty components using the square root sum of squares approach.

Other FIRMS participants explicitly reported standard deviations of replicate analyses rather than measurement uncertainties.

3.2. Participant results and performance metrics

The $\delta^{13}C_{VPDB\text{-}LSVEC}$ values reported by the participants can be found within Table 2 and Fig. 1, together with the reference value from the CCQM study for comparison. The calculated performance metrics can also be found in Table 2. All participants within the FIRMS parallel

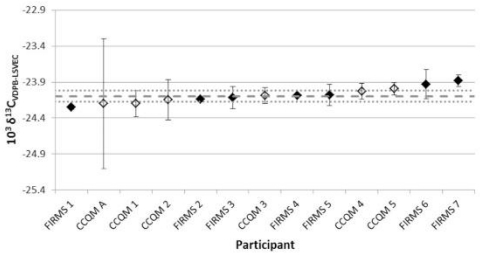


Fig. 1. Reported $\delta^{13}C_{VPDB\text{-}LSVEC}$ values for the honey for all participants within the CCQM study (open diamonds) and FIRMS parallel study (filled diamonds). The error bars represent the reported standard deviations of replicate analyses for the majority of FIRMS laboratories, but expanded uncertainties ($k = 2$) for FIRMS Laboratory 5 and the CCQM laboratories. The dashed and dotted grey lines are the reference value plus or minus its expanded uncertainty ($k = 2$) [7].

study reported the standard deviation of replicate analyses as an indication of measurement uncertainty with the exception of FIRMS Laboratory 5, which estimated a standard uncertainty (Section 3.1.7).

Z-scores within the range $-2 < z < +2$ are satisfactory, within the ranges of $-3 < z < -2$ and $+2 < z < +3$ are questionable while those within the ranges $z < -3$ and $z > +3$ are unsatisfactory. Only one participant, FIRMS Laboratory 7, produced a questionable z-score (+2.01). No participants within the three studies reported results that were unsatisfactory (Table 2). As with z-scores, ζ-scores within the range $-2 < \zeta < +2$ are satisfactory, within the ranges of $-3 < \zeta < -2$ and $+2 < \zeta < +3$ are questionable while those within the ranges $\zeta < -3$ and $\zeta > +3$ are unsatisfactory. If the magnitude of the E_n number, $|E_n|$, is < 1, then the result is satisfactory, where $|E_n|$ is > 1, it is an indication of unsatisfactory performance. Only two participant results produced ζ-scores and E_n numbers that were questionable (FIRMS Laboratories 1 and 7, Table 2).

4. Discussion

4.1. Assessment of performance metrics

It is z-scores that are most commonly applied to provide information of performance during inter-laboratory studies. Indeed, the two commercial PT schemes available for light element isotope ratio analysis both rely on z-scores for the assessment of participant performance. These PT schemes are provided by LGC which organises an isotope ratio PT scheme in collaboration with the FIRMS Network that is accredited to ISO/IEC 17043:2010 [22] and by Eurofins Scientific which organises the Food analysis using Isotopic Techniques – Proficiency Testing Scheme (FIT-PTS) – focussed on official methods of analysis such as those from AOAC [23].

While z-scores are useful performance metrics, they do not take into account the measurement uncertainty reported by participating laboratories. It is therefore possible to report a value that results in a z-score outside of the satisfactory range of $-2 < z < +2$ but for which the measurement uncertainty encompasses part of - if not all of - the reference value range with 95% confidence. This is more likely when the z-score is calculated on the basis of a fixed uncertainty in the reference value than when a parameter such as the robust standard deviation between participants, which may vary between different rounds of the same PT scheme, is used. The use of ζ-scores and E_n numbers, which take into account the uncertainties reported by participants, are therefore beneficial in terms of performance assessment.

Both ζ-scores and E_n numbers increase as either the deviation from the reference value increases or as the uncertainty reported by the participant decreases; therefore larger ζ-scores and E_n numbers can indicate a large bias, an underestimated uncertainty, or a combination of both [24]. These metrics therefore provide avenues for further investigation should poor results be obtained by individual participants. This is not the case for z-scores, where no indication as to the reason for poor performance is given.

While there was one laboratory with questionable z-score (FIRMS Laboratory 7), there were two (FIRMS Laboratories 1 and 7) with questionable ζ-scores and unacceptable E_n number. Naturally, whichever performance metrics are employed to investigate performance within PT schemes or other ILC exercises, there will be subtly different results, particularly if there are laboratories whose results fall close to the thresholds of acceptability for particular metrics.

4.2. CCQM study overview

The published report for the CCQM study [7] contains a detailed discussion of the participant results and therefore only a brief summary is included here. The most significant findings were the lack of agreement in data handling including corrections applied to raw data, specific RMs used for normalisation of results and measurement uncertainty estimation approach. Nevertheless good agreement was observed between all participants with none reporting results leading to questionable performance metrics. Given that the reference value is derived from the CCQM participants (excluding CCQM Laboratory A), this is not altogether surprising.

Measurement uncertainty budgets reported by the metrology institutes were particularly interesting given the large differences in contributions to uncertainty resulting from some parameters. For example, the contribution of uncertainty in the assigned values of the RMs used during scale realisation ranged from approximately 10% to just over 60%. This lack of agreement is perhaps the result of the difficulty in estimating the measurement uncertainty for isotope delta values where correlation between the various input parameters can be difficult to account for coupled to the variety of approaches available for uncertainty estimation. The expanded uncertainties (with $k = 2$) reported by the CCQM-K140 participants were large in comparison to the overall standard deviation of the reported values. The reference value was determined as the arithmetic mean with the value: $\delta^{13}C_{VPDB\text{-}LSVEC} = -24.095 \pm 0.107$‰ (expanded uncertainty, $k = 2.776$, [7]).

4.3. Comparison of approaches between CCQM-K140 and FIRMS participants

There was little difference in instrumental approach between the metrology institutes and the FIRMS laboratories, with EA-IRMS being employed by the majority and two (or more) point scale realisation used by all participants. There were some differences in the corrections applied to raw data, for example only metrology institutes used the CIAAW-recommended ^{17}O correction, while only FIRMS laboratories employed a linearity correction to account for differences in sample mass between replicates. While only CCQM Laboratory 5 used exactly matrix-matched QC materials (in-house honey). The commercial availability of the test material used in these studies, UME CRM 1312 – Honey (Unadulterated) as well as UME CRM 1313 Honey (Adulterated) that was not investigated in these studies, should improve this situation in the future. Exactly matrix matched QC materials facilitate the monitoring of data handling procedures such as corrections applied to raw data and also provide evidence of complete conversion of honey samples to the analyte gas during online or offline combustion. The major difference was in the reporting of measurement uncertainty, with all but one FIRMS laboratory providing standard deviations of replicate analyses (i.e. a precision estimate) rather than an estimate of measurement uncertainty.

FIRMS Laboratory 5 was the only FIRMS participant to estimate a measurement uncertainty (0.30‰ with $k = 2$), which was larger than the uncertainties reported by CCQM Laboratories 1–5 although it was

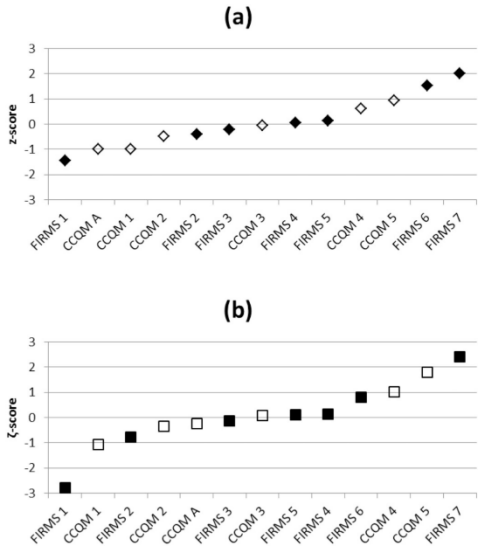


Fig. 2. Performance metrics for all laboratories: (a) z-scores for participants within the CCQM comparison (open diamonds) and the FIRMS parallel study (filled diamonds); (b) ζ-scores for participants within CCQM (open squares) and the FIRMS parallel study (filled squares).

smaller than the uncertainty reported by CCQM Laboratory A. This may be a reflection of the approach used by the FIRMS laboratory (assessing bias from PT results) which is different from the metrological approach. On the other hand, there was no single approach to estimating measurement uncertainty for carbon isotope delta values applied by the CCQM participants, for example, which parameters to include in the uncertainty budget and the relative contributions of these parameters to the uncertainty budget.

In contrast, of the standard deviations (from $n = 5$ to 7 independent replicates, Table 2) reported by FIRMS participants, those of FIRMS Laboratories 1, 2, 4 and 7 were all smaller than the lowest uncertainty reported in the CCQM study (0.084‰), while the standard deviations of FIRMS Laboratories 3 and 6 were within the range of uncertainties reported during the CCQM comparison (note that FIRMS Laboratory 3 was reporting the outcomes of CRDS measurements, which are known to produce approximately three times higher standard deviation than the IRMS-based methods [25]).

Despite these differences, comparison of the results submitted by the participants in the FIRMS study with those from CCQM laboratories shows that there is mostly good agreement between all reported values (Fig. 2), very few instances of questionable performance metrics and no unsatisfactory results (Table 2). The fitness-for-purpose of the reported results in terms of forensic honey analysis is discussed below in section 4.5.

4.4. Discussions of poor performance

Although the normalisation procedures and quality control materials employed by the laboratories that produced one or more questionable performance metrics (FIRMS Laboratories 1 and 7) were very similar to those that did not, there are several factors that might explain why poor performance was observed. Firstly, FIRMS Laboratory 1 used amongst the smallest amount material for analysis (120 ± 50 µg). Secondly, of the two participants that did not directly use secondary reference materials for normalisation (FIRMS Laboratories 6 and 7, Table 1) one produced questionable performance metrics. In addition, the reporting of precision estimates rather than measurement uncertainties by FIRMS participants is likely to be another explanation of the poor performance, particularly for the performance metrics where reported uncertainties are considered. Finally, there is the possibility that the reported results are biased. These factors are discussed in more detail within the following sections.

4.4.1. Sample mass and the "linearity" effect

Sample mass used for analysis certainly has the potential to contribute to variance between reported $\delta^{13}C_{VPDB\text{-}LSVEC}$ values. The so-called "linearity effect" for EA-IRMS analyses is well known and occurs when different isotope ratios for the same material are obtained depending on the mass of sample analysed. This can occur even when the material in question is isotopically homogeneous and could result in poor repeatability of results should there be significant differences in mass of the same analyte. Manifestation of the linearity effect can be avoided by constraining the sample mass range for replicate analyses of the same material or be corrected for via determination of magnitude of the effect using a working reference or quality control material analysed at different amount levels. Both of these approaches were applied by participants within these studies (section 4.1.4).

Fig. 3(a) shows that the CCQM participants' results do not appear to vary with mass of honey analysed. The regression line of best fit accounting for error in both variables by using Williamson's method [26,27] has a gradient of 0.040‰ mg^{-1} with a standard error of 0.052‰ mg^{-1} and is therefore indistinguishable from zero. The FIRMS results presented in Fig. 3(b) however show a larger residual bias with a slope of 0.129‰ mg^{-1} (standard error 0.020‰ mg^{-1}). It is difficult to untangle the effect of smaller ranges in the participant reported delta

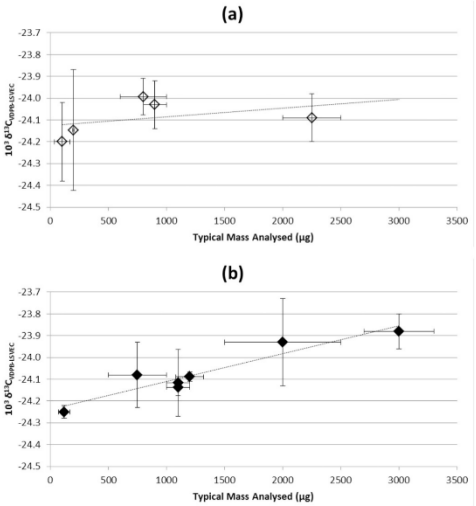


Fig. 3. Relationship between typical mass of honey analysed and reported $\delta^{13}C_{VPDB\text{-}LSVEC}$ value for the participants using EA-IRMS: (a) results for CCQM Laboratories 1–5 (open diamonds); (b) results for the FIRMS laboratories (filled diamonds). Error bars in both plots represent the reported expanded uncertainty (or standard deviation) for the $\delta^{13}C_{VPDB\text{-}LSVEC}$ values and the reported ranges of sample mass. The dashed lines are regression lines of best fit accounting for error in both variables by using Williamson's method [26,27].

values (due to reporting precision rather than measurement uncertainty) from any underlying residual bias from the range of sample mass using the data obtained in this study. Whether the linearity effect has contributed to the poor performance metrics for FIRMS Laboratory 1 is therefore difficult to establish.

Separate from the linearity effect, the degree of homogeneity of the sample material also has the potential to impact the accuracy of isotope ratio measurement results at small amount levels. Therefore, prior to the commencement of the CCQM and FIRMS studies, ten of the units of honey were tested for homogeneity by TÜBİTAK UME. The honey material isotopic composition was found not to be significantly different between vials following ANOVA analysis of the analytical results [7,8]. The honey material was therefore considered to be homogenous in terms of carbon isotopic composition – but only down to the amount level used during the homogeneity study (200–230 µg). Two participants within the CCQM and FIRMS studies, FIRMS Laboratory 1, who returned results with questionable performance metrics, and CCQM Laboratory 1, who did not, used amounts of honey smaller than this. The results from these participants do not indicate that the honey material was the source of the poor performance of FIRMS Laboratory 1. For future inter-laboratory exercises of this type a minimum sample amount to use for each analysis should be recommended based upon the amount used during the homogeneity investigation. This is an aspect that is covered during the production of a reference material under ISO 17034:2016 (previously ISO Guide 34:2009) accreditation [28,29] and a minimum sample amount of 0.2 mg is indeed specified in the TÜBİTAK UME certificate for the honey material [8].

Honey is a liquid and therefore homogeneity in terms of carbon isotope ratio for a bulk sample might be expected. Nonetheless, it is also possible that the viscous nature of honey could result in within-vial variation in carbon isotope ratio, or that some precipitation or crystallisation may go unnoticed during sample storage, preparation and/or analyses. Storage temperature for the honey material was not specified during the study, although it was recommended that vials be kept at "room temperature." No participants reported using a particular storage method for the vials for example at elevated temperature to avoid crystallisation; however no participants reported crystallisation occurring in their honey. The subsequent certificate for the honey reference material provides (+20 ± 5) °C as the storage temperature [8]. Only one of the participants reported using a homogenisation procedure prior to the transfer of honey into tin capsules for analysis (CCQM Laboratory 5). This involved heating, vortex mixing and ultrasonication – a procedure which that laboratory applies for all honey carbon isotope analyses. It is unlikely that the heating resulted in loss of volatile species from the honey as this might be expected to result in lower $\delta^{13}C$ values via preferential loss of ^{13}C-depleted species, while CCQM Laboratory 5 reported a $\delta^{13}C_{VPDB\text{-}LSVEC}$ value that was one of the most ^{13}C-depleted.

4.4.2. Reported measurement uncertainties

Of the corrections performed on the measured isotope ratios (Table 1) it is normalisation and the ^{17}O correction that are essential for every EA-IRMS analysis (and only the former for optical isotope ratio methods); the others - such as blank, drift, linearity and memory corrections - depend on whether there is an analytical need to perform them as indicated by QC materials. While each of these corrections will contribute to measurement uncertainty to some degree (e.g. [21,30]), they will not be reflected in a precision estimate exemplified by the standard deviation of replicate analyses reported by the majority of the FIRMS participants. An estimate of measurement uncertainty that takes into account the effect of normalisation and other corrections performed on raw instrumental data will therefore be larger than simply the precision alone. Furthermore, precision in isotope delta values is sometimes misleadingly reported giving the impression that it is indeed a measurement uncertainty - although in these studies the FIRMS laboratories concerned were all explicitly reporting a standard deviation. Reporting measurement uncertainties that are simply standard deviations can lead to unwarranted confidence in very small variations of isotope delta values and, as stated earlier, an aim of collaboration between the forensic and metrology communities is to change this attitude.

For FIRMS Laboratory 1, if the reported standard deviation (or indeed a reported measurement uncertainty) had been 0.07‰ or larger, the resulting ζ-score and E_n number would have been −1.94 and − 0.88, respectively and fall within the acceptable performance ranges for each metric. Likewise, for FIRMS Laboratory 7, if their reported standard deviation had been larger than 0.1‰, their ζ-score and E_n number would have been +1.99 and + 0.94, respectively and also fall within the acceptable range. These are relatively small increases from the reported standard deviations of approximately 0.03‰ in both cases and therefore the question arises whether simply including the essential data handling processes (^{17}O correction and normalisation) within an uncertainty budget would result in sufficient increase over the reported precision to yield acceptable performance metrics and to provide a more realistic measurement uncertainty that can give a better indication of the significance of small differences in isotopic composition between two materials.

It is unlikely that the ^{17}O correction has a large contribution to measurement uncertainty (Section 3.1.2); however the effect of normalisation is significant and has been previously studied [13,18–21]. As noted in Section 3.1.6, the selection of RMs used for normalisation will impact the achievable uncertainty not only through the Type B contribution from the assigned values (although in some instances such as NBS 19 this is defined as zero), but also a Type A contribution resulting from the uncertainty in their measurement [13]. The Type B contribution to measurement uncertainty reported by CCQM-K140 participants comprised between 10 and 60% of the uncertainty budget depending on the method used to estimate measurement uncertainty. This highlights the potential danger of relying on a precision estimate which does not consider these Type B contributions as these can be significant within the uncertainty budget.

The additional uncertainties arising from (i) normalisation via extrapolation rather than interpolation; and (ii) use of inorganic carbon RMs to normalise organic carbon samples where complete conversion of carbon to the analyte CO_2 has not been demonstrated are also potentially important but not applicable for any of the results reported herein and are therefore not considered further. An extended traceability chain (such as the use of in-house RMs for normalisation that have been themselves characterised against commercially available RMs) will result in a larger measurement uncertainty associated with calibration of measured delta values to the reporting scale. The RMs used by the participants (listed in Section 3.1.6 and Table 1) have correlated assigned values due to being characterised during the same study against the same two reference materials (NBS 19 and LSVEC) [31]. Accounting for this correlation within an uncertainty budget is not straightforward and falls outside of the scope of this manuscript.

It is possible to model the effect of normalisation on measurement uncertainty of the reported isotope delta values from FIRMS Laboratories 1 and 7 provided that some assumptions are made. Firstly it is assumed that normalisation does not alter the raw isotope delta values for sample measurement (i.e. the measured isotope delta values for the RMs are exactly equal to their expected values – although the uncertainty associated with the measured value is different to the uncertainty in the assigned value). As a consequence, the standard deviation of their raw delta values for sample measurements are assumed to be equal to the standard deviations reported; indeed normalisation does not typically alter the reported standard deviation of results by a significant amount. It is also assumed that the replicate analyses of the RMs used for normalisation have the same standard deviation as replicate analyses of the sample material.

The supplementary spreadsheet S1 contains estimations of the measurement uncertainties for FIRMS Laboratories 1 and 7 as

determined by use of the Kratgten spreadsheet approach detailed in the Good Practice Guide for IRMS [13,21,32]. Estimations for FIRMS Laboratory 1 result in a combined standard uncertainty associated with normalisation of 0.05‰ in comparison to the reported standard deviation of 0.03‰. This is still slightly lower than the 0.07‰ required to result in acceptable performance metrics; however FIRMS Laboratory 1 also implemented a linearity correction (Table 1) in addition to normalisation, which is not accounted for. For FIRMS Laboratory 7, the use of in-house RMs with larger assigned uncertainties that take into account the calibrations throughout the traceability chain (expanded uncertainties at the 95% confidence level for their flour and sucrose reference materials were 0.13 and 0.10‰, respectively) resulted in a combined standard uncertainty associated with normalisation of 0.12‰ which would have led to satisfactory performance metrics.

That the differences between the reported precisions and required measurement uncertainty to achieve acceptable ζ-scores and E_n numbers for FIRMS Laboratories 1 and 7 can be accounted for simply by consideration of the uncertainty introduced by normalisation and other corrections to instrumental data highlights the need to report measurement uncertainties rather than simple precision estimates if data compatibility is to be ensured. Moreover, in both of the examples above, the normalisation Type A and B contributions account for over half of the estimated uncertainty. This was also the case for CCQM-K140 participants where some even reported normalisation accounting for over 90% of the measurement uncertainty budget [7]. It is clear that a precision estimate is therefore an overly optimistic measure of the uncertainty.

The best estimate of any result is that its true value lies somewhere within the range defined by its measurement uncertainty; limiting this range to one defined solely by precision data may imply that there is better knowledge of the true value than is justified by the data. This may in turn lead to differences in isotope ratio between two samples being interpreted as significant when this would not be the case if measurement uncertainty were considered, particularly for expanded uncertainties at the 95% confidence level.

4.4.3. Bias in reported values

For FIRMS Laboratories 1 and 7 it is also possible to determine by how much their reported values would need to change (assuming no change to the reported standard deviations) to result in satisfactory ζ-scores and E_n numbers. For FIRMS Laboratory 1 the reported $\delta^{13}C_{VPDB\text{-}LSVEC}$ value would need to be −24.19‰ or higher for an acceptable ζ-score and higher than −24.21‰ for an acceptable E_n number. For FIRMS Laboratory 7 the reported $\delta^{13}C_{VPDB\text{-}LSVEC}$ value would need to be lower than −23.92‰ for an acceptable ζ-score and lower than −23.91‰ for an acceptable E_n number. These are shifts of < 0.05‰ which are small and there is also no indication that the reported results from FIRMS Laboratories 1 and 7 are outliers or not part of a normal distribution of $\delta^{13}C_{VPDB\text{-}LSVEC}$ values. It is therefore unlikely that the poor performance metrics from these participants is the result of reporting biased $\delta^{13}C_{VPDB\text{-}LSVEC}$ values.

4.5. Implications for forensic application of isotope analysis

For an unadulterated honey, $\delta^{13}C_{Honey}$ is generally in the range of −28 to −21‰ while adulteration results in $\delta^{13}C_{Honey}$ values in the range of −21 to −15‰ [33]. Although it is technically possible to observe $\delta^{13}C_{Honey}$ values in the range of −15 to −10‰ these are almost certainly fake "honey" and will consist almost exclusively of sweeteners such as high fructose corn syrup. The $\delta^{13}C_{Protein}$ of an unadulterated honey might be up to 2‰ different to the bulk honey value as a result of bees collecting pollen from different plants to the nectar source of the honey [34]. The proportion of protein within honey is typically 0.1 to 0.2%, although some honeys can contain nearly 1% protein [35].

These indicative values for $\delta^{13}C_{Honey}$ and $\delta^{13}C_{Protein}$ allow a sensitivity analysis of how these terms influence the resultant adulteration percentage, which can be found in supplementary spreadsheet S2. Typically there will be between 5 and 10% increase in reported adulteration level for each permil increase in the numerator of Eq. (1) – this supports the AOAC method whereby 7% adulteration, equivalent to a difference of 1‰, is the lower threshold. Note that this sensitivity analysis simulates how the percentage adulteration might be expected to vary between different honey samples, rather than showing the effect of the measurement uncertainty in the input terms of Eq. (1). The difference in reported $\delta^{13}C_{VPDB\text{-}LSVEC}$ value between the highest (FIRMS Laboratory 7) and lowest (FIRMS Laboratory 1) result was 0.37‰. This equates to a change in reported adulteration of between 2.0 and 3.6%.

The uncertainty in each of the input terms of Eq. (1) will influence the uncertainty in the adulteration percentage obtained. Although the uncertainty in the reference value from CCQM-K140 was 0.107‰ (k = 2.776), we have selected the mean uncertainty of CCQM-K140 participants (0.15‰ with k = 2) as representative of the uncertainty that can be achieved for the $\delta^{13}C_{Honey}$ term. Extraction of the relatively small amount of protein from honey prior to isotope ratio analysis adds additional sample preparation stages that are not required for bulk honey analyses. It is therefore likely that measurement uncertainties associated with the protein carbon isotope ratio measurement are larger than for the bulk honey and consequently the inter-laboratory variability will also likely be larger. We have therefore made the conservative assumption that the spread of results for determination of $\delta^{13}C_{Protein}$ values is double the spread for determination of $\delta^{13}C_{Honey}$ values (i.e. 0.30‰, with k = 2) and used this as an indication of the measurement uncertainty for $\delta^{13}C_{Protein}$.

The two measured terms in Eq. (1) are correlated. For the same honey sample, the $\delta^{13}C_{Honey}$ term includes a contribution from the $\delta^{13}C_{Protein}$ term, although the latter will need to change significantly before the former is affected due to the relatively small amount of protein within bulk honey. For an unadulterated honey the $\delta^{13}C_{HIoney}$ term will be reflected in the $\delta^{13}C_{Protein,}$ and this will be a positive correlation, but on the other hand, for an adulterated honey sample, the $\delta^{13}C_{Honey}$ term will not influence the $\delta^{13}C_{Protein}$ as strongly. The correlation coefficient of these two terms is difficult to establish particularly if both adulterated and pure honeys are considered together. Perhaps the easiest approach is to consider the numerator of Eq. (1) as a single quantity (i.e. a difference) which is independent of the $\delta^{13}C_{Protein}$ term in the denominator and therefore correlation need not be considered. The uncertainty in the numerator difference does still need to be known, however this can easily be estimated as a combination of the uncertainties in the $\delta^{13}C_{Protein}$ and $\delta^{13}C_{Honey}$ terms (neglecting to account for correlation within this combination of uncertainties will only result in an overestimation of uncertainty – which is again a conservative approach).

One of the simplest means to estimate the measurement uncertainty in this case is to use a Monte Carlo simulation [36–38]. The supplementary spreadsheet S2 contains the results of Monte Carlo simulations to estimate the measurement uncertainty in the % C_4 adulteration. Assuming that (i) the standard uncertainties of $\delta^{13}C_{Protein}$ and $\delta^{13}C_{Honey}$ are 0.15 and 0.075‰, respectively and therefore the combined uncertainty in their difference is 0.17‰; (ii) that the numerator of Eq. (1) is allowed to vary from 0 to −10‰; (iii) that $\delta^{13}C_{Protein}$ varies from −28 to −20‰; and (iv) that these values are normally distributed, the Monte Carlo simulations produce an expanded uncertainty in the % C_4 adulteration of between 1.8 and 4.3%. Note that this is not a relative uncertainty – the percentage C_4 adulteration varies between 0 and 97% over the same range of calculated results. This degree of uncertainty is certainly fit-for-purpose for application of the AOAC method as the lower limit of adulteration percentage (i.e. the value minus the expanded uncertainty) for differences of 1‰ or less are all < 7%.

In other forensic applications of carbon isotope analysis, differences in reported delta values between laboratories of up to 0.37‰ or a typical measurement uncertainty of 0.15‰ might be more or less

important – it will depend on the scenario in question and upon stakeholder needs. Where differences in isotope delta of < 1‰ between two materials are to be interpreted (which would not be critical for honey adulteration detection), it is vital that the measurement uncertainty associated to the two results at the 95% confidence level is estimated and considered. While there is still some inconsistency in how measurement uncertainty associated to isotope delta values is estimated as illustrated by the uncertainty budgets reported for CCQM-K140 [7], what is certain is that simply using precision data may be misleading and result in differences between two results being interpreted as significant when this would not be the case considering measurement uncertainty at the 95% confidence level.

4.6. Recommendations for future inter-laboratory studies of isotope delta values

The following points should be considered by organisers and participants of future ILCs of isotope ratio measurements, particularly those involving reporting of isotope delta values:

- Protocols for ILCs should include a minimum amount for sample analysis as is the case with certified reference material certificates.
- Participants should be required to report measurement uncertainties either instead of or in addition to standard deviations – and laboratories should be encouraged to do so whenever reporting results. These should be expanded uncertainties with 95% confidence.
- Measurement uncertainties should include contributions from all correction stages to raw data, particularly normalisation.
- Measurement uncertainties submitted by participants should be included in any interpretation of laboratory performance (i.e. ζ-scores and/or E_n numbers in place of z-scores).
- Reporting of all aspects of the measurement methods employed by participants should be encouraged as this can help identify the source(s) of poor performance and thereby make an ILC into a learning exercise rather than simply a measure of performance.

5. Conclusions

The main role of metrology institutes in the field of chemistry is to provide, directly or by provision of traceability to commercial producers, the chemical standards and matrix reference materials used by field laboratories to calibrate and validate their measurements. In order to do this the metrology institutes which participate in the CCQM often use more elaborate and time-consuming methods than is feasible for field laboratories. The organisation of a FIRMS laboratory ILC for delta values of carbon in the same honey sample in parallel with a CCQM comparison has provided an opportunity to extend the impact of the CCQM's activities by directly assessing the validity of routine forensic measurements. Overall there was good agreement between metrology institutes and forensic laboratories in terms of carbon isotope ratio analysis of bulk honey. The maximum difference in $\delta^{13}C_{VPDB\text{-}LSVEC}$ value for the bulk honey reported by a pair of participants was 0.37‰, while the typical expanded measurement uncertainty (with $k = 2$) reported was 0.28‰ and was > 2.5 times the typical standard deviation reported by the FIRMS participants. The exercise has also enabled the investigation of stable isotope laboratory performance using a range of performance metrics. While different performance metrics highlighted different laboratories as having questionable results, those metrics including the participant reported measurement uncertainty were the most useful. Reporting of standard deviations rather than full measurement uncertainties was highlighted as being a major contributing factor to poor performance metrics and also has the potential to impact interpretation of the results of forensic analyses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scijus.2018.08.003.

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3.3 Scientific paper: "Food matrix reference materials for hydrogen, carbon, nitrogen, oxygen, and sulfur stable isotope-ratio measurements: collagens, flours, honeys, and vegetable oils"



In this chapter, the paper entitled "*Food matrix reference materials for hydrogen, carbon, nitrogen, oxygen, and sulfur stable isotope-ratio measurements: collagens, flours, honeys, and vegetable oils*" by Arndt Schimmelman, Haiping Qi, Philip J.H. Dunn, Federica Camin, Luana Bontempo, Doris Potočnik, Nives Ogrinc, Simon Kelly, James F. Carter, Aiman Abrahim, Lauren T. Reid, Tyler B. Coplen is presented. The paper is accepted in the *Journal of Agricultural and Food Chemistry*.

Stable isotope analysis in food forensics has become a routine and invaluable tool for verification of food authenticity and provenance. Although some methods have been officially validated and acknowledged such as AOAC or EU methods, there are still numerous approaches that are not yet standardized and where a lack of certified isotopic Reference Materials (RMs) limits the mutual comparability of data.

The following paper describes the development, quality-tested, and measured isotope−delta values of ten new food matrix RMs for hydrogen, carbon, nitrogen, oxygen, and sulphur stable isotope-ratio measurements to support food authenticity testing and provenance verification. These new RMs, named USGS82 to USGS91, will enable users to normalize measurements of samples to isotope−delta scales. The new food matrix RMs are chemically complex agricultural plant and animal-derived food matrix materials including (i) honey RMs from Vietnam and Canada spanning different climates, (ii) flour from C_3 and C_4 plants, (iii) vegetable oils from C_3 and C_4 plants, and (iv) collagen powders from marine fish and terrestrial mammal origins. After homogenization of "stock supply", each RM material was divided into multiple aliquots and flame-sealed in round-bottom glass flasks under vacuum. This process was to exclude oxygen in order to extend the shelf life potentially to decades when these RMs are stored at -18 °C and to keep enzyme activity and hydrolysis to a minimum. An errors-in-variables regression model that included the uncertainty associated with the measured and assigned values of the RMs was applied centrally to normalize results and obtain consensus values and measurement uncertainties reported here for the new RMs USGS82 to USGS91.

In this study, I performed all necessary measurements for isotope-delta values of ten food matrix RMs for carbon, nitrogen and sulphur stable isotope ratio measurements. Together, with Prof. Dr. Arndt Schimmelmann I also prepared samples of vegetable oils and flowers.

This work has been presented at the following conferences EGU, IMEKO FOOD, ISEB. The candidate also presented the research (oral presentation) at the 7th Conference of the Forensic Isotope Ratio Mass Spectrometry Network (FIRMS), 16 – 17 September 2019 in San Michele all'Adige, Italy.

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Food matrix reference materials for hydrogen, carbon, nitrogen, oxygen, and sulfur stable isotope-ratio measurements: collagens, flours, honeys, and vegetable oils

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Food matrix reference materials for hydrogen, carbon, nitrogen, oxygen, and sulfur stable isotope-ratio measurements: collagens, flours, honeys, and vegetable oils

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Abstract

An international project developed, quality-tested, and measured isotope−delta values of 10 new food matrix reference materials (RMs) for hydrogen, carbon, nitrogen, oxygen, and sulfur stable isotope-ratio measurements to support food authenticity testing and food provenance verification. These new RMs, USGS82 to USGS91, will enable users to normalize measurements of samples to isotope−delta scales. The RMs include (i) two honeys, from Canada and tropical Vietnam, (ii) flours from C3 (rice) and C4 (millet) plants, (iii) four vegetable oils from C3 (olive, peanut) and C4 (corn) plants, and (iv) collagen powders from marine fish and terrestrial mammal origins. An errors-in-variables regression model included the uncertainty associated with the measured and assigned values of the RMs and was applied centrally to normalize results and obtain consensus values and measurement uncertainties. Utilization of these new RMs should facilitate mutual compatibility of stable isotope data if accepted normalization procedures are applied and documented in publications.

Keywords

Carbon isotope, collagen, flour, food authentication, honey, hydrogen isotope, millet, nitrogen isotope, olive oil, oxygen isotope, peanut oil, reference material, rice, stable isotope ratio, sulfur isotope, vegetable oil.

1. Introduction

Semi-automated and fast stable isotope-ratio analysis (SIRA) in continuous-flow mode with elemental analysis (EA) interfaced with isotope-ratio mass spectrometry (IRMS) has become a routine and invaluable tool for verification of food authenticity and provenance.[1,2,3,4,5] Accurate measurements of relative stable isotope ratios of hydrogen (expressed as δ^2H), carbon (δ^{13}C), nitrogen (δ^{15}N), oxygen (δ^{18}O), and sulfur (δ^{34}S) require normalization using at least two isotopic reference materials (RMs) with contrasting isotopic compositions to (i) anchor δ values to conventional isotope-delta scales and (ii) compensate for differences in responses of analytical instrumentation, which commonly compress isotope−delta scales.[6,7,8,9] To minimize matrix effects, a measurement RM should be chemically as similar as possible to the sample; therefore, food matrix RMs should be used to normalize δ values of food samples. The fundamental "principle of identical treatment of sample and RM" in the analytical process invalidates the use of pulses of pure CO_2, H_2, N_2, CO, or SO_2 "working or monitoring gas" as primary anchors of isotope−delta scales because such gas pulses have not passed through the same analytical inlet system and conversion interfaces before entering the mass spectrometer.[10] Gas pulses from compressed gas reservoirs can only serve to monitor the performance of a mass spectrometer and act as a diagnostic between the introduction of RMs within the same sequence to correct for drift of the IRMS measurements over time, for example during long sequential on-line analyses. Instead, food matrix or other RMs need to be interspersed among test samples and only through them can measured isotope-ratio data be optimally normalized to isotope−delta scales.

This study aims to meet the demand for new international and versatile food-matrix stable isotope RMs and generates synergy with the strategy of the METROFOOD infrastructure (https://www.metrofood.eu/) to provide high-quality metrology services in food, including the development and production of RMs. Recently added RMs are often limited to carbon and nitrogen.[11,12] The current dearth of such RMs can partially be explained by perishability. Long-term storage of food in contact with atmospheric oxygen invariably causes food to degrade *via* slow oxidation, or microbiological spoilage, even during storage at sub-ambient temperatures. Each stock material of our new food matrix RMs has been divided into multiple aliquots that have been flame-sealed into round-bottom glass flasks under vacuum to exclude oxygen and to extend the shelf life potentially to decades when these RMs are stored at −18 °C or lower by reducing enzyme activity and hydrolysis to an absolute minimum. The food matrix RMs, described here, are named USGS82 to USGS91 and are available to end users as 0.5- to 1-g or

0.5- to 1-mL aliquots with isotopic compositions that have been determined as consensus values in a ring-test.

We comply with the International System of Units (SI) when expressing δ values[6,13] and we use the unit mUr instead of ‰ when expressing δ^2H, δ^{13}C, δ^{15}N, δ^{18}O, and δ^{34}S values[14] because the use of the ‰ symbol has been deprecated.[15] A δ value in the traditional form of −25 ‰ can be expressed as −25 mUr.[14] The δ^2H values are normalized to a scale in which δ^2H of VSMOW = 0 mUr and δ^2H of SLAP = −428 mUr;[16] the δ^{13}C values are normalized to a scale in which δ^{13}C of NBS 19 is +1.95 mUr and δ^{13}C of LSVEC is −46.6 mUr (LSVEC is no longer directly used as a RM, yet its assigned δ^{13}C value still defines the carbon VPDB scale);[17] the δ^{15}N values are normalized to a scale in which δ^{15}N of atmospheric nitrogen = 0 mUr and δ^{15}N of USGS32 = +180 mUr; [6,18] the δ^{18}O values are normalized to a scale in which δ^{18}O of VSMOW = 0 mUr and δ^{18}O of SLAP = −55.5 mUr;[19] the δ^{34}S values are normalized to a scale in which δ^{34}S of IAEA-S-1 = −0.3 mUr and δ^{34}S of IAEA-S-3 = −32.49 ± 0.08 mUr.[20,21,22]

2. Materials and Methods

2.1. Honeys, USGS82 from Vietnam and USGS83 from Canada

Vietnamese honey (USGS82) was collected in late October 2018 directly from wild bee hives in the jungle 200 km west of Ho Chi Minh City, Kiên Giang Province (10°0′N 105°10′E). The 8 L of liquid honey were expedited to Indiana University where the honey was filtered twice through multiple layers of fine cotton gauze at ambient temperature in November 2018. The original high moisture mass fraction of ~0.20 g g^{-1} (determined by weight loss after freeze-drying 4.8 g honey) compromised the biochemical stability and would have increased the likelihood of spontaneous sugar fermentation. Honey should ideally contain a maximum of 0.20 g g^{-1} water.[23] Drying of Vietnamese liquid honey was accomplished by placing liter amounts of the filtered honey into pre-annealed Pyrex glass trays for three days each in December 2018 and January 2019 when the laboratory air's relative humidity was ~30 %. The Pyrex trays were shielded from dust by a cover made from cotton gauze. Re-weighing the trays with the partially dried honey enabled determination of the water loss. Subsequently, all Vietnamese honey was pooled into a single collapsible, airtight polyethylene container with a stopcock spout, where it was homogenized while being warmed gently to ~40 °C to reduce viscosity.[24] Freeze-drying of an aliquot of the pooled honey indicated a moisture concentration of 0.14 g g^{-1}.

123 Honey from Saskatoon in Saskatchewan (USGS83), Canada, was purchased in
124 December 2018 from *Three Foragers Bee Company* (https://www.threeforagers.ca/). The 6-kg
125 supply of honey was produced exclusively from hives located in the prairie within a radius of 30
126 km of Saskatoon (52°08′N 106°41′W). The honey arrived at Indiana University as a solid. The
127 manufacturer reported a water concentration between 0.165 and 0.178 g g^{-1}. After liquefaction
128 of the honey during gentle heating in a water bath at 45 °C, the honey was filtered through
129 multiple layers of gauze (cheese cloth) at room temperature in December 2018. The liquid
130 honey was pooled into a single collapsible, airtight polyethylene container with a stopcock spout
131 where it was homogenized at ~40 °C.

132 Aliquots of 1 mL of honeys USGS82 and USGS83 for distribution were sealed in pre-
133 annealed 2-mL amber glass ampoules under argon. For long-term storage, aliquots of both
134 liquid honeys were transferred from their containers through spouts connected to Teflon tubing
135 into pre-annealed 100-, 250-, 500-, and 1000-mL borosilicate glass flasks with pre-constricted
136 necks while minimizing exposure to air. Filled glass flasks were individually attached to a
137 vacuum line, each headspace was evacuated until incipient low-pressure boiling/outgassing at
138 room temperature, and each glass flask was sealed under vacuum with a glass-blowing torch.
139 Both honeys solidified during cold storage. Honey supplies are being stored in freezers at
140 Indiana University and the U.S. Geological Survey (USGS) in Reston, Virginia.

141

142 *2.2. Vegetable oils from C3 and C4 plants cultivated in Italy (USGS84), Peru (USGS85),*
143 *Vietnam (USGS86), and USA (USGS87)*

144 Olive oil (USGS84) produced in Sicily, Italy (37°45'N, 14°15'E) from C3 plants, was
145 obtained from the 2018 harvest. In February 2019, it was filtered through Whatman #1 cellulose
146 filters at room temperature and then stored in a refrigerator until it was sealed in glass in May
147 and June 2019. Temporary storage in a refrigerator did not result in partial solidification and loss
148 of homogeneity.

149 Olive oil from Peru (USGS85) originated from the March 2018 harvest on the family-
150 operated *Chacra Blanca* farm on the desert coast of the Chilca Valley, 60 km south of Lima
151 (12°30'S 76°45'W; http://www.olivico.com/ingles/aboutUs.html). Irrigation water for the farm
152 relies on artesian wells tapping an aquifer that is replenished by precipitation in the high Andes.
153 The 5 L of "Olivico" oil were filtered through Whatman #1 cellulose filters at room temperature in
154 January 2019, followed by sealing in glass. According to the International Olive Council[25] and

the Codex Alimentarius,[26] the concentration of moisture and volatile matter in extra virgin olive oils should not exceed 0.2 wt. %, which is a mass fraction of 0.002 g g^{-1}.

Peanut oil (C3 plant; USGS86) from the Quảng Bình Province in central Vietnam (17°30′N 106°20′E) was freshly squeezed from the 2018 autumn harvest and was supplied in October 2018 by Mrs. Nguyen Thi Hong Thinh at the Institute for Nuclear Science and Technology, Hanoi. After being expedited to Indiana University, it was filtered through Whatman #1 cellulose filters at room temperature in November 2018, followed by sealing in glass.

Corn oil (C4 plant; USGS87) was obtained from the germ of *Zea mays*. The commercially distributed oil was purchased in Bloomington (Indiana, USA) as 5.6 L of "*carlini pure corn oil*" from a retail outlet and filtered through Whatman #1 cellulose filters at room temperature in December 2018, followed by sealing in glass.

Aliquots of 1 mL of vegetable oils USGS84 to USGS87 for distribution were sealed in pre-annealed 2-mL amber glass ampoules under argon. For long-term storage, aliquots of the four vegetable oils were stored in pre-annealed 100-, 250-, 500-, and 1000-mL borosilicate glass flasks with pre-constricted necks. Filled glass flasks were individually attached to a vacuum line, each headspace was evacuated, and each glass flask was sealed under vacuum with a glass-blowing torch. Vegetable oil supplies are being stored in freezers at Indiana University and the USGS.

2.3. Bone collagen powders of marine (USGS88) and terrestrial origins (USGS89)

Marine collagen powder (USGS88) from wild-caught fish was purchased in November 2018 and January 2019 as unflavored powder from single lot #18129 distributed by Zen Principle *via* Amazon. The entire 5.44 kg of collagen powder was pooled in a single large polyethylene bag and homogenized by five minutes of vigorous tumbling prior to sealing of aliquots in multiple glass containers. Prior to sealing of glass flasks, small aliquots of collagen from 10 flasks were selected for testing of homogeneity.

Seventy three aliquots of ~ 40 mL each of porcine (pig) collagen powder (USGS89) from a previous European Framework 6 (TRACE) Integrated Project,[27,28] which had been stored at –20 °C, were pooled in a common container and tumbled five minutes vigorously for homogenization prior to sealing multiple aliquots in glass flasks under vacuum.

For long-term storage, aliquots of the two collagen powders were stored in pre-annealed 100-, 250-, and 500-mL borosilicate round-bottom glass flasks. Filled glass flasks were individually attached to a vacuum line, each headspace was evacuated, and each glass flask was sealed under vacuum with a glass-blowing torch. Collagen supplies are being stored in freezers at Indiana University and the USGS. For distribution to end users, aliquots of 0.5 g of collagen powders USGS88 and USGS89 were sealed in pre-annealed glass vials with aluminum crimp caps and teflon-lined septa.

2.4. Flours from Italian millet (C4 plant, USGS90) and Vietnamese white rice (C3 plant, USGS91)

Five kilograms of millet grains (*Panicum miliaceum*) from the 2016 harvest originated from Tuscany, Italy (43°29'N, 10°47'E). Five kilograms of white rice (*Oryza sativa*) from the 2018 harvest were obtained from rural southern Vietnamese farmers in the Kiên Giang Province, 200 km west of Ho Chi Minh City, (10°0′ N 105°10′ E) in October 2018. Both materials were ground to flour with a model 1093 Cyclotec mill (Tecator™, Höganäs, Sweden) at the Fondazione Edmund Mach in March 2019. Additionally, rice flour was sieved through a 60-mesh (0.25-mm grain size) stainless steel sieve and the fraction with a grain size ≤ 0.25 mm was ball-milled in a Planetary Micro Mill PULVERISETTE 7™ (Fritsch, Idar-Oberstein, Germany) at the Jožef Stefan Institute in May 2019. The combined rice flour fractions with grain sizes ≤ 0.25 mm were used as USGS91. Each flour was pooled in a single polyethylene bag and homogenized by shaking and rolling prior to sealing of multiple aliquots in glass containers. Prior to sealing the glass flasks, small aliquots of flour from 10 flasks were selected for homogeneity testing.

Aliquots of 0.5 g of flours USGS90 and USGS91 for distribution were sealed in pre-annealed glass vials with aluminum crimp caps and teflon-lined septa. For long-term storage, aliquots of the two flours were stored in pre-annealed 100-, 250-, 500-, and 1000-mL borosilicate glass flasks with constricted necks. Filled glass flasks were individually attached to a vacuum line, each headspace was evacuated, and each glass flask was sealed under vacuum with a glass-blowing torch. Flour supplies are being stored in freezers at Indiana University and the USGS.

2.5. Long-term preservation of stock supplies and sealing of aliquots for global distribution

Our aim is to produce food matrix RMs that will retain their compositional and isotopic integrity over decades. It is not practical to sub-divide all food matrix RMs into small aliquots for final distribution owing to cost and the uncertainty of which sample sizes will be appropriate for future methods of analysis. Therefore, relatively large stock supplies of each RM need to be stored appropriately for future subsampling. In the case of waters, carbonates, sulfates, and other relatively stable materials, each RM stock supply is typically kept in a single container to ensure that the entire supply shares a common history during storage.

The supplies of food matrix RMs require a different long-term storage strategy because the more complex food matrix may degrade if repeatedly exposed to atmosphere during subsampling. Furthermore, food may be subject to microbiological spoilage if it is repeatedly removed from cold storage. Although the RMs already contain microbes, opening of containers increases the possibility of further inoculation with microbes of higher activity. An example is honey, which crystallizes during cold storage. Each container of honey will need to be warmed for dissolution of precipitated sugar crystals and homogenization prior to each occasional sub-division into aliquots. The repeated opening of a supply of honey to air would not only add oxygen, but also permit the hydrogen- and oxygen-isotopic exchange of the honey with moisture in air, thus potentially changing the bulk isotopic and chemical composition. Changes in attending personnel over time will add uncertainty in handling procedures. It would be challenging to monitor any slow oxidative, isotopic or microbiological change of a single supply over time.

As an alternative means of storage, the entire supply of each food matrix RM was homogenized in a single container, from which aliquots were loaded expeditiously into multiple pre-annealed borosilicate round-bottom glass flasks with volumes of 100, 250, 500, and 1000 mL. The neck of each glass flask had a further constriction. Individual glass flasks were attached to a vacuum line, head spaces were evacuated, and the flasks were rapidly flame-sealed at their constrictions

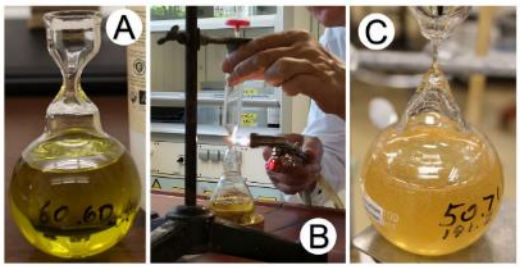


Figure 1 . (**A**) Olive oil prior to evacuation and (**B**) during sealing under vacuum with a torch. (**C**) Honey in round-bottom glass flask sealed under vacuum with incipient outgassing, prior to separation from upper inlet spout.

with a glass blowing torch while taking care that RMs were not heated. The evacuation of headspaces above liquids was brief because (i) honeys began foaming (Fig. 1) and (ii) the loss of moisture from both honeys and vegetable oils needed to be minimized.

For global distribution of RMs, multiple pre-annealed 2-mL glass ampoules were filled with 1 mL of honey or vegetable oil and sealed under argon with a glass blowing torch. Flour and collagen in amounts of 0.5 g were weighed into glass vials and crimp-sealed. All glass containers were engraved, were labeled, and are stored in freezers at Indiana University and the USGS. After storage in freezers, each time a fresh flask is opened and transferred into units for distribution, the isotopic composition of the newly prepared units will be checked against the expected values.

2.6. γ-Irradiation of reference materials for sterilization by Australian Biosecurity

Food matrix and associated international RMs were sent in April 2019 *via* FedEx from Slovenia to Health Support Queensland in Australia. Upon arrival in Australia, the package was quarantined by Australian Biosecurity and sterilized *via* a dose of 30 Gy γ-irradiation from a ^{60}Co source at a "low dose" irradiation level similar to that used to sterilize food or medical equipment (https://www.ansto.gov.au/business/products-and-services/irradiation/gamma-irradiation). Irradiation turned the formerly clear glass of the vials to brown glass (Fig. 2). The irradiated RMs were measured isotopically by Health Support Queensland. In May 2019, subsamples of the irradiated materials were shipped *via* FedEx from Australia to Indiana University, and from there to the USGS for comparative isotopic measurements against their non-irradiated counterparts. The extreme experience of the samples that had been



Figure 2. Comparison of identical types of glass vials with peanut oil from Vietnam as shipped to all ring-test member laboratories (left), and after mandatory sterilization in Australia *via* a dose of 30 Gy γ-irradiation from a ^{60}Co source (right).

air-freighted multiple times with poor temperature control and undergone γ-irradiation served as a test of the short-term stability of food matrix RMs under extreme, yet realistic conditions.

2.7. *Homogeneity of food matrix RMs*

The isotopic homogeneity of food matrix RMs was tested and confirmed at sample sizes commensurate with the abundances of elements in bulk RMs and the sensitivity of current analytical approaches. For example, the hydrogen-isotopic homogeneity of rice flour with grain sizes of ≤ 0.25 mm was established by the USGS *via* dual-H-equilibration of ~0.44-mg samples. Carbon- and nitrogen-isotopic measurements of porcine collagen powder confirmed homogeneity at the 0.35-mg level, whereas the relatively low sulfur concentration of porcine collagen made it necessary to combust 5-mg aliquots at the Food and Environmental Protection Laboratory of the FAO/IAEA Joint Division. As instrumentation becomes more sensitive and able to measure smaller sample sizes, future users of powdered RMs will need to perform tests to establish practical thresholds of isotopic homogeneity. For example, earlier testing of the carbon- and nitrogen-isotopic homogeneity of the porcine collagen by six laboratories participating in the European TRACE project had used relatively large 0.5- to 1-mg aliquots.[27,28]

The original chemical and isotopic homogeneity of filtered liquid vegetable oils was achieved by stirring each supply in a single container at room temperature. After distribution into multiple round-bottom glass flasks, chemical and isotopic homogeneity can be maintained if storage conditions prevent the precipitation of solid phases over time. The elimination of oxygen from sealed glass storage containers and the sealing of oils in silver capillary tubing minimize degradation processes that may introduce heterogeneity. The handling and sealing of honeys in liquid form served the same purpose. Collagens are produced industrially from fish and mammal bones by intermittent solubilization of gelatin, followed by filtration of the aqueous solution and drying to ultimately produce collagen powder. The intermittent solubilization in a common water bath mixes the isotopically diverse collagens from a large number of individual animals with slightly different trophic backgrounds[29] and results in isotopic homogeneity of each industrial batch of collagen. Our collagens derive from single industrial batches.

Rice flour from Vietnam initially presented a homogeneity problem that was detected by the USGS after multiple ~0.44-mg aliquots were equilibrated in water vapor with known hydrogen isotopic composition, followed by drying and analysis for bulk δ^2H composition. Sieving and subsequent analyses of different grain size fractions revealed differences as large

312 as 8 mUr. About one third of the mass of the original bulk rice flour was contributed by grain
313 sizes > 0.25 mm. After sieving, the exclusive use of the finer ≤ 0.25-mm fraction resulted in
314 improved analytical precision and higher H_2 yield owing to (i) greater adsorption of H_2O on the
315 larger surface area of the finer fraction, and (ii) enhanced hydrogen isotopic exchange afforded
316 by the larger surface area per mass unit. Subsequently, the entire supply of Vietnamese rice
317 flour was sieved and only the ≤ 0.25-mm fraction was utilized as an RM. Parallel testing of the
318 millet flour by the USGS did not identify a problem with hydrogen-isotopic homogeneity when
319 samples of ~0.44 mg were used.

320 The homogeneity of rice flour with grain sizes ≤ 0.25 mm, millet flour, and the two
321 collagens was further evaluated by randomly selecting aliquots from 8 to 11 round-bottom
322 flasks, each of which had been filled with respective supplies for long-term storage under
323 vacuum in freezers at a maximum temperature of –18 °C.

324

325 *2.8. Isotopic measurements in participating ring-test laboratories*

326 Five participating ring-test laboratories used a variety of analytical approaches to
327 measure bulk δ^2H, δ^{13}C, δ^{15}N, δ^{18}O, and δ^{34}S values. Their respective on-line methodologies are
328 based on a variety of elemental analyzer (EA) interfaces, some with subsequent gas
329 chromatographic (GC) separation or trap and purge technology, as well as mass spectrometers
330 from different manufacturers (Table 1). Along with 10 food matrix samples in glass containers or
331 silver tubing, each ring-test laboratory received a selection of primary and secondary RMs for
332 calibration (normalization) of isotope-delta scales anchored by VSMOW, SLAP, VPDB, LSVEC,
333 atmospheric nitrogen, USGS32, and VCDT. RMs were used with their most up-to-date δ values
334 and uncertainties assigned to them together with the source/citation for the values, which can
335 be found in Table 2. We did not directly use LSVEC lithium carbonate for normalization of
336 carbon isotope-delta values in this study to avoid the issue that this RM can absorb atmospheric
337 carbon dioxide over time with use in a laboratory, increasing its δ^{13}C value.[30] However, it was
338 necessary to rely on other international RMs (Table 2) whose δ^{13}C values are traceable to
339 LSVEC (as well as NBS 19 and VPDB) for normalization because no alternatives exist at the
340 present time.[8]

341 **Table 1.** Stable isotope on-line approaches and equipment of participating ring-test laboratories.

Laboratory	δ^2H measurements	δ^{13}C and δ^{15}N measurements	δ^{18}O measurements	δ^{34}S measurements
Food and Agriculture Organization / International Atomic Energy Agency, Austria (FAO/IAEA)	No measurements.	Elementar vario PYRO cube® EA connected to Elementar BiovisION IRMS *via* Elementar centrION gas handling unit. EA combustion tube contains tungstic oxide granules supported on a solid quartz spacer at 1020 °C. Reduction tube contains copper wires and a section of silver wool to trap halides at 850 °C. He flow 230 mL min^{-1}, O_2 dosing time 6 sec depending on sample weight, desorption of CO_2 at 180 °C.	No measurements.	Same as for C and N measurements, except 1050 °C combustion temperature, desorption of SO_2 from trap at 230 °C.
Fondazione Edmund Mach, Italy (FEM)	Oils and honeys: Thermo Scientific™ TC/EA, ConFlo IV, glassy carbon reactor 1450 °C, Thermo Delta X PLUS+, GC 1.2 m column at 110 °C, He flow 100 mL min^{-1}. H and O analyses performed separately. Collagens, millet and rice: Samples weighed in silver capsules, pre-dried in desiccator with P_2O_5 under vacuum for 5 days. Dual-water equilibration in 2 separate desiccators under Ar for 13 days in the presence of 0.3 mL H_2O of either USGS49 or USGS53. Repeat drying in 2 separate desiccators with P_2O_5 under vacuum for 5 days, followed by analysis with Thermo Scientific™ TC/EA, ConFlo IV, glassy carbon + chromium (1:1, wt:wt) reactor 1450 °C,[35] Thermo Delta X PLUS+, GC 1.2 m column at 110 °C, He flow 100 mL min^{-1}.	Thermo Scientific™ EA 1112, ConFlo IV, reactor 900 °C, reduction at 680 °C. Thermo Delta X PLUS+, GC 45 °C, He flow 140 mL min^{-1}. C and N analyses performed separately.	Oils, honeys, collagens, millet, rice: Thermo Scientific™ TC/EA, ConFlo IV, reactor 1450 °C, glassy carbon. Thermo Delta X PLUS+, GC 1.2 m column at 110 °C, He flow 100 mL min^{-1}. H and O analyses performed separately. Collagens, millet and rice: Samples weighed in silver capsules, dried in desiccator with P_2O_5 under vacuum for 5 days, followed by TC/EA analysis as described above.	Samples were dried in oven at 50° C for at least 48 h before analysis. EA Elementar vario ISOTOPE cube with backflush connected to Elementar isoprime visION IRMS *via* Elementar centrION gas handling unit. EA combustion tube contains tungstic oxide granules at 1180 °C; reduction tube contains copper wires and final part of silver wool to trap halides, at 630 °C. He flow 230 mL min^{-1}, O_2 dosing time from 25 to 50 sec with flow 38 mL min^{-1} depending on sample weight. Trap desorption of CO_2 at 200 °C, SO_2 at 280 °C.
Health Support Queensland, Australia (HSQ)	Thermo Scientific™ TC/EA coupled to a Delta V PLUS *via* ConFlo IV interface. Alumina reactor at 1400 °C packed with a mixture of glassy carbon/manganese and glassy carbon/chromium. He flow rate 100 mL min^{-1}. GC column at 110 °C.	Thermo Scientific™ FLAH 2000 HT EA coupled to Delta V Plus *via* ConFlo IV interface. Single reactor at 950 °C contained chromium oxide, electrolytic copper and silvered cobaltous oxide. He flow rate 100 mL min^{-1}. C and N measurements performed separately. For N measurements, a Carbo-Sorb®	Thermo Scientific™ TC/EA coupled to Delta V Plus *via* ConFlo IV interface. Glassy carbon reactor at 1400 °C. He flow rate 100 mL min^{-1}. GC column at 85 °C for effective separation of CO and N_2. N_2 was diverted from the mass spectrometer by activating the split flow of the ConFlo IV interface.	No measurements.

		trap was installed between reactor and GC column.		
Jožef Stefan Institute, Slovenia (JSI)	No measurements.	IsoPrime100 IRMS, Vario PYRO Cube (OH/CNS Pyrolyzer/Elemental Analyzer), combustion tube 920 °C, reduction tube 600 °C, He flow 230 mL min^{-1}, O_2 dosing time from 80 to 120 sec depending on sample weight, desorption of CO_2 at 110 °C.	No measurements.	IsoPrime100 IRMS, Elementar vario PYRO cube® (OH/CNS pyrolyzer/EA), combustion tube 1080 °C, reduction tube 850 °C, He flow 230 mL min^{-1}, O_2 dosing time 120 sec, desorption of SO_2 at 240 °C.
U.S. Geological Survey, Reston, Virginia, USA (USGS)	Method 1 targeting non-exchangeable H in solid samples: Drying of samples in Ag cups in a vacuum oven for 3 days at 40 °C, followed by dual-water equilibration.[42,43] Two sets of samples were exposed to ^{2}H-enriched and depleted water vapors for 11 days in two identical desiccators at a pressure of ~87 kPa (650 mm Hg). Samples were dried in a vacuum oven at 40 °C for 5 days prior to isotopic measurement. Method 2 targeting bulk H: (a) Solid samples in Ag cups were dried in a vacuum oven overnight at 40 °C. Chromium-packed high-temperature conversion EA without graphitic crucible. Thermo Scientific™ TC/EA coupled with Thermo Delta XP with ConFlo IV. He flow 100 mL min^{-1}, reactor 1400 °C, GC 90 °C. (b) Liquid samples were introduced in crimp-sealed silver capillary tubes. Glassy carbon-packed reactor utilized a graphite crucible. Thermo Scientific™ TC/EA coupled with Thermo Delta XP with ConFlo IV. He flow 100 mL min^{-1}, reactor 1450 °C, GC 90 °C.	Costech ECS 4010 CHNSO Analyzer coupled to Thermo Delta V+ *via* ConFlo IV. Combustion at 1020 °C, reduction at 650 °C. GC temp. 50 °C, He flow of 110 mL min^{-1}. C and N analyses performed separately. All solid samples were dried overnight at 40 °C after weighing prior to analysis.	Thermo Scientific™ TC/ EA with glassy carbon reactor with graphite crucible. Thermo TC/EA coupled with Thermo Delta XP with ConFlo IV. He flow 100 mL min^{-1}, reactor 1380 °C, GC 90 °C.	EA IsoLink™ coupled to Thermo Delta V+ *via* ConFlo IV. Single reactor at 1020 °C, GC ramping temp. from 70 °C to 240 °C, He flow of 110 mL min^{-1}. Solid samples were dried overnight at 40 °C after weighing prior to analysis.

342

343 **Table 2.** Primary and secondary international measurement RMs used in the ring-test for calibration and normalization of stable
344 isotope ratios along conventional δ scales.

Reference material	*Sources of values*	*Accepted isotope-delta values in mUr with uncertainties*[&]				
		δ^2H_{VSMOW}	$\delta^{13}C_{VPDB}$	$\delta^{15}N_{AIR}$	$\delta^{18}O_{VSMOW}$	$\delta^{34}S_{VCDT}$
GFLES-4 water[$]	42**	+399.8 ± 0.4	n.a.	n.a.	−6.08 ± 0.04	n.a.
IAEA-600 caffeine	43	−156.1 ± 1.3	−27.73 ± 0.04	+1.02 ± 0.05	n.d.	n.a.

IAEA-601 benzoic acid	6	n.d.	−28.81 ± 0.04	n.a.	+23.14 ± 0.19	n.a.
IAEA-N-1 ammonium sulfate	6	n.d.	n.a.	+0.43 ± 0.07	n.d.	n.d.
IAEA-N-2 ammonium sulfate	44*	n.d.	n.a.	+20.41 ± 0.12*	n.d.	n.d.
IAEA-S-2 silver sulfide	22	n.a.	n.a.	n.a.	n.a.	+22.62 ± 0.08
IAEA-S-3 silver sulfide	22	n.a.	n.a.	n.a.	n.a.	−32.49 ± 0.08
NBS 19 calcium carbonate	6	n.a.	+1.95	n.a.	+28.65	n.a.
NBS 22 oil	43	−117.2 ± 0.6	−30.02 ± 0.04	n.a.	n.a.	n.a.
NBS 22a vacuum oil	43	−120.4 ± 1.0	−29.72 ± 0.04	n.a.	n.d.	n.a.
NBS 127 barium sulfate	45	n.a.	n.a.	n.a.	+8.59 ± 0.26	+21.12 ± 0.11
SLAP2 water	6	−427.5 ± 0.3	n.a.	n.a.	−55.5 ± 0.02	n.a.
UCO4 water	42	+113.6 ± 0.5	n.a.	n.a.	+38.95 ± 0.02	n.a.
USGS44 calcium carbonate	42	n.a.	−42.27 ± 0.07#	n.a.	n.d.	n.a.
USGS49 water§	46	−394.7 ± 0.2	n.a.	n.a.	−50.55 ± 0.02	n.a.
USGS53 water§	42	+40.2 ± 0.2	n.a.	n.a.	+5.47± 0.015	n.a.
USGS61 caffeine	43	+96.9 ± 0.9	−35.05 ± 0.04	−2.87 ± 0.04	n.d.	n.a.
USGS62 caffeine	43	−156.1 ± 2.1	−14.79 ± 0.04	+20.17 ± 0.06	n.d.	n.a.
USGS64 glycine	43	n.d.	−40.81 ± 0.04	+1.76 ± 0.06	n.d.	n.a.
USGS65 glycine	43	n.d.	−20.29 ± 0.04	+20.68 ± 0.06	n.d.	n.a.
USGS66 glycine	43	n.d.	−0.67 ± 0.04	+40.83 ± 0.06	n.d.	n.a.
USGS77 polyethylene	43	−75.9 ± 0.6	−30.71 ± 0.04	n.a.	n.a.	n.a.
VSMOW water	6	0	n.a.	n.a.	0	n.a.
VSMOW2 water	6	0 ± 0.3	n.a.	n.a.	0 ± 0.02	n.a.

n.a. = not applicable; n.d. = not determined

* IAEA (1995) TECDOC-825, page 57. The value was normalized by assigning IAEA-N-1 to +0.43 mUr and USGS32 to +180 mUr.

value calibrated using NBS 19 and LSVEC, subject to revision.[47]

δ uncertainties as indicated in sources of values. Uncertainties are either combined standard uncertainties or 1σ standard deviations. Further details about uncertainties offered in Supplemental Information.

§ used for isotopically controlled equilibration of exchangeable hydrogen rather than normalization.

345

The use of reference waters that were crimp-sealed in silver tubes[31,32] (Fig. 3) made it possible to measure δ^2H values of food matrix materials against known δ^2H values of reference waters following the principle of identical treatment.[10] Conventional on-line high-temperature conversion of hydrogen in organic samples to gaseous H_2 in a glassy carbon environment (also termed TC/EA or HTC/EA) generates HCN as a pyrolytic byproduct and fractionates the H_2 analyte when the sample matrix contains nitrogen.[33] Among our 10 food matrix materials, only the two collagens have relatively high nitrogen concentration owing to their proteinaceous nature. In some laboratories, the use of metallic chromium or manganese for high-temperature conversion of organic hydrogen to H_2 [34,35,36] (Table 1) eliminated the bias that has been reported to exceed 10 mUr in collagen.[37,38,39]

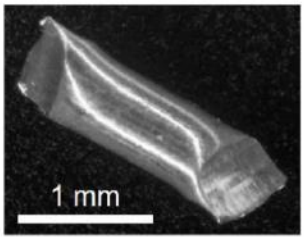


Figure 3. Example of a liquid sample like water, vegetable oil, or honey that is crimp-sealed in a segment of silver tubing. Liquid food matrix RMs and reference waters in silver tubes are available from the USGS, Reston, VA, USA.

This study's most reproducible δ^2H values characterizing non-exchangeable hydrogen in flours and collagens were determined by the USGS and the Fondazione Edmund Mach (FEM) using a dual-water equilibration procedure at room temperature, followed by drying in a vacuum oven for 5 days at 40 °C, and final generation of H_2 in the presence of chromium at 1400 °C.[37,40] For example, the two waters for equilibration at the USGS were USGS49 (δ^2H = –394.7 mUr) and GFLES-4 (δ^2H = +399.8 mUr). Isotopic mass-balance was employed to calculate the δ^2H values characterizing bulk non-exchangeable hydrogen.[40]

The isotopic calibration of future RMs requires utmost care to exclude artefacts and systematic bias. Some participating laboratories initially experimented with analytical sequences where two or more isotope ratios were measured sequentially *via* peak jumping, for example first measuring N_2, then CO_2, and finally SO_2. Isotope ratios of light elements can, however, shift depending on the amount of sample that is employed in on-line oxidative EA and reductive TC/EA analyses. Peak jumping makes it impossible to carefully match the yields of the various analyte gases from samples and RMs entering the ion source unless modern technology is used to dynamically adjust the flow of gas into the ion source.[41] The highest reproducibility was typically achieved when measuring only a single isotope ratio in an analytical sequence, trying to match the analyte yields from samples and RMs, and performing mass-dependent corrections where exact matches were not possible. Isotopic biases introduced by peak jumping and other time-saving practices (e.g., lack of proper pre-drying of powders) reached 0.6 mUr for δ^{13}C, 0.5 mUr for δ^{15}N, 4.2 mUr for δ^{18}O, and 4.5 mUr for δ^{34}S.

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2.9. Statistical evaluation of overall data and arrival at consensus values

To avoid difficulty in combining normalized data from multiple laboratories that may have used the same or similar subsets of RMs for normalization,[43] laboratories submitted measured δ values for centralized normalization. Measured δ values were provided by each laboratory, some of which had undergone some correction processes (e.g., for drift, linearity/sample mass, or blank) while others were simply measured values from proprietary instrumental software. The measured δ values provided only underwent normalization centrally; no other corrections were applied. For all δ values other than the δ^2H values of collagens and flours, the following process was applied:

Centralized normalization and estimation of a consensus δ value by combination of the individual laboratory results was achieved by following the method from Meija and Chartrand using R coupled to Microsoft Excel®.[48] In brief, normalization of each laboratory dataset was carried out using errors-in-variables regression, such that the uncertainties in both the measured and assigned δ values of the normalization RMs were accounted for. Where laboratories reported measured δ values from more than one independent measurement sequence, these were treated separately. The regression also accounted for the typically small number of degrees of freedom associated with measured values of normalization RMs.[49] The normalized values from each laboratory were combined together using a random laboratory effects model to account for any additional variation among the laboratory results that was not accounted for by the reported variance using a restricted maximum likelihood method. Correlations among laboratory results caused by the use of the same

subset of RMs for normalization were taken into account; however, any correlations among assigned values of the RMs were not as these are difficult to estimate. The uncertainty in the consensus value was estimated using a Monte Carlo method. Refer to Meija and Chartrand and Guenther and Possolo for additional details.[48,49]

Prior testing of this approach demonstrated that 1000 replicates were sufficient to estimate the individual laboratory results and associated uncertainties. Given the limited number of measurements contributing to some of the consensus values and their calculated uncertainties, more conservative decisional expanded uncertainties were implemented for the assigned values of the new food RMs of 3 mUr for δ^2H, 0.07 mUr for δ^{13}C, 0.10 mUr for δ^{15}N, 0.25 mUr for δ^{18}O and 0.40 mUr for δ^{34}S based upon previous experience of RM characterization and value assignment. Where the calculated expanded uncertainties in the consensus or recommended values obtained by the Monte Carlo method were larger than these decisional limits, the larger uncertainties were used instead.

For δ^2H of the collagens and flours, two types of measured data were reported by laboratories. For those reporting "bulk" δ^2H values on material "as received" or that had simply been dried prior to analysis, normalization was carried out as above to produce a result with an associated uncertainty for each independent set of data. No consensus value was calculated for these data.

Some laboratories reported measured δ^2H values for the collagens and flours for aliquots that had undergone equilibration with one of two different waters so that the fraction of exchangeable organic hydrogen and adsorbed hydrogen from H_2O could be estimated and accounted for. For these measured δ values, normalization was applied as documented above, but results from each of the two equilibrations were treated independently so that two normalized results together with uncertainties estimated by a Monte Carlo method were obtained for each material. These two results, together with the assigned δ values for the waters used for equilibration (Table 2) were then used to estimate the fraction of labile hydrogen together with the δ^2H value for the non-exchangeable fraction of hydrogen following the calculations described by Qi and Coplen.[40] The uncertainties for this stage of the calculation approach were estimated using the numerical method of Kragten.[50] Again, no consensus value was calculated for these data.

3. Results

3.1. Homogeneity and short-term stability

Unlike the other powdered food matrix RMs, only rice flour expressed initial unsatisfactory

446 homogeneity that was detected by the USGS during hydrogen-equilibration experiments. The
447 presence of coarser grains with a lower mass over surface ratio limited the adsorption of water and
448 the access of H_2O to exchangeable H-sites within grains, thus, contributing to a relatively large
449 standard deviation of 5.9 mUr when measuring δ^2H values of six ~0.44-mg rice flour samples after
450 equilibration with isotopically controlled H_2O. Additional ball-milling and sieving of the rice flour to a
451 grain size of ≤ 0.25 mm improved the standard deviation to 4.4 mUr based on 6 measurements at
452 the same analytical conditions.

453 No evidence was found for isotopic differences of RMs among sealed glass bottles for long-
454 term storage that exceeded the precision of analyses. For example, the δ^{13}C standard deviations of
455 0.07 mUr for 8 and 11 samples of ~0.7-mg aliquots of millet flour and rice flour, respectively, from
456 different round-bottom storage glass flasks were indistinguishable from the standard deviation of
457 0.07 mUr of eight ~0.7-mg aliquots from a single bottle of rice flour (measured *via* on-line EA-IRMS
458 at Indiana University).

459 Adsorption of water by solid RMs can introduce hydrogen- and oxygen-isotopic heterogeneity
460 in the laboratory depending on the handling and exposure to ambient air with variable humidity and
461 seasonally changing isotopic characteristics of atmospheric moisture. For example, extensive drying
462 reduced the bulk hydrogen mass fraction of millet flour USGS90 from 0.0639 ± 0.0004 g g^{-1} (n = 6;
463 Table 3) by ~0.01 g g^{-1}. Thorough drying of aliquots of collagens and flours at 105 °C to constant
464 weight at QH Forensic and Scientific Services determined the mass-fraction of moisture of USGS88
465 through USGS91 to be 0.080, 0.123, 0.083, and 0.105 g g^{-1}. Elemental mass fractions (Table 3) and
466 other analytical results depend on the extent of drying and on the relative humidity of laboratory air
467 at the time of weighing and loading of samples.

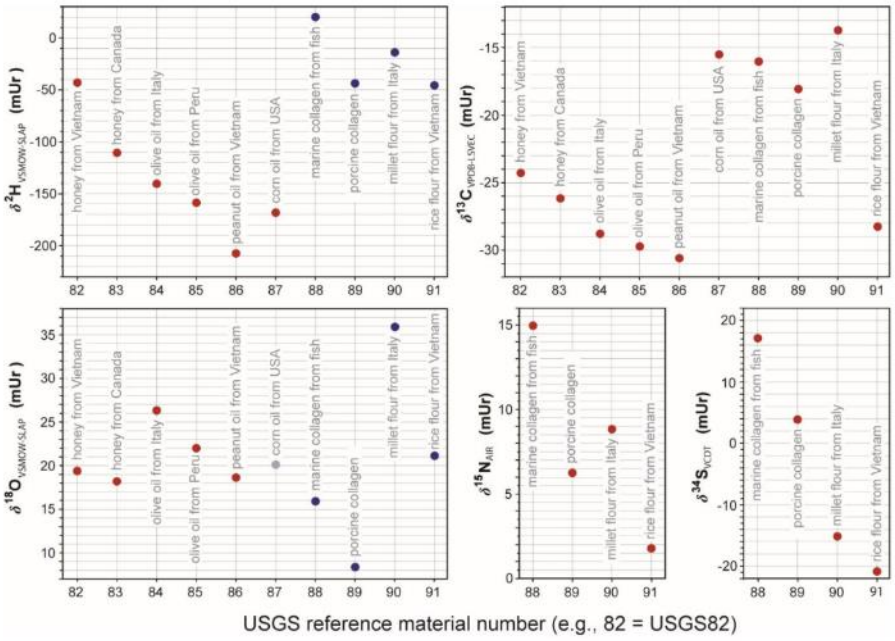


Figure 4. Stable isotope ratios of food matrix reference materials USGS82 to USGS91. Red dots indicate consensus values of bulk materials. Dark blue dots indicate recommended isotope ratios when specific pre-treatment and analytical conditions are applied (see details in text). In the case of hydrogen, blue dots indicate recommended δ^2H values of non-exchangeable hydrogen. A gray dot represents a recommended bulk δ^{18}O value of corn oil based on limited analytical data. The expanded uncertainties of all plotted values are smaller than the size of the dots.

468

469 *3.2. Consensus values*

470 Consensus values with associated expanded uncertainties are listed in Table 4 and graphed
471 in Fig. 4, whereas all detailed data, statistics of particular RMs in individual analytical sequences and
472 overall calculated means and medians are reported in the Supplementary Information. All consensus
473 values are based on isotopic compositions of bulk samples.

474 Owing to the hygroscopicity of powdered organic materials like flour and collagen, any bulk
475 δ^2H or δ^{18}O value would depend significantly on the extent of pre-drying and exposure to laboratory

476 **Table 3.** Hydrogen, carbon, nitrogen, oxygen, and sulfur mass fractions with standard deviations and numbers of analyses of food
477 matrix reference materials determined by the USGS based on gas yields from EA combustions and TC/EA.

Name of Reference Material	*Description*	*Elemental mass fractions (g g^{-1}) with standard deviations*				
		Hydrogen	**Carbon**	**Nitrogen**	**Oxygen**	**Sulfur**
USGS82	Honey from tropical Vietnam	0.0717 ± 0.0006 (n = 6)	0.3301 ± 0.0046 (n = 10)	n.d.	0.5746 ± 0.0116 (n = 6)	n.d.
USGS83	Honey from prairie in Canada	0.0724 ± 0.0003 (n = 6)	0.3412 ± 0.0047 (n = 10)	n.d.	0.5714 ± 0.0068 (n = 6)	n.d.
USGS84	Olive oil from Sicily, Italy	0.1178 ± 0.0023 (n = 6)	0.8024 ± 0.0117 (n = 9)	n.d.	0.1093 ± 0.0031 (n = 15)	n.d.
USGS85	Olive oil from coastal desert, Peru	0.1184 ± 0.0004 (n = 6)	0.7949 ± 0.0117 (n = 10)	n.d.	0.1098 ± 0.0033 (n = 14)	n.d.
USGS86	Peanut oil from tropical Vietnam	0.1178 ± 0.0008 (n = 6)	0.7916 ± 0.0081 (n = 10)	n.d.	0.1130 ± 0.0046 (n = 10)	n.d.
USGS87	Corn oil from USA	0.1152 ± 0.0016 (n = 5)	0.7990 ± 0.0075 (n = 10)	n.d.	0.1119 ± 0.0059 (n = 10)	n.d.
USGS88	Marine collagen from wild-caught fish	0.0632 ± 0.0017[a] (n = 6)	0.4409 ± 0.0071 (n = 15)	0.1666 ± 0.0045 (n = 8)	0.2584 ± 0.0123 (n = 12)	0.0046 ± 0.0002 (n = 12)
USGS89	Porcine collagen	0.0617 ± 0.0001[a] (n = 6)	0.4304 ± 0.0077 (n = 26)	0.1578 ± 0.0019 (n = 6)	0.2516 ± 0.0123 (n = 11)	0.0028 ± 0.0001 (n = 12)
USGS90	Millet flour from Tuscany, Italy	0.0639 ± 0.0004[a] (n = 6)	0.4128 ± 0.0038 (n = 16)	0.0154 ± 0.0003 (n = 8)	0.4325 ± 0.0140 (n = 11)	0.0013 ± 0.0001 (n = 12)
USGS91	Rice flour from tropical Vietnam	0.0609 ± 0.0004[a] (n = 8)	0.3933 ± 0.0062 (n = 16)	0.0150 ± 0.0004 (n = 7)	0.4478 ± 0.0139 (n = 10)	0.0013 ± 0.0001 (n = 12)

478 [a] Flours and collagens were pre-dried in a vacuum oven at 40 °C for at least 5 hours. Note that drying of flours, collagens, and
479 honeys at higher temperature under reduced pressure will decrease the moisture content and affect elemental mass fractions. A
480 chromium-packed reactor was used. The indicated mass fractions are method-specific and are information values, not certified
481 values.

482 n.d. = not determined.

483

484 **Table 4.** Consensus δ values with associated expanded uncertainties of new food matrix reference materials along conventional δ
485 scales.

Name of Reference Material	*Description*	*Consensus δ values in mUr with expanded uncertainties*[a]				
		$\delta^{2}H_{VSMOW}$	$\delta^{13}C_{VPDB}$	$\delta^{15}N_{AIR}$	$\delta^{18}O_{VSMOW}$	$\delta^{34}S_{VCDT}$
USGS82	Honey from tropical Vietnam	−43.1 ± 3.0 [b]	−24.30 ± 0.07	n.d.	+19.39 ± 0.25 [b]	n.d.
USGS83	Honey from prairie in Canada	−110.6 ± 3.0 [b]	−26.18 ± 0.07	n.d.	+18.20 ± 0.25 [b]	n.d.
USGS84	Olive oil from Sicily, Italy	−140.4 ± 3.0	−28.80 ± 0.07	n.d.	+26.33 ± 0.25	n.d.
USGS85	Olive oil from coastal desert, Peru	−158.6 ± 3.0	−29.74 ± 0.07	n.d.	+22.00 ± 0.25	n.d.
USGS86	Peanut oil from tropical Vietnam	−207.4 ± 3.0	−30.62 ± 0.07	n.d.	+18.62 ± 0.33	n.d.
USGS87	Corn oil from USA	−168.1 ± 3.0	−15.52 ± 0.17	n.d.	+20.11 ± 0.28	n.d.
USGS88	Marine collagen from wild-caught fish	(+20.1 ± 3.0)[c]	−16.05 ± 0.07	+14.96 ± 0.10	(+15.91 ± 0.25)[c]	+17.10 ± 0.40
USGS89	Porcine collagen	(−43.7 ± 3.0)[c]	−18.07 ± 0.07	+6.25 ± 0.10	(+8.37 ± 0.25)[c]	+3.86 ± 0.40
USGS90	Millet flour from Tuscany, Italy	(−13.9 ± 3.0)[c]	−13.73 ± 0.07	+8.84 ± 0.10	(+35.90 ± 0.25)[c]	−15.14 ± 0.40
USGS91	Rice flour from tropical Vietnam	(−45.7 ± 3.0)[c]	−28.27 ± 0.07	+1.78 ± 0.10	(+21.13 ± 0.25)[c]	−20.85 ± 0.40

486 [a] Expanded measurement uncertainties at the 95 % confidence level include the uncertainty associated with (i) the measured food
487 matrix RMs, (ii) the uncertainty of assigned values of RMs that had been used for normalization, and (iii) the measured values of
488 those same normalization RMs. With the exception of uncertainties of $\delta^{18}O_{VSMOW}$ values for USGS86 and USGS87, we chose
489 decisional expanded uncertainties that are larger than the respective calculated uncertainties.

490 [b] The sealing of honeys in segments of silver tubing by the USGS eliminated contact with air and enabled direct measurements of
491 bulk δ values against water RMs sealed in silver tubing. This approach adhered to the principle of identical treatment of sample and
492 RM as far as high-temperature thermal conversion and further analytical processing are concerned.

493 [c] The presence of exchangeable organic hydrogen and adsorbed H_2O in flours and collagens causes significant variance depending
494 on sample pretreatment and type of measurement; the indicated $\delta^{2}H_{VSMOW}$ values were determined by the USGS according to a
495 specific double-equilibration procedure[40,51] and represent information values, not consensus values; the indicated $\delta^{18}O_{VSMOW}$ values
496 were determined by the USGS; see Table 1 for analytical details.

497 n.d. = not determined.

498 air with local atmospheric humidity; hence, no consensus values were calculated for flours and
499 collagens. The listed bulk $\delta^{18}O$ consensus values of flours and collagens are valid only for carefully
500 pre-dried samples. The $\delta^{2}H$ values of non-exchangeable hydrogen reported by the USGS for flours
501 and collagens and entered into Table 4 in parentheses are considered to be information values.
502 They were generated using specific pre-treatment, double-equilibration with isotopically known H_2O,
503 and mass-balance calculations.[37,40] Other laboratories should be able to reproduce these results
504 when following the USGS analytical protocol; however, the USGS-reported $\delta^{2}H$ values are neither
505 bulk values nor are they considered to be consensus values.

506 In contrast, the sealing of honeys in silver capillary tubing by the USGS excluded further
507 contact with air and facilitated the determination of bulk $\delta^{2}H$ and $\delta^{18}O$ consensus values, although
508 honeys contain substantial water. The $\delta^{2}H$ and $\delta^{18}O$ data of honeys from laboratories not using
509 USGS-filled silver capillaries were not used for overall statistical evaluation and are displayed in
510 graphs in the Supplementary Information only to document the variance that can be expected from
511 air interaction and different analytical approaches. The $\delta^{2}H$ and $\delta^{18}O$ consensus values of honeys
512 are therefore valid only for USGS-filled silver capillaries and 1-mL aliquots of honeys sealed under
513 argon in glass ampoules. After opening of an ampoule, care must be exercised to strictly limit
514 contact with laboratory air. The $\delta^{13}C$ consensus values of honeys are less influenced by exposure to
515 laboratory air.

516 The statistical basis of consensus values for vegetable oils was not restricted to the use of
517 oils in silver capillary tubing because non-polar oils are not as hygroscopic as powdered
518 carbohydrate and proteinaceous materials.

519 *3.3. Influence of γ-irradiation for mandatory sterilization in Australia*

520 Direct bulk isotopic comparisons at the USGS between original and γ-irradiated food
521 matrix RMs with data sets of n ≥ 3 showed no indication that γ-irradiation had introduced
522 isotopic fractionation larger than the analytical precision of analyses. The exceptions were bulk
523 oxygen-isotopic data of subsamples of honey from Vietnam, porcine collagen, and rice flour that
524 were enriched in ^{18}O by up to 0.8 mUr after their return from Australia to the USGS.

525

526 **4. Discussion**

527 *4.1. Large variance of δ^2H values upon exposure of flour, collagen, and honey to air and*
528 *moisture*

529 The bulk δ^2H values of vegetable oils are unlikely to be significantly influenced by
530 additional drying during handling, adsorption of moisture from laboratory air, or exchange with
531 water vapor because the moisture concentration (mass fraction) of hydrophobic vegetable oils is
532 less than 0.002 g g^{-1} and the vast majority of organic hydrogen in biolipids is bonded to carbon
533 where it is not available for exchange with hydrogen from H_2O.

534 In contrast, major complications arise when honeys, flours, and collagens are exposed
535 to air having different humidities and isotopic compositions of water vapor, for example in
536 laboratories in various countries with contrasting climates. Honeys often contain a mass fraction
537 of water between 0.14 and 0.18 g g^{-1}, and about half of their organic hydrogen is bonded to
538 oxygen in carbohydrates where hydrogen in exposed functional groups can be exchanged with
539 H_2O. Drying, wetting, and simple exchange with atmospheric moisture will affect bulk δ^2H and
540 $\delta^{18}O$ values of honey. Sealing aliquots of honeys in segments of silver tubing by the USGS
541 greatly facilitated the reproducibility of measurements of bulk δ^2H and $\delta^{18}O$ values of honey RMs
542 in different laboratories, even in warm climates with high humidity.

543 Carbohydrate-rich flours and proteinaceous collagen powders offer large surface areas
544 for adsorption of atmospheric moisture and contain isotopically exchangeable organic hydrogen
545 linked to oxygen and nitrogen. Bulk δ^2H and $\delta^{18}O$ values will depend on (i) the isotopic
546 composition of the H_2O that was last in contact with the substrates, (ii) the time and temperature
547 of the exchange (or equilibration), and (iii) the thoroughness and mode of drying prior to
548 analysis. Reproducible measurements of bulk δ^2H and $\delta^{18}O$ values of collagen powders and
549 flours make it necessary not only to control the amount of adsorbed moisture, but also to control
550 its isotopic composition and to pre-equilibrate the isotopically exchangeable organic hydrogen in

an isotopically reproducible fashion. Numerous practical approaches have been published,[40,51,52,53,54,55] yet their non-standardized choices of equilibration temperature, time, and drying conditions result in incompatible bulk δ^2H (and δ^{18}O) values. The authors do not endorse any particular analytical approach. Bulk δ^2H and δ^{18}O values of flours and collagens reported in this study are meaningful only within the context of their applied analytical methodology reported here and represent information values, not consensus values.

4.2. Insignificant isotopic changes from 30 Gy γ-irradiation

Sterilization of aqueous media by γ-irradiation causes radiolysis of water and the production of small amounts of H_2, H_2O_2, and OH· radicals,[56,57] in addition to radiolytic damage to the structure of organic molecules. However, the expected hydrogen-isotopic effect from 30 Gy of γ-irradiation is insignificant.[58] Direct bulk carbon and oxygen isotopic comparisons between original and γ-irradiated food matrix RMs within dedicated analytical sequences at the USGS yielded results that showed no isotopic fractionation within the precision of analyses when sufficiently large data sets of n ≥ 3 were considered. Exceptions were the oxygen isotopic compositions of USGS82 (honey from Vietnam), USGS89 (porcine collagen) and USGS91 (rice flour) that contained relatively large amounts of water and expressed enrichment in ^{18}O by up to 0.8 mUr after small subsamples had returned from Australia to the USA. Instead of γ-irradiation, we attribute this isotopic fractionation to the evaporative and desorptive loss of water from food matrix samples during repeated subsampling and return transport of small aliquots in spacious glass vials. Loss of water from Vietnamese honey was especially severe and resulted in a reduction of its oxygen elemental abundance by ~0.019 g g^{-1}.

4.3. Practical recommendations for users of food matrix RMs and estimated shelf life

Non-sterilized, biochemically complex food matrix RMs contain microorganisms and spores. When exposed to atmospheric oxygen and moisture, they are prone to oxidation and biodegradation, more so than dry, compound-specific organic RMs like cellulose, crystalline sucrose, or L-glutamic acid. All stock supplies of food matrix RMs are being stored frozen in evacuated, sealed glass flasks, which eliminates the possibility of significant oxidation and/or biodegradation over decades of cold storage.

The end user will receive smaller aliquots of RMs that are not sealed under vacuum. The following estimates for shelf lives apply to unopened containers. Liquids like honeys and

583 vegetable oils will be available (i) in glass ampoules sealed under argon at Indiana University,
584 and (ii) 25 to 50-µL aliquots crimp-sealed in silver tubes by the USGS. Sealed glass ampoules
585 can be stored in freezers, which extends their shelf life to an estimated 5 years. In contrast,
586 crimp-sealed silver tube segments must not be frozen to protect the integrity of the crimp-seal,
587 but they can be stored at 4 °C to maximize their lifespan. We estimate the shelf life of liquid food
588 matrix RMs in silver tubes in refrigerators above freezing is ~2 years. Long-term stability
589 monitoring of food matrix RMs is in progress. End users will be informed if any significant
590 problems are discovered.

591 Small aliquots of dry, powdered food matrix RMs will be available to end users in screw-
592 capped or crimp-sealed glass vials where the head- and interstitial space will be filled with air.
593 Freezing will not eliminate slow oxidation, which will likely shorten the shelf life. After opening of
594 any container of food matrix RMs, the remaining shelf life greatly depends on the handling and
595 further storage of RMs. For example, after opening a glass ampoule it is necessary to
596 expeditiously transfer the liquid RM into another suitable container that can be closed tightly and
597 stored at low temperature. In any case, access to atmospheric oxygen decreases the shelf life
598 of RMs.

599 Honey and vegetable oil RMs in glass containers may experience cold temperatures
600 during storage and transport to end users. Honeys and some vegetable oils will have partially
601 crystallized and internally fractionated. The end user is advised to visually inspect each
602 unopened glass container for homogeneity and, if necessary, to warm the unopened container
603 to ~45 °C for at least one hour, then again visually inspect the contents before opening. Specific
604 advice about the handling of honey was provided by Dunn et al.[59,60] For example, one of the
605 participants in the CCQM-K140 comparison first heated honey to 60 °C for ten minutes, then
606 homogenized by vortex-mixing for two minutes, ultrasonicated for five minutes at 60 °C, and
607 finally subsampled from the vial. Before opening any container with RMs, the material should be
608 allowed to stabilize at ambient temperature to avoid condensation of atmospheric moisture
609 when in contact with cold RMs.

610 After opening a glass ampoule with 1 mL of honey, the end user may find it difficult to
611 efficiently transfer the viscous honey to another container while limiting the exposure of honey to
612 room air. Instead, a compact, pea-sized ball of Parafilm® M can be pressed onto the opening of
613 the ampoule for closure and can be temporarily removed for extracting aliquots of honey. Care
614 must be exercised to avoid injury by the sharp glass rim. An additional layer of Parafilm® M
615 wrapped tightly around the ball and the upper part of the opened glass ampoule will further limit

616 the access of room air during storage of honey. Parafilm® M ball and wrapping do not need to
617 be removed when warming honey in an opened ampoule to ~45 °C for re-homogenization.

618 Honeys and vegetable oils sealed in silver tubing by the USGS offer several advantages
619 because (i) silver metal has natural antimicrobial activity,[61,62] (ii) samples are shielded from light
620 and air, (iii) uncertainties in weighing errors and potential contamination and fractionation by the
621 end user are eliminated. Each crimp-sealed segment of silver tube contains vegetable oil or
622 honey with a bulk amount of hydrogen equivalent to the amount of hydrogen in 0.15 µL or 0.25
623 µL of reference water (available from the USGS). When using silver tube segments in an EA
624 carousel, it can be advantageous to wrap the silver tube segment(s) in a tin or silver capsule to
625 prevent jamming of the carousel and to ensure proper dropping of sample(s). The small amount
626 of sample in silver tube segments precludes their use for $\delta^{34}S$ analysis.

627 No polymer vacuum pack technology for RMs can provide a stable vacuum for more
628 than a few years. All plastics allow for slow diffusion of oxygen to eventually reach the sealed
629 organic RMs that will make the RMs "stale" and oxidized. Glass and metal are the only safe
630 options to seal RMs for many years under vacuum. Each consignment of distributed food matrix
631 RMs will be accompanied by clear instructions about recommended opening and storage.

632

633 *4.4. Practical considerations to improve data quality*

634 The mutual comparability of isotopic data from different laboratories greatly improved
635 after the abandonment of the timesaving practice of peak jumping in favor of running analytical
636 sequences that were dedicated to a single isotope ratio, especially when combined with efforts
637 to equalize the signal strengths of normalization RMs and unknowns. Analyzing series of
638 samples with increasing weight as a test for linearity proved beneficial for quality control.
639 Practitioners will benefit from a trend in modern instrumentation to automatically and
640 dynamically control signal strengths during peak jumping.[41]

641 The necessity of reproducible and adequate drying of samples prior to reductive
642 pyrolysis in TC/EA-type interfaces is common knowledge for hydrogen- and oxygen-isotopic
643 measurements. The large hydrophilic surfaces of collagen and flour powders can hold a mass
644 fraction of more than 0.10 g g^{-1} of adsorbed water that is isotopically tracking atmospheric
645 humidity. Fewer practitioners are aware of the influence of elevated water concentrations on the
646 measurement of $\delta^{34}S$ values, especially when low sulfur abundances mandate the combustion
647 of large samples in an EA to generate satisfactory amounts of sulfur dioxide, for example from

collagens and flours. Elevated amounts of water in the analytical train can affect the adsorption and partial loss of sulfur dioxide on surfaces during gas transfer and result in artificially high $\delta^{34}S$ values. Careful pre-drying of samples, proper matching of SO_2 yields from standards and samples, and linearity testing are mandatory for high-quality $\delta^{34}S$ measurements.

A major challenge for hydrogen and oxygen δ measurements in honeys occurred during weighing of small droplets into silver cups owing to interference from atmospheric moisture and partial drying. For example, USGS-generated $\delta^{18}O$ values of Vietnamese honey differed between those of weighed droplets *versus* crimp-sealed honey in segments of silver tubing (i.e. 19.73 ± 0.11 mUr, n=4 *versus* 19.14 ± 0.20 mUr, n=6). The difference for Canadian honey was more dramatic (19.35 ± 0.55 mUr, n=4 *versus* 18.18 ± 0.07 mUr, n=6). We propose that the sample preparation of viscous and sticky organic materials like honeys and syrups is best accomplished by crimp-sealing in segments of metal capillary tubing.

In conclusion, the ten newly developed food matrix reference materials USGS82 to USGS91 will enable the stable isotope community to adhere more closely to established guidelines and recommended practices when measuring stable isotope ratios of food and other materials. It is a requirement for the accurate determination of light stable isotope ratios to employ two or more reliable reference materials for normalization of measured ("raw") data along customary isotope-delta scales. Careful comparisons of various laboratory practices indicated that δ biases in excess of the analytical precision are likely when more than one isotope ratio is being measured with peak jumping and when signal strengths from samples and standards are not matched. In such cases, various stoichiometries of samples and standards cause incompatibility in the yields of analyte gases from samples and standards. Resulting δ biases are not readily detectable unless aliquots of sufficiently homogeneous reference materials spanning different sample weights are included in the analytical on-line sequence. Hence, high-precision data benefit from dedicated analyses for single δ values and the use of a microbalance to approximately match the yields of analyte gases from samples and standards, especially when elemental abundances in samples are low, for example sulfur abundances. Measurements of hydrogen and oxygen stable isotope ratios in food samples are challenging when exposure to atmospheric moisture and the influence of exchangeable hydrogen in samples are not controlled. In addition, the particle size of solid organic samples constrains the accuracy of bulk $\delta^{2}H$ and $\delta^{18}O$ values owing to available surface area for adsorption of water and access to exchangeable hydrogen. It is recommended that (i) the powder of an unknown food sample should have a grain size as close as possible to that of the RMs, and (ii) to expose the unknown samples and RMs to laboratory air at ambient temperature for at least 5 days prior

to analysis. Proper pre-drying of hygroscopic flour and collagen is beneficial to reduce analytical bias during measurements of other isotope ratios like sulfur.

Abbreviations Used

Ar – argon gas
C – carbon
CO – carbon monoxide
CO_2 – carbon dioxide gas
ConFlo – gas partitioning device used in mass-spectrometer design
EA – elemental analyzer
EA-IRMS – elemental analyzer connected to isotope-ratio mass spectrometer
FAO – Food and Agriculture Organization of the United Nations
FEM – Fondazione Edmund Mach
GC – gas chromatography
GFLES – isotope standard (https://isotopes.usgs.gov/lab/referencematerials/GFLES1 to GFLES4.pdf)
H – hydrogen
HCN – hydrogen cyanide gas
H_2 – hydrogen gas
He – helium gas
H_2O – water
H_2O_2 – hydrogen peroxide
HTC/EA – high temperature conversion elemental analyzer
IAEA – International Atomic Energy Agency
IRMS – isotope-ratio mass spectrometry
LSVEC – lithium carbonate stable isotope reference material
N – nitrogen
N_2 – nitrogen gas
NBS – National Bureau of Standards
O – oxygen
O_2 – oxygen gas
P_2O_5 – phosphorus pentoxide
RM – reference material
S – sulfur

716 SIRA − stable isotope-ratio analysis
717 SI − System of Units
718 SLAP – Standard Light Antarctic Precipitation
719 SO_2 – sulfur dioxide gas
720 TC/EA – thermal conversion elemental analyzer
721 USGS – United States Geological Survey
722 VCDT – Vienna Canyon Diablo Troilite
723 VPDB – Vienna Peedee Belemnite
724 VSMOW – Vienna Standard Mean Ocean Water
725

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736 stress-tested our reference materials. Any use of trade, firm, or product names is for descriptive
737 purposes only and does not imply endorsement by the U.S. Government.

738 **Supporting Information description**

739 An Excel file offers 53 sheets of detailed information on (i) individual analytical data from
740 contributing laboratories, (ii) statistical results from individual analytical sequences and overall
741 compiled data, (iii) graphs displaying overviews of isotopic data for the 10 new food matrix
742 reference materials, (iv) elemental mass fraction data, and (v) comparative data on original
743 *versus* γ-irradiated materials. Sheet #1 provides an overview on the contents of all sheets.

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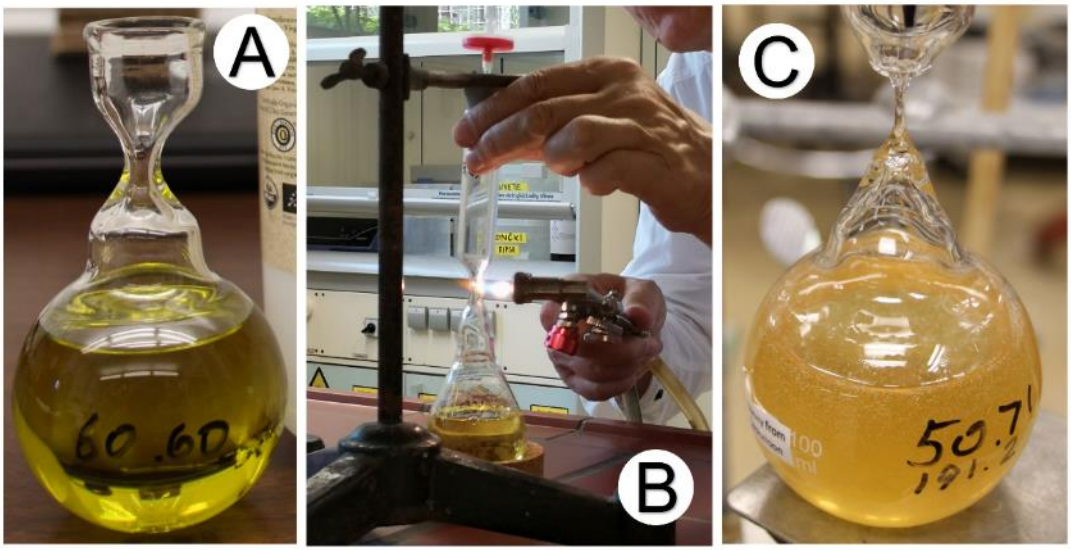
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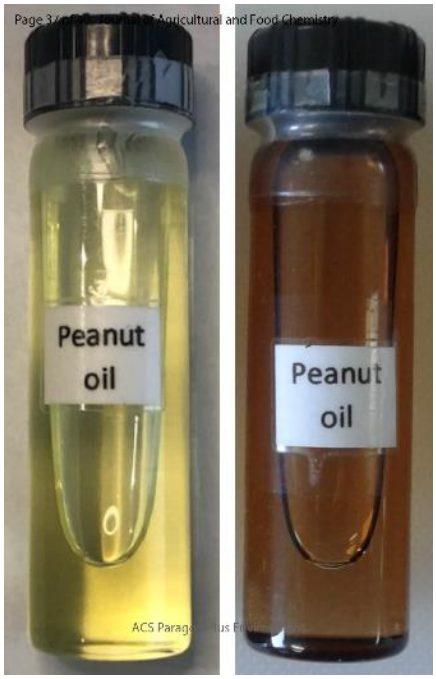
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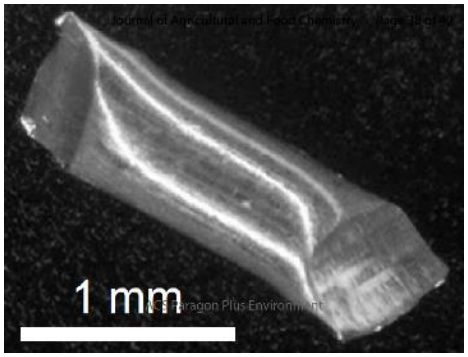
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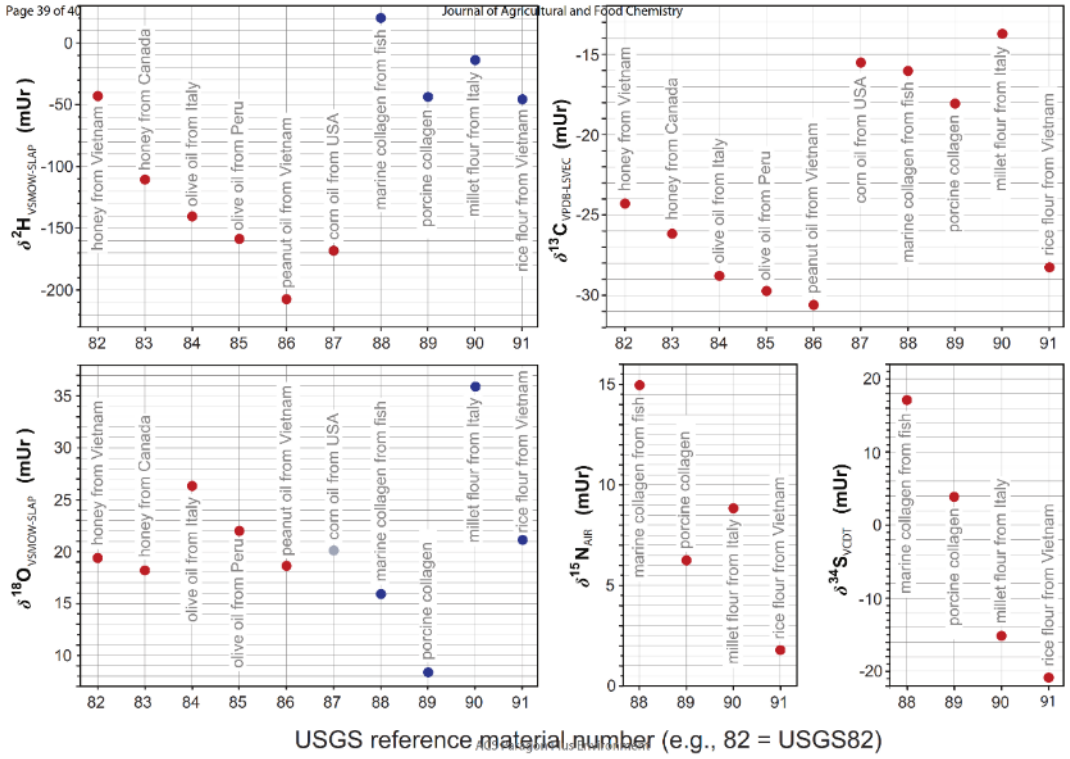
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corn oil from USA
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3.4 Scientific Paper: "Discrimination Between Slovenian Cow, Goat and Sheep Milk and Cheese According to Geographical Origin Using a Combination of Elemental Content and Stable Isotope Data"

This chapter summarizes the paper entitled "*Discrimination between Slovenian cow, goat and sheep milk and cheese according to geographical origin using a combination of elemental content and stable isotope data*" by Marijan Nečemer, Doris Potočnik and Nives Ogrinc. The paper was published in the *Journal of Food Composition and Analysis*, 2016.

The number of recent studies shows how proof of provenance has become an important topic in the context of food safety, quality, traceability and consumers protection. The Protected Designated of Origin (PDO) trademark has been assigned to numerous local products based strictly on their production origin. In Slovenia, the upper Soča/Isonzo valley is a region with a long and rich tradition of cheese making. Throughout history, this area has created two kinds of cheese, "Tolminc" cheese also known as Formaggio di Tolmin and "Bovški" cheese known as Formaggio di Plezzo. Both types of cheese have acquired EU PDO certification. "Tolminc" cheese is made from cow's milk according to traditional technology, while "Bovški" cheese is made from milk of the domestic Bovec sheep, to which can be added up to 20% goat's or cow's milk, but must be correctly declared and labeled. In addition, in the Karst region a traditional half-hard sheep's cheese "Kraški ovčji sir", which is the result of climatic and floristic specificities, also has PDO status.

The objective of this research was the systematic characterization of authentic Slovenian cow's, sheep's and goat's milk and cheese using a combination of different isotopic ratios of bio-elements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$) and elemental composition (P, S, Cl, K, Ca, Zn, Br, Rb and Sr). Based on measured parameters and discriminant analysis (DA), milk and cheese from cow, goat and sheep were classified according to their region of production. During May, June and July in 2012 and 2013, 124 Slovenian milk and cheese samples (n=30) were collected. The isotopic composition of carbon, nitrogen and sulphur of the casein fraction was performed by EA-IRMS, while the isotope composition of oxygen was measure directly in milk using an IRMS coupled to a Multiflow Bio equilibration unit. The elemental composition of the bulk freeze-dried samples was obtained using energy dispersive X-ray fluorescence spectrometry. The data showed that the ability to discriminate between cow's milk according to its production origin was only possible using summer 2012 data. Sheep's milk and cheese samples could be distinguished according to their geographical origin based on the content of P, S, K, Cl, Ca and Zn and on the $\delta^{13}C$ and $\delta^{15}N$ values in the casein fraction with a classification rate of 97%.

Regarding species differentiation, it was possible using stable isotopes parameters ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$) and nine micro and macro elements to distinguish sheep's and goat's milk and cheese from that produced from cow's milk with a prediction ability of 95.2%. Based on the results, isotopic and elemental analysis can help support and protect the existence of the PDO status of "Bovški" and "Kraški" sheep's cheese and "Tolminc" cow's cheese. It will also help protect consumers and will promote local products and contribute to the local economy. These methods can also be incorporated into traceability models to protect PDO dairy products from mislabelling and for the verification of the authenticity of commercial products.

Part of this work was presented (poster) at the 10th Young Researchers' Day CMBE 2016, 31 March 2016, in Ljubljana, Slovenia and as an oral and poster presentation at the 8th Jožef Stefan International Postgraduate School Students' Conference, 31 May – 11 June 2016 in Ljubljana, Slovenia.

In this paper, I was responsible for the collection and storage of milk samples, which were provided by Ljubljanske mlekarne d.d., Pomurske mlekarne d.d., Mlekarna Planika d.o.o. Kobarid and Mlekarna Celeia d.o.o.. I also performed sampling, sample preparation, including isolation of casein fraction from raw milk and cheese, and stable isotope analysis using EA-IRMS. I also collected and in part, analysed the data and jointly prepared the manuscript.

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Original research article

Discrimination between Slovenian cow, goat and sheep milk and cheese according to geographical origin using a combination of elemental content and stable isotope data

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ABSTRACT

The presented work uses stable isotope ratios of major bioelements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) and elemental composition to determine the regional provenance of 124 Slovenian milk and 30 cheese samples collected during May, June and July in 2012 and 2013. The ability to discriminate between cow milk based on geographical origin was only possible in summer 2012, where discriminant analysis identified Cl, Zn, P, Ca and K as the most significant parameters. Both sheep milk and cheese could be distinguished according to their geographical origin based on P, S, K, Cl, Ca, Zn content and $\delta^{13}C$ and $\delta^{15}N$ values in casein and to refuse possible imitations with a correct classification in 97% of cases. Furthermore, stable isotopes of light elements in combination with elemental composition can distinguish sheep and goat milk and cheese from that produced from cow milk with a prediction ability of 95.2%. Such methodology can effectively contribute towards supporting the existence of the Protected Designated Origin of Bovški and Kraški sheep cheese and Tolminc cow cheese and could be incorporated into a traceability system.

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1. Introduction

Proof of provenance has become an important topic in the context of food safety, quality and traceability and consumer protection. The Protected Designation of Origin (PDO) trademark has been assigned to numerous local products based strictly on their area of origin. Milk and dairy products are an important group that has been thoroughly investigated to differentiate between production regions. According to the Food and Agricultural Organization of the United Nations, over 18 million metric tons of cheese per year were produced worldwide (Global Milk Production and Consumption, 2011). The typical characteristics of a PDO dairy product are its production technology, the origin of the milk and materials associated with the animal feeding diet. The techniques employed for differentiation of PDO dairy products have mostly involved stable isotope fingerprinting either on its own or in combination with other techniques used for determining elemental composition. Application of a combination of isotope and elemental composition to verify the authenticity of premium regional products is based on the evidence that these parameters are affected by complementary factors. Stable isotopes mostly depend on climatic or geographical conditions, while elemental composition is affected by geology and pedological characteristics of the soil, excluding "contamination" during processes. Isotopic parameters have been added to the PDO technical specification of Grana Padano Cheese (Italy).

Some studies have used stable isotope ratios to identify animal feed and diet regimes (Kornexl et al., 1997; Camin et al., 2004; Knobbe et al., 2006) and for differentiating products from different countries. Oxygen isotope fractionation patterns in milk water have been used to distinguish cows' milk from different regions in Australasia (Crittenden et al., 2007). Stable isotope ratio of carbon and nitrogen in casein was used to distinguish Pecorino Sardo Cheese from Sardinia from that produced in Sicily and Apulia (Manca et al., 2001) and buffalo mozzarella originating from two areas in southern Italy (Brescia et al., 2005). Together with the

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isotope ratio of other bioelements including ^{13}C, ^{15}N, ^{34}S content of casein and ^{18}O content of extracted glycerol it was possible to classified different European cheeses from Spain, Italy and France (Camin et al., 2004) and Peretta cheese from Sardinia from other competitors cheeses produces in northern Europe.

The elemental composition of dairy products was first investigated particularly in relation to detect the species providing the milk (Fresno et al., 1995; Benincasa et al., 2008) or determining specific elements, mainly toxic, which can be introduced into the human diet in relation to legal and nutritional implication (Moreno-Rojas et al., 2010; Bakircioglu et al., 2011; Gonza'lez-Marti'n et al., 2011). More recently the elemental composition was applied to characterised of milk and cheese. For example Del Signore et al. (2004) by determining the trace-elemental composition (Li, K, Mn, Se, Rb, Cs) of cheese samples from three Italian regions were able to discriminate the origins of the cheese regardless of the type (cow or sheep). Further, it was possible to differentiate buffalo mozzarela from two areas in southern Italy (Brescia et al., 2005) and to separate of Emmental, Edam and caprine cheeses from several EU countries (Suhaj and Korenovska, 2008).

Only a few studies have combined stable isotope and elemental composition in the dairy sector. In 2003, Pillonel et al. (2003) used several trace elements (e.g., Ca, Mg, Na, K, Cu, Mn, Mo and I) and stable isotope analysis (e.g., δ^2H, $\delta^{13}C^*$, $\delta^{15}N^*$, $\delta^{34}S^*$ and $^{87}Sr/^{86}Sr$) to differentiate Emmental cheese produced in Switzerland, Germany, France, Austria and Finland. Sacco et al. (2009) distinguished Southern Italian cow milk from Central European and found $\delta^{13}C$ values to be the most discriminating parameter. Samples from seven typical alpine and pre-alpine cheeses, six with PDO status, were correctly classified according to origin and type by the use of the stable isotope ratios and mineral elements, while $\delta^{13}C$ in casein and glycerol provided an estimate of the maize uptake in the animals diet, which is important for the PDO protocol compliance (Bontempo et al., 2011). The same research group succeeded in differentiating cow dairy products from two mountain sites with different altitudes (δ^2H, $\delta^{18}O$) and different vegetation ($\delta^{15}N$) (Camin et al., 2012). In addition, an international collaborative study was performed to validate the methods for the isotopic and elemental analysis of cheese, in order to support official recognition of their use in authenticity assessment (Camin et al., 2015).

In Slovenia, the upper Soča/Isonzo valley is a region with PDO certification of cow and sheep cheese. "Tolminc" cheese, also known as "Formaggio di Tolmin", is a traditional full-fat hard cheese made from cow's milk that has been produced since 1756. Another higher priced traditional cheese is produced from the milk of the domestic Bovec sheep that have been bred since the 14th century when it was first mentioned as Caseus De Plezio. It is also a full-fat hard cheese registered in 2012 by the EU and protected under PDO name "Bovški sir". Sometimes goat and cow milk is added (no more than 20%), which has to be correctly declared and labeled. Further, in the Karst region a traditional sheep cheese "Kraški ovčji sir" has also local Slovenian PDO name. These products are greatly appreciated by consumers and thus important PDO certification is obtained in order to protect their authenticity. The origin of these products must be declared by law, but at the moment no analytical technique exist that are capable to prove the correct labeling on the products.

This study aims to verify the applicability of a combination of elemental and stable isotope analysis to trace the origin of Slovenian dairy premium products and thus the authenticity of products on the market. Based on measured parameters and the use of discriminant analysis (DA), milk and cheese from cow, goat and sheep were characterised and classified according to their region of production. The possibility of differentiating between different species was also investigated.

2. Materials and methods

2.1. Samples

In total, 124 samples of milk including cow (76 samples), goat (11 samples) and sheep (37 samples) were systematically collected during May, June and July in 2012 and 2013 from various geographical regions: cow milk from Alpine, Dinaric, Pannonian and Mediterranean, while goat and sheep milk production is limited to only few regions in Slovenia namely: Bovec (Alpine), Karst, Vipava, Brkini (Mediterranean), central Slovenian region (Dinaric). Milk samples were stored at −20 °C prior to analysis. In addition seven samples of milk with mixed origin: sheep-goat-cow (1 sample), sheep-goat (2 samples), sheep-cow (2 samples) and goat-cow (2 samples) were also analyzed. In August 2012, 30 cheese samples were collected including sheep (15 samples), goat (6 samples) and cow (9 samples) produced from the milk collected in May from the same producers. Two types of cheese have EU PDO status: Bovški sheep cheese (Bovški ovčji sir) and cow cheese (Tolminc) and one local PDO status Kraški sheep cheese (Kraški ovčji sir). Authentic Tolminc cow cheeses (9 samples) were collected in May, June, September and December in order to include the maximum variability of production and changes in animal diet.

2.2. Stable isotope analysis

The isotopic composition of C and N in milk and cheese was determined in the casein fraction.

2.2.1. Isolation of casein in milk

Analytical preparation of milk samples was carried out according to the standard procedure (Kornexl et al., 1997). Milk fat was removed by centrifugation (Type Centric 322 A, TEHTNICA, Slovenia, 10 min at 3000 × g) and the casein precipitated from the skimmed milk by acidification at pH 4.3 with 2 N HCl (CARLO ERBA, Val-de-Reuill, Italy) followed by centrifugation (10 min at 3000 × g). The precipitate was rinsed with water produced using a Mili-Q system (Milipore, USA) and freeze-dried.

2.2.2. Isolation of casein in cheese

The fresh cheeses were grated and freeze-dried. 10 g of freeze-dried cheese was extracted using a Soxhlet extractor (8 h of extraction) with petrol ether:ethylether (2:1) mixture (Sigma-Aldrich, Germany). After extraction the sample was centrifuge (e.g. 3000 × g for 6 min) to separate the ether from the residue. After lipid extraction, the skimmed cheese was warmed to 40 °C to remove any possible residual ether. Then the residue was washed twice with 20 mL of water, centrifuging each time (e.g. 3000 × g for 3 min). The residue, made up mainly of casein, was freeze-dried and conserved at room temperature until analysis (Bontempo et al., 2012; Camin et al., 2015).

2.2.3. Isotope ratio mass spectrometry (IRMS) measurements

The isotopic composition of oxygen in milk water was measured directly in milk after equilibration with reference CO_2 at 25 °C for 24 h. Measurements were performed on an IRMS (GV Instruments) with a IsoPrime MultiFlow Bio equilibration unit (IsoPrime, Cheadle Hulme, UK).

Dry casein samples were weighed directly into a tin capsule (Sercon, Crewe, UK), closed with tweezers and put into the automatic sampler of the elemental analyzer. All stable C and N analyses were performed separately on a Europa Scientific 20-20 continuous flow mass spectrometer with an ANCA-SL solid-liquid preparation module (Sercon, Crewe, UK). Samples for C, N and S from 2013 were additionally analysed simultaneously on an

IsoPrime100 – Vario PYRO Cube (OH/CNS Pyrolyser/Elemental Analyser) (IsoPrime, Cheadle, Hulme, UK).

Isotope data are expressed using the conventional δ-notation (‰).[1] The results were calibrated against the following international reference materials: NBS 22 (oil; $\delta^{13}C = -30.03 \pm 0.04$‰), IAEA-CH-7 (polyethylene; $\delta^{13}C = -32.15 \pm 0.05$‰) and IAEA-CH-6 (sucrose; $\delta^{13}C = -10.45 \pm 0.03$‰) for carbon; IAEA-N-1 (ammonium sulfate; $\delta^{15}N = 0.4 \pm 0.2$‰) and IAEA-N-2 (ammonium sulfate; $\delta^{15}N = 20.3 \pm 0.2$‰) for nitrogen; SLAP2 (Standard Light Antarctic Precipitation; $\delta^{18}O = -55.50 \pm 0.02$‰) and GISP (Greenland Ice Sheet Precipitation; $\delta^{18}O = -24.76 \pm 0.09$‰) for oxygen; IAEA-S-5 (barium sulphate; $\delta^{34}S = -0.4 \pm 0.2$‰) and NBS 127 (barium sulphate; $\delta^{34}S = 20.3 \pm 0.4$‰) for sulphur with and without added vanadium pentoxide (International Atomic Energy Agency IAEA, Vienna, Austria). Reproducibility of the measurements based on triplicate was ±0.1‰ for $\delta^{18}O$, ±0.2‰ for $\delta^{13}C$ and ±0.3‰ for $\delta^{15}N$ and $\delta^{34}S$. Data quality control charts were systematically recorded throughout the study period. In addition, to ensure the validity and comparability of the isotope results, we regularly participate in the Food analysis using Isotopic Techniques – Proficiency Testing Scheme FIT-PTS organized by EUROFINS three times per year. In this scheme casein and grated cheese are also included.

2.3. Elemental analysis

Elemental composition was performed on the bulk freeze-dried samples. From 0.5 to 1.0 g of sample material pellets were prepared using a pellet die and hydraulic press. As primary excitation sources, the disc radioisotope excitation sources of Fe-55 (25 mCi) and Cd-109 (20 mCi) from Eckert & Ziegler (Eckert & Ziegler, Valencia, U.S.A.) were used. The emitted fluorescence radiation was measured by an energy dispersive X-ray spectrometer composed of a Canberra Si(Li) detector (Canberra Industries, Meriden, U.S.A.), a Canberra M2024 spectroscopy amplifier (Canberra Industries, Meriden, U.S.A.), Canberra M8075 ADC (Canberra Industries, Meriden, U.S.A.) and PC based Canberra MCA S-100 (Canberra Industries, Meriden, U.S.A.). The spectrometer was equipped with a vacuum chamber. The energy resolution of the spectrometer was 175 eV at 5.9 keV. An analysis of the X-ray spectra was made using the AXIL (IAEA, Vienna, Austria) spectral analysis program (Nečemer et al., 2008; Nečemer et al., 2011).

Quantification was performed utilizing the in-house developed QAES (Quantitative Analysis of Environmental Samples) software (Nečemer et al., 2011). The estimated uncertainty of the analysis was 5% to 10%. This high total estimated uncertainty was a result of matrix correction and geometry calibration procedures, which include errors of tabulated fundamental parameters, and contributions from spectrum acquisition and analysis. The accuracy of the results was checked using reference material BCR 151 (Skimmed milk powder) shown in Table 1 (Potočnik et al., 2016).

2.4. Statistical analysis

Statistical calculations and multivariate analysis were carried out using the XLSTAT software package (Addinsoft, New York, USA). Basic statistics included mean values (median and arithmetic mean), standard deviation (SD), minimum and maximum. Simple statistics including analysis of variance of normally distributed data by ANOVA with Duncan's test for comparisons of means and for not normal distributed data one-way analysis of variance by ranks (Kruskal-Wallis test) were carried out in order to find out statistically significant parameters for possible discrimination among three milk and cheese animal specie origin. Further, for determination of key parameters responsible for differentiation of animal specie origin of milk and cheese and for discrimination of geographical origin of milk and cheese production of each animal species, the multivariate analysis by discriminant analysis (DA) was used.

[1] $\delta\,(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$; where R is the ratio between the heavier and the lighter isotope ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) in the sample and standard. Values are reported relative to the following international standards: for carbon the Vienna Pee Dee Belemnite (VPDB), atmospheric N_2 (AIR) for nitrogen, Vienna Standard Mean Ocean Water (VSMOW) for hydrogen and oxygen and Vienna Canyon Diablo Troilite (VCDT) for sulphur.

Table 1
Determination of element concentrations in reference materials skimmed milk powder (ERM-BD 151) by EDXRF.

Element	Certified value	Found value
dry weight (mg/kg)		
Ca	13900 ± 700	13200 ± 1100
Mg	1260 ± 70	–
Mn	0.29 ± 0.03	–
P	11000 ± 600	9600 ± 900
K	17000 ± 800	16200 ± 1300
Se	0.19 ± 0.04	–
Na	4190 ± 230	–
Sr	–	4.10 ± 0.30
Zn	44.9 ± 2.3	43.0 ± 3.4
Cu	5.00 ± 0.23	–
Cl	9800 ± 1200	9000 ± 900
Cd	0.106 ± 0.013	–
Pb	0.207 ± 0.014	–
S	–	3300 ± 300
Br	–	13.6 ± 1.0
Rb	–	17.0 ± 1.4

3. Results

The elemental composition (P, S, Cl, K, Ca, Zn, Br, Rb and Sr) and stable isotope values ($\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) for cow, goat and sheep milk (Table 2) and cheese (Table 3) are reported as the mean, standard deviation (SD), the range (min-max) and compared to the results available in the literature from other EU countries.

3.1. Composition of cow, goat and sheep milk and cheese samples

In the literature data, the average content of Ca, P, K, Cl and Zn was higher in goat and sheep milk than in cow milk, while the concentration of S was slightly higher in cow than in sheep and goat milk. Bromine was higher in goat than in cow milk (Zamberlin et al., 2012). Our results (Table 2) are in agreement with the literature data regarding the content of Ca, P and Zn, whereas for K and Cl we found higher amounts in goat and cow milk than in sheep milk. The average amount of S and Br in our samples was higher in sheep and goat milk than in cow milk. Raynal-Ljutovac et al. (2008) observed higher K and Cl content in goat milk samples compared to cow and sheep milk samples, which was also observed in our study.

The $\delta^{18}O$ values in Slovenian cow milk samples are comparable with reported values for milk produced in northern and central Europe ($\delta^{18}O$ of −11‰ to −2‰) (Kornexl et al., 1997; Rossmann et al., 1998; Pillonel et al., 2003; Renou et al., 2004). Higher values were observed in the Mediterranean region reflecting the comparatively mild and dry climate. The range in $\delta^{18}O$ values (−5.6 to 1.2‰) for sheep and goat milk was also similar. Higher minimum $\delta^{18}O$ values obtained in goat and sheep milk compared to cow milk were mainly related to water intake. Most of the water intake by the goats and sheep is from fresh pasture plants, whereas water intake for some cows is from groundwater water. Results show a large range in $\delta^{13}C$ and $\delta^{15}N$ values in casein of cow, sheep and goat milk. The $\delta^{13}C$ values vary from −28.2 to −18.6‰ for cow

Table 2
Mean, standard deviation (SD) and the range (min-max) of the elemental and stable isotope composition in cow, sheep and goat milk in our study in comparison to the literature data (Coni et al., 1996; Camin et al., 2004; Knobbe et al., 2006; Crittenden et al., 2007; Camin et al., 2008; Raynal-Ljutovac et al., 2008; Chesson et al., 2010; Bontempo et al., 2011; Zamberlin et al., 2012; Chung et al., 2014; Erich et al., 2015; Stevenson et al., 2015; McLeod et al., 2016).

	Cow milk			Sheep milk			Goat milk		
Element	Mean ± SD	Our study	Literature	Mean ± SD	Our study	Literature	Mean ± SD	Our study	Literature
(mg/100 g)									
Ca	104 ± 20	59–144	107–133	129 ± 19	99–195	136–200	141 ± 31	99–189	106–192
P	73 ± 20	40–108	63–102	88 ± 11	68–111	80–145	88 ± 12	69–110	92–148
K	130 ± 31	73–193	144–178	92 ± 22	60–146	174–190	189 ± 36	118–228	135–235
Cl	81 ± 20	42–126	90–106	72 ± 16	49–100	71–92	170 ± 41	93–226	100–198
S	25 ± 6[a]	13–38	32	33 ± 5[b]	28–45	29	31 ± 5[b]	23–38	28
(μg/100 g)									
Sr	23 ± 8	9–43	–	33 ± 13	17–58	48	37 ± 18	19–77	38
Zn	320 ± 70	197–442	210–550	356 ± 47	263–476	415	400 ± 65	302–512	242
Br	147 ± 84	52–332	154–293	151 ± 67	69–325	–	188 ± 72	82–326	411–507
Rb	178 ± 55	82–304	–	326 ± 212	100–759	–	416 ± 190	160–797	–
Isotope ratio (‰)									
$\delta^{34}S$	4.3 ± 1.0	2.3–6.7	7.7–14.8	4.8 ± 0.8	3.5–5.6	–	4.7 ± 1.2	3.1–6.7	–
$\delta^{18}O$	−3.2 ± 2.1	−7.1–0.3	−13.2–3.0	−2.4 ± 1.7	−5.6–1.2	–	1.6 ± 1.9	−5.3–1.2	–
$\delta^{13}C$	−22.7 ± 2.7	−28.2–(−18.6)	−26.4–(−17.5)	−26.6 ± 1.8	−29.3–(−21.8)	–	−26.3 ± 1.5	−29.2–(−23.7)	–
$\delta^{15}N$	4.7 ± 1.1	2.5–6.4	2.9–6.06	4.2 ± 1.0	2.5–6.5	–	4.7 ± 2.7	−1.8–7.5	–

[a,b]Means in the same row with different superscript letters are statistically significantly different according to Duncan' s test ($p < 0.05$).

Table 3
Mean, standard deviation (SD) and the range (min-max) of the elemental and stable isotope composition in cow, sheep and goat cheese in our study in comparison to the literature data (Manca et al., 2001; Pillonel et al., 2003; Camin et al., 2004; Manca et al., 2006; Suhaj and Korenovska 2008; Moreno-Rojas et al., 2010; Bontempo et al., 2011; Bontempo et al., 2012; Camin et al., 2012; Zamberlin et al., 2012; Camin et al., 2015; Stevenson et al., 2015).

	Cow cheese			Sheep cheese				Goat cheese	
Element	Mean ± SD	Our study	Literature	Mean ± SD	Our study	Literature	Mean ± SD	Our study	Literature
(mg/100 g)									
Ca	1370 ± 90[a]	1290–1510	730–1200	1070 ± 150[b]	784–1330	1110–2100	1530 ± 170[a]	1320–1800	200–1410
P	670 ± 60	600–760	100–810	600 ± 120	470–880	700–1000	730 ± 100	580–870	440–820
K	170 ± 40	110–220	77–160	168 ± 48	90–236	90–210	270 ± 90	190–390	190–390
Cl	1310 ± 400	880–2030	480–1440	1950 ± 490	1310–3020	–	1630 ± 500	770–2200	–
S	320 ± 50	280–400		380 ± 70	310–540	–	340 ± 30	290–370	–
(μg/100 g)									
Sr	230 ± 50[a]	190–300	470–630	350 ± 140[a]	140–530	370	450 ± 150[b]	180–590	470
Zn	5640 ± 300[a]	5300–6100	500–5300	3900 ± 900[b]	2200–5300	3800–9430	5050 ± 400[a]	4500–5500	1300–4500
Br	620 ± 120[a]	500–800	–	890 ± 200[b]	500–1200	–	690 ± 200[a]	370–870	–
Rb	300 ± 50	200–300	140–537	410 ± 340	130–1300	–	450 ± 150	180–590	–
Isotope ratio (‰)									
$\delta^{13}C$	−24.6 ± 0.8	−25.2–(−23.3)	−16.9–(−27.1)	−25.2 ± 0.9	−26.5–(−)23.6	−21.3–(−26.9)	−24.7 ± 1.5	−26.3–(−22.8)	−22.7–(−25.4)
$\delta^{15}N$	4.1 ± 0.4	3.5–4.4	3.0–7.7	4.1 ± 0.8	2.7–5.4	4.5–8.9	5.1 ± 1.7	2.9–7.8	5.5–8.9

[a,b]Means in the same row with different superscript letters are statistically significantly different according to Duncan' s test ($p < 0.05$).

milk, from −29.3 to −21.8‰ for sheep milk and from −29.2 to −23.7‰ for goat milk (Table 2). Variations in $\delta^{15}N$ values (Table 2) were similar for cow and sheep milk (2.5–6.5‰), while for the goat milk the variation in $\delta^{15}N$ values was higher (−1.8 to 7.5‰). Significantly higher $\delta^{13}C$ values were observed in regions where maize silage is fed. These results are further supported by higher $\delta^{15}N$ values. Maize is normally harvested fom intensively fertilized fields. Further the lowest $\delta^{15}N$ values observed in some samples could be due to the presence of leguminous plants and the absence of intensive agricultural practices. The $\delta^{34}S$ values are from 2.3 to 6.7‰ for cow milk, 3.5 to 5.6‰ for sheep milk and 3.1 to 6.7‰ for goat milk (Table 2). These values fall in the range found in European dairy products, which are from 3 to 8‰ (Camin et al., 2004; Pillonel et al., 2004; Rossmann et al., 1998, 2000), but differ significantly from the values of 9–15‰ determined in dairy products from various regions within Australasia (Crittenden et al., 2007).

Among above mentioned parameters only S was normally distributed and accordingly to ANOVA test it was found statistically significant ($P < 0.05$) for possible discrimination of cow, sheep and goat milk. The Duncan test revealed that for S it was possible to discriminate between both sheep and goat from cow milk. On the other hand, Kruskal-Wallis test was performed on the remaining not-normally distributed variance. The $\delta^{18}O$, $\delta^{13}C$, $\delta^{15}N$, Ca, K, Cl, P, Br, Rb and Sr values were statistically significant parameters for distinguishing between goat, sheep and cow milk.

Results of cheese samples are graphically presented in Fig. 1. The concentration ranges of the determined elements are in the frame of those reported in the literature (Table 3). The trend in Ca concentrations (goat > cow > sheep) was the same as that observed in our study, whereas the variation in the concentrations of K, P and Zn was different. The trend in concentrations for K, P and Zn in the literature was: goat > sheep > cow for K and sheep > goat > cow for P and Zn, while in our study the trend was as follows: goat > cow > sheep for K and P and the same as in literature sheep > goat > cow for Zn. The literature data for Cl were available only for cow cheese and compared to our data are lower. The reason for these observed

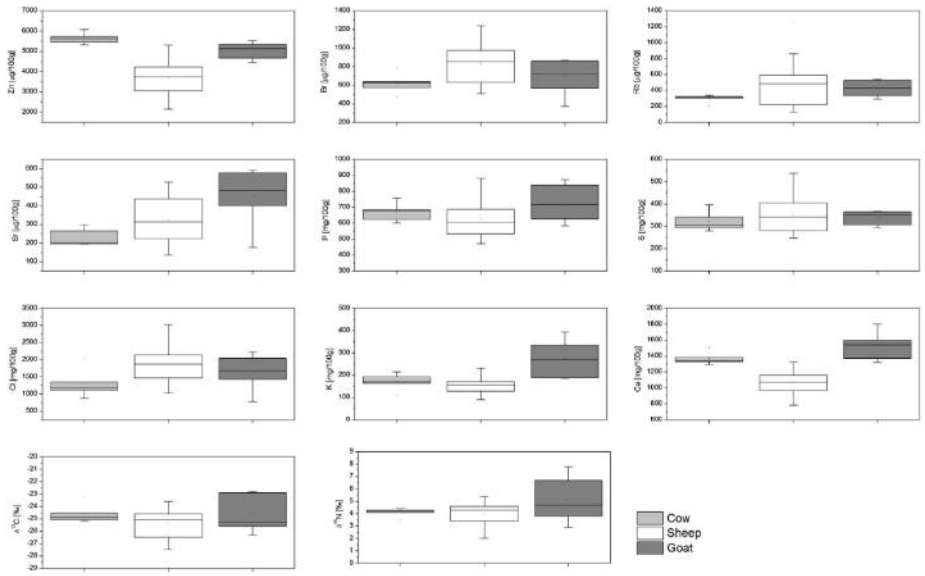


Fig. 1. Box plots of the elemental and stable isotope composition in cow, sheep and goat cheese.

discrepancies in elemental composition could be due to differences in preparation, in particular coagulation conditions, salting, transfer from manufacturing equipment and the concentrates fed to the animals, as well as geographical origin.

Based on the $\delta^{13}C$ and $\delta^{15}N$ values (Table 3) it is not possible to exclude the presence of maize in the diet of some goat and sheep samples (Camin et al., 2008). The highest values were observed in three samples (one sheep, two goat) from Vipava valley, where no limits have been established for maize in the production protocol. In two of the goat samples higher $\delta^{15}N$ content was also observed, which is characteristic for intensive agricultural practice.

Results of ANOVA with the Duncan's test for comparisons of means of normally distributed variances of $\delta^{13}C$, Ca, Cl, P, S, Zn, Br and Sr show that Ca, Zn and Br were significant ($P < 0.05$) in discriminating between the three types of cheeses. Further inspection using the Duncan test reveals that for Ca, Br and Zn, it was only possible to differentiate sheep from both goat and cow cheese. Sr enables the separation of goat from sheep and cow cheese. Parameters with not-normally distributed variances i.e., $\delta^{15}N$, S and Rb were not significant according to the Kruskal-Wallis test.

4. Discussion

Multivariate statistical analysis by DA was applied (Kropf et al., 2010a,b) on the obtained data set (Tables 2 and 3) in order to verify the possibility to discriminate the milk and cheese according to the origin of production. In addition, a multi-elemental and stable isotope approach for differentiating between different species (goat, sheep, and cow) was also tested. $\delta^{34}S$ measurements were performed only on a reduced number of samples and thus they were not included in our statistical evaluation.

4.1. Geographical origin determination

In the case of cow milk, DA modeling was performed on milk samples from four Slovenian geographical regions (Alpine, Dinaric, Pannonian and Mediterranean) in 2012 and 2013. Fig. 2a shows a plot of the first two discriminant functions for the 2012 cow milk samples. Function 1 explains 68.8% of the total variance, while function 2 accounts for 22.4% of the variance. Groups from the Pannonian and Mediterranean regions are well discriminated, while the third group includes a combination of the Alpine and Dinaric regional samples. The main discriminating parameters were Cl, Zn, P and Ca in function 1 and K, Cl, P and Zn in function 2. In the same way we also performed DA treatment of the cow milk data from 2013, however no separation between 4 groups was observed (Fig. 2b). The separation was also not possible when the data from 2012 and 2013 were treated by DA together. One reason is the unusual and unstable weather conditions in May and June 2013, when cattle was not released completely onto pasture and was still on a diet of grass/maize silage.

Fig. 3a shows how it is possible to discriminate between four geographical production locations of 37 sheep milk samples BO-Bovec (15 samples), BR-Brkini (9 samples), KR-Karst (10 samples) and CS-central Slovenia (3 samples). The group of BO, KR and CS samples is well separated between each other, meanwhile BR group of samples slightly overlapped with BO, KR and CS group. Function 1 and 2 explains 53.2% and 30.1% of the variance, respectively. The main discriminating variables were Ca, $\delta^{13}C$, K and Zn for function 1 and Zn, K, $\delta^{15}N$ and $\delta^{13}C$ for function 2. The classification functions reveal that all BO, KR and CS samples were correctly classified (100%) into their original group, while for samples of BR origin 89% correct classification was achieved. Overall correct classification rate was 97%.

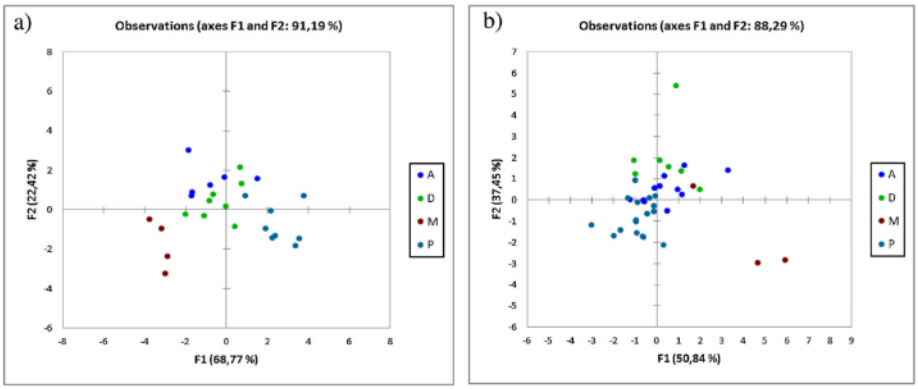


Fig. 2. Results of DA modeling of the data set of 76 samples of cow milk production from Alpine (A), Dinaric (D), Pannonian (P) and Mediterranean (M) geographical region in (a) 2012 in 26 samples, A (N = 6), D (N = 8), P (N = 4) and M (N = 8) and (b) 2013 in 50 samples, A (N = 17), D (N = 7), P (N = 23) and M (N = 3).

Fig. 3b shows the DA plot for the sheep cheese data set (15 samples), according to four production locations (BO-Bovec, BR-Brkini, CS-central Slovenia, KR-Karst). The Groups BO and CS are well separated, while BR and KR form a single group. Function 1 explained 92.6% of the total variance and contains the variables P, S, Zn and $\delta^{13}C$ whereas the parameters that make up function 2, which explains 5.9% of the variance, were: S, P, Cl and Zn. The classification functions reveal that all BO, KR. CS and BR samples were correctly classified (100%) into their original group.

Our results show that an elemental fingerprint is a greater discriminating parameter than stable isotope values for determining the production origin of various types of milk and cheese. These findings are similar to those reported by (Pillonel et al., 2003) who found that stable isotope parameters used to determine the geographical origin of cheese allows the separation of geographically distant regions, while elemental composition allows the discrimination of close regions of production as is the case in Slovenia, where the distances are in the order of a few tens of kilometers up to 150 km.

4.2. Species differentiation

In the case of milk samples, nine macro and micro elements (P, S, Cl, K, Ca, Zn, Br, Rb, Sr) and three stable isotope parameters ($\delta^{18}O$, $\delta^{13}C$ and $\delta^{15}N$) were analyzed by DA (Fig. 4). Similarly the same group of elements as well as the two stable isotope parameters: $\delta^{13}C$ and $\delta^{15}N$, were treated with DA to differentiation between cow, goat and sheep cheeses (Fig. 4), where all 124 samples (76 cow, 37 sheep, 11 goat,) were taking into account. Fig. 4 shows a plot of the first two functions, where function 1 explained 82.2% of the total variance and function 2 the remaining 17.8%. Samples of cow, sheep and goat were well separated, only samples of goat milk show higher dispersion of the data, probably due to the diversity of goat feed contrary to sheep and cow that feed

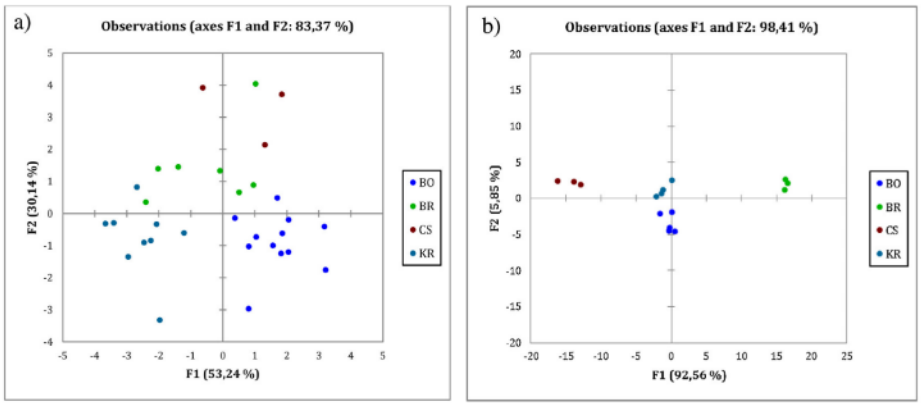


Fig. 3. (a) Discrimination of 37 sheep milk samples according to production origin BO-Bovec (N = 15), BR-Brkini (N = 9), KR-Karst (N = 10) and CS-Central Slovenia (N = 3) (b) DA discrimination of 15 sheep cheese samples according to production location, BO-Bovec (N = 5), BR-Brkini (N = 3), CS-Central Slovenia (N = 3), KR-Karst (N = 4).

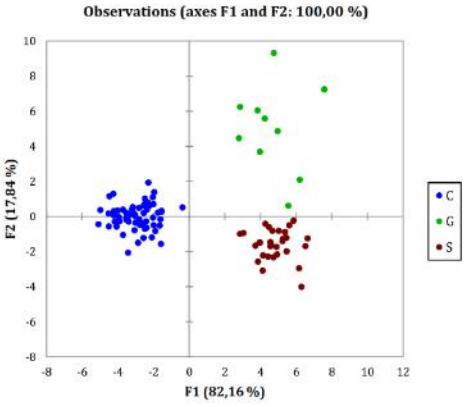


Fig. 4. DA plot of various types of milk (N = 124) of cow (C, N = 76), goat (G, N = 11) and sheep (S, N = 37) species origin.

primarily on grass and clover. Major contributors for such separation were K, Cl, P and $\delta^{18}O$ in function 1 and Cl, S, P and Zn in the second function.

The classification functions can be verified according to the percentage accuracy of each case in its group. All cow and sheep milk samples were correctly classified (100%) into their original group C (cow) or S (sheep), while for samples of goat milk a correct classification of 91% was achieved. The overall correct classification rate was 99%.

The efficiency of the proposed classification procedure was further checked against seven mixed milk samples, sheep-goat-cow (1 sample), sheep-goat (2 samples), sheep-cow (2 samples) or goat-cow (2 samples). These samples were not included in the statistical DA treatment. Classification results are collected in Table 4 and reveal that, with the exception of sample 4, all other mixed samples, with added cow milk, were identified as either pure sheep or goat milk. One mixture of sheep-goat milk was classified as pure sheep milk (number 2) and the other as pure goat (number 6). According to the results, the major part of milk in samples 1, 3, 5 and 7 was goat or sheep milk and only a small amount of cow milk was added, while in sample 4, the majority of the milk is of cow origin. It is likely that more than 20% of cow milk was added to the milk. These results indicated that DA classification could be a useful tool for inspection and identification of the pureness of milk according to its declaration.

Discriminant analysis was also performed on cheese data using the content of nine elements (P, S, Cl, K, Ca, Zn, Br, Rb, Sr) and two stable isotope parameters ($\delta^{13}C$ and $\delta^{15}N$). Fig. 5 shows a plot of the

Table 4
DA classification of seven mixed milk samples: Sheep (S), Goat (G) and Cow (C).

Sample number	Sample description mixture	Predicted group
1	S+G+C	S
2	S+G	S
3	G+C	G
4	G+C	C
5	S+C	S
6	S+G	G
7	S+C	S

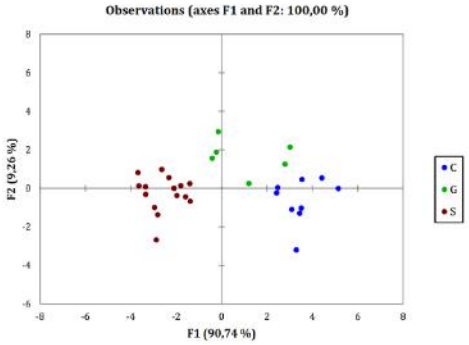


Fig. 5. Discrimination of three type specie origin of 30 cheese samples, cow (C, N = 9), goat (G, N = 6) and sheep (S, N = 15).

first two DA functions for sheep (S), goat (G) and cow (C) cheeses. All three groups show good separation, with function 1 explaining 90.7% of the total variance and function 2 the remaining 9.3%. The major parameters that discriminate the three cheese types were in the first function: Zn, Sr, Ca and Rb and Ca, S Cl and Sr in the second one. Classification functions reveal that all of the goat, sheep and cow samples were correctly classified (100%) into their original G (goat), S (sheep) and C (cow) groups, respectively.

5. Conclusions

The first systematic characterization of authentic Slovenian cow, sheep and goat milk and cheese were defined using a combination of different isotopic ratios of major bioelements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) and elemental composition. This preliminary study highlights the most important parameters, which in DA classification differentiate milk and cheese according to geographical origin and can contribute towards supporting the existence of a Protected Designation of Origin (PDO). It was possible to refuse possible imitations with a correct classification in 97% and 100% of cases of milk and cheese, respectively. Further, results indicated that DA classification could be a useful tool for inspection and identification of the pureness of milk and cheese according to its declaration, since the differentiation between different species was obtained. Based on the obtained data it will be possible to create a traceability model, which could be used to protect our PDO cheeses from mislabeling and to verify the authenticity of commercial products. PDO products are important for the promotion of the local products and in addition to the contribution to local economy, they contribute to the promotion of the culture of the given community as well.

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3.5 Scientific Paper: "Geographical Verification of Slovenian Milk Using Stable Isotope Ratio, Multielement and Multivariate Modelling Approaches"

In this chapter, the paper entitled "*Geographical verification of Slovenian milk using stable isotope ratio, multielement and multivariate modelling approa*ches" by Doris Potočnik, Marijan Nečemer, Igor Perišić, Marta Jagodic, Darja Mazej, Federica Camin, Tome Eftimov, Lidija Strojnik and Nives Ogrinc is presented. The paper was published in *Food Chemistry*, 2020 and represents part of the main body of research performed within the scope of this PhD study.

The work involved determining the geographical origin and authenticity of cow's milk from different geographical regions of Slovenia. A three-year study, from 2012 to 2014, was undertaken and included 277 raw cow's milk samples collected twice per year (winter and summer) from four distinct geographical regions in Slovenia: Mediterranean, Pannonian, Dinaric and Alpine. 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. The milk samples were characterized based on elemental composition (content in Ca, K, Cl, S, P, Zn, Br, Rb, Sr, Mn, Fe, Cu, Se, As, Cd, Pb) and stable isotopes values of δ^{18}O in the cow's milk as well as stable isotope values of δ^{2}H, δ^{13}C, δ^{15}N, δ^{18}O, δ^{34}S in casein.

The objective of this study was to create, implement, update and maintain an isotopic and elemental database of Slovenian cow's milk and study the effect of the production year and season and diet on milk composition in order to differentiate milk according to geographical origin using multivariate statistical methods. The data was also used to develop a model to verify the declaration of Slovenian milk in a real-world application.

It was possible using discriminant analysis (DA) to differentiate milk samples according to production year and season with an overall temporal prediction rate of 84.6%, where δ^{18}O values in milk, S, P, δ^{13}C and δ^{15}N values in casein had the highest discriminatory power. It was also possible to discriminate milk from three geographic regions, although Alpine samples overlap with Dinaric and Pannonian ones. The Mediterranean samples are also well separated from the others due to the higher δ^{18}O values in milk and δ^{34}S values in casein and higher concentrations of Cu. For the Dinaric group, Cl and Br concentrations together with Rb and Se have the highest separation power. The variables that contribute to the separation of the Pannonian group were δ^{2}H values in casein, Mn, δ^{18}O values in casein, Sr, and δ^{13}C values in casein. Prediction ability was the highest for the Pannonian samples (82.1%) and lowest for the Alpine region (26.9%). The overall prediction was 56.4% for regional differences. A model based on data-driven soft independent modelling of class analogy (DD-SIMCA) was also developed and showed that three Slovenian market samples fall out of the acceptance criteria, indicating possible mislabelling. Overall, the valuable information available obtained through this study can be used to increase transparency in the dairy product supply chain.

Parts of this work was presented as an oral presentation at the ISO-FOOD Exploratory workshop – Isotopic techniques in food characterization, 8 – 9 December 2016 in Ljubljana, Slovenia and as a poster presentation at the 9th Jožef Stefan International Postgraduate School Students' Conference and 11th Young Researchers' Day CMBE19 – 20 April 2017 in Ljubljana, Slovenia. The work was also presented as an oral presentation at the 3rd IMECOFOODS International Conference, 1 – 4 October 2017 in Thessaloniki, Greece.

In this study, I was responsible for sample collection, including the samples provided by different Slovenian dairy producers and milk samples available on the market of Slovenian and foreign origin. I also performed all laboratory work, including sample preparation and stable isotope analysis, and I was responsible for conducting the analysis of stable isotope analysis of carbon, nitrogen and sulphur in casein fraction and oxygen in raw milk by IRMS. Further, I prepared samples for hydrogen and oxygen stable isotope measurements at the Fondazione Edmund Mach di San Michele all'Adige in Italy. I was also responsible for analysing, interpreting the data and preparing the manuscript.

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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 (2012–2014). 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 $\delta^{13}C_{cas}$ 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.

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) (Regulation, 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., $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$) 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 et al., 2017) and cereal crops (rice, wheat; Li et al., 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 $\delta^{18}O$ 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 (Chesson, Valenzuela, O'Grady, Cerling, & Ehleringer, 2010; Crittenden et al., 2007). Seasonal variations in isotopic composition are also apparent; for example, in summer, milk water has higher

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$\delta^{18}O$ 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 $\delta^{13}C$ 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 $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$ 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 $\delta^{13}C$ values in milk in combination with other isotopes ($\delta^{15}N$ values) or other analytical parametrs (fatty acids, alfa-linolenic acids, vitamin E) can be used to discriminate between organically and conventionally produced milk (Chung et al., 2018, 2020; Kaffarnik, Schröder, Lehnert, Baars, & Vetter, 2014). The $\delta^{15}N$ values in milk are generally influenced by the intensity of agricultural use, since the $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{34}S$ values generally vary locally and are typical for defined cultivation areas (Crittenden et al., 2007). However, higher $\delta^{34}S$ values are found in arid regions, especially near the sea (high amounts of marine aerosols), or near salt deposits in bedrock (Krivachy et al., 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 of $\delta^{13}C$ and $\delta^{15}N$ values (Scampicchio et al., 2012), while Luo et al. (2016) showed a good separation of milk originating from different countries (Australia, New Zealand, Germany, France, USA and China) using $\delta^{13}C$ and $\delta^{15}N$ in casein and $\delta^{2}H$ and $\delta^{18}O$ in milk water. Further $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ 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 compostion 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, Larcher, Camin, Hölzl, Rossmann, Horn, and Nicolini (2011) successfully distinguished between traditional alpine and pre-alpine Italian cheese. Furthermore, Camin et al. (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 ($\delta^{2}H_{cas}$, $\delta^{18}O_{cas}$, $\delta^{34}S_{cas}$) 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 (Fig. 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

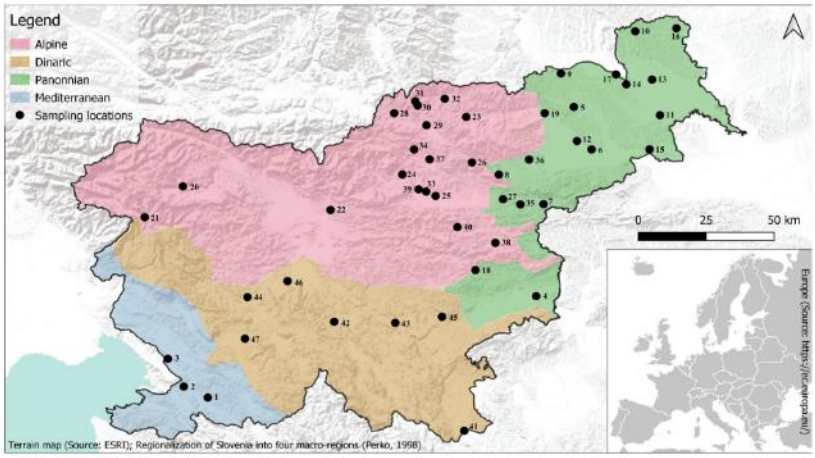


Fig 1. Sampling location of raw cow milk covering different geographical regions in Slovenia.

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 2 M 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 aceton and petrolether (Carlo Erba, Val de Reuil, Italy) and freeze-dried.

2.2.1.1. 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 Eq. (1) (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014):

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

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 ($^2H/^1H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) in the sample (*P-product*) and reference material (*Ref*), respectively. The δ^2H and $\delta^{18}O$ values are reported relative to the V-SMOW (Vienna-Standard Mean Ocean Water) standard, $\delta^{13}C$ values to the V-PDB (Vienna-Pee Dee Belemnite) standard, and the $\delta^{34}S$ sulphur values relative to the V-CDT (Vienna Cañon Diablo Troilite) standard. The $\delta^{15}N$ values are reported relative to AIR.

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

Freeze-dry casein sample from 2012 was weighed to approximately 1 mg in a tin capsule, closed with tweezers and placed into the autosampler of the elemental analyser. Each sample was measured twice and the mean value was considered. All analyses for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ determination were performed separately on a Europa Scientific 20–20 continuous flow IRMS with an ANCA-SL solid–liquid preparation module (Sercone, Crewe, UK). The internal laboratory reference material UreaC (Europa), ($\delta^{13}C$ value of −28.3 ± 0.2‰; $\delta^{15}N$ value of −0.36 ± 0.2‰) was used to normalise the results. The analytical precison, expressed as standard deviation from laboratory reference material, was ± 0.2‰ for $\delta^{13}C$ and ± 0.3‰ for $\delta^{15}N$.

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

The procedure for determining the ratios $^2H/^1H$ and $^{18}O/^{16}O$ in

casein ($\delta^{18}O_{cas}$, $\delta^{2}H_{cas}$) involved transferring 0.2 mg of casein to a silver capsule and analysing the sample simultaneously on DELTA XP IRMS (Thermo Scientific) coupled with a TC/EA pyrolyser (Thermo Finnigan). For normalisation of the results, two internal laboratory reference materials were used: CBS (Caribou Hoof–Standard) with $\delta^{2}H$ values of 157 ± 0.9‰ and $\delta^{18}O$ values of +3.8 ± 0.3‰, and KHS (Kudu Horn Standard) with $\delta^{2}H$ value of −35.3 ± 1.1‰ and $\delta^{18}O$ value of +20.3 ± 0.3‰. 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, Jačimović, & 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.

2.3.2.1. Microwave digestion. An aliquot of dry raw milk (1 mL) was weighed into a quartz tube. To this was added 1 mL of 65% HNO_3, (Merck, Germany, suprapur) and 1 mL of 30% H_2O_2, (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).

2.3.2.2. 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, controle 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 (Potočnik 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, 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; year 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 the new sample and all milk samples that are involved in the study, we can find where it belongs and which milk samples share the most common characteristics. Additionaly, if for some milk sample some of the measurements are missing, we can calculate or assign the values, based on the most similar samples that are obtained from the clustering result. In our case, to calucate the similarity between the milk

Table 1
Mean and standard deviation (SD) of the elemental and stable isotope composition in cow milk in our study from the year 2012, 2013, 2014 in comparison to the min and max range of the literature data (Bontempo, Lombardi, Paolleti, Ziller, & Camin, 2012; Camin et al., 2008; Chesson et al., 2010; Crittenden et al., 2007; Dobrazanski et al., 2005; Griboff et al., 2019; Zamberlin, Antunac, Havranek, & Samaržija, 2012).

	2012				2013			
Mean ± SD	Mediterranean	Pannonian	Alpine	Dinaric	Mediterranean	Pannonian	Alpine	Dinaric
Stable isotopes (‰)								
$\delta^{18}O_w$	−4.9 ± 1.9	−5.9 ± 1.2	−5.2 ± 2.0	−5.2 ± 1.8	−5.6 ± 1.1	−5.9 ± 0.8	−6.2 ± 1.0	−6.1 ± 0.9
$\delta^{18}O_{cas}$					10.1 ± 0.5	10.6 ± 0.8	10.4 ± 0.8	10.4 ± 0.8
$\delta^{2}H_{cas}$					−121 ± 7	−122 ± 10	−125 ± 7	−126 ± 7
$\delta^{13}C_{cas}$	−24.0 ± 3.6	−20.1 ± 1.4	−23.3 ± 2.0	−22.2 ± 2.4	−23.9 ± 2.5	−20.5 ± 1.2	−21.6 ± 1.6	−21.5 ± 2.1
$\delta^{15}N_{cas}$	4.0 ± 0.9	6.0 ± 0.6	5.4 ± 1.3	5.1 ± 1.2	5.3 ± 0.9	6.6 ± 0.6	6.0 ± 0.9	6.3 ± 0.9
$\delta^{34}S_{cas}$					6.3 ± 0.9	4.3 ± 1.5	4.2 ± 1.2	5.1 ± 0.7
Element (mg/100g)								
Ca	86 ± 21	108 ± 29	108 ± 27	103 ± 40	110 ± 10	117 ± 11	119 ± 13	116 ± 12
K	113 ± 30	131 ± 41	136 ± 34	130 ± 39	136 ± 16	147 ± 13	152 ± 16	147 ± 15
Cl	66 ± 18	81 ± 21	84 ± 22	80 ± 25	80 ± 14	84 ± 8	90 ± 9	88 ± 11
S	22 ± 5	26 ± 7	26 ± 7	26 ± 8	25 ± 3	28 ± 4	28 ± 4	29 ± 4
P	66 ± 18	74 ± 20	75 ± 20	72 ± 21	71 ± 13	81 ± 11	78 ± 19	81 ± 11
(μg/100 g)								
Zn	278 ± 65	344 ± 86	331 ± 76	333 ± 125	348 ± 77	401 ± 34	401 ± 44	394 ± 44
Br	159 ± 86	90 ± 30	173 ± 98	170 ± 136	162 ± 90	115 ± 29	152 ± 41	216 ± 66
Rb	133 ± 55	163 ± 46	201 ± 75	186 ± 71	168 ± 69	194 ± 59	203 ± 54	214 ± 56
Sr	23 ± 11	27 ± 10	21 ± 9	19 ± 7	25 ± 6	30 ± 6	32 ± 12	19 ± 7
Mn	1.90 ± 0.64	2.85 ± 0.95	2.16 ± 0.65	2.14 ± 0.67	3.27 ± 0.91	2.89 ± 0.89	2.4 1 ± 0.50	2.70 ± 0.70
Fe	33 ± 11	29 ± 11	30 ± 12	37 ± 20	27 ± 7	26 ± 9	25 ± 5	25 ± 6
Cu	5.83 ± 2.24	5.33 ± 1.38	4.48 ± 1.23	4.79 ± 1.85	5.83 ± 3.23	4.39 ± 1.06	4.06 ± 1.04	5.59 ± 2.99
Se	1.56 ± 0.59	1.77 ± 0.58	1.52 ± 0.49	1.73 ± 0.67	1.80 ± 0.43	1.60 ± 0.36	1.66 ± 0.45	1.91 ± 0.64
As	0.037 ± 0.018	0.032 ± 0.009	0.037 ± 0.014	0.036 ± 0.014	0.034 ± 0.01	0.038 ± 0.014	0.037 ± 0.012	0.046 ± 0.021
Cd	0.006 ± 0.004	0.007 ± 0.006	0.005 ± 0.003	0.012 ± 0.011	0.005 ± 0.003	0.005 ± 0.003	0.005 ± 0.003	0.005 ± 0.003
Pb	0.072 ± 0.044	0.070 ± 0.031	0.071 ± 0.048	0.057 ± 0.031	0.050 ± 0.043	0.051 ± 0.028	0.068 ± 0.037	0.053 ± 0.025

	2014					
Mean ± SD	Mediterranean	Pannonian	Alpine	Dinaric	Our study Min – Max	Literature Min – Max
Stable isotopes (‰)						
$\delta^{18}O_w$	−5.0 ± 1.8	−6.4 ± 0.7	−6.3 ± 1.1	−5.9 ± 1.1	−8.0 to (−)0.3	−13.2 to 3.0
$\delta^{18}O_{cas}$	12.8 ± 1.3	12.4 ± 1.3	11.4 ± 1.1	11.1 ± 0.8	9.0–14.6	9.8–15.9
$\delta^{2}H_{cas}$	−118 ± 9	−121 ± 15	−127 ± 10	−128 ± 10	−149 to 93	−118–(−)79
$\delta^{13}C_{cas}$	−25.5 ± 1.4	−20.8 ± 1.6	−22.3 ± 1.9	−22.0 ± 2.1	28.2 to (−)17.8	−26.4–(−) 17.5
$\delta^{15}N_{cas}$	4.6 ± 0.9	6.0 ± 0.4	5.6 ± 0.8	5.3 ± 0.4	2.5–9.6	2.9–6.06
$\delta^{34}S_{cas}$	4.1 ± 2.3	4.4 ± 0.9	4.3 ± 0.9	5.2 ± 0.6	0.5–7.7	7.7–14.8
Element (mg/100g)						
Ca	107 ± 12	108 ± 8	107 ± 8	107 ± 10	37–169	107–133
K	141 ± 16	140 ± 10	140 ± 10	137 ± 17	57– 195	144–178
Cl	92 ± 18	90 ± 8	95 ± 9	90 ± 12	31–125	90–106
S	31 ± 3	33 ± 4	34 ± 5	32 ± 3	11–58	32
P	110 ± 8	107 ± 13	106 ± 12	105 ± 10	30–118	63–102
(μg/100 g)						
Zn	350 ± 42	404 ± 41	381 ± 39	399 ± 42	121–579	74–530

(continued on next page)

Table 1 (*continued*)

	2014						
	Mediterranean	Pannonian	Alpine	Dinaric	Our study Min – Max	Literature Min – Max
Mean ± SD						
Br	325 ± 45	144 ± 28	165 ± 32	229 ± 56	26–448	52–332
Rb	196 ± 96	197 ± 43	209 ± 68	217 ± 92	39–477	42–88
Sr	23 ± 9	36 ± 9	30 ± 12	24 ± 10	7–61	149–209
Mn	1.91 ± 0.40	2.97 ± 0.79	2.29 ± 0.32	2.15 ± 0.37	0.77–4.79	1.30–6.00
Fe	18 ± 6	25 ± 8	24 ± 7	22 ± 5	13–69	30–80
Cu	3.22 ± 2.59	3.41 ± 0.73	3.11 ± 0.67	3.16 ± 0.48	1.39–10.00	2–60
Se	1.54 ± 0.78	1.75 ± 0.32	1.72 ± 0.42	1.90 ± 0.46	0.59–2.99	0.96–5.30
As	0.023 ± 0.004	0.028 ± 0.006	0.029 ± 0.007	0.026 ± 0.005	0.010–0.099	0.060–5.660
Cd	0.009 ± 0.004	0.007 ± 0.002	0.009 ± 0.004	0.010 ± 0.003	0.0010–0.034	0.173–0.238
Pb	0.057 ± 0.028	0.063 ± 0.016	0.008 ± 0.034	0.063 ± 0.022	0.003–0.225	0.040–0.640

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 & 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 $\delta^{18}O_w$ in the cow milk samples as well as stable isotope values ($\delta^{2}H_{cas}$, $\delta^{13}C_{cas}$, $\delta^{15}N_{cas}$, $\delta^{18}O_{cas}$, $\delta^{34}S_{cas}$) 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 μg/100 g) and the highest in Mediterranean region (5.83 ± 2.24 μg/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 × region; season × 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 $\delta^{18}O_w$ values in raw milk varied over the sampling period from −8.0‰ to 0.3‰, the $\delta^{18}O_{cas}$ values were from 9.0‰ to 14.6‰, and the $\delta^{2}H_{cas}$ values were from −149‰ to −93‰. Statistically significant

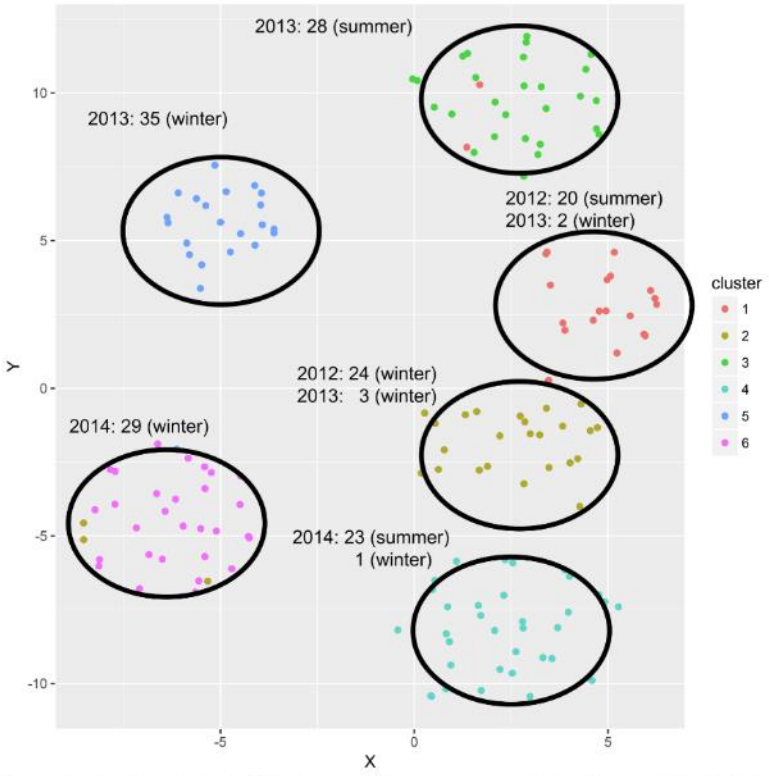


Fig 2. Cluster analysis in milk samples. Results indicate that year and season parameters are the most influence variables for the clustering.

difference was observed in $\delta^{18}O_w$ values according to the year, season, region and interactions therein. The $\delta^{18}O_w$ values were higher in 2012 comparing to 2013 and 2014 and were higher during the summer. Turkey contrasts tests show that the $\delta^{18}O_w$ values were the higest 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 $\delta^{18}O$ 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 (Chung et al., 2020; Crittenden et al., 2007), related to the effect of a high evapotranspiration rate in fresh plant feed and animals during summer. The $\delta^{18}O_{cas}$ values are in agreement with literature values, whereas the $\delta^{2}H_{cas}$ values are lower than literature values. No correlation was observed between $\delta^{18}O_{cas}$ and $\delta^{2}H_{cas}$ 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 et al., 2008). The remaining fraction originates from the diet. No statistically significant difference was observed in $\delta^{18}O_{cas}$ according to the season and region, however in 2013 statistically lower values were observed compared to 2014. For $\delta^{2}H_{cas}$ values the only statistical difference was observed according to the season (p less than 0.05) with the higest values observed in summer and interaction between season and region.

A broad range of $\delta^{13}C_{cas}$ values was observed (Table 1) from −28.2 to −17.8‰. These differences are related to the cows feed – which depends, to a large extent, on the location of the herd. The $\delta^{13}C$ values higher than −23.5‰ indicate the presence of maize (C_4 plant) in the diet (Camin et al., 2008). The $\delta^{15}N_{cas}$ values are also broadly distributed (2.5–9.6‰) during the sampling period. The presence of leguminous plants and the absence of intensive agricultural practices can explain the low $\delta^{15}N_{cas}$ values. Alternatively, the highest $\delta^{15}N_{cas}$ 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 $\delta^{15}N_{cas}$ are supported by the high $\delta^{13}C_{cas}$ 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 $\delta^{13}C_{cas}$ and $\delta^{15}N_{cas}$ values according to the year, season, region and interactions therein. Both higher $\delta^{13}C_{cas}$ and $\delta^{15}N_{cas}$ values were observed in 2013, while during the winter lower $\delta^{13}C_{cas}$ and higher $\delta^{15}N_{cas}$ values were determined. Further, it is also possible to distinguish Mediterranean region from Pannonian, whilst differentiation between the Dinaric and Alpine regions based on $\delta^{13}C_{cas}$ and $\delta^{15}N_{cas}$ values was not possible.

The $\delta^{34}S_{cas}$ values range from 0.5 to 7.7‰ and fall within the range (3–8‰) found in European dairy products (Camin et al., 2016), but are significantly lower than $\delta^{34}S$ values in New Zealand milk that ranged from 7.7‰ to 14.8‰, reflecting seaspray effect (Crittenden et al.,

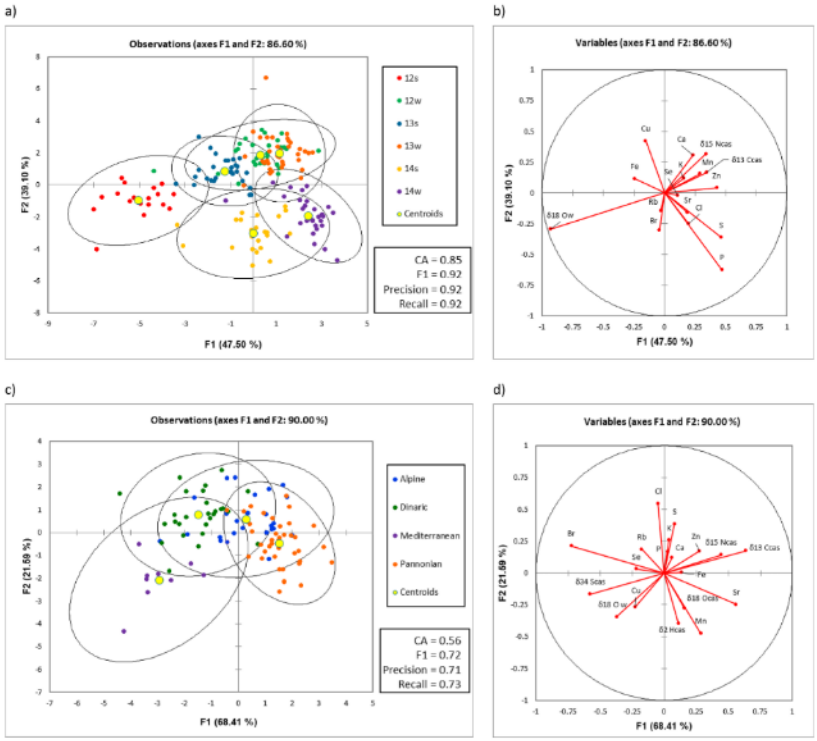


Fig 3. Stable isotope and elemental composition analysis (a): discriminant function score plot of the 176 authentic Slovenian milk samples from summer/winter in 2012 (12s, N = 18, 12w, N = 33), 2013 (13s, N = 31, 13w, N = 37), 2014 (14s, N = 24, 14w, N = 33). Stable isotope and elemental analysis; (b):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

2007). This study found no correlation between $\delta^{15}N_{cas}$ and $\delta^{34}S_{cas}$ values, even though similar factors influence their isotopic fractionation, which suggests that $\delta^{34}S_{cas}$ 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 $\delta^{34}S_{cas}$ values the only statistical difference was observed according to the region and interactions between season and region. Tukey contrasts indicate that based on $\delta^{34}S_{cas}$ 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 $\delta^{18}O_{cas}$, $\delta^{2}H_{cas}$, and $\delta^{34}S_{cas}$ 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 anlysis 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 cal-culated 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 (Fig. 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

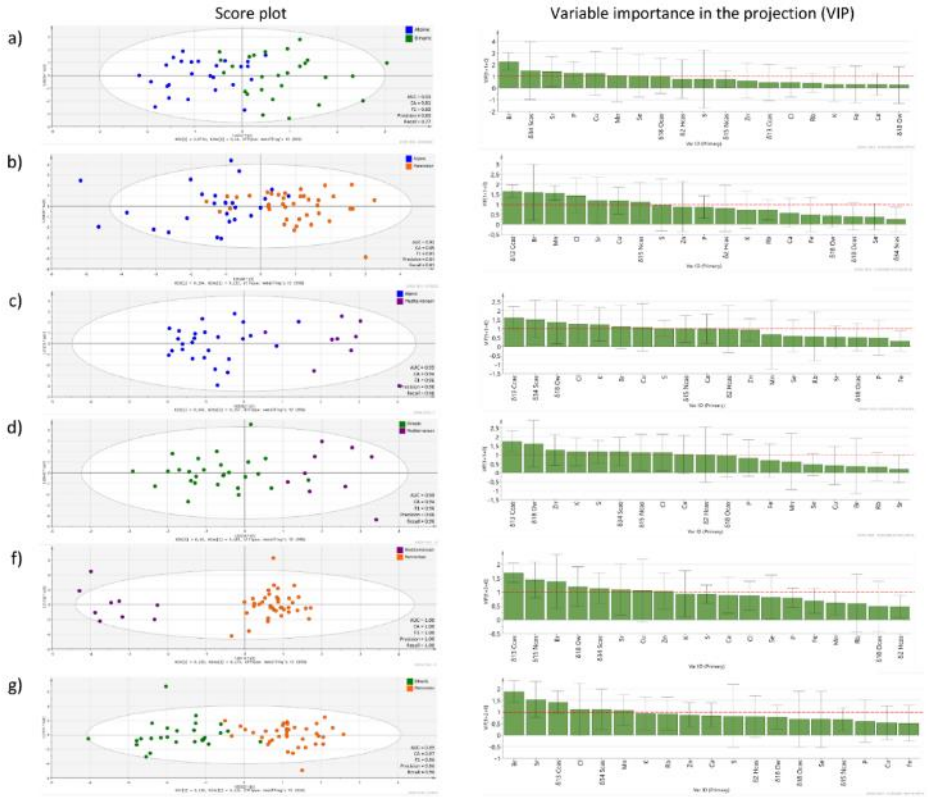


Fig 4. OPLS-DA score plots and VIP values in the pairwise comparison between different region derived from all isotopic and elemental composition data of milk samples. The ellipse on the score plot represents the 95% confidence interval. Red-dotted line indicates a criteria to identify the variable important to the model developed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measurements ($\delta^{13}C_{cas}$, $\delta^{15}N_{cas}$, $\delta^{18}O_w$) and elemental concentrations (P, S, Cl, K, Ca, Mn, Fe, Cu, Se, Zn, Br, Rb, Sr). Fig. 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 $\delta^{18}O_w$ (direction and length of its vector is towards the left part of the biplot). The $\delta^{18}O_w$ 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 (Crittenden 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 $\delta^{13}C_{cas}$ and $\delta^{15}N_{cas}$ 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 perfomed 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 $\delta^{2}H_{cas}$, $\delta^{18}O_w$ and $\delta^{34}S_{cas}$ values. The results are presented in Fig. 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 $\delta^{18}O_w$ and $\delta^{34}S_{cas}$ 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 $\delta^{2}H_{cas}$, Mn, $\delta^{18}O_{cas}$, Sr and $\delta^{13}C_{cas}$ 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%.

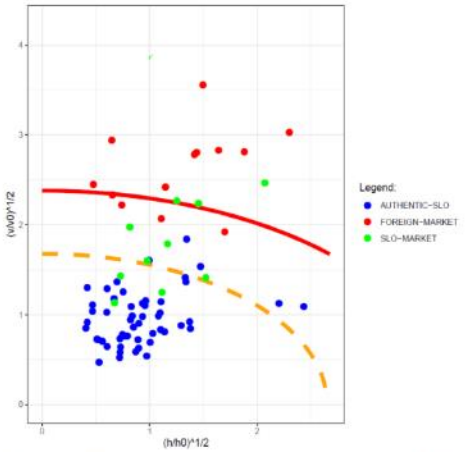


Fig 5. Application of DD-SIMCA for milk authentication. AUTHENTIC-SLO is the target validation set, while FOREIGN-MARKET and SLO-MARKET are two full classes for verification of authenticity. PCA model with 2 PCs. Dotted line is the threshold for $\alpha = 0.05$, for $\alpha = 0.01$.

Further, OPLS-DA tests for pairwise comparisons among all four regions (Fig. 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 $\delta^{13}C_{cas}$ (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 (δ^2H_{cas}, $\delta^{13}C_{cas}$, $\delta^{15}N_{cas}$, $\delta^{18}O_w$, $\delta^{18}O_{cas}$, $\delta^{34}S_{cas}$) 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 authentification 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 declaried as Slovenian were used in the test set. Acceptance areas defined for the give α-values are shown in Fig. 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. Fig. 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 2012–2014, 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.

CRediT authorship contribution statement

Doris Potočnik: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing - original draft. **Marijan Nečemer:** Data curation, Formal analysis, Writing - review & editing. **Igor Perišić:** Investigation. **Marta Jagodic:** Investigation, Validation. **Darja Mazej:** Validation. **Federica Camin:** Investigation, Validation, Writing - review & editing. **Tome Eftimov:** Software, Formal analysis. **Lidija Strojnik:** Formal analysis. **Nives Ogrinc:** Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126958.

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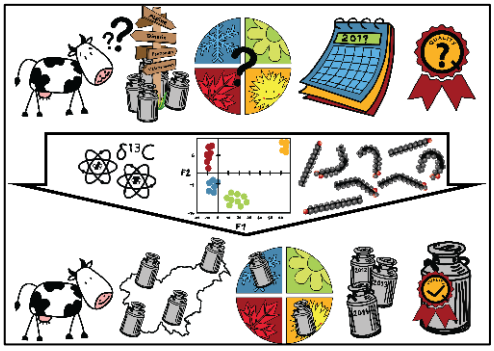
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3.6 Scientific Paper: "Fatty Acid and Stable Carbon Isotope Composition of Slovenian Milk: Year, Season, and Regional Variability"



In this chapter, the paper entitled "*Fatty acid and stable carbon isotope composition of Slovenian milk: year, season, and regional variability*" by Doris Potočnik, Lidija Strojnik, Tome Eftimov, Alenka Levart and Nives Ogrinc is presented. The paper is published in the journal *Molecules* and contains part of the core research of my PhD study.

The main objective of this study was to evaluate both the need and options for origin tracing by focusing on Slovenian milk. In 2016, the Slovenian Ministry of Agriculture, Forestry and Food began a three-year project to promote and raise awareness of the importance of, characterize and to promote understanding and appreciation locally produced and processed foods. During this time, the protective mark "Selected Quality" national scheme and the "Selected Quality - Slovenia" was established. Nowadays, most milk and dairy products, produced and processed in Slovenia, use the "Selected Quality - Slovenia" mark. In this study, two methods: fatty acid composition and isotope ratios of carbon within fatty acids, were applied in order to understand the extent and robustness of using the stable isotope approach for regional traceability. Additionally, the effect of the production year and season on the FA composition of milk was also investigated. Finally, the FA profiles as pertains to the milk's essential and beneficial components was also studied, and a comparative study made to examine the differences in the quality of milk from different geographic regions in Slovenia.

A total of 231 raw cow's milk samples were analysed twice per year, in winter and summer covering four distinct geographical regions: Mediterranean, Alpine, Dinaric and Pannonian. A discriminant analysis model based on FA composition was able to discriminate milk according to the year/season of production (86.9%), while geographical origin discrimination was less successful (64.1%). The stable isotope composition of FAs also proved to be a better biomarker of metabolic transformation processes in ruminants than for discriminating between production origin. Milk from Alpine and Mediterranean regions are healthier due to its higher content of ω-3 polyunsaturated fatty acid and conjugated linoleic acid.

Parts of this work were presented as a poster presentation at the 8th Young Researchers' Days 2014, 18 February 2014 in Ljubljana, Slovenia, as an oral presentation at the workshop "CRP-Projekt-V4-1108: Uporaba specifičnih metod za ugotavljanje in preprečevanje potvorb mleka in mlečnih izdelkov", 12 June 2014 in Ljubljana, Slovenia and as a poster presentation at the scientific 38th International Symposium of environmental analytical chemistry, 17 – 20 June 2014 in Lausanne, Switzerland. It was also given as an oral and poster presentation at the 7th Jožef Stefan International Postgraduate School Students' Conference, 20 – 22 May 2015 in Ljubljana, Slovenia and as a poster presentation at scientific conference 5th MS Food Day, 11 – 13 October 2017 in Bologna, Italy.

My contribution included sample preparation and determining the content and stable isotope composition of individual FA using GC-FID and GC-C-IRMS techniques,

respectively. I was also responsible for evaluating and interpreting the data and preparing the manuscript.

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Fatty Acid and Stable Carbon Isotope Composition of Slovenian Milk: Year, Season, and Regional Variability

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Abstract: This study examined the percentage and stable isotope ratios of fatty acids in milk to study seasonal, year, and regional variability. A total of 231 raw cow milk samples were analyzed. Samples were taken twice per year in 2012, 2013, and 2014, in winter and summer, covering four distinct geographical regions in Slovenia: Mediterranean, Alpine, Dinaric, and Pannonian. A discriminant analysis model based on fatty acid composition was effective in discriminating milk according to the year/season of production (86.9%), while geographical origin discrimination was less successful (64.1%). The stable isotope composition of fatty acids also proved to be a better biomarker of metabolic transformation processes in ruminants than discriminating against the origin of milk. Further, it was observed that milk from Alpine and Mediterranean regions was healthier due to its higher percentage of ω-3 polyunsaturated fatty acid and conjugated linoleic acid.

Keywords: milk; fatty acids; stable carbon isotope composition; GC-FID; CSIA; statistical analyses

1. Introduction

There is an increasing interest in the quality of foodstuffs, and agricultural research continues to focus on improving the nutrient profile of food products. Milk is a valuable commodity that is consumed directly, as well as being a minor and major ingredient in many food products [1]. It is also rich in nutrients, a resource of proteins (2.6–4.2%), vitamins (0.1%), minerals (0.7%), and fatty acids (95–96% of total milk fat), and is considered by many as an essential part of a healthy diet [2,3]. In Europe, milk provides 9% of the dietary energy supply, 19% of the dietary protein supply, and between 12–14% of dietary fat supply [4].

According to the Agriculture and Rural Development, Analysis and Outlook Unit European's farms produced 172.2 million tonnes of raw milk in 2018, a number that they expect to increase to 179 million tonnes by 2030, but at a slower pace (+0.6%) than in 2008–2019, and represents a year-on-year increase of 1.6 million tonnes (+1% per year on average) [5]. In Slovenia, dairy farmers produced 564,000 tons of cows' milk in 2019 (1.2% less than in 2018), and 164,000 tons of drinking milk, which represents a decrease of more than 2% compared to 2018. Despite the decrease, imports increased by almost 13% [6]. Consumers prefer milk with favorable sensory qualities, such as flavor, which depends on animal management factors, including breed, lactation stage, processing techniques, and

diet regime. However, diet is considered the primary and the most sensitive factor responsible for creating a specific milk flavor [7] and also a significant factor affecting its fatty acid (FA) composition. These differences also reflect differences in regional dairy production systems, and linking a milk product to a specific region is of high importance as a recognition of quality [1]. Quality linked to geographical origin has created significant interest in building local and regional food systems across Europe, including Slovenia.

Lipid content and FA profile are considered to have an important role in the human diet [7,8]. For example, certain milk FAs, such as linoleic and α-linolenic FAs (C18:2ω-6 and C18:3ω-3), are essential and cannot be synthesized by the human body [2]. Further, a lower proportion of saturated fatty acids (SFA) and a higher proportion of unsaturated fatty acids (UFA), especially polyunsaturated fatty acids (PUFA), is desirable [9]. The consumption of SFA (especially lauric (C12:0), myristic (C14:0), and palmitic acids (C16:0)) [9] is associated with cardiovascular disease, while UFAs are considered as beneficial for human health. Other functional food components are omega-3 polyunsaturated FA (ω-3 PUFAs), which improve neurological functions and are essential in early human development years, and conjugated linoleic acid (CLA), which has anti-carcinogenic, anti-diabetic, anti-atherogenic effects [10,11].

Farmers can significantly alter the FA composition of milk by managing the nutrition of their dairy cows [12]. Fresh forages (especially grass) in the diet enhance the concentration of unsaturated FA, such as α-linoleic acid and CLA, and decrease the levels of SFA, while adding grass conservation products (hay or silage) increases the proportion of SFA in milk [13]. Besides, the total SFA concentration of milk fat is usually lower in summer compared to winter due to the higher consumption of summer forage crops that have a higher content of PUFAs. The higher content of monounsaturated fatty acids (MUFA) and PUFA in milk from cows grazing in highland regions can be attributed to differences in the botanical composition since higher botanical diversity and energy shortage in cows grazing in mountain pastures modifies the bacterial population and lipid accumulation of UFAs in the rumen [14]. Fatty acid composition of milk fat has also been used as a criterion to detect adulteration of milk, such as the substitution of part of the fat or proteins of plant or animal origin and admixtures of milk of different species. The method to characterize the matrixes according to the animal species can be greatly improved when combined with the content of triacylglycerols [15].

The effects of feeding regime can also be tracked using stable isotope analysis using isotope ratio mass spectrometry (IRMS). In dairy products, the isotope composition is also primarily determined by the cows' diet, as well as their metabolisms [16]. Differences in stable C isotope abundance is mainly the result of the different ingested proportion of feeds derived from C_3 and C_4 plants. The metabolism of C_4 plants, such as corn or sugar, uses a different biosynthetic pathway to fix atmospheric CO_2 compared to C_3 plants (grass and cereals), resulting in the accumulation of the heavier ^{13}C isotope compared to C_3 plants, which leads to higher $\delta^{13}C$ values. These differences also show up in the isotopic composition of FAs [17,18]. Since the spectrum of plants used for feeding purposes depends mainly on local soil and climatic condition, stable isotope composition can also encompass information about the geographical origin of dairy products useful for authenticity control.

Up to now, studies have focused on stable isotope ratio measurements of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values in casein, $\delta^{2}H$, $\delta^{18}O$ in milk water [19–22] and Sr in dry milk samples [21]. One of the most recent significant advances has been the development of compound-specific stable isotope analysis (CSIA) that enables the identification of relevant biomarkers, such as fatty acids, in food authenticity studies. Some studies have even looked at the correlation between long-chain FAs (α-linolenic FA-C18:3ω-3, eicosapentaenoic FA-C20:5ω-3) and stable isotope measurements to distinguish between organic and conventionally produced milk [17,23]. In another study, the authors investigated the relationship between the carbon isotope compositions of CLA in milk and fatty acids in different dietary sources (C_3 versus C_4 plants) to understand the transformation of FA from diet to milk [17]. The results suggested that the $\delta^{13}C$-change in CLA did not originate from the microbial biohydrogenation in the rumen, but most probably from endogenous desaturation of *t*11–18:1.

Only a few studies, however, have focused on $\delta^{13}C$ and $\delta^{2}H$ values in milk fatty acids. Ehtesham and co-workers [24] demonstrated that $\delta^{2}H$ values in precipitation were highly correlated with the $\delta^{2}H$ values in bulk milk and specific fatty acids (butyric, myristic, palmitic, and oleic acid). Separation of milk powder origin to geographic sub-regions within New Zealand has been achieved, with clear discrimination between South Island and North Island and with significant clustering of regions. Further, a linear discriminant analysis (LDA) model based on $\delta^{2}H$ and $\delta^{13}C$ values of butyric, myristic, palmitic, and oleic fatty acids has provided the best separation of samples from the Northern and Southern parts of New Zealand [1]. Ehtesham et al. [25] also focused on the relationship between $\delta^{2}H$ and the FAs in milk and the water component of milk (feed and farm water). The authors found that the $\delta^{2}H$ values of FAs in feed and $\delta^{2}H$ values of farm water were related to the $\delta^{2}H$ values of milk FAs, milk solids, and milk water and could be used as a biogeochemical marker surrogate to environmental conditions. This approach can also be applied to determine the dietary regime and geographical origin of milk products where the water has been removed (milk powder) or is altered in the case of processed milk products (cheese and butter).

The main objective of this study was to evaluate both the need and options for origin tracing, focusing on Slovenian milk. In 2016, the Slovenian Ministry of Agriculture, Forestry, and Food began a three-year project to promote and raise awareness of the importance and characteristics of locally produced and processed foods. During this time, the protective mark "Selected Quality" national scheme and the "Selected Quality-Slovenia" was established. Nowadays, most milk and dairy products, produced and processed in Slovenia, use the "Selected Quality - Slovenia" protective mark. This mark indicates that the products have the following distinguishing properties: (i) the origin of raw milk—the milk and other dairy products are produced entirely in the same country, Slovenia; (ii) milk quality—excellent microbiological quality of milk; (iii) freshness of raw milk—it's 15 h at most from the takeover of milk at the collection site to its acceptance into the processing plant. Therefore, to protect the obtain mark and prevent possible fraud, a robust screening method to determine the authenticity and regional traceability of milk is needed. In this study, two methods (fatty acid composition and isotope ratios of carbon within fatty acids) were applied. Additionally, the effect of the production year and season on the FA composition of milk was also investigated. Finally, the FA profiles pertaining to the milk's essential and beneficial components were also studied, and a comparative study was made to examine the differences in the quality of milk from different geographic regions in Slovenia.

2. Results and Discussion

2.1. Milk Fatty Acids Composition

2.1.1. Influence of the Year and Season of Production and Impact of Feed

The amount of individual fatty acids, as well as SFA, MUFA, PUFA, ω-3 PUFA, ω-6 PUFA, and ω-6:ω-3, according to year, season, and feeding regime (corn/non-corn) are given in Table 1. As expected, SFAs were the most abundant FA groups in milk, followed by MUFA and PUFA. The most abundant FA was C16:0, ΣC18:1 *cis* and *trans* isomers, C18:0, and C14:0, which is in agreement with the published data [26,27]. Time (year/season) was revealed as an important factor influencing fatty acid composition, but not all fatty acids were equally affected. The effect of the year of production (2012, 2013, 2014) on FA composition was investigated using the thirty-five FAs detected in both summer and winter. The results revealed a significant variation in the amount of FA with year except for C12:0, C13:0, C14:0, C14:1, C15:0, C15:0, C17:0, C18:2ω-6, C18:3ω-3, and C22:1ω-9. The year 2012 produced the lowest amount of ∑SFA (67.76 ± 2.49% of total FA) and the highest amount of ∑MUFA (27.26 ± 2.10% of total FAs) and CLA (rumenic acid-C18:2*c*9,*t*11; 0.86 ± 0.28% of total FAs). Furthermore, 2012 also had the lowest percentage of C16:0 (32.60 ± 2.13% of total FAs) and hypercholesterolemia FAs (HFA; C12:0 + C14:0 + C16:0) (48.13 ± 2.97% of total FA) and the highest percentage of *cis* and *trans* isomers C18:1ω-9 (23.7 ± 2.17% of total FA).

Table 1. The percentage of fatty acid (FA) in Slovenian milk according to the year (2012, 2013, and 2014), season (winter (12 w, 13 w, 14 w), summer (12 s, 13 s, 14 s), and type of feed. Influence of the feed type on milk FA composition [9,12,13,28].

		2012	2013	2014	Winter	Summer	Corn	Non-Corn	*p*-Value[1]	
FA (% of Total FA)	**Feed Type**	**Mean ± SD**							**Year**	**Season**
SFA										
C6:0	Corn	1.25 ± 0.26	1.20 ± 0.46	1.30 ± 0.44	1.44 ± 0.32	1.05 ± 0.37	1.30 ± 0.39	1.12 ± 0.40	*	***
C8:0	Corn, concentrates	1.22 ± 0.08	1.36 ± 0.41	1.22 ± 0.34	1.28 ± 0.26	1.25 ± 0.37	1.29 ± 0.33	1.20 ± 0.28	***	*
C10:0	Corn, concentrates	3.03 ± 0.25	3.57 ± 1.02	3.18 ± 0.82	3.28 ± 0.62	3.23 ± 0.95	3.33 ± 0.81	3.07 ± 0.74	**	*
C11:0	Corn, concentrates	0.04 ± 0.03	0.06 ± 0.03	0.08 ± 0.02	0.06 ± 0.02	0.05 ± 0.04	0.06 ± 0.03	0.05 ± 0.03	***	***
C12:0	Corn, concentrates	3.57 ± 0.33	4.10 ± 0.80	3.90 ± 0.82	3.97 ± 0.61	3.74 ± 0.80	3.94 ± 0.67	3.65 ± 0.79	NS	***
C13:0		0.11 ± 0.01	0.11 ± 0.03	0.11 ± 0.03	0.12 ± 0.02	0.10 ± 0.03	0.11 ± 0.03	0.10 ± 0.02	NS	***
C14:0	Corn	12.0 ± 0.65	12.8 ± 1.86	12.9 ± 1.26	12.9 ± 0.94	12.3 ± 1.70	12.6 ± 1.41	12.5 ± 1.39	NS	***
C15:0iso	Hay, grass silage	0.32 ± 0.05	0.34 ± 0.13	0.34 ± 0.05	0.32 ± 0.05	0.35 ± 0.12	0.32 ± 0.09	0.37 ± 0.07	***	***
C15:0aiso	Hay, grass silage	0.51 ± 0.08	0.53 ± 0.09	0.56 ± 0.09	0.50 ± 0.07	0.56 ± 0.10	0.50 ± 0.07	0.61 ± 0.11	NS	***
C15:0	Hay, grass silage	1.17 ± 0.11	1.24 ± 0.13	1.33 ± 0.15	1.24 ± 0.14	1.25 ± 0.16	1.21 ± 0.12	1.34 ± 0.16	NS	NS
C16:0	Calcium salts of palm oil, canola and soy, corn	32.60 ± 2.13	32.7 ± 2.48	33.2 ± 2.11	34.1 ± 1.73	31.6 ± 2.01	32.9 ± 1.86	32.4 ± 3.04	***	***
C17:0iso	Hay, grass silage	0.37 ± 0.07	0.50 ± 0.06	0.53 ± 0.06	0.44 ± 0.10	0.49 ± 0.09	0.45 ± 0.09	0.51 ± 0.10	***	***
C17:0aiso	Hay, grass silage	0.47 ± 0.05	0.44 ± 0.06	0.48 ± 0.07	0.44 ± 0.05	0.48 ± 0.07	0.45 ± 0.05	0.51 ± 0.08	***	***
C17:0	Hay, grass silage	0.61 ± 0.08	0.65 ± 0.11	0.69 ± 0.15	0.63 ± 0.10	0.67 ± 0.14	0.61 ± 0.09	0.73 ± 0.15	NS	***

		2012	2013	2014	Winter	Summer	Corn	Non-Corn	*p*-Value[1]	
FA (% of Total FA)	**Feed Type**				**Mean ± SD**				**Year**	**Season**
C18:0	Fresh herbage, grass silage, concentrates	10.2 ± 1.01	9.87 ± 1.17	9.99 ± 1.66	9.41 ± 0.90	10.6 ± 1.36	9.97 ± 1.06	10.2 ± 1.81	***	***
C20:0		0.18 ± 0.02	0.19 ± 0.15	0.14 ± 0.08	0.16 ± 0.05	0.18 ± 0.13	0.17 ± 0.11	0.17 ± 0.07	NS	***
C21:0		0.03 ± 0.01	0.05 ± 0.07	0.02 ± 0.02	0.04 ± 0.03	0.03 ± 0.05	0.03 ± 0.05	0.03 ± 0.03	***	NS
C22:0		0.07 ± 0.02	0.04 ± 0.04	0.03 ± 0.03	0.05 ± 0.03	0.05 ± 0.04	0.05 ± 0.03	0.05 ± 0.03	***	NS
C23:0		0.03 ± 0.01	0.04 ± 0.05	0.03 ± 0.03	0.04 ± 0.04	0.03 ± 0.02	0.03 ± 0.04	0.04 ± 0.03	***	***
C24:0		0.05 ± 0.01	0.04 ± 0.04	0.04 ± 0.03	0.05 ± 0.02	0.04 ± 0.03	0.04 ± 0.03	0.05 ± 0.03	***	NS
∑SFA	Conserved forage and concentrate rich diet	67.76 ± 2.49	69.90 ± 2.99	70.10 ± 2.74	70.40 ± 2.06	68.04 ± 3.20	69.43 ± 2.61	68.71 ± 3.60	***	***
MUFA	Rapeseed, seeds oils, fresh grass, non-conserved forage									
C12:1		0.12 ± 0.03	0.10 ± 0.03	0.09 ± 0.03	0.12 ± 0.03	0.09 ± 0.03	0.10 ± 0.03	0.10 ± 0.04	***	***
C14:1		1.04 ± 0.11	1.29 ± 1.41	1.17 ± 0.25	1.18 ± 0.18	1.15 ± 1.17	1.19 ± 0.95	1.12 ± 0.29	NS	***
C16:1		2.03 ± 0.11	1.85 ± 0.20	1.94 ± 0.17	1.92 ± 0.14	1.96 ± 0.21	1.95 ± 0.18	1.92 ± 0.18	***	NS
C17:1		0.28 ± 0.03	0.34 ± 0.05	0.37 ± 0.06	0.31 ± 0.06	0.34 ± 0.07	0.32 ± 0.05	0.36 ± 0.07	***	***
C18:1ω-9c/9t	Grass, rapeseeds	23.7 ± 2.17	20.12 ± 2.8	20.7 ± 2.56	20.5 ± 2.01	22.6 ± 3.38	21.4 ± 2.76	21.9 ± 3.42	***	***
C20:1ω-9		0.04 ± 0.01	0.25 ± 0.17	0.14 ± 0.08	0.12 ± 0.09	0.17 ± 0.17	0.14 ± 0.13	0.16 ± 0.16	***	NS
C22:1ω-9		0.01 ± 0.01	0.02 ± 0.02	0.04 ± 0.03	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	NS	***
∑MUFA		27.26 ± 2.10	23.97 ± 2.83	24.42 ± 2.38	24.20 ± 1.87	26.30 ± 3.23	25.10 ± 2.71	25.54 ± 3.17	***	***
PUFA										

		2012	2013	2014	Winter	Summer	Corn	Non-Corn	*p*-Value[1]	
FA (% of Total FA)	**Feed Type**	**Mean ± SD**							**Year**	**Season**
CLA (*c*-9, *t*-11 C18:2)	Grass silage, fish oil, rapeseed, linseed	0.68 ± 0.28	0.61 ± 0.26	0.55 ± 0.20	0.51 ± 0.12	0.72 ± 0.31	0.55 ± 0.19	0.78 ± 0.31	***	***
ω-6 PUFA	Corn, hay, straw,									
C18:2ω-6c	Grassing, red clover silage, corn	2.00 ± 0.22	2.09 ± 0.32	1.98 ± 0.28	1.94 ± 0.23	2.11 ± 0.30	2.05 ± 0.24	1.95 ± 0.35	NS	***
C18:3ω-6		0.11 ± 0.02	0.43 ± 0.29	0.23 ± 0.12	0.20 ± 0.12	0.32 ± 0.30	0.24 ± 0.21	0.31 ± 0.28	***	***
C20:3ω-6	Fish oil	0.08 ± 0.01	0.06 ± 0.04	0.06 ± 0.04	0.06 ± 0.04	0.06 ± 0.04	0.07 ± 0.04	0.06 ± 0.03	***	NS
C20:4ω-6	Fish oil	0.12 ± 0.02	0.03 ± 0.02	0.03 ± 0.03	0.06 ± 0.05	0.06 ± 0.05	0.06 ± 0.05	0.06 ± 0.05	***	NS
∑ω-6 PUFA		2.31 ± 0.24	2.61 ± 0.58	2.30 ± 0.33	2.27 ± 0.26	2.55 ± 0.53	2.41 ± 0.39	2.38 ± 0.54	***	***
ω-3 PUFA	Grassing,									
C18:3ω-3	Grassing, red clover silage, legume silage, cereals, oil seeds	0.62 ± 0.20	0.69 ± 0.33	0.58 ± 0.20	0.54 ± 0.14	0.72 ± 0.30	0.57 ± 0.23	0.78 ± 0.25	NS	***
C20:3ω-3	Fish oil	0.02 ± 0.01	0.10 ± 0.09	0.09 ± 0.03	0.06 ± 0.05	0.07 ± 0.08	0.07 ± 0.07	0.05 ± 0.03	***	NS
C20:5ω-3	Fish oil, linseed	0.06 ± 0.02	0.05 ± 0.05	0.04 ± 0.03	0.05 ± 0.02	0.05 ± 0.06	0.05 ± 0.04	0.07 ± 0.06	***	NS
∑ω-3 PUFA		0.70 ± 0.22	0.84 ± 0.34	0.71 ± 0.21	0.65 ± 0.15	0.85 ± 0.32	0.69 ± 0.25	0.90 ± 0.25	***	***
∑PUFA		3.69 ± 0.50	4.05 ± 0.98	3.55 ± 0.56	3.43 ± 0.33	4.11 ± 0.87	3.65 ± 0.65	4.06 ± 0.86	***	***
ω-6:ω-3		3.64 ± 1.14	3.36 ± 0.79	3.80 ± 3.18	3.71 ± 1.19	3.48 ± 2.58	3.87 ± 2.14	2.87 ± 1.35	NS	***

SFA = saturated fatty acids; MUFA = monosaturated fatty acids; PUFA = polystaurated fatty acids;
[1]NS = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

The percentage of PUFA, ω-6 PUFA, and ω-3 PUFA was higher in 2013 (4.05 ± 0.98%, 2.61 ± 0.58%, 0.84 ± 0.34% of total FAs, respectively) compared to 2012 (3.69 ± 1.14%, 2.31 ± 0.24%, 0.70 ± 0.22% of total FAs, respectively) and 2014 (3.55 ± 0.56%, 2.30 ± 0.33%, 0.71 ± 0.21% of total FAs, respectively), while the ratio ω-6:ω-3 was lower in 2013 (3.36 ± 0.79% of total FAs) in comparison to 2012 (3.64 ± 1.14% of total FAs). The difference in FA composition according to production year depended mainly on diet and altitude, whereas animal-related factors, i.e., lactation stage, breed, milk yield, and proportion of primiparous cows in the herd, were not significant [28].

A lower C16:0 and HFA percentage, as well as a higher amount of CLA and C18:1ω-9, most likely results from increased grazing [9]. However, the amount of each FA depends on lipid metabolism in the rumen and the mammary gland and the release of FAs from body reserves during a period of negative energy balance during early lactation since diet (especially type of forage, lipid supplements, forage:concentrate ratio, starch level, and their interactions) can influence metabolic processes in the rumen and the composition of rumen biome [9]. Variations in FA composition can also be explained, in part, by the micro-environmental conditions influencing the quality of feed. The composition of forage and plant lipids is influenced by factors, such as species, growth stage, temperature, and light intensity. Besides, environmental temperature and humidity can affect both milk yield and FA composition since dairy cows are sensitive to climatic variations. The production of the short-chain FAs (C6:0–C8:0) and C18:0 decrease at the higher temperatures and humidity. In this study, 2013 and 2012 were exceptionally hot and wet years, respectively, and there were no differences in the levels C6:0–C10:0 FAs (Table 1).

The results showed no significant seasonal effects ($p > 0.05$) on the percentage of C15:0, C16:1, C20:1ω-9, C20:3ω-6, C21:0, C20:3ω-3, C20:4ω-6, C20:5ω-5, C22:0, and C24:0, although the percentage of total SFAs was lower in summer (68.04 ± 3.20% of total FA) than in winter (70.40 ± 2.06% of total FA, $p < 0.05$). The percentage of MUFAs of total FA was significantly higher ($p < 0.05$) in summer (26.30 ± 3.23%) than in winter (24.20 ± 1.87%) due to increased amounts of C18:1 in summer compared to winter ($p < 0.05$). The percentage of PUFAs in summer (4.11 ± 0.87% of total FA) was higher than in winter (3.43 ± 0.33% of total FA, $p < 0.05$). On average, amounts of SFA decreased in the summer and increased in the winter, whereas the amount of PUFAs increased in summer and decreased in winter. The percentage of ω-3 PUFA was also higher in summer than in winter. Differences in FA percentage related to the season could be explained by differences in the cow' dietary regimes since during the summer, the cows typically graze on pasture or are fed fresh forage. Replacing grass silages with corn silage increases the concentration of medium-chain saturated FAs (C6:0 to C14:0) and C18:2ω-6 and decreases the concentration of C16:0, C18:0, C18:3ω-3, and C20:5ω-3 in milk fat. Replacing grass silage with corn silage will decrease the amount of total ω-3 PUFA and increase the amount of ω-6 PUFA in milk, leading to an elevated ω-6:ω-3 ratio [12,21,23]. The effect of replacing grass with corn silage on the CLA content of milk is inconclusive. Studies have shown that corn silage-based diets increase the CLA content of milk fat compared with grass-based diets [24,25], while others have found no differences in CLA content of milk fat from cows offered grass or corn silage-based diets [21,26,27]. The present study indicated that the percentage of C18:2ω-6 and CLA was higher during the summer. However, higher environmental temperatures during the summer also affected the FA composition of milk, and milk fat in summer was lower in C16:0 relative to C18:0 than in milk fat from the same cows during winter. Although these changes were related to changes in blood plasma lipids, such observations were confounded by dietary changes.

As part of this study, the data were analyzed using a clustering approach based on the percentage of FA, production year, season, and corn silage and cereals in the diet. The $\delta^{13}C$ values of casein fraction were also used to characterize the presence of corn silage at a threshold limit of −23.5‰, i.e., samples with $\delta^{13}C$ values higher than −23.5‰ indicated the presence of corn in the diet [16]. Before clustering, the Silhouette curve method was applied to estimate the optimum number of clusters [29]. In this study, the best split was achieved using 14 clusters. The partition around medoids (PAM) method was then applied, which divided the milk samples into 14 clusters (Figure 1). The results showed that the production year and season and the presence of corn silage and cereals in the animal's diet played a significant role in the clustering. Figure 1 also shows the combined effects of

year and season. Production year had a more significant effect on FA composition than the season. Specific FAs showed no global pattern of differentiation and were probably influenced by micro-factors, such as temperature, humidity, feeding regime, lactation stage, breed, and processing techniques.

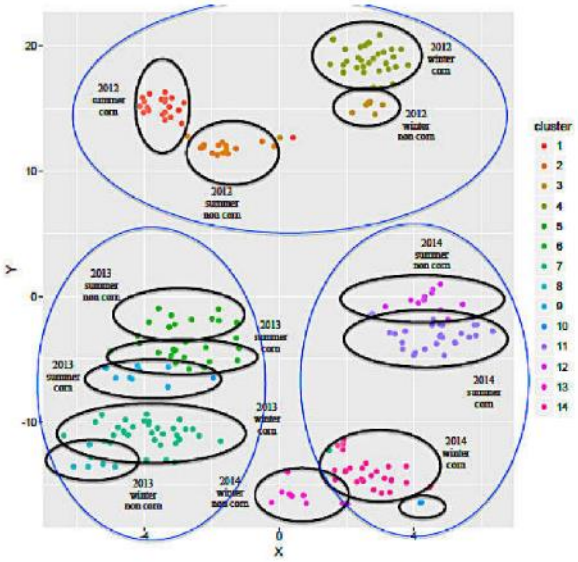


Figure 1. Cluster analysis of the percentage of fatty acid (FA) in Slovenian milk.

Multivariate analysis was used to identify the key factors responsible for discriminating between production year and season. In this case, 231 samples consisting of authentic Slovenian milk samples collected in summer and winter 2012 (12 s, 12 w), 2013 (13 s, 13 w), and 2014 (14 s, 14 w) were statistically evaluated. Figure 2 presents the results of the discriminant analysis (DA) as a discriminant function score plot (a) and a discriminant loadings plot (b). In the functional score plot, each group (centroid) was represented by a scatter plot, while in the loadings plot, they appeared as a set of vectors, indicating the degree of association of the corresponding initial variables with the first two discriminant functions (F1 and F2).

In latter, the degree of distribution of each parameter in the classes was revealed. Except for the 2013 and 2014 winter samples (13 w and 14 w, respectively), good separation between year and season of production was achieved. The most influential parameter for the summer/winter 2012 (12 s/12 w) classes (see top left of biplot a) was C20:4ω-6 (direction of its vector was towards the right part of the biplot), while CLA separated 12 s from 12 w. Winter samples 2013 (13 w) and 2014 (14 w) overlapped, while the separation between summer classes 14 s and 13 s was likely a result of the higher percentage of C17:0iso, C20:1ω-9, C18:3ω-6, C20:3ω-3 (bottom left (a)). A leave-one-out cross-validation (LOOCV) classified 86.9% of the samples correctly.

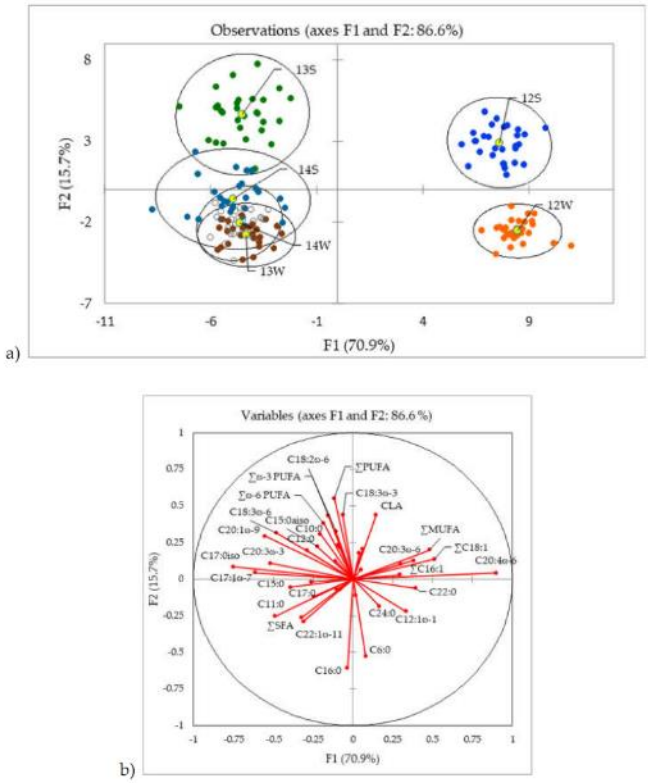


Figure 2. Linear discriminant analysis of FA in milk according to year and season of production: (**a**) discriminant function score plot of 231 authentic Slovenian milk samples from summer/winter in 2012, 2013, and 2014 (12 s/12 w; 13 s/13 w; 14 s/14 w); (**b**) discriminant loading plot of the correlations between the initial variables and the discriminant functions.

2.1.2. Influence of the Geographical Origin on FA Composition

Fatty acid composition combined with various chemometric tools has been successfully applied in different food commodities to discriminate the geographical origin, including fruits [30], oils [31,32], and also milk and dairy products [26,33,34]. In this study, discriminant analysis (DA) was used to discriminate between milk from Alpine, Mediterranean, Pannonia, and Dinaric regions of Slovenia using the whole dataset, including summer and winter samples from 2012, 2013, and 2014. The results are presented in Figure 3a,b. A comparison of the scatter of data points and vector plots indicated that the Mediterranean group (Figure 2a) was a result of high amounts of C15:0, C17:0, C17:0iso, and C17:0aiso FA. For the Alpine region and Dinaric region groupings (Figure 3c: lower left) C18:3ω-3, CLA, C18:3ω-6, and C20:5ω-3 had the most separating power, while the C12:0, C11:0, C21:0, C18:0, and C18:2ω-6 differentiated the Pannonian group. A cross-validation test showed 64.1% correct classification. The classification rate was the highest (80.8%) for Pannonian milk samples and the lowest for Dinaric samples (20.5%). The more precise and efficient separation between the four

regions was obtained when taking into account the season (summer-winter). In this case, the classification rate was > 90%. Figure 3c shows the DA plot for 39 milk samples obtained in winter 2013. The analysis generated four well-separated groups. Separation of the regions was also achieved based on FA composition when the data were treated altogether. For F1 (89.0%), C15:0 and its isomers were the main discriminatory parameters, while for F2, the most discriminatory parameters (10.5%) were C18:3ω-6 and CLA. However, cross-validation was low, and only 35.9% of the samples were classified correctly. The highest rate of classification was for the Mediterranean samples (66.7%) and the lowest for the Alpine samples (8.3%). The low classification rate was related to the low number of samples.

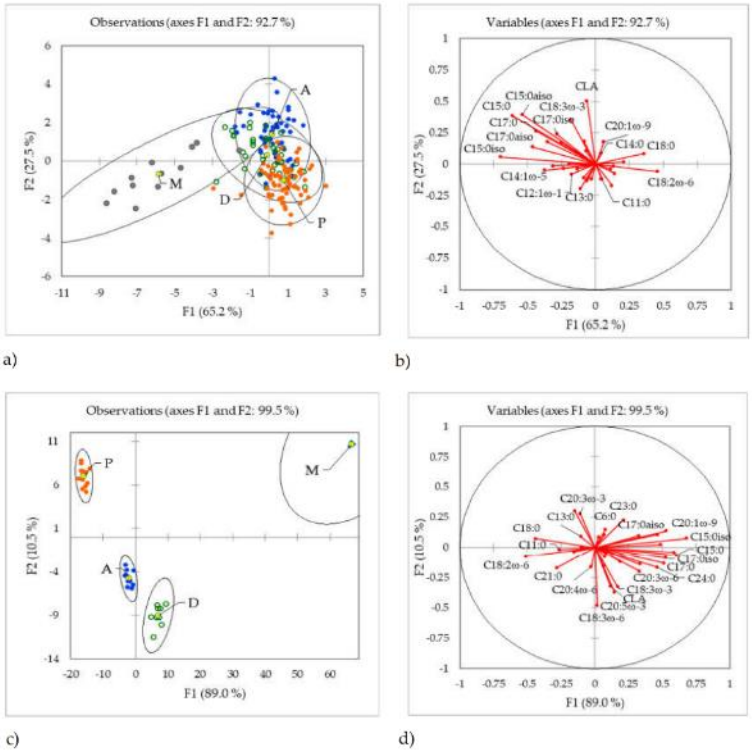


Figure 3. Linear discriminant analysis of FA composition according to the production region: M-Mediterranean, A-Alpine, D-Dinaric, and P-Pannonian; (**a**) discriminant function score plot of 231 authentic Slovenian milk samples from summer/winter: 2012, 2013, and 2014; (**c**) discriminant function score plot of 39 Slovenian milk samples from four regions in winter 2013; (**b**), (**d**) discriminant loading plot, showing correlations between initial variables and discriminant functions.

2.2. Stable Carbon Isotope Composition of FA in Milk

This study used the stable carbon isotope composition of individual FAs to check the variability in production year and season in 2013 and 2014. The differences measured in the isotopic composition between individual milk FA reflected diet regime, the isotopic fractionation during biosynthesis, metabolic rates, and isotope turnover time. Table 2 presents the stable carbon isotope values of individual FAs in cow milk samples and stable carbon isotope values in bulk freeze-dry milk. The given values were the mean and standard deviation (SD). Results were shown as box plots of $\delta^{13}C$ values of milk fatty acids and bulk milk samples (Figure 4). Data points, exceeding two units of SD, were regarded as outliers, and in the cases where measurements were below the 5% confidence level, the data were discarded. Interestingly, FAs formed during biosynthesis had the lowest $\delta^{13}C$ values due to isotope fractionation.

Table 2. $\delta^{13}C$ values (in ‰) in FAs in Slovenian milk according to the year, season, and feeding regime in 2013 and 2014. The source of FAs in milk, which influences its isotopic composition, is summarized by Ehtesham et al. [25], Richter et al. [18], Hanus et al. [9].

		$\delta^{13}C$ (‰) Mean ± SD						
FA	**Source of FA**	**2013**	**2014**	**Winter**	**Summer**	**Corn**	**Non-Corn**	**Average**
C10:0	metabolism	−23.9 ± 2.7	−22.1 ± 2.5	−21.3 ± 2.0	−24.7 ± 2.3	−22.2 ± 2.4	−26.2 ± 1.8	−22.9 ± 2.8
C12:0	metabolism	−24.6 ± 3.2	−22.8 ± 2.6	−21.8 ± 2.2	−25.7 ± 2.5	−22.8 ± 2.6	−27.3 ± 2.0	−23.6 ± 3.0
C14:1	biosynthesis	−27.4 ± 3.2	−24.9 ± 3.0	−24.3 ± 2.5	−28.0 ± 3.0	−25.2 ± 2.9	−29.9 ± 2.2	−26.0 ± 3.3
C14:0	metabolism	−23.5 ± 3.0	−21.9 ± 2.6	−21.2 ± 2.0	−24.3 ± 2.8	−21.9 ± 2.4	−26.2 ± 2.2	−22.6 ± 2.9
C15:0	microbial biosynthesis	−32.1 ± 3.7	−28.8 ± 2.9	−28.2 ± 2.6	−32.6 ± 3.4	−29.5 ± 3.4	−33.9 ± 2.7	−30.3 ± 3.7
C16:1	biosynthesis	−28.5 ± 3.5	−26.0 ± 3.0	−25.2 ± 2.3	−29.4 ± 3.3	−26.3 ± 3.0	−31.3 ± 2.6	−27.2 ± 3.5
C16:0	diet and metabolism	−23.6 ± 2.9	−22.6 ± 2.5	−21.8 ± 2.0	−24.5 ± 2.7	−22.2 ± 2.2	−26.7 ± 1.7	−23.0 ± 2.7
C18:1ω-9c	biosynthesis	−27.2 ± 2.5	−25.9 ± 2.2	−25.6 ± 2.1	−27.5 ± 2.5	−25.8 ± 1.9	−29.8 ± 2.1	−26.5 ± 2.5
C18:0	biosynthesis and diet	−27.8 ± 3.2	−26.6 ± 2.8	−25.6 ± 2.3	−29.0 ± 2.7	−26.4 ± 2.5	−31.0 ± 2.6	−27.2 ± 3.0
Bulk	diet	−21.4 ± 1.9	−21.5 ± 2.2	−20.6 ± 1.7	−22.5 ± 1.9	−20.7 ± 1.4	−24.8 ± 0.9	−21.4 ± 2.0

In all the samples, C15:0 had significantly lower average $\delta^{13}C$ values (−30.3 ± 3.7‰) compared to other FAs. The highest average $\delta^{13}C$ value (−22.6 ± 2.9‰) was observed in C14:0. Higher $\delta^{13}C$ values were found in winter, which reflected the higher amounts of corn in the diet. Although the reported $\delta^{13}C$ values from specific regions, e.g., the Mediterranean region, were influenced by the year of production, significant differences in reported $\delta^{13}C$ values between the summer and the winter season were observed. The fatty acid C18:1ω-9 was an exception with consistently lower values during the summer. It was assumed that this variability was a result of different production practices and the amount of grass and corn forage in a cow's diet. The C18:1ω-9 in milk from cows fed a C_3 diet is enriched in ^{13}C relative to C18:0, whereas no enrichment occurs when cows' are fed on a C_4 diet [17]. In this study, an average enrichment of 1.6‰ was observed in milk sampled during the summer and supported the assumption that a C_3-based diet could result in the transfer of higher amounts of 18:1 ω-9 directly from the feed. As part of a dietary source, C18:0 can be produced by microbial biohydrogenation of C18:0 as the final product of ruminal biohydrogenation [35]. Microbial biohydrogenation can significantly affect the isotopic values of C18:0, leading to a lower $\delta^{13}C$ values

compared to C16:0, which originates in part from dietary lipids and to a significant extent from *de novo* synthesis from acetate, derived mainly from dietary carbohydrates [36]. The $\Delta^{13}C$ value ($\delta^{13}C$-18:0–$\delta^{13}C$-16:0) in the samples were < –3.3‰, which is typical for ruminant fat.

Stable carbon isotope composition of individual FA indicated less variability between season and year than fatty acid composition, which suggested a more distant relationship. There was also no data supporting the correlation between a time-significant percentage of FA and $\delta^{13}C$ values within the same FA. The results of a multivariate analysis of the stable isotope content of FA are presented in Figure 4. The temporal separation between selected groups described 93.8% of the variability. The most important stable isotope parameters that contributed to this separation were: $\delta^{13}C$-16:0, $\delta^{13}C$-18:1ω-9*c*, $\delta^{13}C$-bulk, $\delta^{13}C$-14:1, and $\delta^{13}C$-18:0, and a leave-one-out cross-validation (LOOCV) classified 90.9% of the samples correctly.

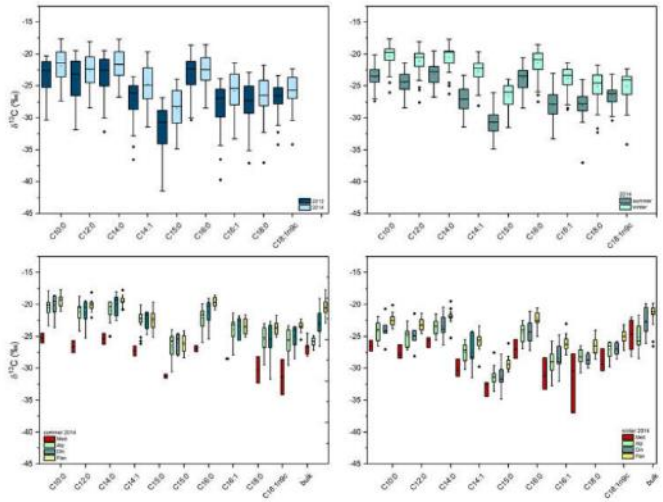


Figure 4. Box plots of $\delta^{13}C$ values of milk fatty acids and bulk milk samples according to the year (2013, 2014), season (summer, winter in 2014), and region of production in summer and winter 2014: Med-Mediterranean, Alp-Alpine, Din-Dinaric, Pan-Pannonian.

Figure 4 shows the summer and winter $\delta^{13}C$ values of different FAs in milk for the four studied regions. The $\delta^{13}C$ values of individual fatty acids (season, year, and production region) are given in Table S1. The $\delta^{13}C$ in FA in milk collected from the Mediterranean region differed significantly ($p < 0.05$) from the other three regions. The differences were more apparent in summer than winter. The Mediterranean region also had more negative $\delta^{13}C$ values, which indicated that cows had a higher amount of grass-based (C_3 plant) forage in their diet, although the higher temperatures in the Mediterranean region could be a factor. The highest $\delta^{13}C$ values were in FA in milk from the Pannonian region. Besides, the $\delta^{13}C$-18:1ω-9*c* determined in the Pannonia region differed significantly from Alpine and Dinaric regions.

2.3. Quality of Milk

Some comparisons can be drawn about the ratios of SFA, MUFA, and PUFA in milk. A healthy diet should contain more PUFA and less of SFA since SFAs are a risk factor for cardiovascular

diseases [37]. Alternatively, MUFA and PUFA, except for trans unsaturated fatty acids, reduce the level of low-density lipoprotein (LDL) cholesterol and increase the level of high-density lipoprotein (HDL) cholesterol [38].

The lowest percentage of SFA (avg. 66.97 ± 2.54 % of total FA) was observed in milk produced in summer in the Alpine region, for all three sampling years, while in winter, there were no significant differences between the regions in the SFA percentage of milk sampled in the summer (Table 3). The feeding regime in the Alpine region is based on the managed grazing of pastures during the summer, while in the winter, the cows' diet consists of a higher amount of cereal concentrates and conserved forages. The feeding of grass conservation products (hay or silage) increases the SFA content of milk fat.

Table 3. Percentage of FA groups in Slovenian milk: 2012 and 2014, based on geographical origin and season.

Fatty Acids	Year	Season	Alpine	Mediterranean	Pannonian	Dinaric
(% of Total FA)			Mean ± SD			
Saturated FA (SFA)	2012	summer	64.15 ± 1.62	69.57 ± 3.30	66.33 ± 0.74	66.74 ± 2.14
		winter	69.57 ± 0.82	71.64 ± 3.10	68.89 ± 0.66	69.52 ± 0.53
		average	66.81 ± 3.09	70.60 ± 3.08	67.69 ± 1.47	68.12 ± 2.05
	2013	summer	68.78 ± 1.60	68.19 ± 7.58	70.20 ± 5.04	70.63 ± 0.71
		winter	70.50 ± 0.75	70.39 ± 3.68	69.63 ± 1.99	70.08 ± 2.46
		average	69.64 ± 1.51	69.29 ± 5.46	69.91 ± 3.77	70.34 ± 1.82
	2014	summer	68.08 ± 1.33	69.55 ± 4.65	68.42 ± 1.22	68.62 ± 3.48
		winter	72.45 ± 1.02	73.23 ± 4.25	70.56 ± 2.76	72.16 ± 0.79
		average	70.26 ± 2.51	71.39 ± 4.47	69.42 ± 2.31	70.49 ± 2.99
Monounsaturated FA (MUFA)	2012	summer	29.93 ± 1.28	24.95 ± 3.35	28.73 ± 0.62	27.96 ± 2.00
		winter	25.79 ± 0.86	23.59 ± 3.26	26.56 ± 0.52	25.94 ± 0.51
		average	27.86 ± 2.37	24.27 ± 3.05	27.57 ± 1.23	26.89 ± 1.72
	2013	summer	24.98 ± 1.38	26.40 ± 7.03	24.36 ± 2.00	23.93 ± 0.86
		winter	23.73 ± 0.84	24.16 ± 3.09	24.16 ± 0.53	23.26 ± 1.13
		average	24.36 ± 1.29	25.28 ± 5.01	24.25 ± 1.42	23.57 ± 1.04
	2014	summer	26.31 ± 0.92	25.53 ± 3.66	26.74 ± 1.14	25.13 ± 2.27
		winter	22.20 ± 1.13	21.59 ± 3.56	23.15 ± 1.37	22.64 ± 0.57
		average	24.25 ± 2.33	23.56 ± 3.89	25.07 ± 2.20	23.81 ± 2.01
Conjugated linoleic acid (C18:2*c*9,*t*11)	2012	summer	1.20 ± 0.30	0.67 ± 0.12	0.62 ± 0.12	0.72 ± 0.20
		winter	0.61 ± 0.09	0.59 ± 0.10	0.48 ± 0.06	0.55 ± 0.10
		average	0.90 ± 0.37	0.63 ± 0.11	0.54 ± 0.11	0.63 ± 0.17
	2013	summer	0.89 ± 0.24	0.67 ± 0.61	0.63 ± 0.34	0.54 ± 0.12
		winter	0.62 ± 0.15	0.52 ± 0.23	0.44 ± 0.07	0.54 ± 0.15
		average	0.75 ± 0.24	0.60 ± 0.42	0.54 ± 0.26	0.54 ± 0.13
	2014	summer	0.86 ± 0.18	0.51 ± 0.31	0.50 ± 0.14	0.58 ± 0.21
		winter	0.47 ± 0.09	0.53 ± 0.20	0.44 ± 0.08	0.43 ± 0.06
		average	0.67 ± 0.24	0.52 ± 0.23	0.47 ± 0.12	0.51 ± 0.16
α-linolenic acid (C18:3ω-3)	2012	summer	0.84 ± 0.14	0.98 ± 0.34	0.52 ± 0.07	0.70 ± 0.16
		winter	0.58 ± 0.09	0.76 ± 0.33	0.45 ± 0.06	0.57 ± 0.16
		average	0.71 ± 0.18	0.87 ± 0.32	0.48 ± 0.07	0.63 ± 0.17
	2013	summer	1.08 ± 0.54	0.46 ± 0.07	0.78 ± 0.30	0.71 ± 0.21
		winter	0.61 ± 0.12	0.56 ± 0.14	0.50 ± 0.05	0.56 ± 0.11
		average	0.84 ± 0.45	0.51 ± 0.11	0.64 ± 0.26	0.63 ± 0.18
	2014	summer	0.69 ± 0.26	0.89 ± 0.24	0.54 ± 0.13	0.66 ± 0.21
		winter	0.60 ± 0.13	0.81 ± 0.08	0.45 ± 0.09	0.45 ± 0.17
		average	0.64 ± 0.21	0.85 ± 0.17	0.50 ± 0.12	0.55 ± 0.22
Eicosapentaenoic acid (C20:5ω-3)	2012	summer	0.08 ± 0.01	0.11 ± 0.02	0.06 ± 0.01	0.07 ± 0.02
		winter	0.06 ± 0.01	0.08 ± 0.02	0.05 ± 0.02	0.06 ± 0.02
		average	0.07 ± 0.01	0.09 ± 0.03	0.05 ± 0.01	0.07 ± 0.02
	2013	summer	0.02 ± 0.03	0.16 ± 0.26	0.06 ± 0.10	0.02 ± 0.02
		winter	0.05 ± 0.04	0.04 ± 0.01	0.03 ± 0.02	0.05 ± 0.02

Fatty Acids	Year	Season	Alpine	Mediterranean	Pannonian	Dinaric
(% of Total FA)				Mean ± SD		
		average	0.04 ± 0.04	0.10 ± 0.17	0.05 ± 0.07	0.04 ± 0.02
	2014	summer	0.04 ± 0.02	0.03 ± 0.03	0.03 ± 0.04	0.03 ± 0.02
		winter	0.04 ± 0.02	0.07 ± 0.01	0.04 ± 0.01	0.07 ± 0.02
		average	0.04 ± 0.02	0.05 ± 0.03	0.03 ± 0.03	0.05 ± 0.03
Polyunsaturated FA (PUFA)	2012	summer	4.59 ± 0.46	3.71 ± 0.18	3.68 ± 0.19	3.90 ± 0.27
		winter	3.40 ± 0.17	3.43 ± 0.25	3.39 ± 0.21	3.32 ± 0.26
		average	4.00 ± 0.70	3.57 ± 0.25	3.52 ± 0.25	3.59 ± 0.39
	2013	summer	4.98 ± 1.04	3.76 ± 1.38	4.58 ± 1.36	4.06 ± 0.61
		winter	3.81 ± 0.38	3.24 ± 0.44	3.44 ± 0.19	3.66 ± 0.29
		average	4.39 ± 0.97	3.50 ± 0.96	4.01 ± 1.12	3.85 ± 0.50
	2014	summer	4.23 ± 0.66	3.64 ± 0.73	3.53 ± 0.39	3.69 ± 0.67
		winter	3.41 ± 0.31	3.21 ± 0.72	3.32 ± 0.31	3.20 ± 0.37
		average	3.82 ± 0.66	3.42 ± 0.69	3.43 ± 0.37	3.43 ± 0.57
ω-3 PUFA	2012	summer	0.94 ± 0.16	1.12 ± 0.37	0.59 ± 0.08	0.79 ± 0.19
		winter	0.66 ± 0.10	0.86 ± 0.35	0.51 ± 0.06	0.64 ± 0.18
		average	0.80 ± 0.19	0.99 ± 0.35	0.55 ± 0.08	0.71 ± 0.20
	2013	summer	1.17 ± 0.54	0.65 ± 0.28	1.00 ± 0.37	0.83 ± 0.16
		winter	0.75 ± 0.14	0.70 ± 0.14	0.63 ± 0.06	0.68 ± 0.12
		average	0.96 ± 0.44	0.67 ± 0.20	0.82 ± 0.32	0.75 ± 0.16
	2014	summer	0.81 ± 0.28	1.01 ± 0.28	0.67 ± 0.16	0.75 ± 0.22
		winter	0.73 ± 0.16	0.95 ± 0.07	0.58 ± 0.10	0.61 ± 0.17
		average	0.77 ± 0.22	0.98 ± 0.18	0.63 ± 0.14	0.68 ± 0.20
ω-6 PUFA	2012	summer	2.45 ± 0.13	1.92 ± 0.16	2.48 ± 0.15	2.38 ± 0.17
		winter	2.14 ± 0.15	1.98 ± 0.08	2.39 ± 0.23	2.14 ± 0.25
		average	2.29 ± 0.21	1.95 ± 0.12	2.43 ± 0.20	2.25 ± 0.24
	2013	summer	2.92 ± 0.70	2.43 ± 1.23	2.94 ± 0.85	2.70 ± 0.51
		winter	2.44 ± 0.18	2.03 ± 0.22	2.36 ± 0.14	2.43 ± 0.21
		average	2.68 ± 0.55	2.23 ± 0.82	2.65 ± 0.67	2.56 ± 0.39
	2014	summer	2.56 ± 0.34	2.12 ± 0.18	2.36 ± 0.30	2.35 ± 0.33
		winter	2.20 ± 0.16	1.72 ± 0.55	2.29 ± 0.22	2.16 ± 0.34
		average	2.38 ± 0.32	1.92 ± 0.43	2.33 ± 0.26	2.25 ± 0.34
ω-6:ω-3 PUFA	2012	summer	2.69 ± 0.55	1.90 ± 0.85	4.26 ± 0.72	3.19 ± 0.94
		winter	3.32 ± 0.61	2.58 ± 1.03	4.79 ± 0.94	3.60 ± 1.08
		average	3.01 ± 0.66	2.24 ± 0.92	4.54 ± 0.87	3.41 ± 1.01
	2013	summer	2.86 ± 1.05	3.64 ± 0.42	3.15 ± 0.86	3.34 ± 0.73
		winter	3.35 ± 0.52	3.01 ± 0.78	3.79 ± 0.39	3.69 ± 0.91
		average	3.11 ± 0.84	3.32 ± 0.66	3.47 ± 0.73	3.52 ± 0.82
	2014	summer	3.01 ± 0.58	2.19 ± 0.48	3.69 ± 0.89	3.30 ± 0.74
		winter	3.13 ± 0.61	1.83 ± 0.67	4.06 ± 0.72	3.32 ± 0.86
		average	3.07 ± 0.59	2.01 ± 0.56	3.86 ± 0.82	3.31 ± 0.69
AI	2012	summer	2.31 ± 0.20	3.25 ± 0.53	2.58 ± 0.10	2.63 ± 0.33
		winter	3.06 ± 0.15	3.51 ± 0.60	2.92 ± 0.10	3.06 ± 0.11
		average	2.69 ± 0.42	3.38 ± 0.52	2.76 ± 0.20	2.86 ± 0.32
	2013	summer	3.03 ± 0.28	2.89 ± 1.82	3.15 ± 0.46	3.33 ± 0.20
		winter	3.30 ± 0.19	3.34 ± 0.62	3.79 ± 0.39	3.71 ± 0.21
		average	3.16 ± 0.27	3.13 ± 1.24	3.47 ± 0.73	3.35 ± 0.18
	2014	summer	2.81 ± 0.27	2.92 ± 0.75	2.84 ± 0.20	3.07 ± 0.43
		winter	3.79 ± 0.25	4.13 ± 1.05	3.50 ± 0.31	3.75 ± 0.19
		average	3.30 ± 0.59	3.53 ± 1.05	3.15 ± 0.42	3.43 ± 0.47
TI	2012	summer	2.69 ± 0.23	3.19 ± 0.23	3.05 ± 0.11	3.03 ± 0.28
		winter	3.47 ± 0.11	3.62 ± 0.28	3.43 ± 0.11	3.46 ± 0.11
		average	3.08 ± 0.43	3.41 ± 0.33	3.25 ± 0.22	3.26 ± 0.30
	2013	summer	3.09 ± 0.34	3.18 ± 1.13	3.19 ± 0.42	3.41 ± 0.20
		winter	3.67 ± 0.14	3.68 ± 0.58	3.64 ± 0.12	3.71 ± 0.21
		average	3.38 ± 0.40	3.43 ± 0.85	3.42 ± 0.38	3.57 ± 0.25

Fatty Acids	Year	Season	Alpine	Mediterranean	Pannonian	Dinaric
(% of Total FA)				Mean ± SD		
	2014	summer	3.25 ± 0.34	3.35 ± 0.71	3.33 ± 0.20	3.46 ± 0.44
		winter	3.82 ± 0.14	3.88 ± 0.79	3.80 ± 0.16	3.95 ± 0.25
		average	3.54 ± 0.39	3.61 ± 0.73	3.55 ± 0.42	3.72 ± 0.42

AI = atherogenic index [(C12:0 + 4 × C14:0 + C16:0)/(∑MUFA + ∑(ω-6) + ∑(ω-3))]; TI = thrombogenic index [(C14:0 + C16:0 + C18:0)/((0.5 × ∑MUFA + 0.5 × ∑(ω-6) + 3 ×∑(ω-3)) + (∑(ω-3)/∑(ω-6)))].

The consensus is that PUFAs, especially ω-3, ω-6, and CLA, have a beneficial effect on human health. Besides, the ω-6:ω-3 ratio, expressed as the concentration of linoleic acid (C18:2ω-6) to α-linolenic (C18:3ω-3) acid, i.e., the most abundant ω-6 and ω-3 FA, as well as atherogenic (AI) and thrombogenic (TI) indices are used to evaluate the nutritional value of milk fat since the milk fat with low AI and TI values could provide protection against coronary heart disease. Further, ω-3 PUFA reduces the risk of cardiovascular disease, hypertension, cancer and has been linked to improved neurological function [5,6,10,13]. The most abundant PUFA in fresh forages is ω-3 (especially α-linolenic acid). In this study, the highest percentage of α-linolenic acid was in the Alpine region in the summer (avg. 0.87 ± 0.38% of total FA) and in the Mediterranean region in winter (avg. 0.71 ± 0.2% of total FA). While the milk from the Pannonian region had the lowest percentage of α-linolenic acid in both summer and winter (avg. 0.62 ± 0.22% of total FA in summer and 0.46 ± 0.07% of total FA in winter). The lower levels of α-linolenic in winter are the result of oxidative losses of PUFA during the wilting of grasses [13]. A correlation also existed between α-linolenic acid and ∑ω-3 FA. The highest percentage of ω-3 FA was present in milk from the Alpine region in summer (avg. 0.97 ± 0.38% of total FA), while in winter, the highest levels of ω-3 FA were observed in the Mediterranean region (avg. 0.83 ± 2.1% of total FA). The lowest percentage of ω-3 FAs was found in milk collected from the Pannonian region in all three production years (avg. 0.76 ± 0.29% of total FA in summer and avg. 0.57 ± 0.09% of total FA in winter). Changes in the ω-3 FA percentage were smaller in the Pannonian region, during both summer and winter compared to the other studied regions. The reason was that the cows' were fed a diet of corn silage (intensive milk production) and conserve forage throughout the year. Low values of ω-3 FA in low altitude regions of Slovenia (Pannonian and Dinaric regions) were consistent with the literature data for lowlands [39,40]. Since a high ω-6:ω-3 ratio can be detrimental to human health and a factor in cardiovascular disease, cancer, inflammatory and autoimmune disease, an optimum ratio in the human diet of 1:1 ratio is considered, while a ratio < 5:1 is recommended [39]. However, a higher ω-6:ω-3 ratio is indicative of higher amounts of cereals (corn, soybean, barley, and oats) in the cows' diet. The ω-6:ω-3 ratio in Slovenian milk is between 4.2:1 and 2.5:1 and is lower in summer compared to winter. The lowest ω-6:ω-3 ratio was in milk produced in the Mediterranean region through the whole season and did not exceed 2.6:1, which was indicative of a fresh forage-based diet. In the Dinaric and Pannonian regions, the effect of feeding cows corn silage and cereals throughout the year increased the ω-6:ω-3 FA ratio from 3.2:1 to 4.2:1. The data of AI and HI indices were comparable to the study performed by Ferlay et al. [41] determined in the milk of Tarentaise and Montbéliarde cows (3.14 and 3.43, respectively), but they were lower than the data reported by Nantapo et al. [42] in the milk of Friesian, Jersey, and Friesian x Jersey cows with values ranged from 4.08 to 5.13. The highest AI indices in our study were determined in the Mediterranean region (2.89–4.13) and the lowest in the Alpine region (2.31–3.79).

Environmental factors related to year and season together with diet are the most important factors influencing the composition of CLA in milk. In this study, the percentage of CLA ranged from 0.52 ± 0.18% of total FA to 0.73 ± 0.31% of total FA in milk from all four studied regions and increased with dietary intake of 18-carbon PUFAs. Increasing the amount of PUFAs in milk could be achieved by feeding cows' a diet based on fresh silage. Milk collected in summer in the Alpine region (avg. 0.99 ± 0.28% of total FA) had the highest percentage of CLA, most likely from grazing on pasture. In the winter, there was no significant difference between the Alpine and other regions since the animal's diet is composed of both corn and grass silage. Because Alpine and Mediterranean milk in summer contained a higher percentage of ω-3 PUFA and CLA, compounds known to have positive effects on human health [40,43], this milk is likely "healthier" than milk produced in other regions in

Slovenia. However, despite the benefits of feeding cows fresh grass silage, the overall effects on the FA composition of milk would not improve human health over the long-term [44].

3. Materials and Methods

3.1. Milk Collection and Storage

During a 3-years study (from 2012 to 2014), in total, 231 raw cow milk samples were collected. Samples were collected twice per year in winter and summer from four geographical regions in Slovenia: Mediterranean (n = 18), Pannonian (n = 90), Dinaric (n = 51), and Alpine (n = 72). Samples were collected from five Slovenian dairies, each supplied by milk from different farms and transported to the laboratory. The geographical characterization of the sampling location: latitude, longitude, altitude, and the number of samples in each location can be found in the supplementary material (Table S2). Each sample was divided into four subsamples and stored in plastic containers at −20 °C. Sampling data is shown in Table 4.

Table 4. Samples collected from different geographical regions in Slovenia.

	Alpine	Dinaric	Mediterranean	Pannonian	Grand Total
2012	24	17	6	30	77
summer	12	8	3	14	37
winter	12	9	3	16	40
2013	24	17	6	30	77
summer	12	8	3	15	38
winter	12	9	3	15	39
2014	24	17	6	30	77
summer	12	8	3	16	39
winter	12	9	3	14	38
Grand Total	72	51	18	90	231

3.2. Chemicals and Standards

Dichloromethane (CH_2Cl_2, for organic residue analysis), methanol (MeOH, for organic residue analysis), and hexane (C_6H_{14}, for organic residue analysis) were purchased from J. T. Baker B. V. (Deventer, Netherlands). Sodium hydroxide (NaOH, puriss. p.a., ACS reagent, reag. Ph. Eur., ≥ 98%, pellets) and boron trifluoride-methanol solution (14% in methanol) were obtained from Sigma-Aldrich (St.Louis, MI, USA). Deionized water (18.2 MΩ) was made using a Mili-Q System (Merck Millipore, Watertown, MA, USA). A standard mixture from Supelco (Bellefonte, PA, USA), named Supelco 37 Component FAME Mix in dichloromethane, containing the methyl esters of 37 fatty acids, was used.

The accuracy of the $\delta^{13}C$ determination was checked with the following international reference materials: polyethlene foil IAEA-CH-7 ($\delta^{13}C = -32.15 \pm 0.04$‰) and sucrose IAEA-CH-6 ($\delta^{13}C = -10.45 \pm 0.03$‰) obtained from International Atomic Energy Agency (IAEA, Vienna, Austria), L-glutamic acids USGS40 ($\delta^{13}C = -26.39 \pm 0.04$‰) obtained from U.S. Geology Survey (USGS, Virginia, USA) and laboratory reference materials: casein IAEA-CRP 2013 ($\delta^{13}C = -20.3 \pm 0.09$‰) (IAEA, Vienna, Austria), UreaC ($\delta^{13}C = -28.3 \pm 0.2$‰), and fatty acid methyl nanodecanoate ($\delta^{13}C = -30.0 \pm 0.1$‰) (Restek, Bellefonte, PA, USA).

3.3. Analytical Procedures

3.3.1. Extraction and Esterification of Milk FAs

Frozen milk samples were thawed (37 °C) and homogenized. Milk fat consists predominantly of triacylglycerides, which were extracted using the in-situ-trans-esterification method without prior extraction of the fat from samples according to the method of Park and Goins (1994). The total lipids fraction was extracted from the milk samples (450 µL) with dichloromethane (300 µL) and 0.5 M

sodium hydroxide in methanol (3 mL), purged with nitrogen and heated at 90 °C for 10 min, after which the samples were cooled rapidly. Fatty acid methyl esters (FAMEs) were formed by adding 14% BF_3-methanol solution (3 mL) and heating the sample at 90 °C for a further 10 min. After cooling, the fatty acids methyl esters (FAMEs) were extracted with hexane (1.5 mL) and transferred to a GC vial and stored at −20 °C.

3.3.2. Gas Chromatography of Fatty Acids

The characterization of FAMEs was performed using Agilent 6890N (Network GC System, Agilent Technology, Santa Clara, CA, USA) gas chromatography with FID detector (GC-FID). The separation was achieved on an Omegawax 320, 30 m × 0.32 mm, × 0.25 µm, on a capillary column (Supelco, Bellefonte, PA, USA). The temperature program was as follows: 50 °C (held 2 min) to 220 °C at 4 °C/min (held 20 min). The carrier gas was helium in constant flow mode (1 mL/min). The injector was set to 260 °C and the detector to 280 °C. The individual fatty acids were identified and quantified by comparing their retention times with those of a standard Supelco 37 component FAME Mix (Supelco) and expressed in weight percent of total fatty acids. Procedural blanks were analyzed with each set of samples. Standard Supelco 37 component FAME mix was analyzed after every ten samples to verify the stability of the analytical system. Method precision based on measurements of replicate (n = 2) real samples was 5%. Results were expressed as the percentage of each fatty acid with respect to the total fatty acids [45]. This percentage was calculated using the peak area of the samples corrected with the respective correction factors, according to Christie and Han [46].

3.3.3. Elemental Analysis-Isotope Ratio Mass Spectrometry (EA-IRMS)

The stable carbon isotope ratio measurements were reported in δ—notation expressed in per mile (‰) relative to the Vienna-Pee Dee Belemnite (V-PDB) standard following Equation (1) [47]:

$$\delta({}^{i/j}E) = \delta^{i/j}E = \frac{{}^{i/j}R_P - {}^{i/j}R_{Ref}}{{}^{i/j}R_{Ref}} \quad (1)$$

where superscripts *i* and *j* denote the highest and the lowest atomic mass number of element E, and R_P and R_{Ref} is the ratio between the heavier and the lighter isotopes (in case of carbon, $^{13}C/^{12}C$) in the sample (*P*) and reference material (*Ref*).

To determine the bulk carbon isotope composition, approximately 1 mg of freeze-dried milk was weighed into a tin capsule, closed with tweezers, and placed into the automatic sampler of the elemental analyzer. All analyses were performed separately on a Europa Scientific 20–20 continuous flow mass spectrometer with an ANCA-SL solid–liquid preparation module (Sercon, Crewe, UK). Analyses were normalized against international standards: IAEA-CH-7 and IAEA-CH-6 with $\delta^{13}C$ values of −32.15 ± 0.04‰ and −10.45 ± 0.04‰, respectively. For independent control, laboratory reference material IAEA-CRP 2013 with $\delta^{13}C$ value −20.3 ± 0.09‰ and UreaC with $\delta^{13}C$ value −28.3 ± 0.2‰ were used. The analytical precision, expressed as the standard deviation of control material, was ± 0.2‰.

The $^{13}C/^{12}C$ ratio of synthetic fatty acid standard C19:0 (methyl nanodecanoate, RESTEK) was determined using a Europa Scientific 20–20 continuous flow mass spectrometer with an ANCA-SL solid–liquid preparation module (Sercon, Crewe, UK). Results were further normalized against two international reference standards: IAEA-CH-7 and IAEA-CH-6. For independent control, the international reference material USGS40 with $\delta^{13}C$ values of −26.39 ± 0.04‰ was used. Analytical precision, expressed as the standard deviation of the control materials, was ± 0.2‰.

3.3.4. Compound Specific Isotope Analysis of $\delta^{13}C$ in Fatty Acids

The Isotopic compositions of individual FA were determined using an Agilent 6890N GC-C system coupled to an IsoPrime GV IRMS (GV Instruments, Manchester, UK)). The separation was achieved using a DB-1MS (60 m × 0.32 mm × 0.25 µm) capillary column (Agilent J&W, USA). The temperature program was as follows: 120 °C (held 1 min) to 300 °C (held 20 min) at 3 °C/min. The carrier gas was helium in the constant flow mode (1 mL/min). The injector temperature was 260 °C,

and the oxidation reactor (Cu/O) in the 6890N GC/C system was 900 °C. Peak identification of individual fatty acids was performed by comparing their retention times with those in the standard Supelco 37 component FAME Mix, which was analyzed in the measurement sequence. Instrument stability was ≤ 0.08‰. C19:0 FAME (methyl nanodecanoate) with $\delta^{13}C$ values of −30 ± 0.1 ‰ (prior determined on EA-IRMS) was used as a laboratory reference material. For data normalization, a single-point normalization method was used [48]. The precision of measurements expressed as the standard deviation of the laboratory reference material was 0.3 to 0.5‰.

3.4. Statistical Evaluation

For statistical evaluation, FAs were considered individually and grouped in classes as follows: saturated fatty acids (SFA), unsaturated fatty acids (UFA), MUFA, ω-3 PUFA, ω-6 PUFA, and CLA. Statistical calculations and multivariate analysis were carried out using the XLSTAT software package (2020.1.3, Addinsoft, New York, NY, USA). The statistical evaluation was performed using R, where non-parametric tests were used, i.e., Kruskal–Wallis followed by a post hoc test. In the case of pairwise comparison, a Mann–Whitney test was used. Basic statistics included mean values (median and arithmetic mean), standard deviation (SD), minimum and maximum, while for multivariate analysis, discriminant analysis (DA) was used.

4. Conclusions

Slovenian milk samples had been classified according to the year, season, and region of production based on the determination of the percentage and stable isotope composition of individual fatty acids. It was found that the FA composition differed significantly, depending more on year and season of production than geographical region. A leave-one-out cross-validation (LOOCV) classified 86.9% of the samples correctly. For geographical origin determination, it was found that the Mediterranean group was separated from other regions based on a high percentage of C15:0 and C17:0 and their isomers. The cross-validation test showed 64.1% correct classification with the highest rate of classification for the Pannonian (80.8%) samples. Although the classification rate according to the year/season of production improved when stable isotope composition of individual FA was included, describing 93.8% of variability, it was found that $\delta^{13}C$ in FA could be more useful for studying metabolic transformation processes in ruminants. It appeared that the natural $\delta^{13}C$ differences in FA depended on both—the diet type and the precursor FA. The "healthiest" milk for human consumption, based on FA composition, was produced in the Alpine and Mediterranean regions. The quality of milk was also affected by summer/winter season production. The results gave proof of the concept that FA composition could help to discriminate the geographical origin of milk and thus identify products of better quality. Finally, to confirm the validity of the markers, future studies should focus on how factors, such as breed and age, lactation period, production level, the farming system, influence the FA and stable isotope composition of milk.

Supplementary Materials: The following are available online, Table S1: $\delta^{13}C$ of fatty acids in milk from a different geographical location in Slovenia during summer/winter in 2013 and 2014; Table S2: Geographical information of the sampling location.

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Chapter 4

Authenticity of Milk and Dairy Products Based on Fatty Acid Composition and PCR Methods

Chapter 4 describes my research relating to authentic samples of milk and dairy products obtained from the Slovenian market, where a combination of PCR data and FA composition was used to determine possible adulteration. This chapter is divided into three parts. The first part describes the theoretical background related to the FA composition and PCR method for analysing dairy products as part of this PhD. The second part describes in detail the sampling of high-value cow's, sheep's and goat's cheese and low-value cow's cheese and laboratory experiments for determining vegetable tallow adulteration based on non-milk fat. The final section discusses the results.

The verification of the authenticity of milk and dairy products is the process by which a food's compliance with its label description is examined. Assessing the authenticity of dairy products is a major factor in consumer choice, and, therefore, important economically as well as from a medical, allergenic and religious perspective. There are several ways that dairy products are adulterated. Among the more nefarious practices are the substitution of fat or proteins of plant or animal origin, the addition of milk from different species, the addition of low-cost dairy products (mainly whey derivatives), and the mislabelling of products protected by a denomination of origin (PDO) (Dennis, 1998; Karoui & De Baerdemaeker, 2007).

A range of analytical methods to detect frauds has been developed, and are continually being modified and reassessed in order to remain one step ahead those who pursue these illegal activities. Methods based on protein testing, such as electrophoretic (capillary electrophoresis, 2-D electrophoresis, isoelectric focusing of milk casein) and immunological techniques (ELISA), as well as molecular methods based on DNA testing and chromatography, have been developed (Danezis et al., 2016). Although milk protein testing can be a very effective method, the main limitation is protein denaturation that occurs during the processing of milk and dairy products. The advantage of using DNA is that it is a relatively stable molecule, which means that products can be tested even after having undergone technological processing (Mafra et al., 2008). Another approach that guarantees security, quality and authenticity of dairy products is the detection of non-dairy fat of plant or animal origin. Determination of admixtures of non-dairy fat with raw milk is possible using multivariate statistical analysis of the FA content. The method is sensitive and can detect as much as 1.2% of added non-dairy fat in the milk (Ulberth, 1995). The FAs composition in milk can also be used to detect the type of milk (goat, sheep or cow's

milk) in the production of the dairy product such as cheese – goat's and sheep's milk exhibit characteristic short-chain FA profiles than cow's milk. The most abundant FAs are C6:0, C8:0 and C14:0 accounting for about 20% of all FAs in goat's milk, while C14:0, C16:0 and C18:1 are the most abundant FAs in sheep's milk (Dennis, 1998).

4.1 Variability in Fatty Acid Composition in Milk and Dairy Products

The FA content of milk is important in determining the nutritional and technological quality of raw milk. Modifying the FA profile has the potential to significantly contribute to the production of milk and dairy products with higher or lower added-value, and can have implications for human health. From a nutritional point of view, a lower proportion of saturated fatty acids (SFAs) and a higher proportion of unsaturated fatty acids (UFAs), especially polyunsaturated (PUFA) ω-3 fatty acids are desirable (Hanus et al., 2018). Cow's milk contains from 3.3 to 4.4% of fat and is composed of lipids, especially triacylglycerols (95 %) that consist of FAs of different length and saturation (Renou et al., 2004). The proportion of SFAs, monounsaturated fatty acids (MUFAs) and PUFAs in milk is mostly determined by the cow's diet, as well as their metabolism, whereas breed and parity have only a small effect (Hanus et al., 2018; Kliem et al., 2013).

Saturated fatty acids are straight-chain organic acids containing from 4 to 18 carbon atoms. In milk, these SFAs account for up to 70 to 75% of the total FAs. The most abundant SFA is palmitic acid (C16:0), which accounts for approximately 33% of total FAs. Other important SFAs are myristic acid (C14:0) and stearic acid (C18:0), which make up 10 to 13% of the total FAs. The amount of short-chain FAs (C4:0 – C10:0) is relatively high when their proportion is expressed in molar percentages, i.e., 10.9% of total SFAs.

Cow's milk fat contains 18 – 24% MUFAs. The main component of *cis* MUFAs is oleic acid (C18:1ω-9) and represents 15 – 21% of the total MUFAs. The *trans* isomer of C18:1 is vaccenic acid (11*t* C18:1) and is also present in fat. Vaccenic acid is the dominant trans-isomer produced by rumen microorganisms and is unique to ruminant fats. Other MUFAs, although present in a lower amount includes C14:1, C15:1, C16:1, C17:1. Vaccenic acid can be converted to conjugated linoleic acid (CLA, 9*c*, 11*t* C18:2), in the mammary gland by the enzyme stearoyl-CoA desaturase. Increased levels of C18:1 in milk fat is the result of a diet based on fresh grass (MacGibbon & Taylor, 2006).

Between 2 and 5% of total FAs in milk are PUFAs, but it is possible to alter these values by modifying the animal's diets. The main PUFAs derive from ω-3, ω-6 and CLA, and can have a beneficial effect on human health. Taking ω-3 FAs can reduce the risk of cardiovascular disease, hypertension, cancer, and have been linked to improved neurological function. It is recommended, that people increase their intake of ω-3 FA with an optimum ratio of 1:1 ω-6 and ω-3 FA. The main ω-3 FA in milk is α-linolenic acid (C18:3ω-3), along with a small amount of docosahexaenoic acid (DHA, C22:6ω-3) and eicosapentaenoic acid (EPA, C20:5ω-3). The principal ω-6 FA is linoleic acid (C18:2ω-6). These FAs are essential and cannot be synthesized by the human body but must be supplied from the diet. Both EPA and DHA are present in low amounts in traditional dairy cow diets, and the transfer of dietary EPA and DHA to milk fat is low (<4%). Their concentration in milk is closely related to the amount that is absorbed in the intestine and hence to dietary PUFA intake (Chilliard et al., 2007; Ellis et al., 2006; Lock & Bauman, 2004; MacGibbon & Taylor, 2006).

As already stated in this thesis, the FA composition in milk differs depending on the species. For example, goat's milk contains a similar amount to cow's milk (3.25 to 4.2% of

total milk fat), whereas sheep's milk contains FAs account for approximately 7.1% of the total milk fat. The FA composition in the milk of three species of dairy animal is presented in Table 4.1.

Table 4.1: Fatty acids composition in the milk of three animal species (% of total FA obtained from literature) (Gaspardo et al., 2010; Hanus et al., 2018; Lindmark Månsson, 2008; Lopez et al., 2019; MacGibbon & Taylor, 2006; Markiewicz-Keszycka et al., 2013; Nájera et al., 2017; Park et al., 2007).

Fatty acids	**Sheep's milk**	**Goat's milk**	**Cow's milk**
	% of total FA (min – max)		
C4:0	3.07 – 3.93	1.97 – 2.60	3.10 – 5.10
C6:0	2.68 – 3.44	2.03 – 2.70	1.45 – 2.90
C8:0	2.10 – 3.27	2.28 – 3.04	0.97 – 1.90
C10:0	5.54 – 9.73	8.85 – 11.0	2.20 – 3.80
C10:1	0.23 – 0.31	0.19 – 0.38	0.15 – 0.40
C12:0	3.48 – 4.92	3.87 – 6.18	2.60 – 4.20
C12:1	0.03 – 0.05	0.10 – 0.40	0.06 – 0.13
C13:0	0.13 – 0.22	0.06 – 0.28	0.19 – 0.13
C14:0	9.85 – 10.7	7.71 – 12.2	9.10 – 12.2
C14:1	0.19 – 0.50	0.17 – 0.20	0.50 – 1.30
iC15:0	0.26 – 0.43	0.12 – 0.15	0.33 – 0.40
aC15:0	0.33 – 0.60	0.17 – 0.20	0.40 – 0.60
C15:0	0.89 – 1.11	0.46 – 0.85	0.90 – 1.40
iC16:0	0.17 – 0.26	0.17 – 0.40	0.34 – 0.40
C16:0	22.5 – 28.8	23.2 – 34.8	23.6 – 34.1
C16:1	0.74 – 1.27	1.00 – 2.70	1.40 – 2.00
iC17:0	0.44 – 0.59	0.24 – 0.52	0.26 – 0.57
aC17:0	0.26 – 0.39	0.30 – 0.50	0.45 – 0.54
C17:0	0.58 – 0.70	0.52 – 0.90	0.58 – 0.71
C17:1	0.17 – 0.22	0.24 – 0.48	0.27 – 0.32
C18:0	8.51 – 11.0	5.77 – 13.2	10.2 – 14.6
C18:1 total	17.8 – 23.0	15.4 – 27.7	14.9 – 24.7
C18:2 total	2.89 – 3.57	2.49 – 4.34	1.20 – 2.67
C18:3	0.52 – 1.04	0.19 – 0.87	0.90 – 1.20
CLA total	0.56 – 0.97	0.32 – 1.17	0.44 – 1.50
C20:0	0.36 – 0.52	0.08 – 0.35	0.15 – 0.35
SFA	61.8 – 73.4	66.7 – 69.8	68.1 – 68.9
MUFA	23.6 – 29.7	22.3 – 24.4	26.9 – 27.6
PUFA	4.82 – 6.20	3.70 – 4.20	2.48 – 4.05
ω-6/ω-3	2.31 – 6.60	5.00 – 5.90	1.69 – 6.21
Total fat (%)	6.27 – 8.60	3.80 – 5.90	3.50 – 4.00

Caproic (C6:0), caprylic (C8:0) and capric (C10:0) acids occur in higher proportions in goat and sheep's milk than in cow's milk. The straight-chain FAs in goat and sheep's milk fat show slightly higher levels of C6:0, C8:0, C12:0 and C14:0 compared to cow's milk, and considerably higher levels of C10:0 in goat's milk. The relatively high levels of medium-chain FAs in goat's milk is responsible for its inferior flavour. Cow's milk is the richest source of oleic FA (C18:1) and makes up around 24% of total FA, while its content in cow and goat's milk makes up on average 18% of total FAs. In cow's milk fat, a significant amount of C16:0 is observed (Jandal, 1996; Lindmark Månsson, 2008; MacGibbon & Taylor, 2006). Sheep's milk, compared to goat and cow's milk, has the lowest amount of

lauric, myristic and palmitic acids (C12:0, C14:0, C16:0, respectively). A characteristic of goat's milk is a lauric/capric acid ratio <0.5, while this ratio in sheep's milk is between 0.5 and 0.6 and in the cow's milk is >1. This parameter is a good indicator of milk falsification. The amount of MUFA in cow, goat and sheep's milk is similar, and it ranges from 20% to 30% of the milk fat. Oleic acid is the most abundant FA from this group and is more abundant in cow's milk (24% of total milk fat), than in goat and sheep's milk (18% of total milk fat). Goat and sheep's milk usually have lower ω-6/ω-3 ratios (Markiewicz-Keszycka et al., 2013), and since the composition of FAs and triglycerides (TAG) in milk fat depends on the species, they are also biomarkers for detecting the adulteration of goat's and sheep's milk cheese with cow's milk. The molecular structure of TAG is characterized by its "partition number" (PN). Thus, it is possible to group TAG according to their PN values: short-chain TAG (PN<34), intermediate-chain (PN=36-40) and long-chain TAG (PN>40). It is known that milk fat form goats and sheep have a higher content of short-chain and medium-chain TAG than cow's milk fat, while cow's milk fat has a higher amount of long-chain TAG (Vieitez et al., 2013). With an increasing amount of cow's milk added to goat's milk, triglycerides with PN values of 38, 40 and 42 decrease, while triglycerides with PN values of 46, 48, 50 tend to increase (Vieitez et al., 2016).

One of the possible adulterations of dairy products appearing on the market that reduces the cost of the product is the admixture of vegetable tallow with raw milk. Detection of this kind of fraud could be obtained based on FA composition, since the FA composition of vegetable fats differs from those of animal fat (Ulberth, 1995). Palm fat, which is often added to raw milk does not contain butyric acid (C4:0) and contains a higher amount of lauric acid (C12:0) compared to milk; thus any addition of palm fat to milk would result in a decrease in the concentration of butyric acid (C4:0) and an increase in the concentration of lauric acid (C12:0). Likewise, by the addition of palm fat, the content of palmitic acid (C16:0) decreases. At the same time, the concentration of C18:1ω-9 (oleic acid) decreases, while the concentration of stearic acid (C18:0) slightly increases.

FA composition of milk fat is also used to detect adulteration with vegetable oils, mainly because short-chain FAs are characteristic of milk fat, whereas vegetable oils contain medium- to long-chain FAs (Molkentin & Precht, 1998; Ntakatsane et al., 2013). Studies indicate that the FA composition has the potential to detect adulteration of milk with plant and animal fats especially when oleic acid (C18:1ω-9) and linoleic acid (C18:2ω-6) profiles are considered along with triacylglycerols profiles (TGs; C52 and C54), and cholesterol levels. These biomarkers enable the detection of as low as 10% adulteration of non-milk fat into 100% pure MF (Kim et al., 2016). Furthermore, Rebechi and co-workers (2016) found that multiple linear regression, when applied to FA profiles, is a valid statistical tool for evaluating the adulteration of milk fat. The authors found that it was possible to detect adulterated milk fat samples at levels >5% for lard and >10% for tallow.

4.2 PCR-Based Method for Determining Adulteration of Milk and Dairy Products

Polymerase chain reaction (PCR) is another technique that can be used to identify adulterated dairy products. PCR relies on the amplification of DNA target sequences by generating thousands to millions of copies of a particular nucleotide acid. The advantages of PCR-based methods are that they are relatively fast and highly sensitive, and are highly selective towards specific DNA, even when other DNA molecules are present. Recently, PCR based methods have become the focus of attention, because DNA is more stable when exposed to the high temperatures, pressures, and chemical treatments that occur during

food processing compared to other target analytes (e.g. lipids or proteins). Also, because DNA can be extracted from thermally processed milk (pasteurized and ultra-pasteurized) and powdered milk, and amplified the adulteration of non-declared milk from different biological sources (cow, goat, sheep, and buffalo) can be easily detected. In the analysis of dairy products, PCR-based methods have already been applied successfully for identifying specific DNA sequences characteristic of particular microorganism or animals (Dalmasso et al., 2011; Di Domenico et al., 2017; Khade et al., 2019; Liao et al., 2017; Mafra et al., 2008).

The identification of milk by PCR amplification of DNA is based on the presence of somatic cells in the milk or by targeting sequences of mitochondrial genes (Klančnik et al., 2012, 2016; Mafra et al., 2008). The lack of quantitative information obtained by conventional PCR has led to the development of two new quantitative detection strategies: quantitative competitive PCR (QC-PCR) and real-time PCR. Real-time PCR involves the use of specific probes or labelled primers and allows the simultaneous detection and conformation of fragments which further increases the reliability of the techniques and the number of applications related to food analysis (Mafra et al., 2008).

However, since food safety and quality have become a real issue, several real-time PCR methods have been developed to determine the authenticity of milk and dairy products. For example, the quantitative detection of cow's milk in commercial Mozzarella cheese (pure buffalo cheese) from local supermarkets, dairy shops or directly from cheese manufacturers were analysed by Lopparelli and co-workers (2007). The authors found that the majority of commercial PDO Mozzarella samples contained cow's milk. López-Calleja and co-workers (2007b) developed a method for quantifying the amount of goat's milk in a mixture goat/sheep's milk in the range of 0.6 – 10% as well as a novel real-time PCR approach using TaqMan probes for measuring cow's milk in raw and heat-treated cow/sheep milk mixtures in the range of 0.5 – 10% (López-Calleja et al., 2007a). More recently, Agrimonti and et al. (2015) developed a quadruplex quantitative real-time PCR (qxPCR) method for the simultaneous detection of DNA of cow, goat, sheep and buffalo milk in dairy products and the quantification of cow's milk in the same products, while Klančnik et al. (2016) designed a rapid, robust and sensitive method for the identification of adulterated goat's and sheep's cheese with cow's milk. The new real-time PCR assays were designed for the specific determination of cow DNA and cow, goat and sheep DNA. Their results showed that 17% of analysed cheeses available from different local Slovenian supermarkets, contained more than 5% of undeclared cow's milk. Until now only a few validated real-time PCR methods have been published (Di Domenico et al., 2017; Lopparelli et al., 2007; Tsakali et al., 2019), nevertheless real-time PCR has proved to be an adequate technique for detecting the presence of milk from different species such as cow's milk in cheese made from goat's or sheep's milk.

4.3 Methods

4.3.1 Case study

The FAs composition was characterized for three reasons: (1) to determine possible adulteration of milk with vegetable fat; (2) to determine possible adulteration of low-value cheese with vegetable fat; (3) to use in combination with PCR analysis FAs were used to determine possible adulteration of sheep's and goat's cheese with cow's milk.

4.3.1.1 Adulteration of raw milk and low-cost cheeses with vegetable fat

Adulteration of dairy products appearing on the market is typically an admixture of vegetable tallow with raw milk. Detection of fraud is based on non-milk fat. Since palm fat does not contain butyric acid (C4:0) and contains a higher amount of lauric acid (C12:0) compared to milk, the addition of palm fat to milk would result in the decrease in the concentration of butyric acid (C4:0) and an increase in the concentration of lauric acid (C12:0). Based on the FA composition, it is possible to determine very low concentrations of an added vegetable fat (1%) (Ulberth, 1995). To check the applicability of FA composition to detect the presence of vegetable palm fat in raw milk laboratory experiments were performed. At the same time, to verify the authenticity of cheese on the Slovenian market, different low-cost cheese products were chosen for the analysis.

4.3.1.2 Adulteration of sheep and goat's cheese with cow's milk

The content of FA composition as well as DNA results for sheep, goat and cow's milk vary significantly, making it is possible to distinguish between dairy products of a specific type. In the production of high-quality cheese, the price depends on the type of milk (goat, sheep, cow's milk), therefore the correct declaration is important. The main focus of this work was to identify the source of milk present in high-quality cheeses on the Slovenian market and whether it corresponds to the label declaration.

4.3.2 Experimental work

4.3.2.1 Sampling of cheese

During 2013, 53 samples of high-value cheese made from sheep or goat's milk, or their mixture without or with cow's milk and nine samples of low-cost cheese made from cow's milk, were collected from different local Slovenian supermarkets. Cheese products were selected randomly from different regions in Europe (Table 4.2). In each case, a layer of approximately 0.5 cm from the surface of the cheese was removed. Each sample was then grated and stored in a freezer at -20°C before analysis.

Table 4.2: Description of cheeses collected from the Slovenian market.

Origin of sample	Sample number	Description
Sheep's cheese	24	High-value
Goat's cheese	17	High-value
Cow's cheese	8	High-value
Cow's and sheep's cheese	3	High-value
Cow's, sheep's and goat's cheese	1	High-value
Cow's cheese	9	Low-cost

4.3.2.2 Adulteration of milk with vegetable palm fat

Adulterated cow's milk with vegetable palm fat was prepared in the laboratory. To raw non-homogenized cow's milk palm tallow was added in the following percentages: 0%, 1%, 2%, 4%, 7%, 9%, 11%, 13%, 16%, 18%, and 20%. Briefly, raw non-homogenized cow's milk and palm fat were first separately heated to 37°C and then mixed in different proportions by pipetting. The mixture (450 µL) was then used in the preparation of fatty acids methyl

esters (FAME), as described in section 4.3.2.3. Each sample was prepared and analysed in triplicate.

4.3.2.3 Extraction and esterification of adulterated milk and cheese fatty acids

Milk fat consists predominantly of triacylglycerides, which were extracted using a modified *in-situ*-trans-esterification method as described by Park and Goins (1994) without the prior extraction of fat from the samples. In brief, frozen grated cheese samples were first thawed, and homogenized by mixing. The total lipids were then extracted from the samples (450 µL) with dichloromethane (300 µL, J. T. Baker B. V. Deventer, Netherlands) and 0.5 M sodium hydroxide (puriss. p.a., ACS reagent, reag. Ph. Eur., ≥98%, pellets, Sigma-Aldrich, St.Louis, MI, USA) in methanol (3 mL, J. T. Baker B. V. Deventer, Netherlands). The samples were then purged with nitrogen and heated for 10 minutes at 90°C, after which, they were rapidly cooled. The fatty acid methyl esters (FAMEs) were then formed by adding 14% BF_3-methanol solution (3 mL, Sigma-Aldrich, St.Louis, MI, USA) and heated again for 10 minutes at 90°C. After fast cooling, the fatty acids methyl esters (FAMEs) were extracted with hexane (1.5 mL, J. T. Baker B. V. Deventer, Netherlands) and transferred to GC vials and stored at -20°C before analysis by gas chromatography.

4.3.2.4 Gas chromatography of fatty acids

The characterization of FAMEs was performed using an Agilent 6890N (Network GC System, Agilent Technology, USA) gas chromatograph fitted with an FID detector (GC-FID). The separation was achieved on an Omegawax 320, 30 m × 0.32 mm, × 0.25 µm, capillary column (Supelco, Bellefonte, PA, USA). The temperature program was as follows: 50°C (held 2 min) to 220°C at 4°C/min (held 20 min). The carrier gas was helium at a constant flow of 1 mL/min. The injector temperature was 260°C, and the detector temperature 280°C. The individual fatty acids were identified and quantified by comparing their retention times with those of the standard Supelco 37 component FAME Mix (Supelco, Bellefonte, PA, USA) and expressed as the weight percent of total fatty acids. Data quality was maintained by preparing a procedural blank with each set of samples. Standard Supelco 37 component FAME mix (Supelco) was analysed after every ten samples to verify the stability of the analytical system. The precision of the method, based on replicate sample measurements (n=2), was ≤5%.

4.3.2.5 Analysis of DNA

The analysis of DNA was performed at the Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana and are described/modified according to (Klančnik et al., 2016). Isolated DNA was obtained using NucleoSpin Food kits (Macherey-Nagel, Duren, Germany) and SmartHelix Complex Samples kits (ExVivon, Ljubljana, Slovenia), following the manufacturer's protocol. The concentration and purities of the obtained DNA were determined using absorbance measurements made with a Lambda Bio spectrophotometer (Perkin-Elmer, Norwalk, CT, USA), with a Hellma TrayCell measuring cell (Helma Analytics, Muellheim, Germany). The DNA was diluted with sterile deionized water and stored at -20°C. Two different assays for identification and quantification of cow's milk in milk and dairy products were designed using the specific Custom-Designed TaqMan Gene Expression services of Life Technologies (Life Technologies, Foster City, CA, USA), namely the Cow1 assay specific for cow DNA and the BoCaOv assay universal for cow (bovine), goat (caprine) and sheep (ovine) DNAs. Due to the possibility of false-negatives, the additional control, the so-called Internal Positive Control (ICP)

(Commercial TaqMan Exogenous Internal Positive Control kits, Life Technologies) was used to detect inhibitors in the amplification to the prevent misinterpretation of the results.

Statistical analysis for the different DNA extraction methods: For the evaluation of experimental data of the different DNA extraction methods (NucleoSpin Food kits and SmartHelix Complex Samples kits), the statistic program SAS/STAT (SAS software; SAS Institute, Cary North Carolina, USA) was used. The data were analysed using a general linear model procedure and least-squares mean test (SAS/STAT), at a 0.5 level of significance. The differences between the percentages of cow's milk in the known cheese samples and the mean data obtained with Cow1 and BoCaOv real-time PCR assays were obtained using a student's *t*-test with 0.5 level of significance.

4.4 Results and discussion

4.4.1 Adulteration of milk with vegetable palm fat

The results obtained from our laboratory experiment are presented in Table 4.3. Since the palm fat does not contain butyric acid (C4:0) and contains a higher amount of lauric acid (C12:0), the percentage of butyric acid decreased from 2.22wt% of total FA in unadulterated milk to 0.55wt% of total FA in 20% falsified milk, while at the same time the percentage of lauric acid increased from 4.78wt% of total FA in unadulterated milk to 41.94wt% of total FA in 20% of adulterated milk. Likewise, by the addition of palm fat, the percentage of palmitic acid (C16:0) and oleic acid (C18:1ω-9) from unadulterated milk to 20% falsified milk, decreased from 35.61wt% of total FA to 12.79wt% of total FA and from 18.91wt% of total FA to 7.05wt% of total FA, respectively.

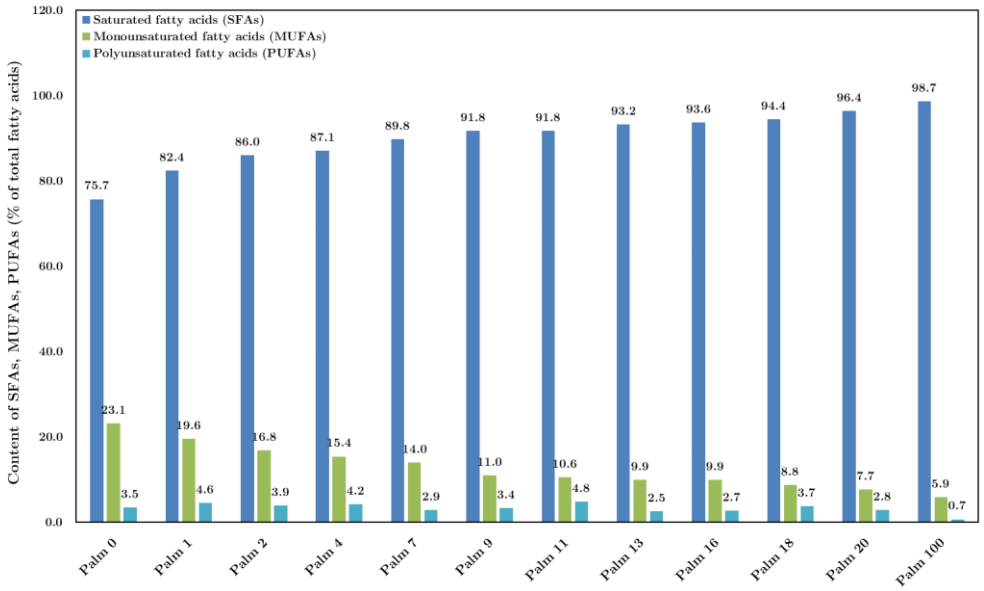
Table 4.3: Percentage of individual fatty acids in a sample of non-homogenized raw milk with and without the addition of vegetable palm fat.

Fatty acids	C4:0	C6:0	C8:0	C12:0	C16:0	C18:0	C18:1ω-9
				% of total FA			
Palm 0	2.22 ± 1.43	2.06 ± 0.67	1.62 ± 0.45	4.78 ± 0.78	35.61 ± 1.07	7.42 ± 0.90	18.92 ± 2.26
Palm 1	2.01 ± 0.78	1.92 ± 0.48	2.37 ± 0.58	15.13 ± 2.98	29.87 ± 1.23	8.13 ± 0.82	15.66 ± 1.87
Palm 2	1.70 ± 0.87	1.43 ± 0.30	2.46 ± 0.37	20.69 ± 1.45	25.56 ± 1.31	8.79 ± 0.78	13.65 ± 1.22
Palm 4	1.10 ± 0.31	1.18 ± 0.10	3.11 ± 0.56	29.15 ± 3.02	22.29 ± 1.81	10.69 ± 0.29	11.95 ± 0.85
Palm 7	1.10 ± 0.31	1.18 ± 0.10	3.11 ± 0.56	29.15 ± 3.02	22.29 ± 1.81	10.69 ± 0.29	11.95 ± 0.85
Palm 9	0.70 ± 0.11	0.76 ± 0.04	3.36 ± 0.25	36.87 ± 1.17	16.94 ± 0.85	12.63 ± 0.34	9.27 ± 0.64
Palm 11	0.73 ± 0.16	0.69 ± 0.08	3.05 ± 0.24	34.11 ± 2.21	16.16 ± 1.41	12.35 ± 0.84	8.94 ± 0.82
Palm 13	0.55 ± 0.13	0.64 ± 0.11	3.48 ± 0.48	38.89 ± 2.79	15.28 ± 0.34	12.89 ± 1.32	8.45 ± 0.14
Palm 16	0.73 ± 0.40	0.62 ± 0.10	3.57 ± 0.43	39.04 ± 2.13	14.25 ± 0.65	13.46 ± 0.44	7.89 ± 0.39
Palm 18	0.39 ± 0.06	0.50 ± 0.06	3.33 ± 0.47	39.38 ± 3.95	14.31 ± 1.11	14.21 ± 1.44	8.18 ± 0.94
Palm 20	0.55 ± 0.20	0.58 ± 0.10	3.95 ± 0.61	41.94 ± 3.56	12.79 ± 0.66	13.53 ± 1.30	7.05 ± 0.44
Palm 100	0.00 ± 0.00	0.37 ± 0.12	4.93 ± 0.97	50.85 ± 3.64	9.26 ± 0.91	14.69 ± 1.93	5.92 ± 0.73

Palm 0 – unadulterated homogenized milk, Palm 1 – adulterated milk with 1% palm fat, Palm 2 – adulterated milk with 2% palm fat, Palm 4 – adulterated milk with 4% palm fat, Palm 7 – adulterated milk with 7% palm fat, Palm 9 – adulterated milk with 9% palm fat, Palm 11 – adulterated milk with 11% palm fat, Palm 13 – adulterated milk with 13% palm fat, Palm 16 – adulterated milk with 16% palm fat, Palm 18 – adulterated milk with 18% palm fat, Palm 20 – adulterated milk with 20% palm fat, Palm100 – 100% palm fat.

An important parameter for determining adulteration with palm fat is the sum of specific FAs. In this case, the content of MUFA in adulterated milk decreases with increasing vegetable fat, while the content of SFA increases. Figure 4.1 shows how the adulteration of unadulterated milk with vegetable fat can be detected as low as 1% of added vegetable fat. Adding different amounts of palm fat to raw milk increases the content of SFAs, and decreases the content of MUFAs and PUFAs (especially ω-3 and ω-6 PUFAs). The content of total SFAs in unadulterated homogenized milk was 75.7 wt% of total FA,

while the content of SFAs in 1% falsified milk with palm fat is 82.4 wt% of total FA. In the sample containing 20% of palm fat, the SFAs content was 96.4 wt% of total FA and differed significantly from non-adulterated milk. Also, the total amount of MUFAs reduced from 23.1 wt% of total FA in non-adulterated milk to 7.7 wt% of total FA in 20% adulterated milk. The total MUFAs content in unadulterated milk was 23.2 wt% of total FA, while in 1% adulterated milk it accounts for 19.6 wt% of the total FA.



Palm 0 – unadulterated homogenized milk
Palm 1 – adulterated milk with 1% palm fat
Palm 2 – adulterated milk with 2% palm fat
Palm 4 – adulterated milk with 4% palm fat
Palm 7 – adulterated milk with 7% palm fat
Palm 9 – adulterated milk with 9% palm fat
Palm 11 – adulterated milk with 11% palm fat
Palm 13 – adulterated milk with 13% palm fat
Palm 16 – adulterated milk with 16% palm fat
Palm 18 – adulterated milk with 18% palm fat
Palm 20 – adulterated milk with 20% palm fat
Palm 100 – 100% palm fat

Figure 4.1: Changes in fatty acid composition with the addition of palm fat

4.4.2 The authenticity of commercial dairy products on the Slovenian market

To verify the Slovenian market situation and check the authenticity of commercial dairy products various high-value cow's, goat's, and sheep's cheese, as well as low-cost cow's cheese, were analysed using real-time PCR and FA profiling.

4.4.2.1 Detection of cow's milk in sheep's and goat's milk and cheeses using fatty acid composition and real-time PCR

Randomly selected high-value cheeses, from different geographical regions in Europe (Slovenia, Spain, Netherlands, Croatia, Italy, France and Bulgaria) available on the Slovenian market were analysed to obtain their FA composition and by real-time PCR assay using the Cow1 and BoCaOv systems. Determination of authenticity and declaration of individual cheeses based on their FA composition was related to the type of milk in the cheese. The determined FAs in cheese samples were also compared with literature data and with data from my analyses of authentic raw cow's, sheep's and goat's milk in 2013. The results from the 2013 study are presented in Table 4.4. (Chilliard & Ferlay, 2004; Chung et al., 2018; Collomb et al., 2008; Gaspardo et al., 2010; Luna et al., 2005; Markiewicz-

Keszycka et al., 2013; Park et al., 2007). The content of palmitic acid (C16:0) in cow's milk ranged between 28.27 – 36.91 wt% of total FA but was higher than in goat and sheep's milk, which varies between 22.84 – 28.55 wt% of total FA and 20.28 – 26.06 wt% of total FA, respectively. The difference between goat's, sheep's and cow's milk is also reflected in the total content of caproic (C6:0), caprylic (C8:0) and capric (C10:0) acid, which varied between 4.15 – 9.45 wt% of total FA in cow's milk, from 8.56 – 13.39 wt% of total FA in sheep's milk, while in goat's milk the values varied between 10.48 – 16.01 wt% of total FA. Significantly higher content of capric acid (C10:0) was detected in goat's milk. The most significant difference between cow's and sheep's cheese was in the total amount of caproic (C6:0), caprylic (C8:0) and capric (C10:0) acid. The sum of these FAs was lower in cow's cheese, while the content of palmitic acid (C16:0) increased.

Table 4.4: Mayor fatty acids in milk from various species compared with values reported in the literature (Chilliard & Ferlay, 2004; Chung et al., 2018; Collomb et al., 2008; Gaspardo et al., 2010; Luna et al., 2005; Markiewicz-Keszycka et al., 2013; Y. W. Park et al., 2007).

	Our results in 2013			**Literature data**		
Fatty acids	**Cow's milk**	**Goat's milk**	**Sheep's milk**	**Cow's milk**	**Goat's milk**	**Sheep's milk**
	% of total FA (min-max)					
C6:0	0.29 – 3.03	1.85 – 4.51	1.26 – 2.90	1.13 – 2.02	2.03 – 3.37	1.87 – 2.94
C8:0	1.04 – 3.88	1.38 – 3.94	2.08 – 5.52	0.94 – 1.39	2.00 – 3.68	1.87 – 3.44
C10:0	2.65 – 6.40	10.44 – 12.93	6.59 – 8.84	2.23 – 3.03	6.10 – 11.11	5.54 – 9.73
C12:0	3.21 – 6.76	4.22 – 4.75	3.96 – 4.54	2.59 – 3.79	2.70 – 6.18	3.48 – 4.92
∑ (C6:0, C8:0, C10:0)	3.97 – 13.31	11.82 – 18.42	9.93 – 15.65	4.67 – 5.76	10.10 – 18.16	10.32 – 16.44
C12:0/C10:0	1.05 – 1.21	0.35 – 0.44	0.46 – 0.60	1.11 – 1.25	0.38 – 0.50	0.53 – 0.63
C16:0	28.83 - 37.65	22.84 – 28.55	20.28 – 26.06	25.62 – 32.45	17.80 – 31.10	22.50 – 28.20

The authenticity of individual high-value cheeses was further evaluated based on the suggested ratio between lauric (C12:0) and capric (C10:0) acid (C12:0/C10:0 ratio), which is until now the most appropriate method for evaluation of adulteration of sheep's and goat's cheese with cow's milk (Iverson and Sheppard, 1989). The relationship between FAs ratios in pure products and mixed products can be expressed using equation 4.1:

$$AX + BY = C \tag{4.1}$$

where A is the theoretical ratios of C12:0/C10:0 in cow's cheese obtained from the literature, X is the percentage of cow's cheese in the test sample divided by 100, B is the theoretical C12:0/C10:0 FAs ratio of sheep or goat's cheese obtained from literature, Y is the percentage of sheep or goat's cheese divided by 100, and C is the ratios of C12:0/C10:0 FA in the test samples. For cheese prepared by mixing two types of milk, equation 4.2 was used.

$$X + Y = 1 \tag{4.2}$$

The mean ratios of C12:0/C10:0 FAs from literature was 1.18 for cow's cheese, 0.58 for sheep's cheese and 0.44 for goat's cheese. This ratio was used to determine the percentage of cow's milk in cheese labelled as sheep's or goat's cheese. The major FA compositions are presented in Table 4.5. A comparison with the results obtained in milk from the same species (see Table 4.4) gave a good correlation. However, the only parameter that was consistent within the milk from different species was the ratios of C12:0/C10:0 FA.

Table 4.5: Mean fatty acids values for cow's, sheep's and goat's cheese samples and calculated percentage of cow's milk in sheep's and goat's cheese.

Cheese samples	C6:0	C8:0	C10:0	C12:0	C12:0/C10:0	∑(C6:0, C8:0, C10:0)	C16:0	Percentage of cow's milk
	% of total FA					% of total FA		
Cow's cheese 1	3.21	1.95	4.03	4.38	1.09	9.19	31.76	
Cow's cheese 2	3.02	1.81	3.69	3.95	1.07	8.51	30.44	
Cow's cheese 3	2.41	1.76	4.12	4.87	1.18	8.29	32.87	
Cow's cheese 4	3.61	2.07	4.15	4.39	1.06	9.83	31.11	
Cow's cheese 5	2.79	2.00	4.76	5.00	1.05	9.55	29.87	
Cow's cheese 6	2.39	1.69	3.77	4.29	1.14	7.84	34.38	
Cow's cheese 7	2.87	1.86	4.02	4.48	1.11	8.75	35.91	
Cow's cheese 8	2.27	1.47	3.26	3.76	1.16	7.01	33.62	
Sheep's cheese 1	3.83	3.18	8.51	4.45	0.52	15.52	25.75	
Sheep's cheese 2	2.96	2.81	7.86	4.34	0.55	13.63	25.34	
Sheep's cheese 3	3.00	2.89	8.53	4.91	0.58	14.42	27.97	
Sheep's cheese 4	3.39	3.24	9.11	5.01	0.55	15.74	24.19	
Sheep's cheese 5	2.20	2.17	6.25	3.66	0.58	10.62	28.60	
Sheep's cheese 6	4.75	4.42	11.05	5.35	0.48	20.22	22.56	
Sheep's cheese 7[a]	2.78	2.92	8.71	5.23	0.60	14.42	27.19	3
Sheep's cheese 8	4.48	4.25	11.91	6.12	0.51	20.64	27.02	
Sheep's cheese 9	3.71	3.40	9.77	5.31	0.54	16.88	27.08	
Sheep's cheese 10[b]	2.97	1.55	2.65	3.12	1.18	7.17	32.44	100
Sheep's cheese 11	2.60	3.13	9.57	5.54	0.58	15.30	26.00	
Sheep's cheese 12[b]	2.12	2.62	9.41	6.23	0.66	14.15	29.54	14
Sheep's cheese 13[a]	2.53	2.35	6.80	4.08	0.60	11.68	27.95	3
Sheep's cheese 14	4.48	3.40	7.73	4.34	0.56	15.61	26.44	
Sheep's cheese 15[b]	0.61	0.61	2.11	1.83	0.87	3.33	31.20	48
Sheep's cheese 16[b]	3.25	2.30	5.06	3.85	0.76	10.61	29.10	30
Sheep's cheese 17	2.82	3.12	9.25	5.06	0.55	15.19	25.31	
Sheep's cheese 18	4.56	3.84	9.94	5.03	0.51	18.34	25.43	
Sheep's cheese 19	3.98	3.56	9.29	4.68	0.50	16.83	24.59	
Sheep's cheese 20[a]	2.70	2.50	7.23	4.27	0.59	12.43	25.54	2
Sheep's cheese 21[c]	2.39	2.08	5.89	3.66	0.62	10.36	25.82	7
Sheep's cheese 22	2.43	2.11	5.59	3.22	0.58	10.14	25.11	
Sheep's cheese 23	3.25	3.29	9.40	5.16	0.55	15.95	24.90	
Sheep's cheese 24	3.34	3.35	10.30	5.87	0.57	16.99	24.55	
Goat's cheese 1	2.93	3.67	12.46	4.90	0.39	19.07	27.85	
Goat's cheese 2	2.49	3.18	10.58	4.67	0.44	16.25	28.60	
Goat's cheese 3	2.78	3.43	10.93	4.77	0.44	17.14	26.67	
Goat's cheese 4	2.80	3.08	10.52	4.49	0.43	16.41	27.72	
Goat's cheese 5	2.71	2.94	8.88	3.78	0.43	14.53	24.82	
Goat's cheese 6[b]	2.78	3.05	10.21	5.53	0.54	16.04	33.10	14
Goat's cheese 7[b]	3.20	3.41	10.60	6.21	0.59	17.21	29.51	20
Goat's cheese 8	3.84	3.85	10.91	4.54	0.42	18.60	26.19	
Goat's cheese 9[b]	2.48	2.70	9.31	6.10	0.66	14.49	30.15	29
Goat's cheese 10[b]	2.37	2.90	10.07	5.53	0.55	15.34	32.32	15
Goat's cheese 11	3.98	4.41	13.17	5.34	0.41	21.57	26.85	
Goat's cheese 12[b]	3.02	3.33	10.65	6.91	0.65	17.00	28.52	28
Goat's cheese 13[b]	2.89	2.86	8.87	5.16	0.58	14.62	31.08	19
Goat's cheese 14[c]	2.57	3.24	11.07	5.32	0.48	16.88	26.63	6
Goat's cheese 15[b]	2.49	2.88	10.38	7.28	0.70	15.75	30.07	35
Goat's cheese 16[b]	1.60	2.18	8.71	4.83	0.55	12.49	34.07	15
Goat's cheese 17[b]	2.83	3.19	11.00	6.56	0.60	17.03	32.20	21
Cow's and sheep's cheese 1	2.23	1.71	4.32	4.30	1.00	8.26	33.78	69
Cow's and sheep's cheese 2[b]	2.44	1.57	3.43	3.96	1.15	7.44	34.49	96

Cheese samples	C6:0	C8:0	C10:0	C12:0	C12:0/C10:0	∑(C6:0, C8:0, C10:0)	C16:0	Percentage of cow's milk
	% of total FA					% of total FA		
Cow's and sheep's cheese 3	1.89	2.17	7.12	3.77	0.53	11.19	28.95	81
Cow's and sheep's and goat's cheese 1	3.62	2.91	7.51	4.79	0.64	14.04	30.53	

[a)] contains traces of cow's milk
[b)] adulterated sample with cow's milk
[c)] suspected adulterated sample with cow's milk

In the analysed samples, the C12:0/C10:0 FA ratio in cow's cheese varied from 1.05 – 1.18, in sheep's cheese between 0.48 and 1.18, while for goat's cheese, the ratio was between 0.39 – 0.70. Among the 24 sheep's cheeses sampled <5% of cow's milk was present in three samples (Sheep's cheese 7, 13, 20; Table 4.5), 7% of cow's milk was detected in one sample (Sheep's cheese 21; Table 4.5), and in four samples (Sheep's cheese 10, 12, 14, 16; Table 4.5) the percentage of cow's milk exceeded 10%. Among the 17 goat's cheese samples, one sample (Goat's cheese 14; Table 4.5) contained 6% of cow's milk, and nine samples (Goat's cheese 6, 7, 9, 10, 12, 13, 15, 16, 17; Table 4.5) contained >10% of cow's milk. Among the mixtures of cheese made from cow's, sheep's or goat's milk, the mixed cow's and sheep's cheese contained more cow's milk than sheep's milk. However, since the declaration does not include the amount of cow and sheep's milk, these samples cannot be treated as adulterated. Those cheese samples with a ratio of C12:0/C10:0 FAs in the range expected for sheep's or goat's milk (0.58 and 0.44, respectively), indicates that there is no cow's milk present; thus the evaluation of the presence of cow's milk was not necessary.

The addition of >5% of cow's milk was taken as the limit for non-labelled cow's milk in sheep's and goat's milk cheese samples. This limit was evaluated based on the analytical precision of FA extraction and the gas chromatographic analysis. The cheese samples that contained more than 5% of cow's milk are considered as adulterated and were further divided into two groups, namely suspected adulterated cheeses, which contained between 5 and 10% of added cow's milk and adulterated cheeses, which contained >10% of added cow's milk.

Due to inconsistencies between measured FA content and the data on the declaration (Table 4.5), there is sufficient reason to believe that four sheep's cheeses (8%) and nine goat's cheeses (17%) were adulterated, since the ratio of C120/C10:0 FAs increased and exceeded 0.58 and 0.44 values for sheep's and goat's milk, respectively. In these samples, >10% of cow's milk was detected. In total, at least 25% of high-value cheese available on the Slovenian market does not correspond to the data on the declaration and can be considered as adulterated. Other cheeses that contained <10% of cow's milk (9%) might represent a risk to people who are allergic to cow's milk.

Sheep's and goat's cheese as well as cheese prepared from a mixture of different types of milk, including cow's milk, were also analysed using real-time PCR assays. Detection of cow's milk in sheep's and goat's cheese was obtained using the Cow1/BoCaOv system. Among the 24 samples of sheep's cheese, cow's milk was detected in 11 samples with the Cow1 assay. In one sample, cow' milk was below the LOD, four samples contained <5% of cow's milk, while five samples of sheep's cheese contained from 6.2 – 58.0% of cow's milk. Since cow's milk was not declared, these five cheese samples can be considered as adulterated. Among the 17 goat's cheese samples, cow's milk was detected in five samples with Cow1 assay, in two samples cow's milk was <LOD, one sample contained <5% of cow's milk, while two samples contained 7.5 ± 0.45% and 17.0 ± 0.9% of cow's milk and can be considered adulterated.

The addition of >5% of cow's milk was used as the limit of the fraudulent addition of cow's milk to sheep's and goat's cheese. The obtained results with real-time PRC analysis indicated that 15% of sheep's cheese samples contained <5%, while 12% of sheep's cheese samples contained >5% of undeclared cow's milk and can be considered adulterated. Among the goat's cheese samples, 7% contained <5% cow's milk, and 5% contained >5% of undeclared cow's milk.

A comparison between FA analysis and real-time PCR of analysed cheese samples is presented in Table 4.6. Samples, where the methods disagree are highlighted in blue. Differences between the obtained results may occur since various factors affect both the FA composition in milk as well as real-time PCR quantification. For example, the variability of FAs in milk is influenced by breed, animal genetics, farm management practices, animal nutrition and stage of lactation. The verification of the FA profile in milk is dependent on the analytical methodology, which includes sample preparation and extraction of FAMEs (Hanus et al., 2018). However, the applicability of quantitative real-time PCR for determining cow's milk in mixed-milk cheese samples may be hampered by factors such somatic cell counts in milk, the concentration of DNA molecules in the samples, degradation of DNA, optimal selection of primers (oligonucleotides) and probes as well as amplification efficiency and the specific instrumentation platform (Klančar 2015; Mayer et al., 2012). In addition, several technological parameters in ripened commercial cheeses such as homogenization, filtration, centrifugation and heating during pre-treatment of cheese milk, may influence the final DNA concentration (Mayer et al., 2012). The sample preparation of DNA is also a factor affecting PCR analysis and its sensitivity since DNA is usually extracted from somatic cells (SC) that are present in milk in large but generally variable numbers. Many factors contribute to elevated milk somatic cell count, such as breed, stage of lactation, parity, milk yield, management practice, and the hygienic condition of milk production on farms (Volk 2012). In the case of cow's milk somatic cell counts range from 100,000 SC/mL – 150,000 SC/mL for a healthy animal to >1000,000 SC/mL during bacterial infection. Similar somatic cell counts are observed in sheep's milk, ranging from 500,000 SC/mL to 1,000,000 SC/mL, while somatic cell counts in milk from goats are higher than somatic cell counts in milk from cow and sheep and range from 750,000 to 5,400,000 SC/mL.

Dabrowska and co-workers (2010) found that the relative quantity of cow DNA changes significantly with somatic cell counts. They found that a 5% addition of cow's milk with 800,000 SC/mL produces the same relative quantity of DNA compared to 20% addition of cow's milk with 50,000 SC/mL.

Table 4.6: Percentage of cow's milk present in different kinds of cheese obtained using fatty acid analysis and real-time PCR assay.

	% of cow's milk in cheese sample	
Sample	**Fatty acid analysis**	**Real-time PCR**
Sheep's cheese 1	–	–
Sheep's cheese 2	–	–
Sheep's cheese 3	–	+ (>5%)
Sheep's cheese 4	–	–
Sheep's cheese 5	–	–
Sheep's cheese 6	–	+ (<5%)
Sheep's cheese 7	+ (<5%)	+ (below LOQ)
Sheep's cheese 8	–	–
Sheep's cheese 9	–	–
Sheep's cheese 10	+ (>5%)	+ (below LOQ)
Sheep's cheese 11	–	+ (below LOQ)
Sheep's cheese 12	+ (>5%)	–
Sheep's cheese 13	+ (<5%)	+ (>5%)
Sheep's cheese 14	–	–
Sheep's cheese 15	+ (>5%)	+ (>5%)
Sheep's cheese 16	+ (>5%)	+ (>5%)
Sheep's cheese 17	–	+ (>5%)
Sheep's cheese 18	–	–
Sheep's cheese 19	–	+ (below LOQ)
Sheep's cheese 20	+ (<5%)	+ (<5%)
Sheep's cheese 21	+ (>5%)	–
Sheep's cheese 22	–	–
Sheep's cheese 23	–	–
Sheep's cheese 24	–	–
Goat's cheese 1	–	+ (<5%)
Goat's cheese 2	–	–
Goat's cheese 3	–	–
Goat's cheese 4	–	–
Goat's cheese 5	–	+ (>5%)
Goat's cheese 6	+ (>5%)	–
Goat's cheese 7	+ (>5%)	–
Goat's cheese 8	–	+(>5%)
Goat's cheese 9	+ (>5%)	–
Goat's cheese 10	+ (>5%)	–
Goat's cheese 11	–	–
Goat's cheese 12	+ (>5%)	–
Goat's cheese 13	+ (>5%)	–
Goat's cheese 14	+ (>5%)	+ (below LOQ)
Goat's cheese 15	+ (>5%)	–
Goat's cheese 16	+ (>5%)	+ (below LOQ)
Goat's cheese 17	+ (>5%)	–
Cow's and sheep's cheese 1	+ (>5%)	+ (>5%)
Cow's and sheep's cheese 2	+ (>5%)	+ (>5%)
Cow's and sheep's cheese 3	–	–

A comparison of the results obtained based on FAs composition and real-time PCR assay indicated that in 26 kinds of cheese (59%), both methods are in good agreement. In 10 cheese samples (23%), two sheep's cheese and eight goat's cheese samples, the cow's milk was determined only by their FA composition, while in eight cheese samples (18%), six sheep's cheese samples and two of the goat's cheese samples the cow's milk was identified only by real-time PCR. The percentage of sheep's and goat's cheese that contained cow's milk or cow DNA obtained by FA analysis and real-time PCR are presented in Figure 4.2.

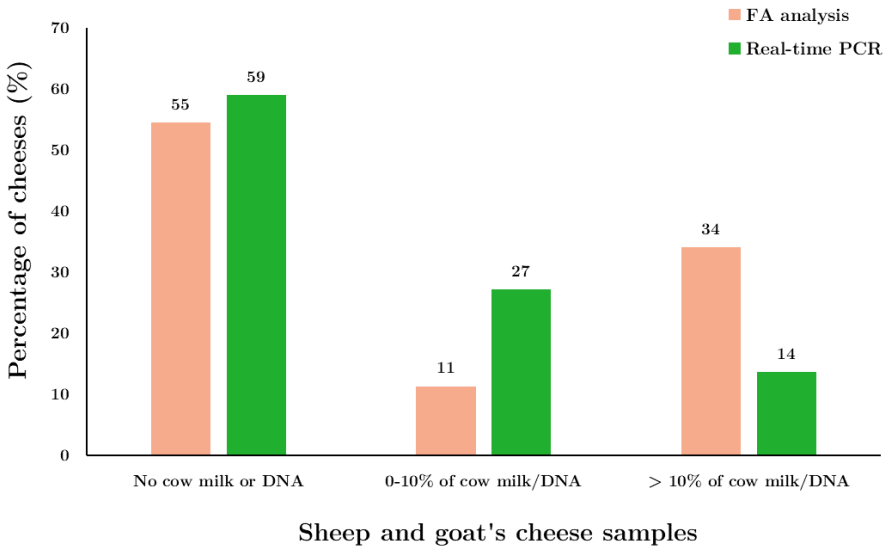


Figure 4.2: Percentage of sheep and goat's cheese samples containing cow's milk or cow DNA determined by their fatty acid content and real-time PCR.

4.4.2.2 The authenticity of low-cost cheeses available on the Slovenian market

Low-cost cheese made only from cow's milk, available on the Slovenian market, were analysed to verify for the addition of non-milk fat and to check if the corresponding declaration is correct. In nine cheese samples from Slovenia, Austria, and Germany, 36 FAs were determined, and the sum of the total content of SFAs, MUFAs, and PUFAs in cheese samples was calculated. Based on the FA composition in milk, it was found that non-low-cost cheeses, available on the Slovenian market in 2014, were unadulterated with cheaper vegetable fat since the FAs composition of SFAs, MUFAs and PUFAs corresponds to the composition of FAs in raw cow's milk. Milk fat can be altered depending on the nutrition of dairy cows; thus the FA composition of SFAs, MUFAs and PUFAs in raw milk varies between 67 – 75%, 18 – 27%, and 2 – 5% of total FAs, respectively (Collomb et al., 2008; Elgersma et al., 2006; Ellis et al., 2006; Lock & Bauman, 2004). In analysed cheese samples the content of SFAs varied between 75.8 – 80.9% of total FA, the content of MUFAs ranged between 20.7 – 25.4% of total FA, while the content of PUFAs was between 2.6 and 4.2% of total FA. The results are presented in Figure 4.3. All analysed samples are in agreement with the data for raw cow's milk and correspond to the label declaration.

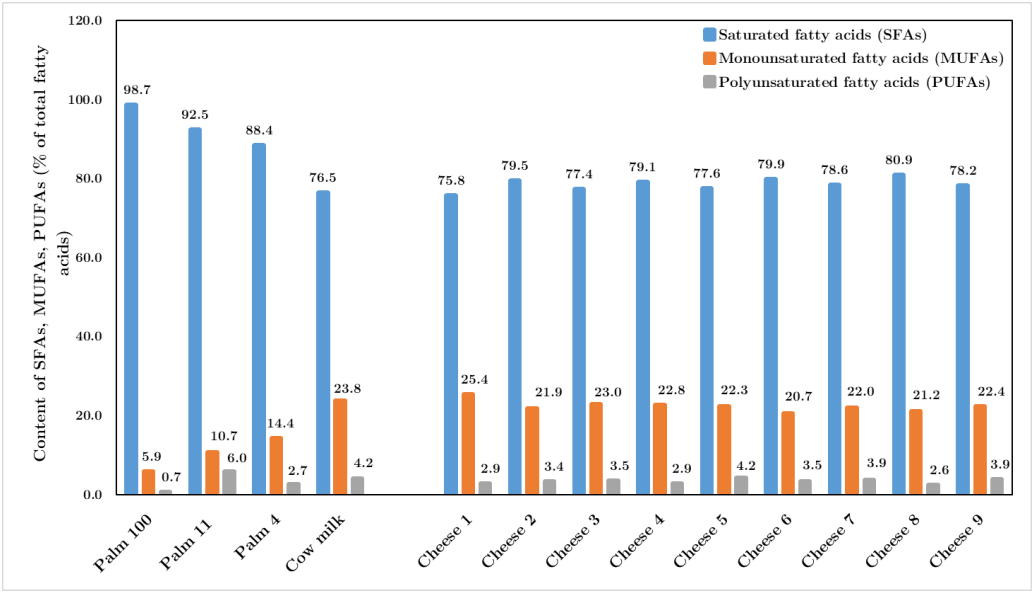


Figure 4.3: Comparison of saturated, monounsaturated and polyunsaturated FAs between palm fat, adulterated raw milk and unadulterated milk and low-cost cheeses available on the Slovenian market. Palm 100 represents 100% palm fat; Palm 11 represents adulterated milk with 11% of palm fat, and Palm 4 represents adulterated milk with 4% of palm fat.

Chapter 5

Conclusions

The availability of reliable analytical methods, which can ensure the safety, quality and authenticity of food, play an important role in modern society. This work has established a large-scale database of stable isotopic ratios of light bio-elements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$), elemental compositions of micro and macro elements, FA compositions, and stable carbon isotope ratios of individual FA in milk. This dataset will form the basis for the development of novel approaches for determining the authenticity of milk and dairy products, which could be included in new regulations. The database was further used to verify the authenticity of commercially available milk samples and for determining of the geographical origin of cow, sheep and goat's milk and cheese, which contribute towards supporting the existence of two PDO Slovenian cheeses: "Bovški" and "Kraški". To achieve the overall goal of this thesis the validation of the methods in the area of elemental composition, the JSI participated in an inter-laboratory comparison at the highest metrological level and development of food matrix certified RMs for stable isotope ratio measurements had been considered. The proposed dissertation will help the competent authorities to protect the quality of dairy products and at the same time to protect both consumers and producers from fraudulent practices and maintain brand reputation. The contribution to the science performed with this study has resulted in six published papers in SCI international journals. The work has been presented at 21 international conferences (see Bibliography) with 11 oral presentations, among which I presented the work in 14 international conferences with five oral presentation.

The main conclusions of the present dissertation can be summarized into four interrelated research topics as follows:

1. **The development of suitable methods and protocols for the determination of elemental and stable isotope measurements:** This work forms the basis of the first three investigations. Sample preparation and analytical methodology were critically tested and evaluated in terms of uncertainty, accuracy, limits of detection (LOD), while new food matrix reference materials of plant and animal origin for stable isotope-ratio measurements were developed and characterized. Also, validation of the methods used for multi-elemental analysis in milk samples was performed. Three techniques, namely EDXRF, k_0-INAA and ICP-MS, were compared and the data obtained used to create an element profile of milk to provide information about nutritional uptake, levels of trace and toxic elements, and authenticity and geographical origin. Quality assurance proved entirely satisfactory for all involved measurements, and an intercomparison exercise between EDXRF and k_0-INAA revealed good agreement between the two methods. Method precision was also satisfactory with relative standard deviations of 1 – 10%. Except in the case of Se, As, Cd and Pb were

the precision was >10%, since the measured concentrations were close to the detection limit. The simple, fast, and inexpensive EDXRF method in combination with ICP-MS, were found to be the most appropriate techniques for the analysis of Mn, Fe, Cu, Se and toxic elements such as As, Cd and Pb. These methods were used to obtain multi-elemental data obtained for Slovenian milk samples. The data was then used to create a linear discriminant model for discriminating milk according to geographical origin. However, only the partial separation of milk samples was possible using elemental content with an overall prediction rate of 66.7%.

The second investigation dealt with carbon isotope ratio measurements in a food matrix as part of an inter-laboratory assessment. Achieving comparability of results is one of the fundamental aims of any analytical laboratory. To verify the performance of the measurements for stable isotopes analysis, our laboratory participated in the international CCQM-K140 inter-laboratory comparison exercise. The results of bulk honey sample analysis obtained by national and designated metrological institutes from five countries were briefly reviewed and compared to those obtained by forensic stable isotope laboratories connected via the Forensic Isotope Ratio Mass Spectrometry (FIRMS) Network. It was found that:

- The majority of the participants employed elemental analyser-IRMS for determining the $\delta^{13}C_{VPDB\text{-}LSVEC}$ value and two (or more) point scale realization for normalization of the results. There were some differences in the corrections applied to the raw data since only metrology institutes used CIAAW-recommended ^{17}O correction, while only FIRMS laboratories employed a linearity correction to account for differences in sample mass between replicates. However, only one CCQM laboratory used exactly matrix-matched quality control materials (in-house honey) and took into account the principle of identical treatment of samples and reference material.
- The results revealed a good agreement between metrological institutes and forensic laboratories regarding carbon isotope ratio analysis of bulk honey since all participants ensured traceability and provided measured uncertainty.
- The main difference was in reporting uncertainty since almost all FIRMS laboratory provided standard deviations rather than an estimate of measurement uncertainty.
- The maximum difference in $\delta^{13}C_{VPDB\text{-}LSVEC}$ value for the bulk honey reported by a pair of participants was 0.37‰, while the typical reported expanded measurement uncertainty was 0.28‰ (k=2) and was >2.5 times the typical standard deviation reported by the FIRMS participants.
- Reporting of standard deviations rather than full measurement uncertainties can be a major factor for poor performance metrics. It can also affect the interpretation of the results, where low-level adulteration can only be detected by methods that result in suitable small measurement uncertainties, i.e., 1‰ or less.

The third investigation looked at the correct usage of reference materials (RMs) for performing accurate measurements and providing reliable data for stable isotope ratios of light elements in food forensics. Due to the lack of certified RMs in the field of food authenticity testing and food provenance verification, ten new food matrix RMs for hydrogen, carbon, nitrogen, oxygen and sulphur were developed, quality tested and measured for isotope-delta values. The following conclusions of the research are as follows:

- The RMs include (i) two types of honey from tropical Vietnam (USGS82) and Canada (USGS83), (ii) four vegetable oils from C_3 and C4 plants cultivated in

Italy (USGS84), Peru (USGS85), Vietnam (USGS86) and USA (USGS87), (iii) bone collagen powder of marine (USGS88) and terrestrial origins (USGS89) and (iv) flours from Italian millet (C_4 plant USGS90) and Vietnam white rice (C_3 plant USGS91).

- The new RMs will allow users to normalize measurements to the isotope-delta scale and span a range of δ^2H_{VSMOW} values from -207.4 to -43.3‰, for $\delta^{13}C_{VPDB}$ from -30.60 to -13.72‰, for $\delta^{15}N_{air}$ from -20.25 to +17.49‰, from $\delta^{18}O_{VSMOW}$ from +18.20 to +26.33‰ and for $\delta^{34}S_{VCDT}$ from -20.25 to +17.49‰.
- Comparison of various laboratory practices indicate that δ biases in excess of the analytical precision are likely when more than one isotope ratio is measured during a sequence via peak jumping (for example first measuring N_2, then CO_2 and finally SO_2) and when signal strengths of the samples and standards are not matched. The highest reproducibility was achieved when measuring only a single isotope ratio in an analytical sequence, trying to match the analyte yields from samples and the RMs. Measuring of hydrogen and oxygen stable isotope ratios, especially in honey, collagen, and flour are challenging when exposed to atmospheric moisture and the influence of exchangeable hydrogen in the samples is uncontrolled. The particle size of solid organic samples can result in significant bulk δ^2H variance, due to available surface area for adsorption of water and access to exchangeable hydrogen. It is recommended that the particle size of unknown samples should be similar to that of the RMs and that the RMs should be exposed to laboratory air at ambient temperatures (acclimated) for at least five days prior to analysis. Also, honey and vegetable oil RMs sealed in silver tubing by the USGS greatly facilitated the reproducibility of measurements of bulk δ^2H and $\delta^{18}O$ values of reference materials in different laboratories, even in warm climates with high humidity.
- All "stock supplies" of food matrix RMs should be stored in glass under vacuum or noble gas to exclude oxygen and to extend the shelf life to decades when stored at -18 °C in the dark at the USGS, Indiana University and Jožef Stefan Institute.

2. **The establishment of a database of authentic Slovenian milk and dairy products and the development of traceability models**: The database established as part of this work includes stable isotope composition of light elements, elemental composition and FA content and isotopic composition of individual FAs in milk and cheese. Milk and cheese from different animal species (cow, sheep, goat) are also included. The first systematic characterization of authentic Slovenian cow, sheep, and goat's milk and cheese were defined using a combination of isotopic ratios of major light elements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$) and elemental composition (P, S, Cl, K, Ca, Zn, Br, Rb and Sr). These were then used to trace the origin of premium Slovenian dairy products and contributed towards supporting the existence of Slovenian PDO cheese, and the protection of consumers and producers form fraud. In this research, 124 samples of Slovenian milk and 30 cheese samples collected during May, June and July from 2012 and 2013 were analysed. The results showed:
   - Discrimination of cow's milk according to production origin was only possible in summer 2012, where discriminate analysis (DA) identified Cl, Zn, P, Ca and K as the most discriminating parameters.
   - Sheep's milk and cheese from four geographical production locations could be distinguished according to their geographical origin based on the P, S, K, Cl, Ca, Zn content and $\delta^{13}C$ and $\delta^{15}N$ values in the casein fraction, with an overall classification rate of 97% and 100% in the case of milk and cheese, respectively.

- When differentiating between species, linear discriminant analysis (LDA) revealed that samples from sheep, goat and cow's milk were well separated; only the results for goat's milk were more highly dispersed. The most discriminating parameters were K, Cl, P, $\delta^{18}O$, S and Zn. All cow and sheep's milk samples were correctly classified (100%) into their original groups, while for samples of goat's milk, a correct classification rate of 91% was achieved. LDA of the cheese data indicated that all of the sheep's, goat's and cow's cheese samples were correctly classified (100%) into their original groups, where Zn Sr, Ca, Rb, S and Cl had the most discriminating power.
- The results suggest that LDA is a useful tool for the inspection and the authentification of milk and cheese according to its declaration and that such a methodology can effectively contribute towards supporting the existence of the Protected Designation of Origin status for "Bovški" and "Kraški" sheep's cheese and "Tolminc" cow's cheese and its incorporation into a traceability system.

In the next study, the use of stable isotopes of light elements and elemental composition for determining the geographical origin of authentic cow's milk from four geographical regions; Alpine, Dinaric, Mediterranean, Pannonian in Slovenia were investigated. In total, 277 samples of cow's milk were collected during summer and winter from 2012 to 2014. The conclusion of the study can be summarized as follows:

- The first isotopic and elemental database of authentic Slovenian milk was used for classifying samples according to the year, season and production origin. These included stable isotope values of $\delta^{18}O$ in milk water; $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{34}S$ in casein isolated from milk, and the elemental composition of the whole milk (Ca, K, Cl, S, P, Zn, Br, Rb, Sr, Mn, Fe, Cu, Se, As, Cd, and Pb).
- Discrimination of milk according to the year and season using LDA based on stable isotope and elemental parameters was most successful when taking into account the following parameters: $\delta^{18}O$ in milk water, $\delta^{13}C$ and $\delta^{15}N$ in casein fraction and elemental concentrations of P, S, Cl, K, Ca, Mn, Fe, Cu, Se, Zn, Br, Rb and Sr. The overall temporary prediction variability was 84.6%, with the most discriminating parameters being $\delta^{18}O$ in milk water, P, S, $\delta^{13}C$ and $\delta^{15}N$ in casein fraction, and Ca, Zn, and Mn.
- LDA revealed that it was possible to discriminate milk from three of the four geographical regions (Mediterranean, Pannonian and Dinaric), while the Alpine group overlaps with the Dinaric and Pannonians regions. The best separation was obtained for 2013 and 2014 since the results also include $\delta^{2}H$, $\delta^{18}O$ and $\delta^{34}S$ values in casein fraction. The most discriminating parameters for separation were $\delta^{18}O$ in milk water, $\delta^{34}S$ in casein fraction, Cu, Cl, Br, Rb, Se, and $\delta^{2}H$ in casein fraction, Mn, $\delta^{18}O$ in casein fraction, Sr and $\delta^{13}C$ in casein fraction. Prediction ability was the highest for the Pannonian region (82.1%) and lowest for the Alpine region (26.9%), with an overall prediction ability of 56.4%.
- Orthogonal projection to latent structure-discriminant analysis (OPLS-DA) for pairwise comparison among all four regions displays good regional predictability ($\geq$0.77). It was possible to differentiate among the four geographical regions based on $\delta^{13}C$ values in casein and the Br content of milk.

The sixth investigation was oriented to the characterization of Slovenian milk based on FAs content and stable isotope ratios of carbon within FAs to study variability related to year, season and region. During a 3-years study (from 2012 – 2014) a total of 231 raw cow's milk samples were collected twice per year in summer and winter, covering the same four distinct geographical regions as studied previously. This work

contributed to the development of new analytical tools to evaluate both the need and options for origin tracing of milk, focusing on Slovenian milk. Based on the obtained results, the conclusions are summarized as follows:

- During the 3-years study, results indicated that season and year of production have a higher impact on the FAs composition of milk than production region. Every milk sample was characterized by its FAs profile, together with year and season of production and the presence of corn silage and cereals in the animal's diet and analysed using a clustering approach. Results revealed 14 different clusters and those variables that play a significant role in defining the clusters.
- LDA model was useful in discriminating milk according to the year and season of production with overall prediction ability of 86.7%, where the most discriminating parameters were C20:4ω-6, CLA, C17:0i, C20:1ω-9, C18:3ω-6, C20:3ω-3.
- Geographical origin discrimination of 230 authentic Slovenian milk samples based on FAs composition using a LDA model was less successful compare to temporal variability. Based on the LDA model, the Mediterranean group was separated from the other regions based on the highest values of C15:0 and C17:0, C17:0i and C17:0a FAs. The most discriminating parameters for the separation of the Alpine and Dinaric groups were C18:3ω-3, CLA, C18:3ω-6 and C20:5ω-3 FAs, while for the Pannonian group it was ∑C18:1 *cis* and *trans* isomers, C12:0, C11:0, C21:0, C18:0 and C18:2ω-6. The overall prediction ability was 44.7%. Also, regional discrimination was most successful when the data were divided according to season. The LDA analysis of 39 milk samples collected in winter 2013 produced a good separation of all four geographical regions, where the C15:0, C15:0i, C15:0a, C18:3ω-6 and CLA had the most discriminatory power. However, the leave-one-out cross-validation (LOOVC) test is low, and only 35.9% of the samples were classified correctly. Overall, the content of FAs alone could not discriminate between production regions.
- Stable carbon isotope composition of individual FAs indicates less variability between season and year than the FA composition. The LDA according to the year/season of production was improved when stable isotope composition of individual FA was included. A LOOCV classification rate slightly improved from 80.5% (only FA composition) to 83.7% (FA composition and stable carbon isotope composition of individual FA).
- Stable carbon isotope composition of individual FAs could only discriminate between the two geographical regions: Mediterranean and Pannonian. The data suggest that $\delta^{13}C$ values in FA could be more useful for studying metabolic transformation processes in ruminants. It appears that the natural differences in the $\delta^{13}C$ values of FA depend on both diet and the precursor FA.
- It was also observed that the most suitable milk for healthy human consumption originates from the Alpine and Mediterranean regions, due to the higher percentage of ω-3 PUFAs and CLA. Quality of milk was also affected by production season. Differences in milk quality support the assertion that control over the geographical origin of milk is needed.

3. **Development of the methods to identify the authenticity of dairy products:** Two different methods were tested and compared to identify the authenticity of dairy products, namely FA composition and real-time PCR. The fatty acid composition was used to check two types of adulteration: the substitution of part of the fat of plant origin and admixtures of milk of different species.
   - A possible adulteration on the market that reduces the cost of the products is an admixture of vegetable tallow with raw milk and can be detected based on the

sum of the individual FAs. It was observed that adding different amounts of palm fat to pristine milk resulted in an increased content of SFAs, decreased content of MUFAs and decreased content of PUFAs (especially ω-3 and ω-6 PUFAs). Adulteration of raw milk with vegetable fat could be detected when at least 1% of vegetable fat was added to milk.

- In the production of high-quality cheeses, the price depends on the type of milk (goat's, sheep's, or cow's milk), therefore the correct declaration is very important. The authenticity of individual high-value cheese made from sheep's and goat's milk was evaluated based on the ration between lauric (C12:0) and capric (C10:0) FAs, which is now considered an appropriate method for the evaluation of adulteration of sheep's and goat's cheeses with cow's milk. Based on the analytical precision of FA determination, the addition of >5% of cow's milk was taken as the limit for non-labelled cow's milk in sheep's and goat's cheeses.
- Real-time PCR could also be used for the identification of the addition of cow's milk, i.e., if the content of cow's milk is >0.1% and for a semi-quantitative analysis when the content of cow's milk is >0.5%. The proposed method provides a rapid, robust and sensitive tool for the identification of adulteration of goat's and sheep's milk cheeses with cow's milk. The parallel investigation of goat and sheep's cheese by real-time PCR and FA composition indicate that both methods are comparable when the addition of cow's milk is >5%.

4. **The application of developed models and methods to control the authenticity of milk and dairy products on the Slovenian market:** The real-world application of the methods developed as part of this work was implemented on milk and low and high-value cheese products. The conclusions are as follows:
   - The authenticity of commercial milk samples available on the Slovenian market was checked based on the database of authentic Slovenian cow's milk samples with a model based on data-driven soft independent modelling of class analogy (DD-SIMCA). The results showed that three market samples labelled as Slovenian fall outside of the acceptance criteria and indicate possible mislabelling.
   - Based on FA composition in low-cost cow's milk cheese, it was found that none of the cheeses sampled in 2014, were adulterated with vegetable fat since the FAs composition of SFAs, MUFAs and PUFAs corresponds to the FA composition in raw cow's milk.
   - The study found inconsistencies between the measured FA content and the label declaration of sheep's and goat's milk since in many cases the ratio of C120/C10:0 FAs increased and exceeded the values of 0.58 and 0.44, respectively. The data suggests that at least 25% of high-value cheese products (8% of sheep and 17% of goat) do not correspond to the data on the label declaration and can be considered adulterated. The results of real-time PCR analysis indicated that among the 24 sheep's cheese samples, cow's milk was detected in 11 samples, while in 17 of the goat's cheese samples cow's milk was detected in five samples using the Cow1 assay. Since the presence of >5% of cow's milk was used as the limit of the fraudulent addition of cow's milk to sheep's and goat's cheese, 12% of the sheep's cheese samples and 5% of goat's cheese samples can be considered as adulterated.

     The results obtained for 26 cheese samples (59%) by both FAs composition and real-time PCR assay were in good agreement. In 10 cheese samples (23%), two sheep's cheese samples and eight goat's cheese samples, the cow's milk was determined using only the FA composition, while in eight cheese samples (18%), six sheep's cheese and two goat's cheese, the presence of cow's milk was identified only using real-time PCR. For the identification of adulteration of goat and sheep's

cheese with cow's milk, the results suggest that PCR is the method of choice since it is rapid, robust and sensitive. However, its limitations concerning quantification and standardization must be taken into account.

This thesis proves that stable isotope ratios of light elements and elemental composition, as well as molecular methods such as real-time PCR and the chromatographic determination of FAs, can make an important contribution in determining the authenticity and provenance of food products, which has been verified using milk and dairy commodities. This work also represents the first systematic characterization of Slovenian cow, sheep and goat's milk and cheese and has led to the construction of a database for developing a traceability model for the protection of PDO status cheeses from fraudulent activities such as mislabelling and adulteration. In the future, it is expected that based on these datasets, the generation of isotopic and multi-element maps of food types from different geographical locations could be established which would further improve traceability systems. Greater consideration should also be given to the use of GC-C-IRMS for the determination of carbon isotope ratios in FAs. This method allows specific compounds to be targeted that are important to the overall make-up of a particular foodstuff. While the foundation of this technique can be traced to the very beginnings of mass spectrometry, modern-day applications require sophisticated and precisely calibrated instrumentation and attention to detail concerning sample preparation. Nevertheless, it is expected that this technique will become central to the detection of food fraud, either on its own or in combination with other analytical methods. Overall, information available through this study should be used to increase the transparency of the dairy product supply chain and provide information relating to a product's authenticity in order to better protect the consumer, producer and human health.

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Biography

Doris Potočnik began her studies at the Faculty of Chemistry and Chemical Technology at the University of Ljubljana, Slovenia in 2006. In 2013 she was awarded a Diploma degree in Chemistry and then in 2016 a second degree in Chemical education, for the thesis entitled "*Enantioselective reduction of 2-benzilidenecycloalkanones*" In 2013, she enrolled as a doctoral student in the Ecotechnology programme at the Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, under the supervision of Prof. Dr. Nives Ogrinc at the Department of Environmental Sciences. The focus of her research is on the development of methods for determining the authenticity and provenance of milk and dairy products. During this time, she has become proficient in different isotopic techniques for determining stable isotope ratios of hydrogen, carbon, nitrogen, oxygen and sulphur as well as in multielement analysis and chromatographic methods. In her specialist field, she has paid special attention to the determination of the isotopic composition of lipids using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). During her studies, she has had the opportunity to collaborate in an inter-laboratory comparison at the highest metrological level and helped develop food matrix reference materials. This experience has allowed her to be actively involved in the department's accreditation scheme at the Jožef Stefan Institute, where she is currently employed. She has also attended several workshops on isotopic measurement techniques and presented her work at international conferences, and collaborated closely with the Institute Fondazione Edmund Mach di San Michele all'Adige under the supervision of Assoc. Prof. Dr. Federica Camin, where she strengthened her knowledge of sample preparation in the field of stable isotope analysis.