TRACING ORIGIN OF FOOD USING STABLE ISOTOPES OF LIGHT AND HEAVIER ELEMENTS

Staša Hamzić Gregorčič

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Prof. Nives Ogrinc, Jožef Stefan Institute, Ljubljana, Slovenia
Co-Supervisor: Ass. Prof. Tea Zuliani, Jožef Stefan Institute, Ljubljana, Slovenia

Evaluation Board:
Prof. Milena Horvat, Chair, Jožef Stefan Institute, Ljubljana, Slovenia
Prof. Katarina Vogel-Mikuš, Member, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia
Prof. Paola Iacumin, Member, University of Parma, Parma, Italy

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
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UPORABA STABILNIH IZOTOPOV LAHKIH IN TEŽJIH ELEMENTOV ZA SLEDENJE IZVORU HRANE

Doktorska disertacija

Supervisor: Prof. Nives Ogrinc

Co-Supervisor: Ass. Prof. Tea Zuliani

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To all positive changes in a world full of opportunity.

''Life is not about how hard of a hit you can give. It is about how many you can take, and still keep moving forward.'' – Rocky Balboa

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Abstract

This dissertation examines two main topics. The first topic is focused on the potential of strontium (Sr) isotope analysis to characterise milk and truffles as selected food commodities, traditionally important for Slovenia. The $^{87}Sr/^{86}Sr$ ratio for determining the provenance of bovine milk and truffles originating from different regions of Slovenia has been applied for the first time. Previous studies included stable isotope analysis of light elements and multi-elemental composition to determine the geographical origin of milk and dairy products and the data of the Sr isotope ratios in milk can improve the verification of their origin.

Slovenia has a rich geological diversity making it an ideal setting in which to investigate the potential of the Sr isotope analysis. In pursuit of this, the range of bioavailable $^{87}Sr/^{86}Sr$ values in milk and truffles was established to create an isotopic database. Truffles are a good example where isotope ratios of Sr reflect directly that in the soil, while in milk the primary source of Sr is the cattle's diet (feed and drinking water). While considerable variability of $^{87}Sr/^{86}Sr$ ratios found in milk reflects the substantial heterogeneity of the geological background of its origin, multi-element and multi-isotope approach using cross-validation of truffles resulted in a 77 % correct classification rate according to geographical origin and a 74 % correct classification rate for species. The results, although promising, cannot rule out possible inter-annual or annual variation of the Sr isotopic composition of milk. The underlying geologic complexity and presence of multiple Sr inputs (air, water), including commercial feeding regime of the cattle, can undermine the strength of the provenance assignment of milk. Overall, the results indicate the limitations and directions of the use of $^{87}Sr/^{86}Sr$ ratios as a tool in food authenticity and traceability. The $^{87}Sr/^{86}Sr$ ratio has the potential to distinguish between different milk production areas as long as these areas are characterised by geolithology and land use. Study performed on truffles also indicated significant variations in $^{87}Sr/^{86}Sr$ ratios connected to geology. Based on the preliminary results, it is hypothesised that the $^{87}Sr/^{86}Sr$ ratio is controlled by the carbonate fraction of soil, which makes Sr a helpful tool for tracing the origin of truffles grown in areas without limestone. Both studies show that $^{87}Sr/^{86}Sr$ ratios can be a powerful tool for determining the geographical origin of food originating from countries with more homogeneous geology, while any interpretation based on $^{87}Sr/^{86}Sr$ ratios can be challenging for countries with heterogeneous geology, such as Slovenia. To investigate broader geographical origin, the Sr method requires detailed knowledge of the geology and the land use.

The second topic examines the possibility to detect milk adulteration with water using lactose as an internal standard. Several experiments were prepared, where milk was diluted with different proportions of water and the lactose isolated according to the standard procedure. The procedure utilizes the $\delta^{18}O$ value of the lactose extracted from milk in comparison to the water $\delta^{18}O$ value of the same milk sample. It has been found that the $\delta^{18}O$ of lactose is correlated to that of the $\delta^{18}O$ of the milk water and can be considered as a reliable internal reference.

Povzetek

Ta disertacija obravnava dve glavni temi. Prva tema se osredotoča na potencial analize izotopov stroncija (Sr) za karakterizacijo mleka in tartufov kot izbrani dobrini, tradicionalno pomembni za Slovenijo. Prvič je uporabljeno razmerje $^{87}Sr/^{86}Sr$ za določanje porekla kravjega mleka in tartufov, ki izvirajo iz različnih regij Slovenije. Prejšnje študije so vključevale analizo stabilnih izotopov lahkih elementov in elementarne sestave za določitev geografskega porekla mleka in mlečnih izdelkov, podatki o izotopski sestavi Sr v mleku pa lahko izboljšajo natančnost določanja njihovega izvora.

Slovenija je geološko zelo raznolika, zaradi česar je idealno okolje za raziskovanje potenciala uporabe izotopske sestave Sr za določanje geografskega porekla hrane. Določili smo razpon vrednosti $^{87}Sr/^{86}Sr$ v mleku in tartufih z namenom, da bi ustvarili izotopsko bazo podatkov. Tartufi so dober primer pridelka, kjer je izotopska sestava Sr neposredno povezana z zemljo, medtem ko je v mleku primarni vir Sr prehrana goveda (krma in pitna voda). Medtem ko znatna variabilnost razmerij $^{87}Sr/^{86}Sr$, ki jih najdemo v mleku, odraža precejšnjo heterogenost geološkega ozadja njegovega izvora, pa večelementni in večizotopski pristop z uporabo navzkrižne validacije pravilno razvršča tartufe s 77 % stopnjo glede na geografski izvor in 74 % stopnjo k vrstam tartufov. Rezultati, čeprav obetavni, ne morejo izključiti možnih medletnih ali letnih sprememb izotopske sestave Sr v mleku. Temeljna geološka zapletenost in prisotnost več vnosov Sr (zrak, voda), vključno s komercialnim režimom krmljenja goveda, lahko spodkopava moč določitve izvora mleka. Na splošno rezultati nakazujejo omejitve in smeri uporabe razmerja $^{87}Sr/^{86}Sr$ kot orodja pri pristnosti in sledljivosti živil. Razmerje $^{87}Sr/^{86}Sr$ lahko razlikuje med različnimi območji proizvodnje mleka, če so za ta območja značilni geolitologija in raba tal. Tudi v študiji na tartufih so bile ugotovljene pomembne razlike v razmerjih $^{87}Sr/^{86}Sr$, povezane z geologijo. Na podlagi predhodnih rezultatov lahko domnevamo, da je razmerje $^{87}Sr/^{86}Sr$ pod vplivom karbonatne frakcije prsti, zaradi česar je Sr koristno orodje za sledenje izvora tartufov, gojenih na območjih brez apnenca. Obe študiji kažeta, da so razmerja $^{87}Sr/^{86}Sr$ lahko močno orodje za določanje geografskega porekla živil, ki izvirajo iz držav z bolj homogeno geologijo, medtem ko interpretacija rezultatov razmerij $^{87}Sr/^{86}Sr$ predstavlja večji izziv za države s heterogeno geologijo, kot je Slovenija. Za pravilno interpretacijo rezultatov pri raziskovanju širšega geografskega izvora je torej potrebno dobro poznavanje geologije in rabe tal.

Druga tema preučuje možnost odkrivanja ponarejanja mleka z vodo z uporabo laktoze kot internega standarda. Pripravljenih je bilo več poskusov, kjer smo mleko razredčili z različnimi razmerji vode in iz teh redčenih vzorcev mleka izolirali laktozo po standardnem postopku. V postopku se primerjajo vrednosti $\delta^{18}O$ laktoze, izolirane iz vzorca mleka z vrednostmi $\delta^{18}O$ vode istega vzorca mleka. Laktoza se je izkazala kot zanesljiva izotopska referenca, saj so njene vrednosti $\delta^{18}O$ v korelaciji z $\delta^{18}O$ vode v mleku.

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Abbreviations

IJS	...	Jožef Stefan Institute
C	...	characterisation
GO	...	geographical origin
RO	...	regional origin
A	...	authenticity
V	...	vintage
Y	...	year
S	...	season
RV	...	regional variability
SO	...	species origin
BO	...	botanical origin
AP	...	agricultural practice
SV	...	species variability
GV	...	geographical variability
GC-C-IRMS	...	gas chromatography combustion isotope ratio mass spectrometry
ICP-OES	...	inductively coupled plasma optical emission spectroscopy
ICP-MS	...	inductively coupled plasma mass spectrometry
SNIF-NMR	...	site-specific natural isotope fractionation by nuclear magnetic resonance
INAA	...	instrumental neutron activation analysis
EDXRF	...	energy dispersive X-ray fluorescence
FID	...	flame ionization detector
CSIA	...	compound specific isotope analysis
MC-ICP-MS	...	multicollector ICP-MS
(I)SCIRA	...	(internal) stable carbon isotope ratio analysis
HPLC	...	high-performance liquid chromatography
TXRF	...	total reflection XRF
HS-SPME	...	headspace-solid phase microextraction
CPANN	...	counter-propagation artificial neutral network
PCA	...	principal component analysis
LDA	...	linear discriminant analysis
RDA	...	redundancy analysis
OPLS-DA	...	orthogonal projections to latent structures discriminant analysis
DD-SIMCA	...	data driven soft independent modelling of class analogy
ANOVA	...	analysis of variance
SPR	...	supervised pattern recognition

TIMS	...	thermal ionisation mass spectrometry
ICP-Q-MS	...	quadrupole inductively coupled plasma mass spectrometry
SHRIMP	...	sensitive high resolution ion microprobe
RIMS	...	resonance ionization mass spectrometry
SIMS	...	secondary ion mass spectrometry
REE	...	rare earth element

Chapter 1

Introduction

1.1 World's Battle Against Food Fraudsters

Given the growing awareness of impact of food frauds on food safety and consequently on human health, the public's interest is increasingly focusing on the quality of foods and food production. A lot of attention is paid on where their food came from and what is in it. As consumers cannot always know what processing steps are taken in food production and what ingredients are used in these steps, they want to ensure that the food they buy is safe, healthy, sustainable, and of high and consistent quality. In particular, premium food products have a higher value due to the time required for a certain process or the rarity of any genuine ingredients. In fact, consumers expect to receive exactly what the product is advertised as, otherwise consumers will pay a higher price for a product that is falsely claimed as authentic. On the other hand, food manufacturers often become victims of frauds committed in their supply chains, which contributes to negative outcomes such as financial losses resulting from consumer rejection of foods associated with the manufacturer's brands. One such example is Manuka honey from New Zealand. In 2013, statistics revealed that 1,700 tons of this expensive honey are harvested each year while global sales were as high as 10,000 tons, indicating widespread fraudulent selling of ordinary honey as the expensive Manuka version [1]. Building trust between a consumer and a brand is just as important as any other relationship. If trust is lost, everything the industry is trying to achieve will become more of a challenge. In this way, food fraud becomes not only a public health issue, but also a socio-economic one.

1.2 Food Fraud as a Global Issue

Food fraud comes in different forms, such as *substitution*, *dilution*, *mislabeling*, *artificial enhancement* and *counterfeiting* [2]. The common denominator to commit such frauds is economic gain. There is no current system that collects media reports on food safety scandals and concerns, but several larger scales of food fraud incidents have actually been perpetrated. Among these are the melamine incident in China [3], presence of horsemeat in burgers and ready meals in supermarkets in the United Kingdom and later in many other European countries, including Slovenia [4], [5], vodka spiked with bleach and high levels of methanol [6], or tuna treated with carbon monoxide to enhance its colour and then sold as fresh fish [7]. Other life-threatening or even fatal cases include food allergens or undeclared spices entering foods undetected [8], [9], [10], [11], [12]. Also milk is overtaking nuts as the top food allergy threat, since it can cause severe allergic reactions in infants and children. In 2016, the UK Food Standards Agency (FSA) issued warnings over mislabeled coconut juice drinks after the Australia's Department of Agriculture tested coconut milk for presence of foreign additives (e.g. cow milk) as a result of a reported case

of the death of a 10-year old boy from an allergic reaction to coconut milk imported from Taiwan [13].

The above incidents illustrate vulnerabilities in food regulatory systems; however, these cases represent only a small fraction of global food fraud [14]. It is only a matter of time before the next big fraud case is discovered. As global cases of food adulteration and misrepresentation become more complicated, so are the risks to populations around the wolrd, affecting international food trade due to safety and quality issues [15]. Although it is difficult to quantify how widespread food fraud is throughout the supply chain, experts estimate that its impact on the global food industry is between $30 billion to $40 billion annually [16]. Based on the Joint Research Centre (JRC) food fraud monthly reports for the period from January 2020 to February 2024, fish/seafood and alcoholic beverages (including wine), followed by meat/meat products, tend to be the most adulterated food commodities (Figure 1.1).

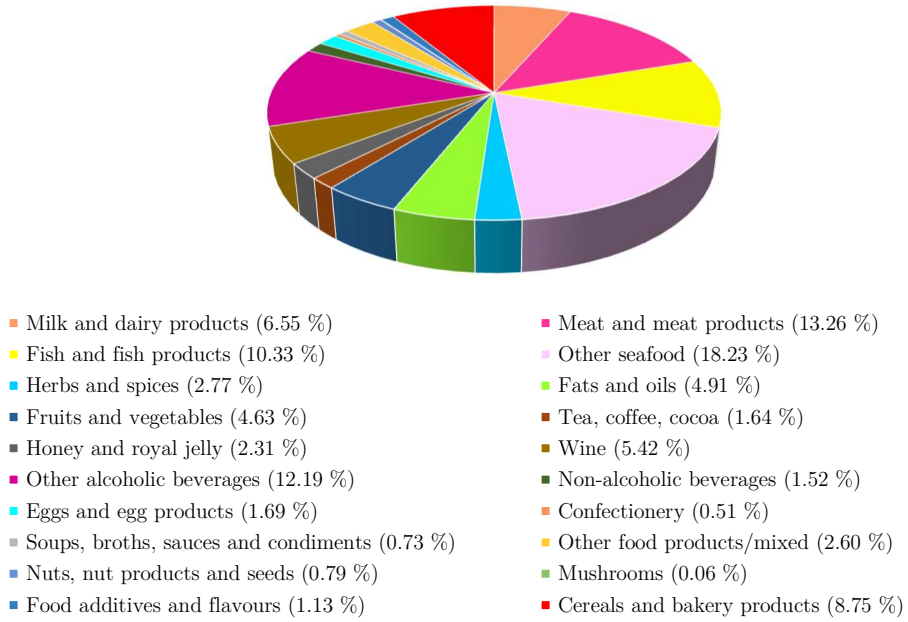


Figure 1.1: Food fraud cases reported during the period from January 2020 to February 2024. (Source: JRC monthly food fraud reports).

The most common adulteration type is mislabelling, which is mostly related to substitution of a valuable food ingredient with cheaper one, or masking the defects of fresh food products with (possibly) harmful substances (Figure 1.2). In more than 40 % of cases discovered during the period from January 2020 to February 2024, there was a lack of traceability documentation or documents were forged in order to cover crimes such as smuggling and/or mislabelling of the product (brand name, origin, ingredients, hygienic requirements, expiration date).

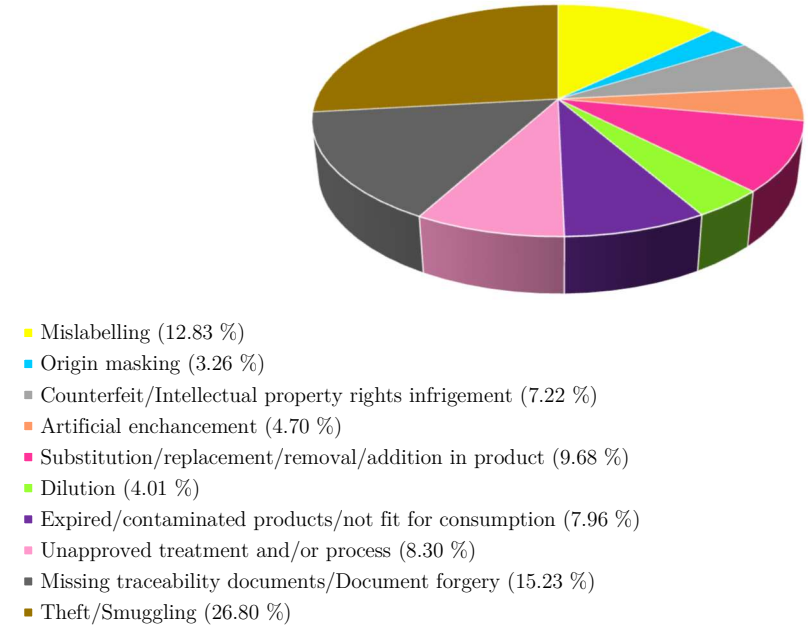


Figure 1.2: Food fraud issues discovered in the period from January 2020 to February 2024 (Source: JRC monthly food fraud reports).

1.3 Actions Against Food Fraud in the European Union

In the aftermath of the horsemeat scandal in 2013, the EU Food Fraud Network (EU FFN) – a group composed of the Commission's Directorate General for Health and Food Safety (DG SANTE), the European Union Agency for Law Enforcement Cooperation (Europol) and Member State liaison bodies – was established to fight food fraud. The goal of this network was to strengthen the EU system and restore consumer confidence, thereby allowing competent authorities to assist and coordinate with one another, exchange information and cooperate in matters where they are confronted with violations of the EU agri-food chain legislation of a cross-border nature. The network works in close consulation with the European Commission's (EC) Knowledge Centre for Food Fraud (in the JRC), which provides expertise in food science, including research on the authenticity and quality of food supplied in the EU. The Regulation (EU) No 1169/2011 encompasses the legal act for the creation of food information throughout the EU (compliance with it is one of the basic preconditions for smooth trade inside the EU) [17], while the Regulations (EC) No +-178/2002 and (EC) No 2019/1381 ensure more transparency and sustainability of the EU risk assessment in the food chain [18], [19]. Under the General Food Law, the European Food Safety Authority (EFSA) was set up by the EU, which covers all matters with a direct or indirect impact on food and feed safety, including animal health and welfare, plant protection and plant health and nutrition. In that way, the agency provides scientific and technical support to the EC and EU countries in all areas impacting on food safety and helps consumers avoid food-related risks [20]. Thanks to the EU food safety standards set in place, the Rapid Alert System for Food and Feed (RASFF) can help to avert many food safety risks before they could become harmful to European consumers [21].

Within the EU legislative framework, the concept of food authenticity refers to the product being genuine and intact, implying that the food or its contents correspond to the label description. Authenticity also includes information that is characteristic of a particular grade of product: origin (specific, geographic or genetic), production

management system (conventional, organic, or traditional practice, free-range) and processing technology. For example, Regulation (EU) No 1151/2012 has been enacted to protect premium food products with geographical indications such as Protected Designation of Origin (PDO), Products Geographical Indication (PGI), or traditional speciality guaranteed (TSG) [22]. These protected products by the EU quality schemes must be distinguishable from similar ones regarding their characteristics, production process and composition, responsible for their quality [22]. Also, the consumption of premium foods prepared by local people using ingredients and local practices typical for that region is considered authentic. Geographical indications can create positive socio-economic benefits to producing regions as these indications allow producers to obtain market recognition and often at a premium. A comprehensive list of registered and protected food products and beverages in the EU can be found in the geographical indications register, called eAmbrosia. In addition to the geographical origin, the botanical origin is also an important parameter of authenticity as it can influence the market price. A more common problem is adulteration of food with different raw materials during production. Such a loss of the identity of the geographical origin of food can expose consumers to many risks arising from cultivation processes due to the globalization of the market and the easier circulation of food products [23]. In this context, food traceability is seen as a food safety and management crisis tool to comply with legislation and meet safety and quality requirements that include (1) ensuring the quality of the raw material entering the food chain, (2) the certification and accreditation of products, (3) quickly locating trouble spots and implementing control systems, (4) preventing food fraud and unfair competition between producers [24], [25], [26]. Traceability is defined as "*the ability to follow the movement of a food through specified stages of production, processing, and distribution*" [27]. It is primarily based on paper records that are easily falsified or insufficient to meet specific requirements. For example, sector-specific legislation applies to certain categories of food products (fruit and vegetables, beef, fish, honey, olive oil), so that consumers can recognize their origin and authenticity.

Traceability is even more difficult in international trade and commerce, as different countries have different regulatory systems and control is limited [28]. As a result, much food fraud goes undetected, especially if it does not pose a threat to public health. The EC is therefore actively working on a harmonized testing methodology to improve comparative tests of food products, which will then enable law enforcement agencies to conduct market tests that include product comparisons across different regions and countries. In this context, the development and/or optimisation of analytical tools for verifying the compliance of foods with the description on the label has become a priority [29], [30], and the characterisation of foods is feasible only by a combination of reliable analytical methods with advanced chemometric tools [31]. Although chemometrics can offer many advantages (speed in obtaining real-time information from data, obtaining quality information from less separable data, improving measurements, improving knowledge of existing processes), due to its complex nature, it also requires knowledge of a problem-solving approach [32].

Considering the problems of the global food market, the purpose of this doctoral thesis is to evaluate the potential in the development of different chemometric approaches using stable isotopic and elemental data to determine possible discriminatory markers for Slovenian and non-Slovenian food products with the aim of combating false declaration of food products. The main topic of the thesis is specifically focused on the role of isotope analysis for identifying food products to aid in confirming their authenticity and provenance, with emphasis on the Sr isotope analysis since it has never been applied for food products grown or produced in Slovenia. The overall objective is to verify how $^{87}Sr/^{86}Sr$ ratio can be used as a tracer to determine the origin using milk and truffles as example commodities, and to set the first database on the Sr isotopic composition of both

commodities for further explorations. Truffles are an example where you can get the direct impact and transport of Sr from the soil, while dairy feed and drinking water are the main source of Sr. First, in Section 1.4, we will discuss the possibilities of using a combination of multi-element and stable isotopes of light elements for tracing the authenticity and origin of foods, and in Section 1.5, we will evaluate the current examples of foods on the Slovenian market. We will then review the basic principles of stable isotopes of Sr, followed by a brief discussion of the behavior of Sr in the environment. Section 1.6 refers to the analysis of the $^{87}Sr/^{86}Sr$ isotope ratio. An overview of the use of $^{87}Sr/^{86}Sr$ isotopic ratio in traceability studies is presented in Section 1.7. The final Section 1.8 is devoted to the presentation of two case studies: milk and truffles.

1.4 Multi-Element and Multi-Isotope Approach

For tracing the geographical origin of foods, multielemental composition and stable isotopes of light elements (*hydrogen* ($^{2}H/^{1}H$), *carbon* ($^{13}C/^{12}C$), *nitrogen* ($^{15}N/^{14}N$), *oxygen* ($^{18}O/^{16}O$), *sulphur* ($^{34}S/^{32}S$)) and heavier elements (*strontium* ($^{87}Sr/^{86}Sr$)) have been widely explored [30], [33]. Geologic background, seasonal variations, altitude, latitude and precipitation produce natural geographic variations in isotopic ratios that are consistent within a given location. Hydrogen and O isotopes, including Sr isotopes, can provide information on the origin of food on a large scale [34], [35], [36], [37], [38], while C, N and S isotopes are useful to a smaller extent [39], [40]. Similar to stable light isotopes, the content of elements in foods is influenced by regional environmental conditions such as soil composition, agricultural practices and local climatic conditions (temperature, precipitation, floods, drought, *etc.*). In addition, elements are usually more stable in commodities than in organic food components, so they can be useful markers for classification of geographical origin [41]. Understanding how and why isotopes and the content of elements in our foods vary is important in terms of the advantages and limitations of these parameters as markers of food origin.

1.4.1 Brief introduction to isotopes and their role in food traceability

The term *isotope* was coined by Scottish doctor and writer Margaret Todd [42] from the Greek *iso* (same or equal) and *topos* (place) in a suggestion to the British chemist Sir Frederick Soddy [43], referring to the fact that isotopes occupy the same place in the periodic table. Isotopes are variations of the same element but with a differing number of neutrons and therefore atomic mass. Most elements have multiple naturally occurring isotopes, which have similar chemical properties, regardless of whether they are *stable* or *radioactive* (i.e. decaying with time).

The mass differences of stable isotopes produce fractionation, which is defined as differentiation of isotopes (when the ratio of light to heavy isotopes in the involved molecules changes) by their mass between two phases [44]. If we want to describe isotopic fractionation in plants and animals, this is best understood by beginning with the individual physical, chemical, and biological processes that contribute to the overall isotopic fractionation. Generally, in every chemical (e.g. photosynthesis, oxidation, combustion) or physical reaction (e.g. vaporisation, condensation, evaporation), lighter isotopes react at a slightly higher rate than heavier ones, because the bonds in a molecule between lighter atoms vibrate with a higher frequency, and therefore they split easier than those between heavier atoms. In the products of a chemical reaction are therefore, at least at the beginning, more lighter isotopes than in the reactants. As a consequence, some substances in nature are more enriched in heavier isotopes than the others, depending on their formation pathway. A good example is the isotopic fractionation of water. When

water condensates (an equilibrium fractionation) [45], the heavier isotopes ^{2}H and ^{18}O become enriched in the liquid phase while the lighter isotopes ^{1}H and ^{16}O tend toward the vapour phase. Also, considerable variations in the isotopic composition of plants occur during photosynthesis [46], where plants preferably absorb ^{12}C than ^{13}C and therefore are enriched in ^{12}C (or depleted in ^{13}C) compared to the atmospheric CO_2.

In nature, elements continuously circulate, hence mixing of sources with different isotopic compositions is a natural process. Because of this continuous conversion in local biogeochemical cycles, the isotopic composition of elements may vary from place to place, from product to product. For example, isotope ratios of $^{2}H/^{1}H$, $^{18}O/^{16}O$ and $^{13}C/^{12}C$ in grape and wine compounds differ with regards to the origin of sugar, the geographical area of production and the year of production. Reasons for these differences lie in the biotic (i.e. mechanism of sugar photosynthesis, grape variety, alcoholic fermentation, yeasts) and abiotic (i.e. geographical origin, general climatic conditions, temperature, rainfall during veraison, date of harvest, oenological treatments) fractionation effects [47]. Abiotic factors differ considerably between continental and coastal areas since climatic conditions such as average monthly air temperatures and monthly precipitation between ripening of grapes influence the evapotranspiration of the vine, which determines the isotopic fractionation of grape compounds. To summarize, when we collect information on the isotopic composition of the assumed sources (water, air, soil) from the environment and their contribution to the overall isotopic composition of plant or animal, we can read into the relative influence of each individual source to the mixture [33], [48]. However, replacing an authentic ingredient in the food product with a similar cheaper one can be difficult to detect, especially when small amounts of an adulterant are added to the food. Dilution makes the problem even more challenging. Indeed, food products are often complex mixtures, and without the reference, it can be challenging to pinpoint any changes in food composition.

This case is more complex for foods of animal origin. If different animals are fed the same diet (plants and drinking water), isotope ratio values of a particular body component may not be the same for all species. If animals consume different diets, the extent of isotopic variability may increase significantly. Thus, isotopic variability among these animals may reflect metabolic and dietary diversity [49]. Understanding the integration of dietary and physiological signals in the isotopic composition of animal's body tissues requires understanding the body size, tissue growth and turnover rates, and fractionation associated with synthesis reactions [50]. In an attempt to quantify the diet-body offset in animals, a large number of controlled feeding studies have been carried out [51], [52]. Mixing models can be used to relate the proportion of plant to animal components (e.g. proteins) incorporated in the tissue if the isotopic offset (i.e. isotopic difference) between tissue and dietary proteins owing to metabolism is considered [53]. In fact, the sum of all isotopic inputs of the diet consumed equals all isotopic outputs, i.e. respired breath, sweat, urinary and faecal excretion.

In contrast to the light stable isotopes, Sr keeps its original isotopic fingerprint unchanged up to the end food product due to its high mass, even after processing, with no isotopic fractionation [54]. The natural variability in Sr isotope ratios is due to radioactive decay of rubidium (Rb) (see Subsection 1.6.1). The $^{87}Sr/^{86}Sr$ ratios within environmental components have strong regional variations that are commonly controlled by the underlying geology, thereby Sr can be used as a geographical tracer to determine its origin [55], [56].

Establishing an isotopic signature is accomplished by measuring the ratios of stable isotopes of a variety of elements (Table 1.1). The table shows the types of different isotopic fractionations in natural materials and the way in which they can be used for food authentication.

Table 1.1: Stable isotopes and their interpretation in foods and beverages origin and authenticity.

Isotope ratio[1]	Fractionation	Information	Reference
$^{2}H/^{1}H$ $\delta^{2}H$	water cycling, latitude, altitude, season	geographical origin, dilution	[45], [48]
$^{13}C/^{12}C$ $\delta^{13}C$	photosynthesis (C3, C4, CAM pathways)	plant species, geographical origin, adulteration	[39]
$^{15}N/^{14}N$ $\delta^{15}N$	N cycle including biological fixation, mineralization, nitrification/denitrification, assimilation, nitrogen fixation	agricultural practice, mislabelling (organic vs conventional product)	[57]
$^{18}O/^{16}O$ $\delta^{18}O$	water cycling, latitude, altitude, season	geographical origin, dilution	[45], [48], [58]
$^{34}S/^{32}S$ $\delta^{34}S$	local soil conditions, proximity to coast, microbial processes	agricultural practice, geographical origin	[40], [59], [60]
$^{87}Sr/^{86}Sr$	age of the rock, Rb/Sr ratio	underlying geology, geographical origin	[61]

Data interpretation also requires an adequate database of food samples against which the unknown food sample can be compared. Samples should be authentic, representative of the population concerned and accurately reflect the natural variation in isotopic and elemental compositions caused by biotic and abiotic factors, collected on a monthly or annual basis to capture data variability at the time of harvest/production. In addition, each region or country has a specific "fingerprint", so these regional or continental baseline maps can be a valuable aid in the traceability studies, as they can provide useful information on climatic conditions, soil classification, intensity of agricultural practices, ground/surface water and vegetation cover [56], [62], [63]. Differences in growth conditions and assimilation of elements in a food product are directly related to the climate of the growing/production area [64]. Several studies show that elements in foods also depend on the accumulative effects of chemical fertilisers [65].

Thanks to extensive ecological and environmental studies and measurements of isotopic ratios in various plant and animal materials, it is possible to determine the local isotopic background of the environment, which is often characterised by predictable isotopic signatures. Stable isotopes can also serve as a valuable diagnostic tool to identify adulterants with chemical properties similar to authentic components [66], [67].

[1] Isotope data are expressed with the conventional δ-notation using the general formula:

$$\delta E_i = \left(\frac{R(E_i/E_j)_{sample}}{R(E_i/E_j)_{standard}} \right) - 1$$

where E is the element (H, C, N, O, S), R is the isotope ratio between the heavier "i" and the lighter "j" isotope ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) in the sample and relevant internationally recognised reference standard. The delta values are multiplied by 1000 and expressed in units "per mil" (‰). For hydrogen and oxygen, Vienna Standard Mean Ocean Water (V-SMOW) is used as a reference standard, the Vienna Pee Dee Belemnite (V-PDB) for carbon, atmospheric N_2 (AIR) for nitrogen, while for sulphur Vienna-Canyon Diablo Troilite (V-CDT) is used.

Isotopic compositions of strontium are typically reported as R_{sample}, with no conversion to δ-notation (e.g. $^{87}Sr/^{86}Sr$). Although reference standards are not needed to report Sr isotope ratios in δ-notation, there are standard reference materials available for calibration and verification of measured Sr isotope ratios (e.g. NIST SRM 987).

1.5 Tackling Food Fraud in Slovenia

Slovenia is a culinary country with plenty of tradition and customs, which are reflected in the variety of local and traditional foods. That raises the question of how to identify products that are authentic and protected in Slovenia.

The Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection (AFSVSPP) was established in 2013 by merging the Veterinary Administration of the Republic of Slovenia, the Phytosanitary Administration of the Republic of Slovenia, the Institute of Food Safety and the Inspectorate for Agriculture, Forestry, Hunting and Fisheries with the aim of ensuring the safety of the entire food chain "from farm to fork". Accordingly, AFSVSPP focuses on stricter control of traceability, adequacy or labelling of agricultural products and foodstuffs, including genetically modified foods. Although legislation on food safety is exhaustive, there is no comprehensive legal framework covering food fraud neither at the EU level nor in Slovenia. Nonetheless, Slovenia pays great attention to protecting its traditional products and currently protects 17 wines and 26 registered food products. In accordance with these measures and campaigns, which are still being carried out, Slovenian experts regularly monitor and verify products in order to successfully deal with the problem of food fraud. The AFSVSPP has conducted 721 inspections between 2013 to 2017, finding 117 violations, i.e. incorrect use of logos, misleading labelling, an invalid certificate, non-compliance in online sale and the sale of organic products by uncertified operations [68].

There is still low awareness of EU quality labels, since Slovenia has a weak tradition in using geographical indications [69]. Slovenian consumers tend to pay more attention to the taste of the food products, its positive impact on their health, and the ingredients [69], while the awareness and use of packaged food labelling information was higher among consumers with secondary education. Results of another survey demonstrated that the price is the most powerful driver of consumer preferences for cheese and honey, while it is origin for ham [70].

On the other hand, through the support of the Joint Centre's Food and Environmental Protection Laboratory (FEPL) and the Slovenian Ministry of Agriculture, Forestry and Food (MAFFS), the Slovenian milk SITE (Stable Isotope and Trace Element) database developed was applied to control Slovenian milk in a real-world application. It has also been used to verify the authenticity of fruit juices on the Slovenian market. Currently, the technology is being transferred to high value truffles, fruits and vegetables. To promote and raise awareness of the importance and characteristics of locally produced and processed foods, the MAFFS began a five-year project "Naša super hrana" (eng. Our super food) in 2019. In the framework of this project, different sectors of producers and processors of meat (beef and poultry), milk (milk and dairy products) and fruit (fruit and processed fruit products) can be presented, making it easier for local consumers to buy food directly from them. To help consumers to easily recognize local quality products, the "Selected Quality" national scheme and the "Selected Quality-Slovenia" protective mark were established. Nowadays, most milk and dairy products, produced and processed in Slovenia, use the "Selected Quality-Slovenia" protective mark. This mark is conferred to the milk and dairy products that are (1) produced entirely in Slovenia, (2) of excellent microbiological quality, and (3) raw milk not older than 15 h is used in the process [71]. To protect the obtained mark and prevent possible fraud, a robust screening method to determine the authenticity and regional traceability is needed.

In 2014, the ERA Chair ISO-FOOD project was established with the aim of ensuring the quality, safety and traceability of food and feed in Slovenia. Project ran its course until 2019 and during this period Slovenian researchers and other experts were actively involved in multidisciplinary research aimed at innovation and the development of new solutions in

the field of analytical techniques in the fields of food characterisation, food safety and food traceability. Within this project, existing databases on food and their consistuents were also upgraded and expanded, as they are fundamental to many areas of research, policy, food production and consumer behaviour and thus play a key role in food safety management.

Additionally, a new way – an ISO-FOOD ontology – was proposed to connect different data coming from different datasets and extract relevant knowledge from these data. To support scientists in the research of isotope knowledge for food science, this ontology focuses on enumerating certain stable isotopes and chemical elements within food compounds in a way that scientists can more easily find answers to open questions about the quality of measurements in food commodities, isotopic characteristics of food of plant and animal origins, food provenance and authenticity [72].

1.5.1 Tracing local foods back to its source

The geographical diversity and different, often locally limited, climatic conditions in Slovenia cause a great variety of relief and vegetation. This diversity means that individual types of food (e.g. Karst Teran wine) can only be produced in certain natural regions, and differences in some parameters can be found between those foods (e.g. olive oil, milk, honey) that are produced in two or more regions. So far, several studies have been conducted to determine the origin and authenticity of various local foods (Table 1.2).

Table 1.2: Examples of studies dedicated to authenticity of various Slovenian food commodities.

Food commodity	Target analysis	Measured parameters	Instrumental technique	Statistical analysis	References
olive oil	C	δ^{13}C (fatty acids)	GC-C-IRMS	-	[73]
wine	GO	Li, Be, Sc, Ti, V, Cr, Co, Ni, Ga, Ge, As, Se, Y, Al, B, Ca, Cu	ICP-OES ICP-MS	PCA CPANN	[74]
wine	RO, A, V	δ^{18}O, δ^{13}C, (D/H) ratios	IRMS SNIF-NMR	PCA LDA	[75]
pumpkin seed oil	GO, A	fatty acids, δ^{13}C	GC-C-IRMS	PCA RDA	[76]
milk	GO	Ca, Mn, P, K, Se, Na, Sr, Zn, Cu, Cl, Cd, Pb, S, Br, Rb, Fe, Ni, Mo, As	k0-INAA EDXRF ICP-MS	LDA	[77]
milk	Y, S, RV	δ^{13}C (fatty acids)	GC-FID CSIA	LDA	[71]
milk	GO, A	P, S, Cl, K, Ca, Zn, Br, Rb, Sr, Cd, Pb, As, Se, Mn, Cu, Zn, Fe, δ^{2}H, δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S	ICP-MS EDXRF IRMS	OPLS-DA DD-SIMCA	[78]
milk	GO	^{87}Sr/^{86}Sr	MC-ICP-MS	DA OPLS-DA	[79]
milk, cheese	SO, GO	P, S, Cl, K, Ca, Zn, Br, Rb, Sr, δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S	EDXRF IRMS	DA	[80]
milk	A	δ^{18}O, δ^{2}H	IRMS	ANOVA	[81]
hops	GO	Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Zn, Br, Rb, Sr	EDXRF	DA	[82]

hops, beer	AP	prenylflavonoids (XN, IXN, 8-PN), δ^2H, δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S	LC-MS/MS IRMS	OPLS-DA	[83]
honey	BO, GO, A	δ^{13}C, δ^{15}N	SCIRA ISCIRA	LDA	[39]
apple	BO, GO, AP	chemical and physical parameters P, S, Cl, Ca, K, Mn, Fe, Cu, Ni, Zn, Br, Rb δ^2H, δ^{18}O, δ^{13}C, δ^{15}N	HPLC TXRF IRMS	LDA	[84]
apple juice	GO	P, S, Cl, K, Ca, Rb, Mg, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Sb, Tl, Pb δ^2H, δ^{18}O, δ^{13}C, δ^{15}N	TXRF ICP-MS NMR IRMS	LDA	[85]
commercial fruit juice	A, BO	δ^{18}O, δ^{13}C, $(D/H)_I$ and $(D/H)_{II}$	IRMS SNIF-NMR	PCA	[86]
potato	GO	Na, Mg, P, S, Cl, K, Ca, Mn, Fe, Ni, Cu, Zn, Mo, Br, Rb, Sr, rare earth elements (Sc, Y, Nb, La, Ce, Pr, Nd, Dy, Er) δ^{13}C, δ^{15}N, δ^{18}O, δ^{34}S	EDXRF ICP-MS IRMS	ANOVA SPR	[87]
tomato	GO	chemical parameters P, K, Ca, S, Cl, Zn, Br, Rb, Sr δ^{13}C, δ^{15}N, δ^{18}O, δ^{34}S	EDXRF IRMS	DA	[88]
garlic	GO	P, S, Cl, K, Ca, Zn, Br, Rb, Sr δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S	XRF IRMS	DA	[89]
commercial vegetables	GO	P, S, Cl, K, Ca, Mn, Fe, Zn, Br, Rb, Sr δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S	EDXRF IRMS	DA	[90]
truffles	SV, GV	aromas	HS-SPME/ GC-MS	LDA	[91]
truffles	SV, GV	Al, As, Ba, Ca, Cd, Co, Cr, Cs, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, Rb, S, Sr, V, Zn Rb/Sr, Sr/Ca, Mg/Ca ratios δ^2H, δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S, ^{87}Sr/^{86}Sr	ICP-MS XRF IRMS MC-ICP-MS	DA	[92]
fresh and freeze-dried truffles	SV, RV	volatile profiles	HS-SPME/ GC-MS	DA	[93]

dietary supplements (Spirulina)	A, GO, AP	P, Ti, Zn, Si, Br, S, Cl, Mn, Rb, Sr, K, Ca, Fe δ^2H, δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S	EDXRF IRMS	PCA DA OPLS-DA	[94]
strawberries	GO	As, Ba, Ca, Cd, Co, Cs, Cu, Fe, Hg, K, Mg, Mo, Na, Ni, P, S, Sr, Zn δ^{18}O, δ^{13}C, δ^{15}N, δ^{34}S	ICP-MS GC-C-IRMS	PCA, LDA OPLS-DA DD-SIMCA	[95]

Abbreviations: C (characterization); GO (geographical origin); RO (regional origin); A (authenticity); V (vintage); Y (year); S (season); RV (regional variability); SO (species origin); BO (botanical origin); AP (agricultural practice); SV (species variability); GV (geographical variability); GC-C-IRMS (gas chromatography combustion isotope ratio mass spectrometry); ICP-OES (inductively coupled plasma optical emission spectroscopy); ICP-MS (inductively coupled plasma mass spectrometry); SNIF-NMR (site-specific natural isotope fractionation by nuclear magnetic resonance); INAA (instrumental neutron activation analysis); EDXRF (energy dispersive X-ray fluorescence); FID (flame ionization detector); CSIA (compound specific isotope analysis); MC ICP-MS (multicollector ICP-MS); (I)SCIRA ((internal) stable carbon isotope ratio analysis); HPLC (high-performance liquid chromatography); TXRF (total reflection XRF); HS-SPME (headspace-solid phase microextraction); CPANN (counter-propagation artificial neutral network); PCA (principal component analysis); LDA (linear discriminant analysis); RDA (redundancy analysis); OPLS-DA (orthogonal projections to latent structures discriminant analysis); DD-SIMCA (data driven soft independent modelling of class analogy); ANOVA (analysis of variance); SPR (supervised pattern recognition).

Although Slovenia is an ideal area for the study of natural factors that regulate the isotopic distribution in local foods, the use of light stable isotopes had some limitations in determining the origin of, for example, Slovenian milk [78]. As already mentioned (see Subsection 1.4.1), stable isotopes of light elements follow seasonal variations that affect their isotopic composition during physical and chemical processes in the environment, thus limiting their potential. In addition, the proximity of macro-regions in Slovenia and the climate changes that affect these regions make it difficult to distinguish between milk samples of different origin, especially if they originate from dairy farms that are located in the area between two regions and therefore have a similar or identical isotopic signature [78]. In this case, strontium isotopes can be used to interpret the origin of plant or animal material based on soil geology and pedological characteristics of the local area where the material is grown or produced, making it a powerful tool for geographic traceability.

1.6 Strontium Isotope Ratio ($^{87}Sr/^{86}Sr$) as Geographical tracer

In the last decades, the $^{87}Sr/^{86}Sr$ isotope ratio gained interest in investigation of archaeological, environmental, medical, forensic and food traceability studies [38].

1.6.1 Strontium isotopes

Strontium is a Group II alkaline earth metal found in rocks, soil water and air. It comprises 0.02 – 0.03 % of the Earth's crust, from where the Sr of water derives [96]. Elemental Sr is soft, silver-gray metal that behaves similar to the other alkaline metals in group II of the periodic table. They have similar properties forming divalent cations in minerals and solution. For example, Sr has an ionic radius of 1.32 Å, which is slightly larger than that of calcium (Ca^{2+} = 1.18 Å). Because of similar isotopic radii and the same valency, Sr^{2+} closely mimics the behaviour of Ca^{2+}, for example, by substituting for Ca^{2+} within the mineral lattice (e.g. plagioclase feldspar, calcite, dolomite, aragonite, gypsum, apatite), and also replacing Ca^{2+} in the cell walls of plants and animals. Strontium occurs naturally in the Earth's mantle as a mixture of four stable isotopes, i.e. ^{84}Sr, ^{86}Sr, ^{87}Sr and ^{88}Sr [44]. Of these, ^{88}Sr is the most prevalent form, comprising 82.58 % of natural strontium. The relative abundances of the other three stable isotopes are ^{84}Sr (0.56 %), ^{86}Sr (9.86 %) and ^{87}Sr (7.00 %).

How Sr isotopes are geographically distributed in the biosphere is determined mainly by how ^{87}Sr has evolved in geological systems. Given the Rb-Sr decay system (Figure 1.3), different minerals and rocks in a given geological terrain can have different $^{87}Sr/^{86}Sr$ ratios due to the different ages of the rocks. Specifically, the $^{87}Sr/^{86}Sr$ ratio in a rock mineral depends on (1) the $^{87}Sr/^{86}Sr$ at the time the rock crystallised (time zero, t_0), (2) the $^{87}Sr/^{86}Sr$ ratio, which is directly proportional to the Rb/Sr ratio in most cases, and (3) the time t elapsed since formation [61]. The relative abundance of strontium isotopes (^{87}Sr and ^{86}Sr) in a rock is linked to the initial presence of the radioactive isotope of rubidium (^{87}Rb).

Rubidium is an alkali element with a similar ionic radius to that of potassium (K^+), such that Rb^+ often substitutes for K^+ in K-rich minerals, e.g. potassium feldspar, muscovite, biotite and illite [97]. Only ^{87}Sr isotope is radiogenic; it is formed from the β-decay of ^{87}Rb with a half-life of approximately 4.88 x 10^{10} years.

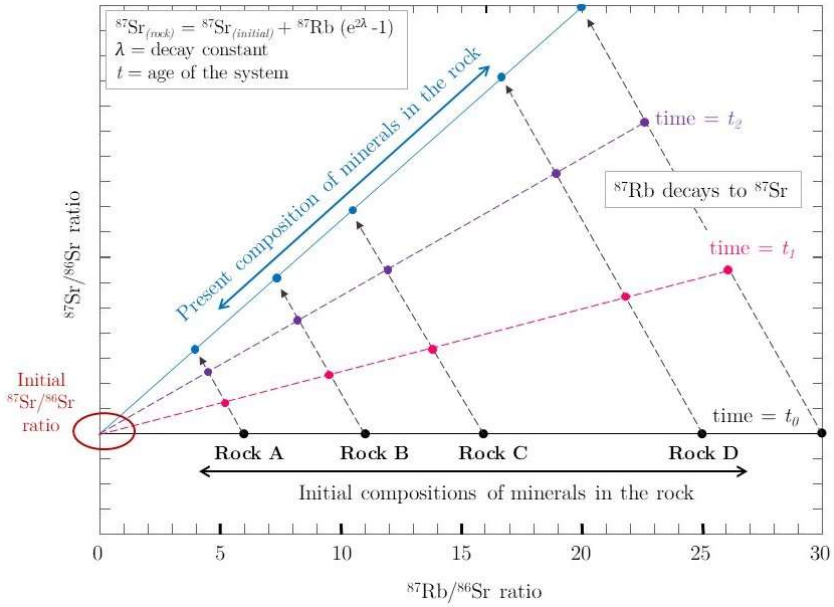


Figure 1.3: Rubidium – strontium decay system. Figure adapted from Dr. Roger C. Wiens [98].

Basically, the starting point for the evolution of strontium isotopes is the $^{87}Sr/^{86}Sr$ ratio at the time of the formation of the Earth. The stable isotope ^{86}Sr is used as a reference isotope, as it is not affected by any radioactive process and remains constant over time.

Enrichment of ^{87}Sr is high in old continental rocks (granites), but low $^{87}Sr/^{86}Sr$ ratios are found in the mantle and rocks derived from it (basalts), resulting from significant elemental fractionation (i.e. fractional crystallisation) during early separation of the continental crust and upper mantle. Strontium tends to concentrate during crystallisation in the crust, while rubidium remains in the liquid phase. The loss of rubidium from the mantle and its enrichment in the continental crust resulted in very different strontium isotope patterns in these two geological domains due to their different Rb/Sr ratios (Table 1.3). This means that the $^{87}Sr/^{86}Sr$ ratio will differ significantly between the current geological terrains. This difference becomes more pronounced with time, and consequently the origin of a sample (from the mantle or crust) can be constrained by using its Sr isotopic signature.

Table 1.3: Typical ranges of strontium and calcium concentrations (expressed in ppm), and Rb/Sr ratios for various types of materials [97].

	Material	Sr	Ca	Rb/Sr
Geological	Sandstone	20	40,000	3
	Low-Ca granite	100	5,000	2
	Deep-sea clay	180	30,000	0.6
	Shale	300	20,000	0.5
	High-Ca granite	440	25,000	0.3
	Ultramafic rock	1	25,000	0.2
	Basalt	500	75,000	0.07
	Deep-sea carbonate	2000	300,000	0.005
	Carbonate	600	300,000	0.005
Soils	Soil minerals	10 - 1000	24,000	
	Labile soil minerals	0.2 - 20	1,000	
	Soil moisture	0.001 - 0.07	1 - 4	
Water	Seawater	8	400	
	River	0.006 - 0.8	15	
	Rain	0.001 - 0.4	1 -100	
	Snow	0.00001 - 0.001	0.01 - 0.1	

According to Faure [99] and Capo et al. [61], Precambrian granitic bedrock and alluvial sands derived from felsic rocks have high $^{87}Sr/^{86}Sr$ ratios (0.710 – 0.716), reflecting the age of the continental crust and high Rb/Sr ratios, from which these materials originated. Himalayan rocks have remarkably high $^{87}Sr/^{86}Sr$ ratios (> 0.74) due to their old age and form from crustal material having high Rb/Sr ratios [100]. Oceanic crust is, in comparison, younger and mainly basalt. As a result, they have lower $^{87}Sr/^{86}Sr$ ratios (0.703 – 0.704). Whereas limestone have intermediate $^{87}Sr/^{86}Sr$ values (0.706 – 0.709), young oceanic basalts and their sediments exhibit the lowest values (0.702 – 0.705). The isotope ratios that characterise a specific geology theoretically pass from the source rocks into soil, groundwater and plants, and eventually up the food chain. The $^{87}Sr/^{86}Sr$ in marine sedimentary rocks reflect that of seawater, which depends on the relative inputs from different weathering and hydrothermal sources (e.g. between continental rocks and basalts). Numerous $^{87}Sr/^{86}Sr$ measurements in ocean water have demonstrated $^{87}Sr/^{86}Sr$ ratios (~ 0.7092) which are constant throughout the world's oceans [101].

In addition, some minerals in a single rock can have quite variable $^{87}Sr/^{86}Sr$ ratios. For example, granite may contain two feldspars with different $^{87}Sr/^{86}Sr$ ratios. The plagioclase feldspar in such granite contains mainly calcium and strontium with a low amount of Rb, resulting in a low $^{87}Sr/^{86}Sr$ value near 0.70. In contrast, K-feldspars, as the most common minerals in granite, are high in rubidium and low in strontium, with $^{87}Sr/^{86}Sr$ ratios above 1.0 [97].

1.6.2 Cycling of strontium in the environment

Given that the $^{87}Sr/^{86}Sr$ ratio in geological strata is a function of mineral composition rather than age, significant geographical variations in $^{87}Sr/^{86}Sr$ ratios across the continents can be used to assign possible provenance [56], [102], [103]. The isotopic composition of Sr enables the characterisation and identification of different sources of Sr in natural systems and enables a reliable description of degree of their mixing when all the end entities are known (Figure 1.4). In this section, the factors affecting the variability of the $^{87}Sr/^{86}Sr$ ratios in the environment are discussed in order to explain their applicability for determining the provenance of milk and truffles.

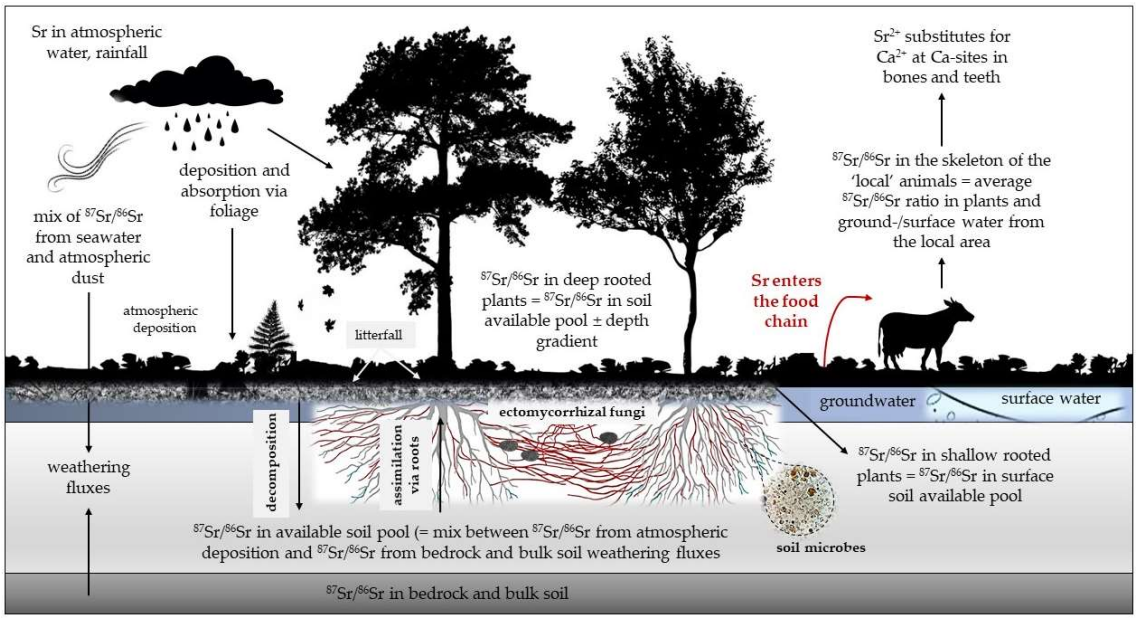


Figure 1.4: Strontium cycle in the soil – air – water compartments and linking the $^{87}Sr/^{86}Sr$ ratio to the geographical origin for *milk* and *truffles* as ectomycorrhizal fungi. Figure adapted from Söllner et al. [104], Hajj et al. [105], and Burger & Lichtscheidl [106].

The presence of Sr in the atmosphere is the result of natural and anthropogenic activities; it can be transported and redeposited on plants and soil by dryfall or rainfall [106]. Strontium leaves the oceans, which are the largest reservoir of dissolved Sr, by deposition in marine carbonate sediments. Some Sr is transferred from the oceans to the atmosphere in sea spray, which is returned to the terrestrial environment in precipitation [61]. Aerosols derived from the ocean have a similar $^{87}Sr/^{86}Sr$ value to seawater [101]. A similar case applies to rainwater located near the coast, which in turn artificially increases the contribution of Sr from the ocean [107]. However, the concentration of Sr in rainwater is low, ranging from 0.001 to 0.383 ppm, compared to a typical seawater Sr concentration of 8 ppm [61]. Although the low concentrations of Sr in rainwater mean that atmospheric deposition is unlikely to significanlty affect $^{87}Sr/^{86}Sr$ marine catchments, it may be important in local hydrological environments and regions heavily influenced by anthropogenic inputs [108], [109]. However, there may be exceptions where atmospheric deposition overrides the bioavailable Sr isotope fingerprint of local bedrock, especially if the rate of Sr deposition into soil exceeds the rate of bedrock weathering [107]. This is the case for older, deeply weathered soils, while basalt weathering dominates the Sr isotope abundance of the youngest soils [110], [111], [112]. Interestingly, the opposite pattern was observed for soils in New Mexico, where atmospheric deposition of bioavailable Sr measured in plants dominated in young soils and decreased with age [113].

Groundwater chemistry in coastal areas is controlled by marine influences through seawater intrusion and water-rock interactions [114]. Strontium released to the water by chemical weathering of rocks is essentially a function of both the geologic age and Rb/Sr ratios of rocks contributing to the system and the stability of the aquifer bedrock forming minerals. Minerals dissolve at different rates [115], so the Sr isotope signature imprinted in water is predominantly controlled by the most easily weathered minerals and does not always reflect the isotopic composition of the parent rock. When the water reaches the surface, the original $^{87}Sr/^{86}Sr$ ratio should remain constant, unless the waters mix with

waters of a different isotopic composition. Since $^{87}Sr/^{86}Sr$ ratios do not fractionate during geochemical reactions, differences in isotope ratios reflect mixing processes [116], [117], [118].

However, soil is a complex environmental matrix where minerals, organic matter, water, and air are constantly mixed and thus provide a suitable substrate for the growth and development of soil biota, including microorganisms, plants and fungi. In particular, weathering of underlying bedrock is an important source of Sr for soils, from where Sr is released, diffused and then circulated in the environment.

After precipitation, Sr is affected by various physical and chemical processes in the soil-water-plant surfaces. Due to the continuous interplay between soil, water and air, plants are the primary recipients of major and trace elements from the environment, taking up elements through roots and leaves [119].

Plant uptake and biological cycling processes represent the main flow of essential and trace elements in complex ecosystems, where major internal fluxes (i.e. litter decomposition and root uptake) are tightly balanced over time. High uptake of trace elements has been found to be associated with high soil temperature, where dry soils conditions are often responsible for low element availability [120]. Microbiological activity is also mainly controlled by temperature, but it can also be changed by the redox state, the type of microorganisms, the amount of organic matter, soil pH, moisture and porosity. Furthermore, because Rb^+ replaces for K^+ in minerals and rocks, they have different K/Ca ratios and different Rb/Sr ratios. Rubidium is easily mobilized in soil and readily transferred to plants. The ratio of K/Rb in the plant to K/Rb in the soil is constant but varies between different rock and soil types and can be a good indicator of geographic identity [121].

The Ca (calcium)/Sr ratio has been used as a chemical tracer in geochemistry, hydro-geochemistry and bioavailability studies [122]. Plants usually contain the same Ca/Sr ratio as in the soil, which it varies greatly with depth. This is not the case for the exchangeable Ca/Ba (barium) ratio [123]. A high Mg (magnesium)/Ca ratio is associated with dolomite weathering, while a low Mg/Ca ratio, typically less than 0.1, corresponds to calcite weathering [124].

Strontium can remain on the plant, be washed off, or absorbed directly into the plant through the leaves. The mobility and accessibility of Sr to plant roots in soil is controlled by external factors such as physicochemical and mineralogical characteristics of the soil and pH, temperature and agricultural tillage, and plant-microbial networks constructed by soil microbial communities. The translocation of Sr in plants is influenced by the particular species and growth rate of the biomass. The most metabolically active parts will accumulate higher Sr concentrations [106], resulting in lower Sr uptake through leaves than roots [125]. The total content of Sr in plants is the result of a multi-year accumulation process due to the time-dependent input of minerals and precipitation into the plant tissue [104]. Once absorbed by the plants, the $^{87}Sr/^{86}Sr$ isotope ratio in water and rocks is in isotopic equilibrium with plants, so they have similar isotopic ratios for Sr [126]. Therefore, the Sr isotopic signature is representative of the soil in which the plants were grown. Plants absorb only the bioavailable fraction of Sr and then allocate it to other plant parts (leaves or fruits), thereby entering the food chain [127]. More importantly, the $^{87}Sr/^{86}Sr$ isotopic fingerprint remains unchanged to the final product, even after processing [128], providing a unique and well-established geographical tracer for several types of plant-based food products such as rice [129], [130], vegetables [131], [132], [133], [134], cereals [135] and mushrooms [136].

The combination of $^{87}Sr/^{86}Sr$ values with Sr, Rb, Ca and Mg concentrations can provide additional information on the geographic origin of truffles, as fungi and plants inherit their

Sr isotopic signature from their geological and pedological environment. Access to water also depends on the porosity of the soil. Tree roots can alter soil Sr isotope ratios by depleting soil minerals over time. Studies have shown that the isotopic composition of bioavailable Sr is influenced by root depth and plant-specific cycling [137]. Tree species such as spruce (*Picea abies*) and pine (*Pinus sylvestris*) differ in their root morphology, and both exhibit different $^{87}Sr/^{86}Sr$ ratios at the same location. Spruces typically have a shallow root system [138] and cycle up to 12 times more strontium in the litter than pines [137], which are able to develop deeper roots [139].

Based on the typical soil types (Figure 1.5), the differences in isotope content of pine and spruce are probably the result of differences in the soil material, where the trees penetrate deep into soil layer[2] with their roots [141].

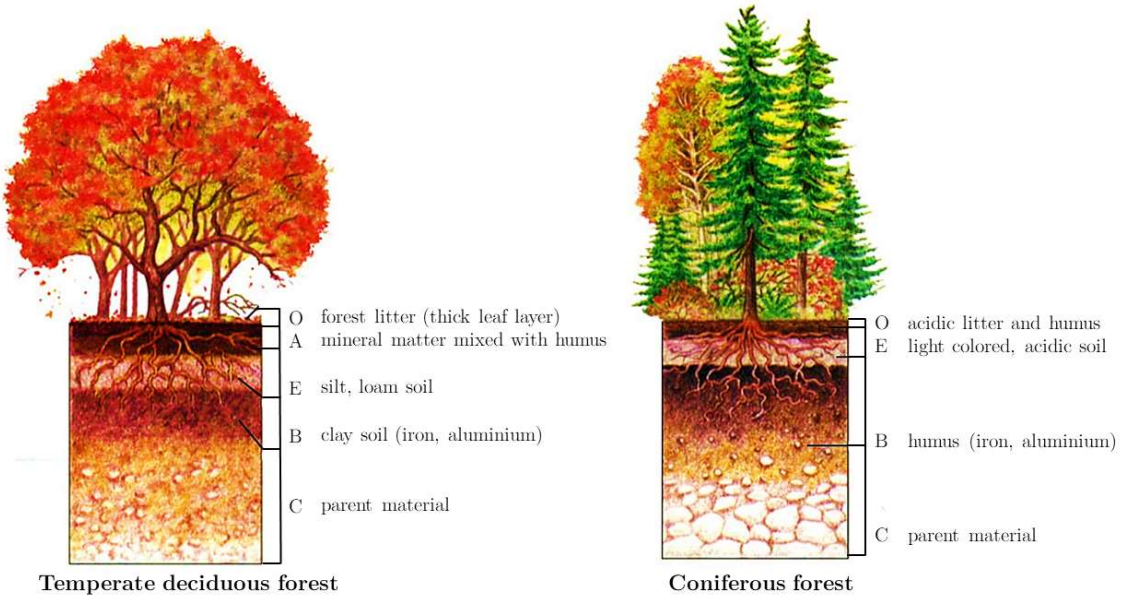


Figure 1.5: General soil profile characterisation for terrestrial ecosystems, i.e. deciduous and coniferous forests. Figure adapted from Chambers [142].

Although it is accepted that Sr keeps its isotopic fingerprint unchanged until the final product, the situation is more complicated in milk and milk products. The availability of the Sr isotope analysis of food products of animal origin hinders a limited understanding of the degree to which biologically relevant materials vary in their spatial averaged $^{87}Sr/^{86}Sr$ ratios, and how these differences may be influenced by lithological complexity. Strontium is incorporated into the human or animal skeleton by exchanging Ca^{2+} ions in the hydroxyapatite crystal lattice. Experimental evidence suggests that low doses of Sr reduce bone accretion and increase bone Ca uptake and promote bone formation [143]. Milk is produced by complex biochemical processes during metabolism. The $^{87}Sr/^{86}Sr$ isotopic ratio in animal body water is always a mixed isotopic ratio of Sr from plants and drinking water, reflecting different environmental sources (i.e. air, water, soil) [104]. Furthermore, it has been found that plants with shallow root systems (e.g. grass) tend to reflect topsoil Sr

[2] Soil horizons: O – organic horizon, topmost layer with high percentage (> 20%) of fresh to partly decomposed organic matter (leaves, stems, seeds, needles); A – leached horizon, highly decomposed organic matter in a mineral matrix, coarser mineral horizon from which iron, aluminium, clay minerals, and carbonates have been removed to lower horizons; E – zone of eluviation, typically light colored, leached horizon; B – accumulation horizon, fine organic material and clay minerals derived from the A horizon, accumulations of clays and iron oxides; C – partially weathered horizon, which is heavily decomposed parent material near the top of horizon, grades down into partially weathered to unweathered material at base. At the bottom of the soil profile presented is unaltered parent material [140].

ratios, while shrubs and trees with deeper root systems tend to reflect Sr ratios in deeper soils [144], [145]. This effect was prominent in northern Israel and the Golan regions, and the authors hypothesize that it is possible to distinguish between grazing and browsing animals based on the difference in their Sr isotope ratio [144]. However, in a study conducted in France, no significant differences were observed between plants with different root systems [146]. Plants are assumed to reflect bioavailable Sr depending on soil type and rainfall regime. Dietary Sr is assimilated in animal tissues at different times depending on the rate of Sr turnover [147]. Therefore, tissue-stored radiogenic ^{87}Sr may provide a unique dietary signature from a specific feeding area with heterogeneous geochemistry. It would be possible to use $^{87}Sr/^{86}Sr$ ratios to distinguish between different food supplies in a given area.

1.7 $^{87}Sr/^{86}Sr$ Isotope Ratio Analysis

Since the radioactive decay of isotopes leads to tiny differences in the isotopic composition of an element, only a few techniques have the ability to correctly perform isotope ratio measurements. The isotope ratios of Sr can be measured via thermal ionization mass spectrometry (TIMS), multicollector inductively coupled plasma mass spectrometry (MC-ICP-MS), or quadrupole ICP-Q-MS. Also, the potential of secondary ion mass spectrometry (SIMS), sensitive high resolution ion microprobe (SHRIMP) and resonance ionization mass spectrometry (RIMS) obtaining Sr isotopic compositions has been investigated [148], [149], [150]. However, the latter are mostly used for analysis of solids, especially geological materials and bones.

TIMS is the classic isotopic technique for food studies, however, MC-ICP-MS overtook TIMS in popularity more than two decades ago as it can analyse isotope ratios of many elements at lower cost, and it is faster and has equivalent or better precision [151], [152]. Both MC-ICP-MS and TIMS enable Sr isotopic analysis with very high precision down to 0.002 %, while ICP-Q-MS offers relatively poor precision ($\geq$ 0.05 %) [153], [154], [155]. Moreover, TIMS allows elimination of rare earth elements (REEs) present in the Sr-fractions obtained after chromatographic extraction, based on the difference between their respective ionization temperatures, at $>$ 1700°C for REEs and $<$ 1300°C for Sr. The considerable difference of ionization temperature existing between ^{87}Sr and ^{87}Rb ($<$ 800°C) allows evaporation and the removal of Rb residues before beginning Sr data acquisition. MC-ICP-MS, on the other hand, allows the analysis of elements that are difficult or impossible to analyse by TIMS (e.g. W, Hf), due to their first ionization potentials [156]. TIMS also has some disadvantages, such as limited ionization efficiency for elements with ionization energies above 7.5 eV, extensive sample preparation and long measurement times [157]. MC-ICP-MS is actually a hybrid of ICP-MS and TIMS and is becoming an increasingly attractive alternative for researchers due to its ability to accurately measure both radiogenic and stable isotopic compositions in a wide variety of geological and biological materials [158]. The development of MC-ICP-MS also brought several advantages over other techniques such as SIMS, RIMS and SHRIMP in the area of extremely low-abundant isotope analysis as this technique is rapid and less expensive with high sensitivity and versatile sample introduction systems well established in ICP spectrometry and, since isotopic determinations occur simultaneously, no time-dependent mass fractionation as observed in analysis using other techniques [158].

However, due to the chemical separation of the target analyte from the sample matrix and various quality control tests, measurements with MC-ICP-MS are almost as time-consuming as with TIMS. TIMS, on the other hand, is less prone to matrix effects and

presents a lower instrumental isotopic fractionation [159]. Thus, other factors such as robustness, running costs and element-specific advantages become decisive.

This section will discuss procedures for sample preparation of food samples and effects on measurement conditions using mass spectrometers.

1.7.1 Accurate and precise $^{87}Sr/^{86}Sr$ isotope ratios results: Key factors to consider

Quality control and safety in the food supply chain requires a reliable methodology that is fast and easily transferable. Determining the origin of a food product represents a problem that can involve multiple analytical approaches. In general, the analytical determination of $^{87}Sr/^{86}Sr$ ratios mainly consists of the following steps: (1) decomposition of organic matter, (2) extraction of the bioavailable Sr fraction from the sample matrix, (3) measurement of element concentration by mass spectrometry analysis, (4) Rb/Sr separation procedure, (5) isotope ratio measurements, and (6) mass-bias correction and data reduction. Factors that may be encountered in obtaining precise and accurate isotope ratio data are discussed in the following sections.

1.7.1.1 Sample pre-treatment

Elemental analysis of foodstuffs presents a challenge because of the variety of food types and range of element concentrations that need to be measured. Although sample preparation is routine, it is a critical step in ensuring accurate laboratory results. To reduce uncertainty in sample preparation, the following factors should be considered. Solid samples must be completely dissolved and digested into a homogeneous liquid sample. In food samples, organic matter can be a problem, which significantly influences trace element contents' determination. Therefore, attention should be paid to choosing the optimal digestion procedure [160]. These include checking the accuracy and completeness of digestion (with certified reference materials), reagent selection, sample matrix composition, consideration of elements, interferences, sample losses and contamination. The removal of organic matter is generally achieved by dry or wet ashing and microwave-assisted acid digestion [160]. The complexity and diversity of food samples mean that digestion is highly dependent on the composition of the sample and the reagents used to digest the sample. The most common digestant is nitric acid (HNO_3), especially for organic samples, due to its easy purification and its ability to oxidise organic compounds. The advantage of using HNO_3 is that it converts metal ions into their highly soluble nitrate salts. Most analysts also prefer nitric acid matrices for ICP-MS analysis because they are relatively free of the chemical and spectral interferences compared to acids containing Cl (chloride), S (sulphur), F (fluoride), or P (phosphorus). A mixture of HNO_3 and H_2O_2 (hydrogen peroxide) is widely employed as they both mineralise organic matter effectively [161], [162]. Perchloric acid ($HClO_4$) is sometimes added to HNO_3 to enhance the oxidation and digestion efficacy; however, nitric-perchloric acid method is discouraged because $HClO_4$ is potentially hazardous during digestion and recovers relatively little heavy metal [163]. Other strong acids such as sulphuric acid (H_2SO_4) and hydrochloric (HCl) acid are not recommended since interference derived from S- and Cl-containing species may occur. These species may give rise to spectral interferences in ICP-MS (e.g. $^{35}Cl^{16}O^{1}H^{+}$, $^{34}S^{18}O^{+}$, $^{35}Cl^{17}O^{+}$ on ^{52}Cr). For some techniques that involve pneumatic nebulization process (e.g. ICP-MS, ICP-OES), non-spectral interferences may occur. In fact, the evaporation rates of these acids are different and can affect the atomization and ionization process in the plasma, as well as the dispersion of the sample aerosol, which affects the detection sensitivity [161], [162], [163], [164].

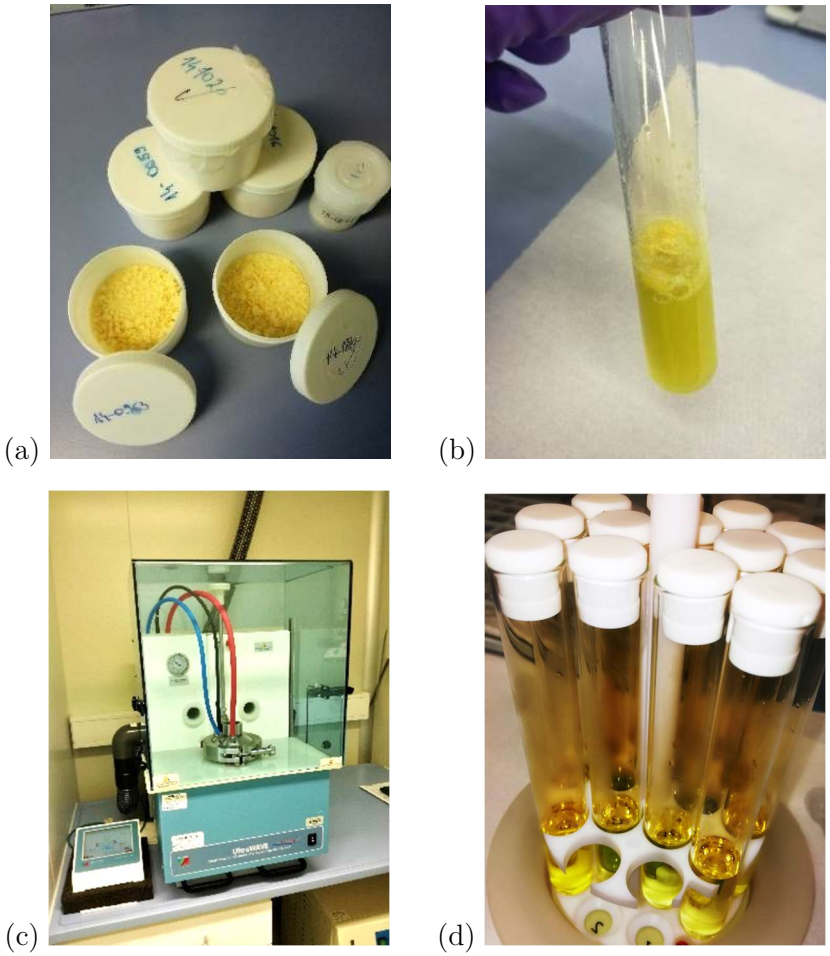


Figure 1.6: Pre-treatment of Slovenian milk samples: (a) lyophilized milk samples, (b) addition of HNO_3 to the milk samples prior to the sample pre-treatment process, (c) UltraWave MILESTONE microwave-assisted digestion system used for milk sample pre-treatment, (d) digested milk samples after completed microwave digestion process. (Photo: S.H.G., IJS Reactor Centre, 2018)

1.7.1.2 Possible interferences on the strontium masses in ICP-MS analysis

For accurate and precise isotope ratio determination, it is essential to avoid, or at least accurately correct, spectral and non-spectral interferences. The factors affecting the Sr isotope ratios have been discussed in detail by Vroon et al. [168].

Spectral interferences refer to the ion species with the same mass-to-charge (m/z) ratio as analyte [169]. These interferences can be caused by another element isotope, i.e. (1) *isobaric interferences*: ^{87}Rb on ^{87}Sr; ^{86}Kr (krypton) on ^{86}Sr, (2) *polyatomic ions*: e.g. ^{40}Ca^{46}Ar, ^{43}Ca^{44}Ar on ^{86}Sr, or (3) *doubly charged ions*: e.g. rare earth elements (REE) such as ^{172}Yb (ytterbium), ^{174}Yb and ^{174}Hf (hafnium) on ^{87}Sr. Traces of ^{86}Kr in the argon (Ar) plasma gas can also affect Sr measurements. For this reason, the Ar plasma gas is continuously monitored (corrected) after optimization and before the analysis [168].

Polyatomic interferences result from the combination of two or more atomic species in the ICP source and can be avoided by (1) sample purification for the removal of ion species that cause polyatomic interferences, (2) use of blank correction as these interferences occur with using Ar plasma gas or with water and acids used for sample preparation, or (3) using collision cells with appropriate reaction gases.

Since high mass resolution ($m/\triangle m$; ~ 300,000) is required to resolve the isobaric overlap, and modern quadrupole ICP-MS instruments allow resolutions of up to 10,000 [170], a mathematical correction is often required. However, this method may result in higher measurement uncertainties [171].

Alternatively, isobaric interferences can be resolved using reactive gases such as O_2 [172], but in some cases side reactions can cause new unwanted interferences with other ions extracted from the sample. Consequently, it may not be possible to remove all of these interferences [173]. An experimental strategy using ICP-MS/MS with a flow of reactive N_2O gas for in-line separation of ^{87}Sr from interfering ^{87}Rb without the need for prior chemical separation was proposed by Murphy et al. [174]. Although the method is feasible for the analysis of low Sr samples, it may not be suitable for samples with complex matrices such as milk and milk products. Dairy foods are well known for their nutritional value, especially major minerals such as Ca^{2+}, Ba^{2+}, Mg^{2+}, Na^{+} and K^{+}, and their high-fat content.

There is also the possibility that the sample pre-treatment methods are not always effective in completely destroying organic material such as a fat, which can lead to problems with sample introduction into the ICP-MS and to the occurrence of spectral interferences arising from the physical properties and high carbon content of the organic sample matrix. The best way to deal with isobaric and matrix interferences is to use a chemical separation that quantitatively separates Sr from its concomitant sample matrix.

1.7.1.3 Interference removal: Use of selective resins and acid chemistry in elution procedures

Strontium can be isolated from the sample solution using selective resins, which bind a particular element until a specific eluent is added. In this way, the analyte is obtained free from interfering species, and the matrix of the sample can be matched to that of the standard solutions.

Classical methods for separating strontium from barium and calcium rely on differences in the solubility of Ba, Ca and Sr precipitates in acidic solutions [175]. For example, Ca-nitrate is soluble in strong HNO_3, while Ba- and Sr-nitrates precipitate under the same conditions. Other methods are based on HCl, oxalate ($C_2O_4^{2-}$) and sulphate (SO_4^{2-}) separations. Although these separations give satisfactory results, they require numerous steps, repeated precipitations to recover all the Sr, or are affected by a large amount of Ca in the sample [176], [177]. Alternate methods for separation have therefore been introduced. Among these is the sorption of Sr on an ion-exchange resin from a chelating agent solution such as DCTA or EDTA [178], cation exchange chromatography using a AG 50W-X8 resin in HCl medium [131], [179], [180], extraction chromatography using specific resin for Sr [181], [182], liquid-liquid extraction using benzo-crown ethers, or selective extraction of strontium with supercritical fluid carbon dioxide (CO_2) [183]. Each of these methods, however, suffers from shortcomings such as low recovery, poor elemental selectivity, or usage of large reagent quantities.

When it comes to the sample analysis using MC-ICP-MS or TIMS, the most commonly used resins for isolating Sr are produced by Eichrom (i.e. Sr-Spec resin), which typically consist of crown ethers immobilized on an inert matrix.

Generally, dissolved samples are acidified with HNO_3 before the separation procedure, and Sr is loaded onto the resin from a HNO_3 solution. Although excellent separation can be obtained compared to previously stated traditional ion exchange techniques, crown ether columns also have some limitations. For example, small amounts of sample must be used, which means additional pre-treatments, resulting in potential analyte loss or contamination. Several authors performed various experiments in chromatographic extraction studies to investigate the elution behaviour of different elements on the Sr-Spec

resin, matrix effects and the resin capacity for Sr [181], [182]. It has been demonstrated that the Sr-Spec resin tolerates high levels of Sr, iron (Fe), aluminium (Al) and Mg. According to Horwitz et al., Sr is strongly retained on the resin with the increasing HNO_3 concentration of the sample and eluting solution. While 3M HNO_3 has been shown to adequately separate ^{87}Sr from ^{87}Rb during chromatographic extraction separation from biological, environmental, and nuclear waste samples, 8M HNO_3 was shown to maximize the separation of other matrix elements such as Ba and Ca. Barium can be easily removed by adjusting the concentration of eluent used, while to avoid calcium interference, the use of a longer column has been suggested. Potassium shows a minimal affinity for the resin. However, its high concentration (> 0.01 M) in the sample decreases retention of Sr onto the resin [182].

Although various methods have been used to date to isolate Sr from the sample matrix of various food products, there is no standard protocol for determining the Sr isotope ratio in a specific food product. The choice of method to isolate Sr from the sample matrix is important, otherwise sample preparation bias can occur if different procedures are used. For example, Zhu et al. [153] used sonication to remove Sr from peanut seeds using only pure water, whereas most analysts use chromatographic extraction followed by dry or wet digestion.

It should be noted that the complex nature of many food matrices is a major potential source of error for many types of analytical measurements. The level of difficulty associated with dealing with a matrix usually depends on the complexity of the matrix and/or degree of concentration and the analytical technique used. An example are untreated food samples, which usually contain dissolved organic material derived from vegetation and water. This material can be irreversibly adsorbed into the resin beads and thus reduce its exchange capacity (Figure 1.7). Therefore, sample pre-treatment is one of the key factors affecting the elution peak and Sr recovery.

Figure 1.7: Yellow colour on the upper ring of the Sr-resin indicates that organic matter (milk fat) adsorbed on the resin. Sample pre-treatment was further optimised. (Photo: S.H.G., IJS Jamova, 2017)

In addition, finding the optimal sample size can also be a challenge. If the concentration of Sr in the total sample is too high, serial dilutions can be performed, but this method is associated with high measurement uncertainty. In some cases, samples must be preconcentrated before starting the column procedure (Figure 1.8), especially when dealing with samples containing low Sr content [184]. For each sample solution, the total Sr concentration in the sample solution must be determined to calculate the final Sr concentration required for the isotope ratio measurement, which depends on the performance of the instrument [174].

Figure 1.8: Sample preconcentration: Evaporation of the digested milk sample solutions to near dryness on sand bath at T ≤ 90°C (*left*) and dissolution of dried samples in 1 mL 8M HNO_3 followed by sonication for 10 min (*right*). (Photo: S.H.G., IJS Jamova, 2019)

The use of nitric acid as an eluent for strontium can be very beneficial in ICP-MS applications and further sample changes are not required, thus reducing the risk of contamination and analyte loss. Although changes in stable isotope ratios have been observed after acidification [185], effects on strontium isotopes during chromatography using Sr-Spec resin have not been reported. The cumulative isotope ratio of the eluent after chromatography shows no detectable isotopic fractionation [186]. Interestingly, the isotopic fractionation in cation-exchange chromatography was three times higher than during extraction chromatography [187]. Enrichment with heavier strontium isotopes has also been observed when using crown ether resins, specifically for benzo-15-crown-5-ether resin [188]. However, the effects of isotopic fractionation can be minimized by using small resin beds and an adequate amount of eluent.

Memory effects are also a problem when using these resins, as their lower regeneration levels lead to a small proportion of exchange sites that allow ions to pass through the column. Some studies suggested washing the resin with ultrapure water after soaking the resin in nitric acid for an extended period of time. Among the binder resins selected, Sr-Spec resins are popular in food authentication applications.

Extraction chromatography can be time-consuming and often requires many repetitions to obtain a good recovery of strontium, which has led to the search for faster and more accurate methods such as automated separation by direct analysis. For example, to improve Rb-Sr separation, an online flow injection Sr-matrix separation method coupled with MC-ICP-MS has been proposed [189], [190]. Compared to off-line separation, it offers the following advantages: (1) reduces sample preparation time as the conditioning, washing and elution steps are performed automatically, (2) reduces the risk of sample contamination, (3) reduces analyte loss due to evaporation, (4) reusable columns, (5) increased sensitivity (small sample volume < 1 mL of the sample), (6) reduced waste, and (7) reduced exposure to toxic reagents. Nevertheless, studies using online detection are rare as the method still has some drawbacks, such as optimising the preconcentration and elution procedures to achieve good precision in one step for different elements, e.g. co-elution of matrix interferences.

1.7.1.4 Isotope ratio measurements

For Sr, the isotopic composition of a sample is typically reported as R_{sample}, with no conversion to δ-notation (see Subsection 1.4.1).

In order to optimize the data quality with MC-ICP-MS, some factors should be considered before measuring samples. These factors include in particular the reduction of isobaric interferences and the control of environmental conditions surrounding the mass spectrometer. Instrumental isotopic fractionation refers to mass bias depending on the instrumental setup, ICP conditions, and voltage settings. Such fractionation between elements is most evident during sample introduction (e.g. evaporation, diffusion, transport efficiency) and ionisation (e.g. different ionisation efficiency) and is matrix-dependent. It also represents a primary source of systematic errors [191]. Therefore, an adequate correction of mass bias is a prerequisite for an accurate isotope ratio determination.

In our study, Sr was isolated from concomitant elements in the sample matrix, i.e. to remove potential isobaric interferences, using column chromatography with a Sr-specific resin. After separation, total Sr and Rb were measured using ICP-MS to control Sr recovery and check whether Rb was effectively removed. Several studies have shown that matrix matching and analyte separation are mandatory when performing external mass bias correction [192], [193]. It has been reported that on-column isotopic fractionation can occur for some elements such as Cu and Zn [194] or for Sr when using cation-exchange chromatography in HCl medium [186]. For this reason, obtaining a quantitative recovery is crucial to prevent an isotopic bias introduced during the separation procedure. The calculated Sr recovery represents the ratio between the Sr concentrations measured in the eluent after chromatography and the initial extract before evaporation. A good agreement was obtained, allowing the conclusion that no detectable isotopic fractionation of Sr was introduced by the extraction chromatographic isolation procedure using a Sr-specific resin (Appendix A). The $^{87}Sr/^{86}Sr$ isotope ratios in pure Sr fractions were then determined by MC-ICP-MS. In our study, Sr isotope data were collected using a Nu II MC-IPC-MS (Nu Instruments, Ametek Inc., UK) fitted with an Aridus IITM desolvating nebulizer system (Teledyne Cetac, Omaha, Nebraska, USA). Typical instrumental conditions, measurement parameters, and all necessary corrections used are reported in the paper by Zuliani et al. (2020) [195]. The operating parameters of MC-ICP-MS were optimized daily using a standard NIST SRM 987 solution (strontium carbonate, $SrCO_3$; certified value $^{87}Sr/^{86}Sr = 0.71034 \pm 0.00026$; National Institute of Standards and Technology, Gaithersburg, USA) to achieve the maximum sensitivity and stability for Sr beam. To ensure high precision and accuracy, the concentration of Sr in the final extract was not lower than 50 ng/mL.

Instrumental isotope fractionation was corrected by internal normalization using the ratio $^{86}Sr/^{88}Sr = 0.1194$, and Rb and Kr interferences were corrected for mathematically using $^{87}Rb/^{85}Rb$ and $^{86}Kr/^{83}Kr$ ratios of 0.38567 and 1.50566, respectively. Measurements were performed following the standard-sample-standard bracketing method using a NIST SRM 987 as the standard. In sequence, blank solution (HNO_3, 5% w/w) was allocated in the sequence before and after five samples. The average of the measured intensities of the blanks was calculated and, if necessary, subtracted from the measured intensities of the respective standards or samples.

Finally, any variability related to natural fractionation processes in samples can be detected.

1.8 $^{87}Sr/^{86}Sr$ Isotope Ratio in Traceability Studies

Over time, the development of new advanced analytical techniques made it possible to determine the geographical origin of most foods by analysing their elemental and isotopic composition.

Many applications of the $^{87}Sr/^{86}Sr$ isotope ratios include paleomobility studies [196], [197], [198], weathering reactions and water flow path studies [199], [200], and lately origin authenticity studies of biological matrices [38]. All of these studies are based on reference maps and databases that allow very broad mapping of bioavailable Sr isotope signatures in natural materials (e.g. water, plants, soils, animal and human tooth enamel and bones) as well as major and trace element concentrations for soil, water and plant samples in local or regional areas. These applications are mainly based on the principle that the $^{87}Sr/^{86}Sr$ fingerprint of natural materials can reflect the dominant source of Sr, which is largely controlled by the underlying geology [105]. Although archaeologists have used Sr isotope analysis for decades to answer many questions related to the mobility of (pre)historic human and animal populations, it is only in the last few years that researchers have collected the necessary scope of isotopic data needed to establish Sr isotope baselines that enable or even improve large-scale inter-regional comparisons. Some of these include plants of a specific lithology in Great Britain [201], surface water in Denmark [202], grazing and agricultural soils in Europe [56], plants and topsoil leachate from France [146], plants at various locations in Ireland [203], soil leachates, plants, groundwater and surface waters in Cyprus [204], and water and plant samples from different regional sites, local animal and human samples from archaeological sites in China [205]. Furthermore, Delattre et al. [206] have expanded the study dealing with the feasibility of using radiogenic ^{87}Sr ratio for discriminating the origin of aquatic plants at a worldwide scale for the first time. The proposed model accounts for the small variations in $^{87}Sr/^{86}Sr$ ratios of aquatic plant species and allows a discrimination between producers using different agricultural practices.

Minerals with different Sr concentrations and $^{87}Sr/^{86}Sr$ ratios weather at different rates, therefore the baseline map of $^{87}Sr/^{86}Sr$ variations within a region is not always sufficient to predict the $^{87}Sr/^{86}Sr$ ratio entering the environmental Sr cycle, but it can be used as a reference map to determine if there are unexpected isotopic variations [207]. Recently, Thomsen & Andreasen [208] highlighted the influence of agricultural lime on the $^{87}Sr/^{86}Sr$ ratios of bioavailable Sr in low-calcareous to non-calcareous soils, as sediments similar to the studied deposits are usually subjected to intensive farming and liming. The authors hypothesized that if agricultural lime can significantly alter the isotopic composition of bioavailable Sr in regions with low to non-calcareous soils, then this calls for the re-evaluation and development of accurate $^{87}Sr/^{86}Sr$ baseline maps. Only with a uniform, high-density sampling approach it is possible to properly model geologically complex areas [209].

1.8.1 Mineral waters

The waters used for bottling originate from different parts of the hydrological cycle and have a distinct hydro-geochemical fingerprint [202]. Mineral waters show a wide range of $^{87}Sr/^{86}Sr$ values, reflecting the wide range of diverse underlying bedrock in each area [195], [210], [211], [212]. Water is completely bioavailable to plants and animals and can be transferred to any organic material without significant fractionation of Sr isotopes. In order to verify the correlation between the surface geology and the isotopic signature of Sr in natural mineral waters, a comparison with a geological map can be made [55]. The authors showed a good correlation between food products (honey and wheat) and surface water samples. However, some deviations in the Sr isotopic signature were observed, especially in food samples originating from coastal areas, probably due to the influence of sea spray

coming from the Atlantic Ocean [55]. While baseline maps based on soil, plant and water samples can be useful in multi-scale predicting of $^{87}Sr/^{86}Sr$ variability, other factors (e.g. atmospheric Sr sources, dust deposition, intensive agriculture) must be considered when interpreting results, which affect the isotopic composition of Sr.

1.8.2 Plant-based foods

The elemental composition of plants is very complex and reflects the composition of the soil in which they grow. Various factors (e.g. seasonal variations, plant physiology, soil characteristics, acidity, humidity, porosity, humic complex, microbial activity, soil fungi etc.) influencing the uptake of elements into plants have been studied by several authors [213], [214], [215], [216], [217]. It is assumed that environmental sources and the content of elements in the soil are different in different regions, which causes differences in the content of elements in foods of different origins. The role of each element in the soil-plant interaction is therefore key to understanding whether a particular element can act as a marker for food classification. Elements such as Cs (cesium), Rb and Sr have been proved to be good indicators of the geographical identity of most plants [34], [218].

Strontium and trace elements are absorbed into plants in the same proportions as they occur in soil and precipitation, so the isotope ratios in plant material depend on the geology of the region in which the plant grew. Consequently, geographically based differences in $^{87}Sr/^{86}Sr$ ratios in plants have been particularly useful in determining the origin of plant foods [126].

1.8.2.1 Wine and other alcoholic beverages

Wine is the most investigated food commodity in the area of geographical origin authentication of alcoholic beverages [219]. Indeed, Europe is the largest producer of wine and it accounts for 50 % of wine consumption worldwide.

Production practices, such as winemaking processes, are important in determining the total availability of elements for plant uptake, as they can influence the content of major and trace elements in wines [220]. Li (lithium), Be (beryllium), Mn (manganese), As (arsenic), Rb, Sr and Cs seem to be potential discriminatory parameters and are therefore more important for verifying the geographical origin of wines [220]. It was found that the content of several elements can decrease (Li, Ca) or increase (Mn, Na, Rb, REEs) during fermentation and fining of wines. The concentration of certain elements in wines can also be significantly affected by bentonite fining treatments [221]. Therefore, the elemental profile must be considered with caution before ensuring the significance of the elemental pattern in the assessment of the wine's provenance. Nevertheless, some elements such as Li, B (boron), Mg, Ca, Rb, Cs and Pb (lead) were found to remain almost unchanged throughout the winemaking process and independent of the time of bentonite addition [222]. Similarly, bentonite does not appear to affect the $^{87}Sr/^{86}Sr$ ratio value, as observed in Tenerife wines [109].

Ariyama et al. [223] investigated the influence of fertilisation, including other various conditions (year, variety, provenance), on the variation of element concentrations in onions. Such information can be helpful in the development of methods for determining the plant geographical origin by multi-element composition. An example is fertilisers, which are often used in viticulture, as they can improve the quality of the vineyard areas. Soils and climates vary from one region to another, so the choice of the type of fertiliser required depends on many factors (e.g. soil nutrient availability, effect on soil pH, suitability for delivery by fertigation, cost per unit metal, etc.). Rooting pattern and depth varies between species and hybrid rootstocks, which also affects aspects of vine nutrient uptake efficiency. An example is the use of water-soluble calcium fertiliser ($Ca(NO_3)_2 \times 4H_2O$), which can

significantly increase the Ca content in grapevine leaves, but decrease it in stems and fruits [224]. The influence of fertiliser use on the wine and grape properties showed that the use of fertilisers containing Mg, Fe, Mn, Cu (copper), Zn (zinc), and B can increase not only the yield of wine grapes, but also the content of tannins, total phenols, and anthocyanins [224]. However, in a comparative study of Italian wine, no significant differences were observed in the mineral content of organically and commercially produced wines, except for a higher Ni (nickel) content in organic wines [225]. Similar findings were reported in another study of organic and conventional crops (wheat, barley, faba beans and potatoes) grown in three geographical areas [226]. Only further improvements and the inclusion of additional measured trace elements made it possible to distinguish the geographical origin between organic and conventional wheat, barley and potatoes. The inclusion of non-essential elements in the profile appears to greatly improve discrimination, probably due to impurities in fertilisers typical of conventional agriculture. Regarding the topic at hand, Capuano et al. [227] provide a comprehensive review of potential biomarkers for the authentication of organic products of plant and animal origin. Nevertheless, some questions about biased authentication of foods remain open. Not all technological processes or agricultural practices are the same and elemental concentrations vary from sample to sample. Therefore, there is no certainty that these elements could be successfully used in provenance studies in other regions or countries.

Based on the scope of wine investigations using the Sr isotope analysis in the past several years, common conclusions have been made following a series of published studies: (1) the $^{87}Sr/^{86}Sr$ ratio of wine is strongly correlated with the $^{87}Sr/^{86}Sr$ ratio of rocks, soils and their respective grape juices [228], [229]; (2) the $^{87}Sr/^{86}Sr$ ratio of wine is not altered during the winemaking processes [230]; and (3) Sr isotope composition of wine remains constant for different vintages [180], [230]. The latter agrees with similar findings by Cellier et al. [231], who stated that no variation in the Sr isotopic signature could be observed for Champagne wines produced between 1983 and 2016. The results also showed a remarkable homogeneity of Sr isotopic ratios between different brands of Champagne, which can be partially explained by the homogeneity of the bedrock of the production area. Although observations on the influence of the production year or winemaking process on $^{87}Sr/^{86}Sr$ values in Pomerol wines were found in a study by Epova et al. [232], the studied wines were successfully distinguished from other authentic Bordeaux wines. These authors also demonstrated effective discrimination of geographic origin using a combination of strontium isotopic and elemental signatures of wines.

Interestingly, Gabel [233] proposed a new approach in the determination of $^{87}Sr/^{86}Sr$ ratios in vines and terroir. He concluded that the average isotopic composition of the substrate cannot be a reliable indicator for the wine's origin authenticity, but the isotopic composition of the pore water, which at different stages washes out various mineral phases and passes into the vine root system. A method was proposed for the preparation and treatment of soil samples and consequently must and wine samples [233]. It should also be taken into account that a high ethanol content in wine can affect the accuracy of the analytical measurement, as it can increase the signal intensity of the analyte. Thus, Moreira et al. [234] proposed the use of nanofiltration, a membrane process with several applications in oenology, to remove ethanol from wine, since this method does not affect the $^{87}Sr/^{86}Sr$ ratio in wines.

Like wine, different types of beer are sold around the world. The Global Beer Market was valued at $593 million in 2017 and is expected to reach $685 million by 2025 [235]. Beer is a very popular drink not only in the world, but also in Slovenia, which has a strong and rich brewing industry. Moreover, Slovenia is among the top 10 countries in Europe in terms of beer consumption. About 1.7 million hectolitres of beer are produced in Slovenia, and much of it is also exported. The trend is in the variety of beers, especially in specialty

beers, which generally sell for much higher prices than regular beers due to special treatments or the use of atypical components to produce them. Compared to wine, limited studies have been conducted focusing on the Sr composition of beer. Bong et al. [236] investigated beers imported into South Korea from various countries, which were analysed for C, O and Sr isotopes, and multi-element composition. The analysis of the data made it possible to differentiate the beers according to their geographical origin in four countries (America, Australia/Oceania, Asia, and Europe). Five elements (S, Li, Cr (chromium), Ni, and Sr) and isotopes ($\delta^{13}C_{DIC}$, $\delta^{18}O$ and $^{87}Sr/^{86}Sr$) were particularly useful for distinguishing beers.

1.8.2.2 Coffee and tea

Leaves are more enriched in ^{87}Sr than soils and rocks. Fertilisers are often assumed to be potential soil contaminants as they are used in viticulture to improve the quality of the terroir. Aguzzoni et al. [237] demonstrated a significant shift in Sr isotopic value due to external Sr inputs (fertilisers), which is probably due to higher Sr contents and high Sr ratios in these products. Therefore, it is recommended to carry out an analysis of the Sr isotopic composition of fertilisers to verify whether leaf enrichment is related to the addition of these products. However, a study that aimed to show the actual effect that modern fertilisers have on soil in Denmark showed that the concentrations of Sr in the fertilisers used are often so low that their effect is minimal and often not even measurable [202]. Similar observations were made for Hawaiian coffee beans, where Sr isotope values were lower than the range of values reported for fertilisers. This suggests that the latter were not the main source of Sr [238], allowing the identification of the geographical origin of the coffee beans. Although the soils of the Hawaiian islands are generally of young geological age, the ages of individual islands are quite different. Accordingly, the $^{87}Sr/^{86}Sr$ ratios in coffee beans grown on these soils vary according to cultivation sites [239], and the combination of $^{87}Sr/^{86}Sr$ ratios with $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values allowed a complete differentiation between coffee beans of different regional origins. Further, Liu et al. [240] extended the use of Sr isotopes to origin coffee beans and showed that coffee from 14 countries in Africa, America and Asia could be distinguished based on $^{87}Sr/^{86}Sr$, $\delta^{11}B$ and $\delta^{18}O$ values.

Other studies involved the characterisation of genuine tea samples grown in different production areas. Tea originating from four different regions (i.e. Assam, Darjeeling, Munnar and Kangra) in India [241] was characterised based on isotopic signature of Sr and C, while the multi-element composition and isotopic ratios of B and Sr were determined in tea samples from Taiwan [242]. Darjeeling tea samples ($^{87}Sr/^{86}Sr$ = 0.726 – 0.829) were found to be more radiogenic than other tea samples, in which Sr ratio values ranged from 0.711 to 0.732. Furthermore, significant differences in $\delta^{13}C$ were observed in teas from Munnar ($\delta^{13}C$ = -25.17 ± 0.57 ‰) and Kangra ($\delta^{13}C$ = -29.02 ± 0.67 ‰) regions. Samples from Assam ($\delta^{13}C$ = -26.69 ± 0.56 ‰) and Darjeeling ($\delta^{13}C$ = -27.02 ± 0.58 ‰) had similar $\delta^{13}C$ values. In addition, the $^{87}Sr/^{86}Sr$ ratios in Indian teas were higher than tea samples from Taiwan, with $^{87}Sr/^{86}Sr$ values ranging from 0.70482 to 0.71462. For samples coming from a similar geological background, additional parameters (elemental content and stable isotope ratios of light element) should be considered for better geographical discrimination.

1.8.2.3 Edible oils

According to the EC JRC, Europe is the leading producer and consumer of olive oil, producing 73 % and consuming 66 % of the world's olive oil. According to the outlook for agricultural markets in the EU, olive oil production is expected to increase until 2030 [243]. In order to increase the reputation of European olive oils, methodologies in the fight against

fraudulent practices are crucial in quality control and data validation, as well as the transfer of knowledge on the isotopic analysis of olive oils to the laboratories and control authorities of the Member States [244]. Various physical and chemical analyses have shown that olive oils have sensory and nutritional properties that are often related to the geographical origin and variety of the olive fruit used [245]. Despite the complex high-fat and low Sr matrix, a method using Sr isotopes was developed and subsequently validated to trace the origin of olive oils [184], [246]. The proposed method was used in a study by Techer et al. [247], who investigated the role of cultivation practices on the bioavailable $^{87}Sr/^{86}Sr$ ratio of Aglandau and Picholine olives grown in two locations, approximately 1 km apart, on similar soils but with different cultivation practices. Although the isotopic composition of Sr in trees has been found to be related to the mineral composition of the soil, this is not the case for irrigation practice, as it can cause perturbations in the intrinsic isotopic properties of Sr in both exchangeable and mobile fractions. In conclusion, distinguishing between land use changes and changes in land management intensity could be useful in differentiating between organic and conventional crop production practices.

1.8.2.4 Vegetables and fruits

Multi-element and strontium isotope ratio analyses were performed to distinguish the geographic origin of cabbage grown in Korea and China [248]. To determine the geographical origins of the samples, 21 parameters were analysed: $^{87}Sr/^{86}Sr$, Ca, Mg, Na, K, Mn, Cu, Sr, Al, Fe, Ba, P, S, Co (cobalt), Cr, Li, Ni, Ti (titanium), V (vanadium), Zn, and Zr (zirconium). The values of the measured $^{87}Sr/^{86}Sr$ ratios were from 0.70814 to 0.72018, while the range for Chinese samples was from 0.71059 to 0.71457. Among all parameters measured, the combination of Sr, Ca, and Mg showed the possibility of discriminating between Chinese and Korean cabbage samples, while the further measurement and use of Ti concentrations allowed an improved discrimination of some samples that were less discriminated by Sr, Ca and Mg alone.

Although studies on berries are not new [249], blueberry samples were first investigated using multi-element and Sr isotopic ratio analysis [250]. Fisher's function analysis was used to test the feasibility of distinguishing blueberries based on Sr isotopes alone. The combined accuracy of the three regions (Ziyun, Majiang and Huangping) was 81.3 %, indicating that the Sr isotope ratios can be used to trace the origin of blueberries. Mineral content combined with $^{87}Sr/^{86}Sr$ ratios, however, significantly improved origin discrimination with an accuracy of 95.8 % (93.8 % in the case of cross-validation).

From the point of view of traceability studies based on the $^{87}Sr/^{86}Sr$ ratio, sampling is very important especially when harvesting fruits or other horticultural products that are only available seasonally [251]. By comparing different parts of the tree, the variability of the $^{87}Sr/^{86}Sr$ ratios was observed within the tree and in the orchard [252]. Although the variability of $^{87}Sr/^{86}Sr$ values in orchards does not preclude its use as a geographic tracer, the authors urge researchers to properly design the sampling campaign or generalize the results.

1.8.2.5 Cereals

Geographically based differences in plants $^{87}Sr/^{86}Sr$ ratios were particularly useful in determining the provenance of winter wheat from three regions in China [253]. The authors investigated the discrimination effects of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{2}H$, alone or with $^{87}Sr/^{86}Sr$. An overall correct classification rate of 77.8 % was obtained for wheat discrimination based on light stable isotopes, while a better classification rate of 98.1% was achieved with the combination using $^{87}Sr/^{86}Sr$. In Japan, agricultural products such as brown rice were analysed using the binary isotope signature of $^{11}B/^{10}B$ and $^{87}Sr/^{86}Sr$ [254]. Rice samples

were collected from 44 different locations in Japan, 4 in China, 3 in Australia, and 1 each in Vietnam and USA. The results showed that Japanese rice can be distinguished from other samples based on both isotopic parameters. Ariyama et al. [130] extended the application of Sr isotopes, including lead isotope ratios and multi-element concentrations to source rice, originating from the above-mentioned countries. This approach allowed the separation of rice from Japan, USA, China, and Thailand based on $^{87}Sr/^{86}Sr$, $^{204}Pb/^{206}Pb$, $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, Rb, Ba, and Co.

On the other hand, secondary factors (i.e. environmental conditions, production practices, animal metabolism or anthropogenic sources) also contribute sequentially to the interaction between soil and plants, which can further influence the elemental content of foods. It is important to know which elements are easily influenced and how much these concentrations vary according to various factors. In traceability studies, ideal tracers are those elements on which concentrations are unaffected by secondary sources. Major and minor elements seem to perform better in authentication studies based on varietal discrimination or production practice, while trace and ultra-trace elements are mostly suitable for geographic discrimination, which is also consistent with their role as geochemical markers [255]. REEs are considered interesting as tracers because they do not appear to play an active role in plant physiology. There is considerable scientific evidence for the fact that plants usually absorb rare earth elements from soil with little or no fractionation, and this behaviour can also be useful in food traceability studies [255], [256].

1.8.3 Animal-based foods

Compared to foods of plant origin, determining Sr isotopes in foods of animal origin is more challenging, as factors affecting the composition of foods (e.g. milk and dairy products, meat) are more complex and cannot always be controlled (see Subsection 1.9.1).

The isotopic composition of milk and cheese is prone to large variations in the $^{87}Sr/^{86}Sr$ values according to the lithology in a given region. The $^{87}Sr/^{86}Sr$ ratios in milk and cheese from Quebec vary over a wide range of values, from 0.70961 up to a maximum of 0.71447, indicating a relative enrichment with radiogenic isotope ^{87}Sr in Proterozoic and Paleozoic carbonate intrusive and calcareous rocks that make up the St. Lawrence Platform [257], [258]. The high variability of Sr ratios in dairy products reflects the great diversity of underlying bedrock and soils that formed from them. Therefore, the widely scalable results of Sr ratios reflect the significant heterogeneity of the geological background of its origin. In a study of milk from Australia and New Zealand [179], $^{87}Sr/^{86}Sr$ ratios also varied sufficiently between milk samples; however, no good correlation was observed between milk samples and the respective soils of a particular production region.

In many cases, groundwater is used for drinking water for grazing animals, which may have different Sr isotopic signals due to the dissolution of differing minerals. Groundwater geochemistry is influenced by the $^{87}Sr/^{86}Sr$ ratio of bioavailable Sr in soil and the degree to which different rocks are susceptible to different mineral weathering, deposition types, and climate. However, if the interaction time is long enough, the water in the plants tends to reach the isotopic equilibrium with the rock. In some cases, groundwater and plants need to be treated independently, especially when there is chemical contamination or geochemical changes due to modern land use. These factors can make it difficult or impossible to directly attribute a sample to its source. In this case, the parent rock samples must be analysed to determine the natural basis for the strontium isotope ratios. To avoid misinterpretation of the results, other potentially influencing factors should also be taken into account, such as the addition of salt to cheese [259], [260], [261], or if the animal feed (e.g. imported feed, supplements) is from geographically distant sources.

Despite all these limitations, strontium isotope analysis works, especially if the feeding regime of the cattle is known or controlled. An excellent agreement of $^{87}Sr/^{86}Sr$ values was found between soils, milk and cheese samples from the same location, which confirms the role of Sr isotopes as soil tracers [257] (Figure 1.9). The blue line in Figure 1.10 represents the ratio of milk to soil ($r^2 = 0.98$), indicating that milk, cheese, and soil samples from the same farm have similar Sr isotopic compositions.

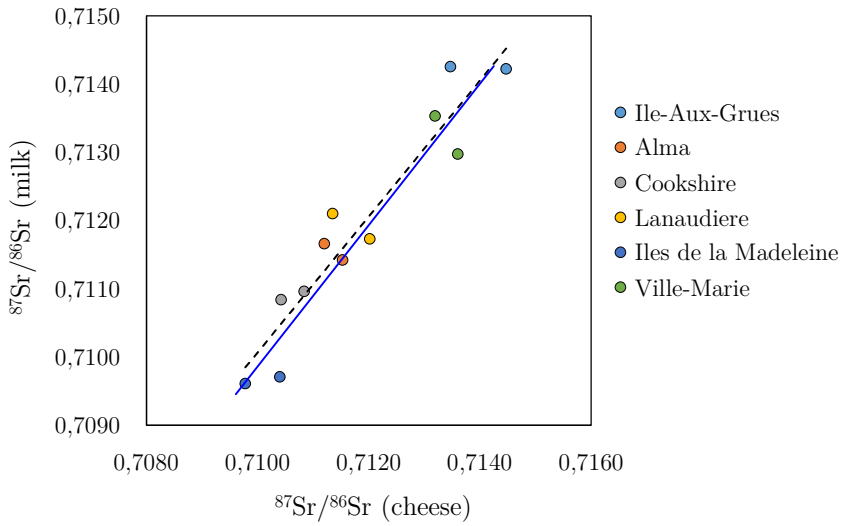


Figure 1.9: Correlation between $^{87}Sr/^{86}Sr$ ratios for milk and cheese collected from six dairy farms (Ile-Aux-Grues, Alma, Cookshire, Lanaudiere, Iles de la Madeleine, and Ville-Marie) in Quebec, Canada ($r^2 = 0.90$) [257].

Franke et al. [262] analysed poultry and dried beef of different origin in order to determine their provenance based on a total of 72 different elements. Poultry samples were collected from six different countries (Switzerland, France, Germany, Hungary, Brazil, and Thailand), while beef samples were produced in Switzerland, Austria, Australia, USA, and Canada out of raw meat originating either from these or from other countries. Statistical analysis showed that As, Na, Rb, and Tl were significant for poultry, while B, Ca, Cd (cadmium), Cu, Dy (dysprosium), Eu (europium), Ga (gallium), Li, Ni, Pd (palladium), Rb, Sr, Te (tellurium), Tl (thallium), Tm (thulium), V, Yb, and Zn were significant for beef. Unfortunately, it was not possible to distinguish sufficiently between the origins. Heaton et al. [263] combined a multi-element fingeprinting approach with stable isotope analysis. Among the parameters identified, six key variables (Sr, Fe, Rb, Se, $\delta^{13}C$ (defatted dry mass) and $\delta^{2}H$ (lipid)) allowed to distinguish between beef samples from Europe, South America, Australia and New Zealand. In this context, differentiation according to the geographical origin could be even more effective if multi-element content is combined with stable isotope analysis [33]. In a further attempt to confirm the possibility of identifying the origin of the meat, other authors used $^{87}Sr/^{86}Sr$ and $\delta^{18}O$ ratios in another study [264]. Samples of poultry meat and dried beef were obtained from the mentioned countries. Similarly, small differences between meat samples did not facilitate the distinction of geographical origin. The authors recommended that future studies should test combinations of the $\delta^{18}O$ method with multi-element analysis or with different stable isotope abundances (e.g. $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$). For example, measurement of $\delta^{13}C$ and $\delta^{15}N$ has shown potential in differentiating between beef samples originating from Japan, Australia and the USA [265] and beef samples originating from Europe and the USA [266]. A combination of multi-

element and stable isotope analysis appears to be suitable for distinguishing the origin of beef [267]. The latter was demonstrated by Baroni et al. [268], where differentiation of beef between three areas in Argentina was possible. Linear discrimination analysis allowed 100 % classification of meat samples from these regions based on Rb, Ca/Sr ratio, $\delta^{13}C$, $\delta^{15}N$ and $^{87}Sr/^{86}Sr$.

Strontium isotopic ratios in plants, soils, and waters cover a wide range of values, and these components are commonly used as environmental proxies to determine the local range of bioavailable strontium isotope composition in archaeological, forensic, and environmental studies. The complexity of the geology and the type of proxy (proxies vary widely depending on the type and origin of the samples) affect the baseline value of bioavailable strontium, which explains the high variability of $^{87}Sr/^{86}Sr$ values in food matrices. However, the use of natural abundance biogeochemistry in determining food provenance is limited. If regional variability in $^{87}Sr/^{86}Sr$ ratios is large, they are more likely to overlap with $^{87}Sr/^{86}Sr$ values of the same food products originating from other regions. In practice, it is difficult to distinguish between soils that show a similar geological background, so a combination of elemental content and isotope ratios of light and heavier isotopes can be useful in these cases.

1.9 Case Studies: Milk and Truffles

In this chapter, subsections are given on the general characteristics and typical matrix composition of the milk and truffle samples. Milk was chosen as a suitable commodity due to its simple processing processes, high level of trade and use as an ingredient in sensitive products such as infant formula. At the same time, truffles represent a Mediterranean high-quality product that must be certified and protected.

1.9.1 Milk and dairy products

Milk is highly nutritious and contains all essential nutrients as carbohydrates, proteins, fat and vitamins [269]. Unfortunately, the increased demand for dairy products can lead to widespread counterfeiting, as milk and dairy products represent such a large market. In this way, milk adulteration is an easy way to reduce production costs and increase profit margins, which often results in the adulteration of milk and dairy products in various ways (Table 1.4).

Table 1.4: Target analytes for the detection of adulteration of milk and dairy products.

Milk component	**Adulteration**	**Target(s)**
Fat	addition of vegetable oil or animal fats to milk fat	fatty acids, triglycerides, phospholipids, sterols, fat-soluble vitamins
Protein	mixing of different species' milk, addition of non-dairy proteins	casein, whey proteins, denatured proteins
Lactose, minerals	addition of water	freezing point

One of the main questions regarding the authenticity of milk and dairy products is the deliberate dilution with water or substitution with cheaper types. Although these counterfeiting practices are less common nowadays, they are still a huge and potentially dangerous problem for many overpopulated countries like China and Sudan [270], Brazil [271], Bangladesh [272], India [273] and Pakistan [274]. Quantifying the level of adulteration of milk with water is of particular interest in Indian markets, as 79 % of branded milk

available in the market was adulterated, as reported by the Consumer Guidance Society of India [275]. When milk is diluted with water, its nutritional value is reduced, and chemicals are added to balance the density and colour after dilution, posing a potential risk to human health [276]. In addition, there are strong economic arguments for reducing the permitted amount of water added to milk, since the price of milk is based on the milk solids content. Milk processing also allows manufacturers to add water to preserved milk above acceptable limits, which is illegal. Since the amount of added water is not mandatory on the label, some companies take advantage of this legal loophole.

Water in foods is already present, and if adulterated with extraneous water, it is difficult to detect with routine laboratory techniques and equipment. While modern methods have some limitations [277], [278], [279], [280], several studies using $\delta^{18}O$ isotope ratios have been successfully used to detect illegal watering of different types of food matrices such as wine [281], fruit juices [282], and concentrated spirits [283]. Hydrogen and oxygen isotope fingerprints are not only useful for detecting food adulteration; they are also an effective means of determining the geographical origin of foodstuffs. Delta ^{2}H and $\delta^{18}O$ values in the original food can provide key information on water origins (e.g. local precipitation, groundwater), climate (ambient temperatures during condensation and precipitation) and the degree of evapotranspiration [45], [284], [285]. Therefore, adding water from other sources changes this fingerprint enough for isotope ratio mass spectrometers to detect the dilution.

All food products have a unique isotopic composition, which is reflected in the metabolic turnover of the plant or animal species that compose them. Compared to plant metabolism, the transformations that take place in animal bodies are more complex and in some cases still unexplained. Perhaps the most challenging feature of H and O isotopes for animal food research is that the isotopic composition of animal tissues reflects not only the isotopic composition of the diet, but a mixture of the isotopic composition of the feed and body water, and in the case of oxygen, also molecular O_2 [286]. The relationship between milk – animal – diet (feed and drinking water) – soil – fertiliser is interesting but complicated to authenticate (Figure 1.11). Since the process of milk synthesis takes place through certain biochemical pathways [287], the relative content of elements and isotope in milk constituents (e.g. casein, lipids, lactose) must show characteristic values and differences. These differences can aid in authenticating milk [288], [289], [290], [291].

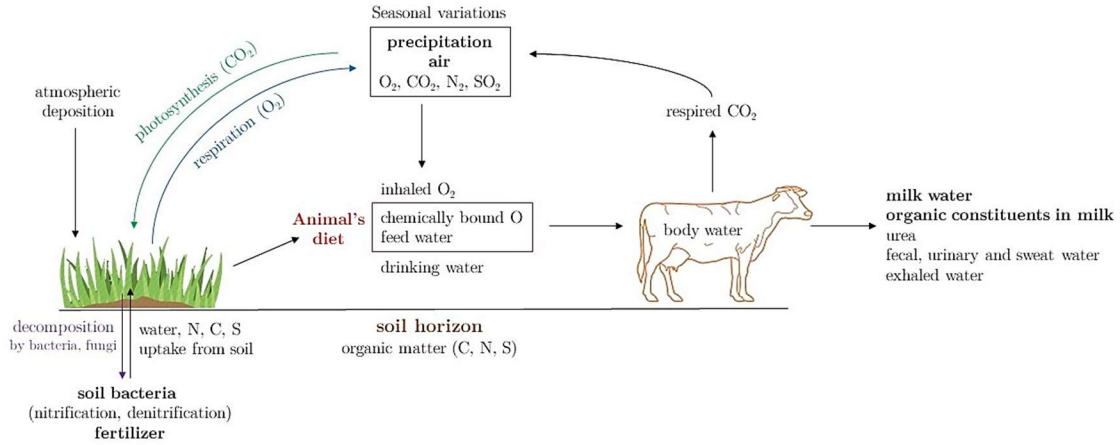


Figure 1.10: Main fluxes of elements from precipitation and diet through the animal's body water to the final product – milk.

1.9.1.1 Stable isotopes and the dietary background of milk

The isotopic composition of carbon in milk is directly related to the composition of pasture and forage, whose $\delta^{13}C$ values depend on geographical and climatic factors. Differences in C_3 and C_4 plants are evident in the form of a gradient of decreasing $^{13}C/^{12}C$ ratios in plant material from the equator to the poles, with C_3 plants predominating at higher latitudes and C_4 being more common in warmer climates at lower latitudes (e.g. trophic level), which can be used as a proxy for determining geographical origin [292]. Milk from regions dominated by grassland typically shows relatively negative $\delta^{13}C$ values, while in regions dominated by crop cultivation, the $\delta^{13}C$ values are more positive. While $\delta^{13}C$ values lower than -23.5 ‰ indicate the presence of C_3 plants in the animal's diet, C_4 plants with higher ^{13}C fractionation usually fall below values from -16 ‰ to -10 ‰ [46]. When animals eat plants, they incorporate the carbon from those plants into their tissues. Tieszen et al. [293] demonstrated that the whole body of an animal is, on average, enriched in $\delta^{13}C$ by about 1 ‰ relative to its diet. This difference in $\delta^{13}C$ values between cattle and their diet is small compared to the average difference in $\delta^{13}C$ values between C_3 and C_4 plants, so diet is the main factor in the variation in $\delta^{13}C$ values in milk [294]. In animals, the metabolism of sugars produces alcohol, which becomes isotopically enriched and differs from similar processes in plants. A good correlation was observed between casein and glycerol for $\delta^{13}C$ determined in cheese, and both constituents were strongly correlated with the amount of maize in the animal's diet [295].

Another possibility is that the isotopic composition of nitrogen in plants and soil depends mainly on soil nutrition. They are also influenced by the climatic conditions, which determine the turnover of organic matter in the soil and the intensity of agricultural practices, especially the type of fertilisers used [296], [297]. The synthetic fertilisers have $\delta^{15}N$ values close to that of atmospheric N_2 ($\delta^{15}N$= 0‰) [298] and conventionally grown crops typically have similar $\delta^{15}N$ values, ranging from -5 ‰ to +5 ‰ [57]. In contrast, organic fertilisers have higher $\delta^{15}N$ values, which can help determine the authenticity of an organically labelled product. The $\delta^{15}N$ values in milk reflect the animal's diet (plants eaten by cattle) and the isotopic composition of the original soil [299], [300]. In addition, plants that fix dietary nitrogen may produce lower $\delta^{15}N$ values in milk and dairy products because these plants use atmospheric and soil nitrogen as a source of nitrogen, resulting in lower ^{15}N content than plants that rely only on nitrogen in the soil [288].

1.9.1.2 Stable isotopes and the geographical origin of milk

Local agricultural practices and animal diet affect $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios, respectively. In case of the carbon, the $^{13}C/^{12}C$ ratio in foodstuffs is directly linked to their botanical origin. The ^{13}C content is particularly good for distinguishing organic products originating from C_3 and C_4 plants [46]. The majority of plants are C_3 plants (e.g. wheat, rye, oats) which use the Calvin photosynthetic pathway to assimilate CO_2. During this process, the plants discriminate against ^{13}C and therefore possess relatively lower $^{13}C/^{12}C$ ratios than C_4 plants (e.g. corn, maize) that utilize the more energy-efficient Hatch-Slack pathway. The enzyme responsible for binding and fixing CO_2 in photosynthesis, RuBisCO, has a strong preference for CO_2 bearing the lighter ^{12}C isotope, resulting in lower ^{13}C values in plants relative to atmospheric CO_2. Many plants native to dry environments evolved a CO_2-concentrating mechanism that reduces water loss from the leaf and reduces the extent to which RuBisCO can discriminate against ^{13}C [294]. C_3 plants contain approximately 14 ‰ less ^{13}C than C_4 plants. In addition to the CO_2 fixation pathway, the isotopic compisition of plants is also influenced by other factors (i.e. local atmosphere CO_2 concentrations, plant variety, and factors affecting plant physiology and the nutritional status of cells, enzyme levels, plant growth rate, water-use efficiency, decomposition of soil organic matter and

cultivation practice) [301], [302], [303], [304]. Therefore, the isotopic composition of carbon can record the source of plants and their geographical origin.

As the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios of milk constituents (e.g. casein) are mainly influenced by dietary intake, the use of both isotopes in milk origin may be limited by animals consuming feed that are isotopically similar in C and N. For example, higher values of $\delta^{13}C_{casein}$ in 80 % of the Slovenian milk samples indicate an approximately 30 to 40 % share of maize (which originates mainly from the Pannonian region) in the cattle's diet [78]. Higher values of $\delta^{13}C_{casein}$ were also accompanied by higher values of $\delta^{15}N_{casein}$, which indicates a higher content of maize in the cattle's diet. Maize is usually cultivated in intensively fertilised fields and no correlation was observed between $\delta^{15}N_{casein}$ and $\delta^{34}S_{casein}$ values, indicating that $\delta^{34}S_{casein}$ values depend more on geology than on feeding regime. However, the local soil/feed fingerprint can be combined with the fertiliser fingerprint, thus information on elemental fingerprint and geographic origin of fertilisers is also important. As a result, it may not be possible to distinguish between milk of different origins [305]. The $\delta^{13}C$ and $\delta^{15}N$ values of casein were reported for milk originating from Australia and New Zealand ($\delta^{13}C$ = -25.9 ‰ to -10.2 ‰ and $\delta^{15}N$ = +5.2 ‰ to +7.3 ‰), Germany ($\delta^{13}C$ = -29.4 ‰ to -26.5 ‰ and $\delta^{15}N$ = +3.5 ‰ to +5.0 ‰), Ireland ($\delta^{13}C$ = -30.5 ‰ to -20.3 ‰ and $\delta^{15}N$ = +4.7 ‰ to +8.9 ‰), Italy ($\delta^{13}C$ = -24.0‰ to -17.2 ‰ and $\delta^{15}N$ = +3.0 ‰ to +5.9 ‰), and Slovenia ($\delta^{13}C$ = -28.2 ‰ to -17.8 ‰ and $\delta^{15}N$ = +2.5 ‰ to +9.6 ‰) [305]. In this scenario, isotope analysis of O and H can be particularly useful because $\delta^{2}H$ and $\delta^{18}O$ stable isotopes in milk represent the relationship between the isotopic signature of milk and drinking water in regions at different latitudes and altitudes [179], [306]. The exchange of H and O between organic molecules and animal's body water due to metabolism and biosynthesis was studied. The results show that H isotopes carry a signature related to the animal's feeding habits, while the O isotopic signature is more reflective of the animal's physiological water balance [37].

The stable isotopes of hydrogen and oxygen depend on the latitude. The relationship between $\delta^{2}H$ and $\delta^{18}O$ values in the hydrosphere across continents, known as the meteoric water line (MWL; $\delta^{2}H = 8 \cdot \delta^{18}O + 10$), was first defined by Craig [307]. In addition to the "latitude" effect, precipitation gradually deplets in ^{18}O and ^{2}H isotopes with increasing elevation ("altitude" effect) or with the movement of vapour masses over the continents from the coast to inland ("continental" effect due to the distance from the sea; a global average decrease of -2.8 ‰ $\delta^{18}O$/1000 km from the coast)[308]. There are also seasonally different patterns indicating different variations in $\delta^{2}H$ and $\delta^{18}O$ values: precipitation in the summer regime is enriched in ^{2}H and ^{18}O, especially inland [308].

Groundwater produced by precipitation is the main source of drinking water for animals and its isotopic composition depends on geographical factors such as altitude, latitude and distance from the sea, but not on the season. In plants, which are the main components of animal's feed, the isotopic composition of water is positive relative to the corresponding soil water because there is generally no isotopic fractionation during water uptake by roots [309]. In addition, $\delta^{18}O$ values in plants reflect evapotranspiration enrichment arising from leaves and isotopic exchange between plant water and organic molecules [310], [311]. The average value of $\delta^{18}O$ in the body water of most domestic animals is about 3 ± 1 ‰ more positive than the value in drinking water [290]. As a result, enrichment with ^{2}H and ^{18}O is observed in milk, where metabolism during milk synthesis causes additional isotopic fractionation. Also, analysis of individual milk constituents instead of bulk values (of whole milk sample) can provide information on the possible existence of correlations between these constituents, which can be used to understand the relationships between milk constituents. For example, a close correlation between the measured $\delta^{18}O$ of milk water and that of lactose in milk can aid in the authenticity of milk [81]. In general, the isotopic composition of milk depends on the species, drinking water and respiration rate [290],

season, farm conditions, breed and physiological state of the animal [288], [291]. Dairy species with different thermoregulatory physiology should have different evaporation-related water isotope fractionation in body fluids, as the vapour is more depleted in heavy isotopes than other body fluids [312], [313]. Goat milk has a higher proportion of calcium than cow milk, which is related to the higher metabolic rate of smaller animals [314]. Similarly, a mammal is prone to evaporative water loss due to body surface area relative to body mass according to Bryant & Froelich [314] and Podlesak et al. [315]. Further, Kornexl et al. [300] described the relationship between $\delta^{18}O$ in milk water and season, resulting from seasonal changes in the $\delta^{18}O$ of forage plants and animal's body, associated with evapotranspiration.

The $^{87}Sr/^{86}Sr$ isotope ratios can be useful in determining provenance because Sr is not appreciably fractionated by chemical and biological processes. Ideally, for food products coming from different regions within the same climate zone (and therefore having similar $\delta^{2}H$ and $\delta^{18}O$ values), additional information on the isotopic composition of Sr can contribute to the correct interpretation of geographical origin if there are different lithologies between regions (Figure 1.11) [78], [79], [179], [257].

Water in plants is usually in isotopic equilibrium with the local source rock, thus sharing similar $^{87}Sr/^{86}Sr$ ratios. The $^{87}Sr/^{86}Sr$ ratio reflected in animal tissues is close to that of food consumed, which is the largest source of dietary strontium for cattle and consequently milk [257].

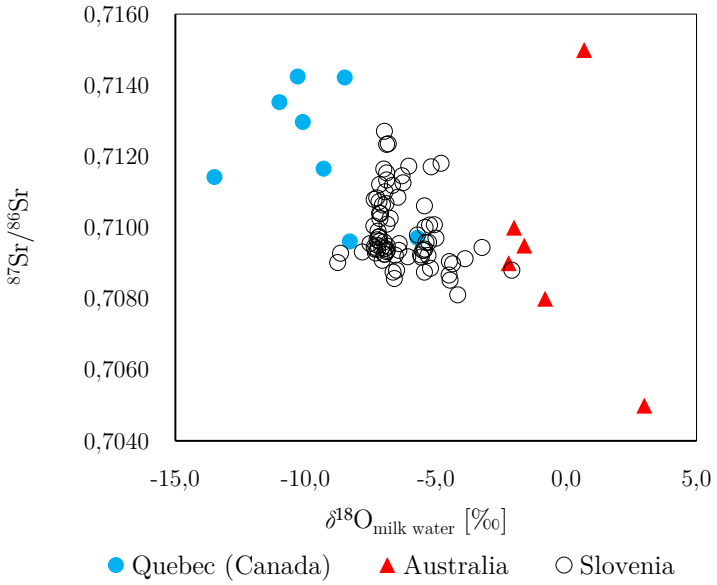


Figure 1.11: Plot of $\delta^{18}O$ values versus $^{87}Sr/^{86}Sr$ values in milk from Canada (blue), Australia (red) and Slovenia (black circles).

Supplementary information can be obtained by measuring $^{34}S/^{32}S$ ratios, as they may reflect ecological changes. The source of sulphur of any animal tissue is the S contained in plants. Given that the sources of S in agricultural systems are SO_2 emissions, sulphate-containing fertilisers, and soil erosion, organisms exposed to S-based pollutants tend to be enriched and more variable in their $^{34}S/^{32}S$ ratios. In this way, researchers can use S isotope ratios to trace pollutants from their anthropogenic sources into and through different ecosystems and to define the degree of impact on plant and animals. In addition, specific effects regarding $^{34}S/^{32}S$ isotopic ratios are also evident in agricultural products originating from coastal areas where marine sulphate, which is highly enriched in ^{34}S compared to soil

sulphate, is spread by sea spray. Sulphate in seawater has $\delta^{34}S$ values of +21 ± 0.2 ‰ across the world's oceans [316], while sulphate in soil has $\delta^{34}S$ values of +10 ‰ [317]. Therefore, soil or sea spray influenced by proximity to the sea can provide useful information on geographic origin [59].

1.9.1.3 Specific compounds in milk as biomarkers of dietary background

The identification of fatty acids (FAs) by chromatography combined with chemometric methods is suggested as an excellent tool for detecting changes in milk composition due to adulteration and for determining geographical origin. Milk fat is usually replaced with cheaper vegetable oils or animal fats [318].

One of the most significant advances has been the development of compound-specific stable isotope analysis (CSIA), which is a useful method for controlling the authenticity of milk and dairy products, especially in identification of relevant biomarkers such as FAs [319]. Isotopic signatures of FA in the milk reflect the dietary regime of the milk-producing animal as well as its metabolism [288], [300]. Isotopic fractionation and physico-chemical reactions lead to large variations in the $\delta^{2}H$ of individual fatty acid compounds [320] and this variation can help determine the geographical origin of milk and dairy products [321]. The authors confirmed the potential of fatty acids as biomarkers in milk traceability [321]. Further, Potočnik et al. [71] demonstrated that the stable carbon isotope composition of fatty acids can be better biomarker of metabolic transformation processes in ruminants than milk origin discrimination. In their study, the discriminant analysis model based on FA composition was effective in differentiating milk according to year and season of production (86.9 %), but differentiating by geographical origin was less successful (64.1 %). Overall, the results have shown that FA composition could help to discriminate the geographical origin of Slovenian milk and thus identify products of better quality. In an extended study by Xu et al. [322] to determine the geographical origin of milk from Australia, New Zealand and Austria, a multivariate model based on stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$), 51 elements and 35 FAs was successfully constructed. Significant parameters for verifying the milk origin were Rb, Tl, Ba, Mo, Sr, Cs, As, Eu, K, Ca, including $\delta^{15}N$ and $\delta^{18}O$, and C20:4n6, C13:0 and C16:1n7. However, future studies should focus on how factors (e.g. breed and age, lactation period, production level, the farming system) influence the FA and stable isotope composition of milk.

1.9.1.4 Trace and rare earth elements

Considering that the concentration of organic compounds in food is related to the geographical origin and depends on, e.g. seasonal changes and climate conditions in the year of cultivation/production, it can be useful to look at the elemental composition of foods. The results collected in the study by Benincasa et al. [323] showed that there is no correlation between the multi-element composition of the diet (drinking water and feed) and the composition of milk. Therefore, when it comes to milk traceability, the feeding regime should be seriously considered. Indeed, the interactions between minerals and other substances in the diet, which consists of drinking water (river and pool), forage (fresh and dried), and/or feed supplements, are complex, which can affect the elemental composition of milk [324]. This may be less complicated with sheep and goat milk compared to cow milk, as sheep and goats are mostly raised outside and mostly kept on local pastures, with little feed being imported [325]. To put this in context, the goat and sheep would reflect local elemental fingerprint. A study carried out on dairy products showed that there are significant differences in Fe, K, Mg, Mn, P and Zn contents in cheese depending on the dairy animal species [326]. 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

milk than in sheep and goat milk [327]. In the case of Slovenian milk, the average content of S and Br was higher in sheep and goat milk than in cow milk [80]. Finally, using stable isotope parameters ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$) and nine micro- and macroelements, it was possible to distinguish sheep and goat milk and cheese from that produced from cow milk with a predictive ability of 95.2 %. [80]. In a similar comparative study conducted by Potočnik et al. [77], it was possible to distinguish Slovenian milk according to regional origin using a multi-element fingerprinting. The authors used the LDA approach taking into account the elements Ca, S, P, K and Cl, which showed a correct classification of 66.7 % of the cow milk samples according to the region of origin. Although the separation was relatively poor, such discrimination was improved by including the stable isotope values of the milk samples [78].

1.9.2 Truffles

Truffles (*Tuber* spp.) belong to the ectomycorrhizal (ECM) fungi that undergo a complex life cycle associated with different forest species.

Truffles are among the most valued ingredients in the culinary world and can fetch from one hundred to one thousand Euros per kilogram, depending on the type and size [328]. The purchase price of black truffle is from €200 to €300 per kilogram. White truffles are much more valued and currently cost up to €5,000 per kilogram, but can also go up to €8,000. This kind of culinary experience has a positive effect on regional economies, as better promotion of truffle products leads to their higher market value and higher profits for producers. Europe represents 85 % of the global export market, where the most sought-after black and white truffles grow in France, Italy, Croatia, Slovenia, and Hungary. According to TrendEconomy data (Figure 1.12) [329], the largest export destinations of mushrooms and truffles, prepared or preserved otherwise than vinegar or acetic acid, from Slovenia in 2020 were Italy, Croatia, Austria, Czech Republic, Hungary, Bosnia and Herzegovina, Slovakia, Germany, Chile and Switzerland. The value of exports of mushrooms and truffles from Slovenia in 2019 amounted to $1.09 million.

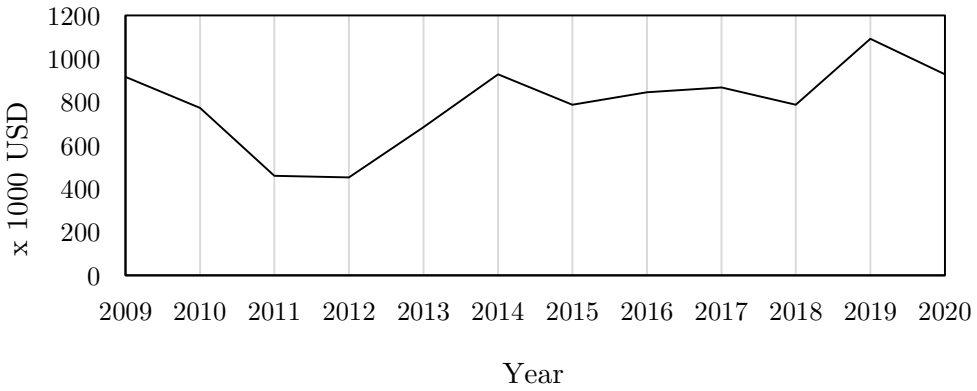


Figure 1.12: The value of exports of mushrooms and truffles, prepared or preserved otherwise than by vinegar or acetic acid, from Slovenia from 2009 to 2020 (Source: TrendEconomy).

Truffles play an important role in controlling the functioning of the forest ecosystem. Almost the entire life cycle of truffles takes place in the soil, which means that the environment of truffle is basically characterised by the properties of the soil, the presence of a suitable host plant and the microbial community associated with the host plants. In temperate climates, truffles are primarily related to hazel, oak, and pine trees, while desert

truffles are more closely related to shrubs. On the other hand, soil properties are closely related to the nature of bedrock, climate and vegetation cover. Certain ecological conditions contribute to the growth of mycelium, the formation of mycorrhizal communities and ultimately the formation of the truffle [330]. This suggests that symbiosis drives the evolution of truffle diversity.

At the early development phase of the symbiosis, the truffle mycelium forms a relationship with the roots of certain trees, which involves the exchange of water, mineral nutrients and organic carbon [331]. In this way, underground networks connecting ectomycorrhizal fungi and their host plant roots shape these ecosystems, affecting the global carbon cycle as well as the plant health and resistance to stressors such as drought or disease [332]. Studies have shown that ectomycorrhizal fungi can slow soil carbon cycling by limiting nitrogen [333]. Due to their hidden underground life cycle, complex host symbiosis and yet unknown distribution, several studies have been carried out to date to better understand the natural occurrence of the species and their role in promoting the uptake of nutrients and water into the host plants, thereby maintaining the aboveground primary productivity of forest ecosystems. Some studies are evaluated in the following Subsections 1.9.2.2, 1.9.2.3 and 1.9.2.4.

Among the different species of truffle, only three are commercially important: the white truffle (*Tuber magnatum*), the black truffle (*Tuber melanosporum*) and the summer truffle (*Tuber aestivum*). Geographically, *Tuber melansporum* is more restricted than *T. magnatum* and *T. aestivum*. The distribution of *T. melanosporum* is relatively exclusive to sites covered with oak (*Quercus* spp.) and hornbeam (*Carpinus* spp.) trees in France, Spain and Italy. These species prefer burnt areas (French brûlé) around the host tree, characterised by sparse vegetation and a temperate Mediterranean climate [334]. *Tuber magnatum* is found in Italy, Switzerland-Ticino, Romania, Hungary, and some parts of the Balkans. In its native range in Italy and Croatia, *T. magnatum* competes and produces best in climatic zones with abundant annual rainfall with very short dry periods. The most productive hosts are oaks (*Quercus* spp.), poplars (*Populus* spp.) and willows (*Salix* spp.) in the presence of calcareous and porous soils with moderately alkaline pH [335]. Interestingly, *T. magnatum* prefers habitats without traces of calcium carbonate and lower pH (6.8 – 7.5), in the presence of *Quercus robur*, *Populus* spp. and *Fraxinus angustifolia*. *Tuber aestivum* is widely distributed throughout Europe due to its high commercial success in cultivation, as these species can easily adapt to different soil parameters, climate and rainfall, and to a wide range of hosts [336].

1.9.2.1 Mycodiversity in Slovenia

The territory of Slovenia is characterised by a high ecosystem and species diversity due to its specific geographical position in combination with a diverse relief structure, complex geology, significant water resources and a modified Mediterranean, continental and mountain climate [140]. The combination of these factors contributes to the rather rich mycodiversity in the country.

Experts estimate that there are 16 types of truffles in Slovenian forests, including the white truffle *Tuber magnatum*, *Tuber borchii* and *Tuber asa*, and the black truffles *Tuber aestivum* Vittad, *Tuber ubicatum* and *Tuber melanosporum* [337]. Based on the map of potential natural habitats in Slovenia for *T. aestivum*, there are 44 % of suitable areas with very good natural potential for truffle growing [338] (Figure 1.13). The higher the suitability index, the greater potential the location has for natural growth of truffle.

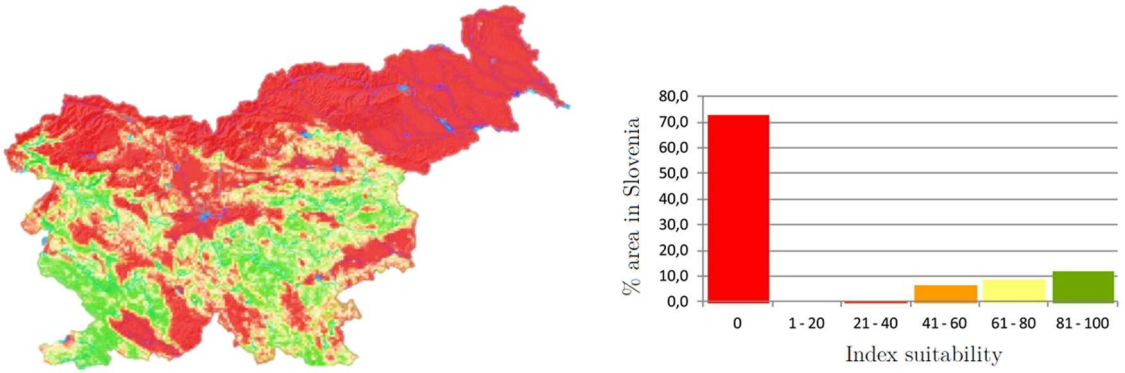
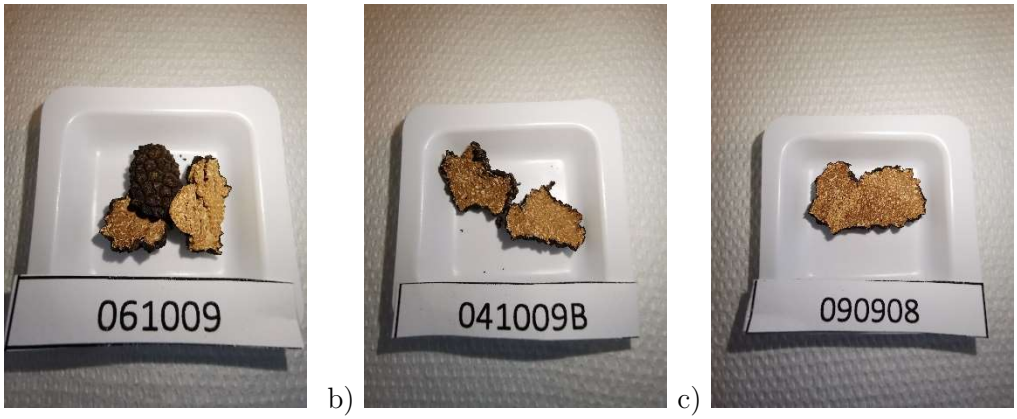


Figure 1.13: The most potential grow areas of *Tuber aestivum* in Slovenia are coloured in green. Figure taken from Bergant et al. [338].

Among the suitable areas are the less suitable areas of the pre-Alpine hills and pre-Alpine valleys and basins. The most suitable areas are very common in the sub-Mediterranean regions, especially in the karst plains and foothills, such as the Karst, in the area of the Dinaric Karst foothills and plains and the southern part of the Posavsko hills. A smaller concentration of very suitable potential areas is visible in the area of Kozjansko and the western part of Bela Krajina [338].



b) c)

Figure 1.14: *Tuber aestivum* samples collected from different natural habitats in Slovenia (*from left to right*): Žlebič, Jasnica, and Rakitovec. (Photo: S.H.G., IJS Reactor Centre, 2019)

1.9.2.2 Truffle authentication

Due to the lack of regulations, it is essential to prevent fraud in the truffle market [339]. High truffle prices have led to several forms of adulterations. For example, 15 % of French truffles tested in 2012 were found to be common cheaper truffle species, originating mainly from China. *Tuber borchii* can be mistakenly identified as *Tuber magnatum*, as it is visually similar to the latter and it can be sold as the latter. Other fraudulent practices involve the use of unripe fruiting bodies of cheaper species in processed foods [340]. Since fraudulent activities can have a profound impact on the local economy, there is an urgent need to protect the truffle market and establish clear authenticity information on truffle products. To date, this has been achieved mainly through molecular approaches [340], [341], [342] and the analysis of volatile organic compounds [91], [339], [343], [344]. Apart from their

unique aroma, different chemically valuable compounds such as fatty acids and sterols can be characteristic of different species and origin [345], [346].

The compositional details of truffles vary from species to species and from area to area and can therefore contribute significantly to authentication studies. Recently, the identification of *Tuber* species using stable isotopes and elemental fingerprints has become increasingly important in determining authenticity and geographic origin [347], [348]. Studies using stable isotope techniques were mainly orientated towards investigating carbon isotope fractionation during sucrose decomposition [349], the eco-physiological relationship between truffles, soil and host plants [350], or assessing the mycorrhizal versus the saprophytic status of fungi using the natural abundance of carbon and nitrogen stable isotopes [351]. Habitats with different nutrient inputs and plant communities can significantly differ in overall $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotope ratios [352], [353], [354], [355]. Geographical differentiations involving stable isotope composition have been performed for other fungal species. Based on the $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$ ratios in mushroom (*Agaricus bisporus*), it was possible to differentiate between specimens from six regions in Korea [356], while Puscas et al. [357] were able to distinguish samples from different regions of Transylvania using $^{13}C/^{12}C$ isotope ratios of bulk fungi, and the $^{2}H/^{1}H$ and $^{18}O/^{16}O$ isotope ratios in water extracted from the samples. In the study of Krauß & Vetter [347], Chinese truffles were well separated from all European samples using their significantly ($p < 0.05$) more negative $\delta^{2}H$ values. The authors also found that European *T. magnatum* were more enriched in ^{15}N and more depleted in ^{13}C than the remaining species (*T. aestivum*, *T. melanosporum*, *T. indicum*, *T. albidum*, and *T. moscatum*). Further, it has been suggested that high $\delta^{15}N$ values ($> +12$ ‰) combined with low $\delta^{13}C$ values (< -27 ‰) could be indications for this truffle species.

1.9.2.3 Elemental composition as potential markers of species and geographical origin

Elemental analysis in ECM fungi was used mainly to determine the environmental contamination of fungi [358]; however, some studies of elemental truffle composition were also aimed at determining its authenticity [348], [359], [360]. Rossbach et al. [359] highlighted the possibility that host plants control the transport of nutrients to the different parts of fruiting bodies, as the peridium was enriched in elements compared to gleba. Further, Bontempo et al. [360] demonstrated the possibility to distinguish white truffles based on elemental and isotopic profiles despite the proximity of some of the production areas investigated in their work. Although the results were encouraging, further investigations involving a wide range of authentic *T. magnatum* samples are needed to improve the characterisation of these truffles. Segelke et al. [348] developed a protocol for the authentication of fresh truffles, which allows the determination of provenance based om elemental composition. However, some questions need to be answered before testing the adequacy of the protocol, what are the factors that affect the rate of maturation during storage, and how host trees and type of cultivation (wild or artificial) affect the elemental profile of truffles. To date, only three studies have examined the elemental composition of *T. magnatum* to assess potential differences in their ability to assimilate and accumulate nutrients from the environment [361], [362]. *Tuber magnatum* appears to be the most competitive among the different cultivars as it can effectively assimilate/accumulate Cu, K, Na, P, and Zn. At the same time, *T. brumale* was more successful in sulphur accumulation/assimilation. Segneanu et al. [362] investigated the antioxidant activity, total organic carbon, and the levels of As, Cu, Pb, Zn, Mn, Fe and Ni in *T. magnatum* and *T. melanosporum*. Their results show that *T. melanosporum* contains a higher amount of C and Fe than *T. magnatum* Pico, while there was no difference in the levels of other elements. Distinguishing between European and Chinese truffles is facilitated because the

Chinese black truffle species such as *T. indicum*, *T. himalayense* and *T. sinense* [363] are endemic to China, which means that the determination of geographical origin can be made already at the species level. Therefore, it is necessary to look for specific markers related to the species and geographical origin of truffles and their products.

1.9.2.4 Volatile organic compounds as markers of geographical origin

Also, specific structures of amino acids and volatile compounds can be characteristic of a particular food type. While some aromas are common to many truffle species, others are more species-specific, or are limited to particular species [364]. For most white and black truffle species, volatile organic components (VOCs) such as alcohols (1-octenol-3-ol, 3-methyl-1-butanol), aldehydes (2-methyl-butanal, 3-methyl-butanal), ketones (2-butanone, 3-octanone), and sulphur compounds (dimethyl sulphide, dimethyl disulphide, methylsulphonylmethane) are common [91], [343], [365], [366], [367]. Esters were found to be the most abundant in *T. melanosporum* compared to other truffle species [366]. Among esters identified in *T. melanosporum*, only butan-2-yl formate and 2-methylpropyl formate can also be found in *T. indicum*, *T. macrosporum*, *T. rufum* and *T. brumale* [84]. While 2,4-dithiapentane is exclusive to *T. magnatum* [91], [365], 3-methyl-4,5-dihydrothiophene seems to be specific to *T. borchii* [368].

Many authors have suggested that the aroma might vary according to the geographical origin of truffles of the same species [369], [370]. Strojnik et al. [84] extended the study to different truffle species. The authors determined the aroma profile of fresh ascocarps of truffle species (*T. aestivum*, *T. magnatum*, *T. melanosporum*, *T. mesentericum*, *T. brumale*, *T. excavatum*, *T. rufum*, *T. indicum* and *T. macrosporum*) from eleven countries (Slovenia, Croatia, Bosnia and Herzegovina, Macedonia, Italy, Spain, France, United Kingdom, Germany, Poland and China). Using the LDA model, truffle species were 96.9 % correctly classified by their aroma profile, confirming that the discrimination model can identify samples of unknown or suspicious origin. 2-butanone and 3-octanone were found to be specific markers for *T. aestivum*.

The LDA analysis of five Slovenian truffles (*T. aestivum*, *T. mesentericum*, *T. magnatum*, *T. brumale* and *T. melanosporum*) showed a 95.8 % correct classification rate according to their geographical origin [84]. On the other hand, the degree of geographic discrimination of *T. aestivum* grown in four natural habitats in Slovenia (Sežana, Bloke, Žlebič, and Spodnje Blato) was very low (50.5 %), which is probably due to insufficient sample size. Since there are different lithologies within these natural sites of the Slovenian truffle *T. aestivum*, it would be possible to obtain additional information from $^{87}Sr/^{86}Sr$ to confirm the geographical origin.

More recently, Niimi et al. [371] reported that volatile profile of *T. magnatum* varied more within sites than across the geographical area, and bacterial communities associated with these species were partially explained by provenance. These authors also indicated that aroma of these fungi is not dependent on the maturity level. However, previous studies concluded that maturity has an influence on the aroma of *T. melanosporum* and *T. aestivum*. It has been observed that with fruiting body maturation, the levels of monosaccharides and some free amino acids increased in the case of *T. melanosporum* [372], while in *T. aestivum*, the contents of phenols and tannins decreased [345]. In addition, the choice of sample preparation method should be taken into account, as it has been confirmed that a simultaneous lyophilization process can significantly change the aroma profile compared to fresh truffles [93]. Nevertheless, their study showed that the transformation of VOC during freeze-drying is species-specific, and the authors were able to discriminate between all four freeze-dried truffle species as successfully as fresh samples using

multivariate discriminant analysis, confirming the applicability of the approach in food fraud detection.

Chapter 2

Aims and Hypothesis

Historically, food products have always been linked with a specific geographical origin. Food-consumption habits were created by the local natural resources and the social or cultural factors of the community. Stable isotope and elemental fingerprinting have become increasingly important in establishing the authenticity and geographical origin of food products. Isotopic ratios apply to food authentication because they change with the climatic conditions, geographical origin, soil pedology, and geology of the locations of food ingredients. Characterisation of Slovenian milk was performed using a stable isotope of light elements in combination with elemental composition. As a primary indication, H and O isotopic data in milk are linked to the H and O isotope data of water from the source region with geographical variability. The $\delta^{13}C$ values in milk are mainly dependent on the animal's diet, while $\delta^{15}N$ are generally influenced by the soil nutrition and the intensity of agricultural practices, since the $\delta^{15}N$ values in milk and dairy products reflect the isotope composition of the original soil. In the case of sulphur, its isotope fractionation in milk is related more to the geology of the region, fertiliser application, SO_2 emissions and the distance from the coast (due to the seaspray). Correlations between the mode of production and $\delta^{13}C$ or $\delta^{34}S$ signatures have not been established, and $\delta^{2}H$ and $\delta^{18}O$ are not always suitable markers due to the overriding effect of climate on the isotopic composition of milk of different origin. As there is an overlap between the isotopic signatures of milk and dairy products, the use of the Sr stable isotope ratio can increase the probability of a geochemical fingerprint of a given region on its food products, as it is strongly related to the geological characteristics of the area.

Thus, its overall goal, the PhD builds on the following specific objectives:

OBJ1: The optimisation of a method for Sr stable isotope measurements using multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) for analysing milk and truffles.

OBJ2: To couple $^{87}Sr/^{86}Sr$ ratios with other geographical tracers such as stable isotopes of light elements (H, C, N, O and S) and elemental composition and perform multivariate statistical analysis to classify truffles according to species and geographical origin.

OBJ3: To evaluate the possibility of applying lactose as an internal standard for determining the presence of added water in milk.

Within this dissertation, the following hypotheses will be tested:

1. Dry ashing and microwave-assisted acid digestion as pre-treatment techniques give similar results for Sr stable isotope ratios.

2. The $^{87}Sr/^{86}Sr$ isotopic fingerprint of the local environment is reflected in the fruiting body of a truffle.

3. The combined use of stable isotopes and elemental composition, and multivariate analysis will significantly improve geographical origin determination compared to either method alone.

4. Falsification identification using lactose as an internal standard can detect < 15 % of water addition to milk.

The proposed dissertation represents a part of the Era Chair ISO-FOOD for isotope techniques in food quality, safety and traceability, IAEA Project under Contract No. 17897 entitled "*The use of stable isotopes and elemental composition for determination of authenticity and geographical origin of milk and dairy products*" as a part of CRP D5.20.38 "*Accessible technologies for the verification of the origin of dairy products as an example control system to enhance global trade and food safety*" and REALMed project: *Pursuing authenticity and valorisation of Mediterranean traditional products*. The research was also supported by the Slovenian Research Agency within programme P1-0143.

Chapter 3

The Provenance of Slovenian Milk Using $^{87}Sr/^{86}Sr$ Isotope Ratios

This chapter summarizes the paper entitled "The Provenance of Slovenian Milk Using $^{87}Sr/^{86}Sr$ Isotope Ratios" by Staša Hamzić Gregorčič, Nives Ogrinc, Russell Frew, Marijan Nečemer, Lidija Strojnik, and Tea Zuliani. The paper was published in the *Foods* journal, 2021.

Authenticity and fraud issues affect a wide range of food products; not only high-value foods, but also milk and dairy products that millions of people depend on daily. To protect consumers and producers, analytical methods must be validated to prevent food fraud and mislabelling and to ensure the origin of milk and dairy products. Although the combination of multi-element analysis and stable isotope ratio analysis is proven to be effective in detecting adulteration and mislabelling, there are some limitations in determining the origin of Slovenian milk. The composition of milk is mainly influenced by the feeding regime of cattle as a function of geological, climatic and agricultural factors that influence the environment in which they live. These factors affect the composition of milk, which can lead to uncertainty in predicting the origin of milk. As there are different lithologies in Slovenia, the isotopic ratios of strontium ($^{87}Sr/^{86}Sr$) could provide additional information about the origin of the milk, which is not obscured by the local climate or biological processes.

Determination of $^{87}Sr/^{86}Sr$ composition in complex food matrices often requires extensive sample pre-treatment and isolation steps prior to instrumental analysis. In the absence of standard protocols for sample preparation, preservation and analysis, a methodology was needed to accurately measure the isotopic ratios of Sr in milk. The proposed method for determining the $^{87}Sr/^{86}Sr$ isotopic ratio in milk was optimized in this work (optimization of the method is described in Appendix A). Since no matrix-certified reference material exists for Sr isotope analysis, method validation was performed by comparing the $^{87}Sr/^{86}Sr$ results for the IAEA-153 reference material (milk powder; International Atomic Energy Agency, Vienna, Austria) obtained using the optimized method with those obtained in an independent laboratory. The method has proven to be suitable for determining the origin of milk and can be used in real applications.

The aim of this research was to determine the variation of $^{87}Sr/^{86}Sr$ ratios in an authentic Slovenian milk sample (n = 77) and whether milk samples could be separated according to the region of origin using a combination of $^{87}Sr/^{86}Sr$ ratios together with different isotopic ratios of bio-elements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$) and multi-elemental composition (Mn, Fe, Cu, Rb, Sr, Ca, K, Cl, S, P, Zn, Br), including elemental ratios (1/Sr, Rb/Sr, Ca/Sr and K/Rb). The results showed that the $^{87}Sr/^{86}Sr$ ratio has the potential to distinguish between different milk production areas if these areas are characterised by different lithology. Despite the promising results, inter-annual or annual

variations of the Sr isotopic composition in milk cannot be neglected. The $^{87}Sr/^{86}Sr$ ratios of groundwater and surface waters are in good correlation with milk, indicating that the Sr isotopic fingerprint in milk is reflective of cow drinking water. Stable isotope ratio and elemental composition parameters combined with the discriminant analysis classification model showed that $^{87}Sr/^{86}Sr$ ratio together with $\delta^{13}C_{cas}$ and $\delta^{15}N_{cas}$ values have the main discriminating power to distinguish the Quaternary group from the others. The Cretaceous (carbonate rocks and flysch) group is associated with Br content, 1/Sr and $\delta^{18}O_w$ values. The overall predictive ability was found to be 63.5 %. Diet and geological parameters were found to be significant for separation according to pairwise comparisons using OPLS-DA.

Future research should focus on the $^{87}Sr/^{86}Sr$ ratio in feed, water and soil originating from the exact location as the milk to better understand their influence on the elemental and Sr isotopic composition of milk, which could improve milk provenance determination. The database of the $^{87}Sr/^{86}Sr$ values in soil and water in Slovenia could also be useful for future studies of local foods from the perspective of food traceability, where it would serve as a reference map for identifying the authenticity of individual foods, or to determine whether unexpected isotopic variations occur.

In this paper, I was responsible for the sample collection and preparation, including an optimisation of method for precise and accurate determination of the $^{87}Sr/^{86}Sr$ isotope ratios by MC-ICP-MS. I also collected and, in part, analysed the data and prepared the manuscript. The work was also presented at the 10^{th} Jožef Stefan International Postgraduate School Students' Conference and 12^{th} Young Researchers Day in Piran, Slovenia (10 May 2018 – 11 May 2018), under the slogan "*Decoding science: Science communication, dissemination and research*". I received a special ERA Chair Isofood award for the best poster presentation, in which the importance of optimizing the method for the separation of Sr from the sample matrix for the reliable determination of $^{87}Sr/^{86}Sr$ isotope ratio by MC-ICP-MS in milk was emphasized. Results of the study were also presented at the European Winter Conference on Plasma Spectrochemistry 2019 in Pau, France.

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The Provenance of Slovenian Milk Using $^{87}Sr/^{86}Sr$ Isotope Ratios

Staša Hamzić Gregorčič [1,2], Nives Ogrinc [1,2], Russell Frew [3], Marijan Nečemer [2], Lidija Strojnik [1,2] and Tea Zuliani [1,2,*]

[1] Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia; stasa.gregorcic@ijs.si (S.H.G.); nives.ogrinc@ijs.si (N.O.); lidija.strojnik@ijs.si (L.S.)
[2] Jožef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia; marijan.necemer@ijs.si
[3] Department of Chemistry, University of Otago, P.O. Box 56, Dunedin 9016, New Zealand; russell.frew@otago.ac.nz
* Correspondence: tea.zuliani@ijs.si; Tel.: +386-1-477-3542

Abstract: This work presents the first use of Sr isotope ratios for determining the provenance of bovine milk from different regions of Slovenia. The analytical protocol for the determination of $^{87}Sr/^{86}Sr$ isotope ratio was optimised and applied to authentic milk samples. Considerable variability of $^{87}Sr/^{86}Sr$ ratios found in Slovenian milk reflects the substantial heterogeneity of the geological background of its origin. The results, although promising, cannot discount possible inter-annual or annual variation of the Sr isotopic composition of milk. The $^{87}Sr/^{86}Sr$ ratios of groundwater and surface waters are in good correlation with milk, indicating that the Sr isotopic fingerprint in milk is reflective of cow drinking water. The $^{87}Sr/^{86}Sr$ ratio has the potential to distinguish between different milk production areas as long as these areas are characterised by geo-lithology. Discriminant analysis (DA) incorporating the elemental composition and stable isotopes of light elements showed that $^{87}Sr/^{86}Sr$ ratio together with $\delta^{13}C_{cas}$ and $\delta^{15}N_{cas}$ values have the main discrimination power to distinguish the Quaternary group (group 6) from the others. Group 1 (Cretaceous: Carbonate Rocks and Flysch) is associated with Br content, 1/Sr and $\delta^{18}O_w$ values. The overall prediction ability was found to be 63.5%. Pairwise comparisons using OPLS-DA confirmed that diet and geologic parameters are important for the separation.

Keywords: geographical origin; milk; cow diet; $^{87}Sr/^{86}Sr$; discriminant analysis

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1. Introduction

Proof of provenance has increased in relevance over the past decade because of its positive impact on food safety, quality and consumer protection per national legislation and international standards and guidelines. This trend also coincides with an increase in consumer demand for local and regional food, which is considered higher quality, safer and more sustainable. This has created interest in building local and regional food systems across Europe, including Slovenia. Most milk and dairy products, produced and processed in Slovenia, now use the "Selected Quality—Slovenia" mark, which indicates that the product is of Slovenian origin. The characteristics of milk are highly dependent on the farming practices and the soil where cattle graze, thereby, from the geographical region, reflecting specific and peculiar geologic information. Consequently, the geographic origin of milk and dairy products is an important factor affecting quality.

The geographical origin of milk and especially dairy products has been frequently traced by using stable isotope analysis of light elements (δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$ and $\delta^{34}S$) [1–4] or in combination with the multi-elemental analysis [5–8]. Recently, isotopic information from heavy elements in soil and food has been explored for its potential to serve as reliable geographical tracers for food origin. In particular, the isotopic composition of strontium (Sr) has proven to be a promising tool for discriminating at a regional level. It

has been found that the Sr retains its original isotopic ratio unaltered up to the end product, even after processing (e.g., mixed biological processes involved in soil-plant interaction), with no isotopic fractionation [9]. The $^{87}Sr/^{86}Sr$ isotope ratio, therefore, provides a unique and highly efficient geographical tracer for several types of food products such as asparagus [10], rice [11,12], tea leaves [13,14], coffee [15,16], orange juice [17], and wine [18–20]. The authors of these studies highlighted two main applications of the $^{87}Sr/^{86}Sr$ isotope ratio: (i) to characterise the Sr isotope composition of a specific agricultural area, creating a valuable database for subsequently verify products cultivated in that area; and (ii) to discriminate with certainty different production areas of a specific product.

Although Sr isotopic analysis has led to significant advances in food traceability, there is still a lack of standard protocols for sample preparation, preservation and analysis, making data interpretation and cross-study comparisons difficult and confusing. The $^{87}Sr/^{86}Sr$ isotopic analysis in complex food matrices often requires extensive sample pretreatment and/or isolation steps before instrumental analysis, especially when multi-collector inductively coupled plasma mass spectrometry (MC ICP-MS) and thermal ionisation mass spectrometry (TIMS) are used [21,22]. The $^{87}Sr/^{86}Sr$ isotope ratio determination is influenced by the isobaric interference of ^{87}Rb, leading to incorrect isotope ratio determination [23]. Among four stable isotopes of Sr (^{84}Sr, ^{86}Sr, ^{87}Sr, and ^{88}Sr), only ^{87}Sr is radiogenic and formed by β-decay of ^{87}Rb. Thus, ^{87}Rb and ^{87}Sr are often co-present in environmental samples. Chemical separation of ^{87}Rb from ^{87}Sr is required to assure accurate results and is usually accomplished using separation techniques such as extraction chromatography. Among the extraction chromatographic resins developed for the isolation of Sr from the complex sample matrix, Sr resin seems to be the most popular one due to its high selectivity for Sr [10,12,17,24]. The extractant contains 4,4′(5′)-di-t-butylcyclohexano 18-crown-6 (crown ether) in 1-octanol on inert polymeric support with the working capacity of 8 mg Sr per 2 mL column. As the column and resin size are dependent on the concentration of the elements present in the sample matrix under study, the efficiency of Sr-matrix separation also depends on the volume and molarity of nitric acid (HNO_3). Horwitz et al. (1992) [25] show that the Sr capacity factor increases with increasing HNO_3 concentration and decreases with increasing concentration of other cations such as barium.

Milk is a complex matrix with a higher organic load (fat and proteins) than most other food products previously cited, making the determination of Sr isotope ratios challenging. Although there are studies on the $^{87}Sr/^{86}Sr$ isotope ratio in dairy products [24,26–28], to our knowledge, only a limited number of studies on the $^{87}Sr/^{86}Sr$ isotope ratio determination in milk has been published [29,30]. Since no standard separation procedure for milk exists, there was a need for a methodology for accurately measuring Sr isotope ratios. To overcome the lack of standard protocols for sample preparation and analysis needed to enable comparability of cross studies and data interpretation, a proposed method for the $^{87}Sr/^{86}Sr$ isotope ratio determination in milk was optimised within this work. The method was shown to be fit-for-purpose for determining milk provenance and could be used in real-world applications.

Therefore, our study aimed to determine the $^{87}Sr/^{86}Sr$ isotope ratio in milk using an optimised and validated analytical method and apply it to Slovenian milk samples originating from different geological regions to test its applicability in traceability studies.

2. Materials and Methods

2.1. Sampling

Milk samples were collected from 43 dairy farms located in different geological areas in Slovenia in 2014 (Figure 1).

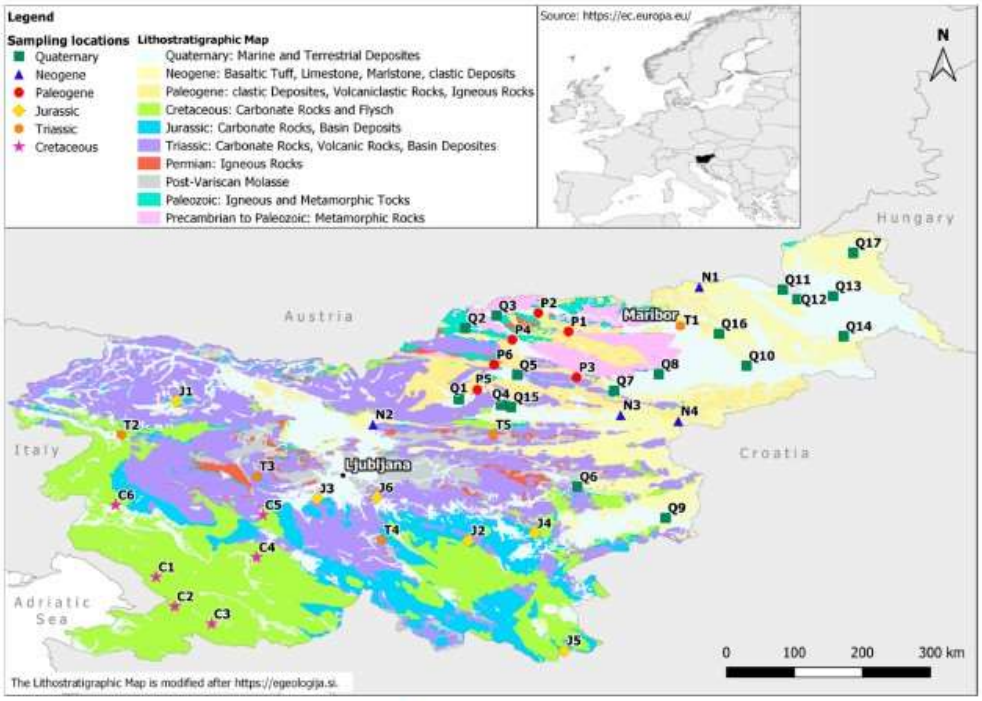

Figure 1. Geological map of Slovenia as indicated [31] with dairy farm locations (Table 1). The map was prepared by J. Vrzel.

The climatic conditions in Slovenia do not allow year-round grazing on outdoor pastures. Additionally, the landscape is diverse, where not all areas allow the growing of appropriate feed, so the geographical origin of winter feed may change. Both circumstances are responsible for the change in the cow's diet. To evaluate these changes, milk samples were sampled during summer and winter in 2014 and the winter of 2015. Further, the elemental and stable isotopic composition of light elements (H, C, N, O and S) of authentic milk samples was determined to characterise authentic Slovenian milk.

2.2. $^{87}Sr/^{86}Sr$ Isotope Ratio in Authentic Slovenian Milk

Although milk contains about 87% of water, its proteins, carbohydrates, and especially fat make its matrix very complex in the Sr isotope analysis. If not adequately destroyed, organic remnants can irreversibly adsorb on the extraction resin, thus reducing its exchange capacity and leading to a reduction in the Sr recovery and possibly to isotopic fractionation. The method was optimised in terms of completeness of mineralisation, chemical recovery of Sr isolated from the sample matrix (Table S2), minimal contamination, and turnaround time. The blanks of analyte-free media were prepared using the same materials and reagents as for the samples. A procedure for optimisation and validation of the analytical method for accurate $^{87}Sr/^{86}Sr$ isotope ratio measurement in milk is fully described in Supplementary Materials with Tables S1–S5, while the analytical protocol is presented in Figure 2.

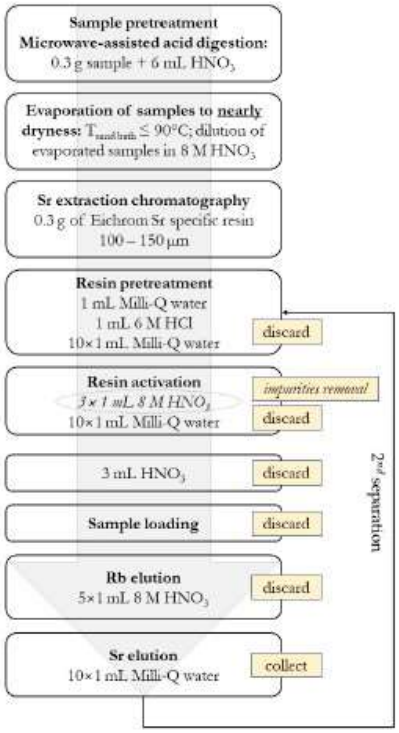

Figure 2. Analytical protocol for effective Sr isolation from the sample matrix.

In summary, as presented in Figure 2, for pretreatment of the milk samples, 0.30 g were subjected to microwave digestion and then evaporated to dryness and redissolved in 1 mL of 8M HNO_3. A column (2 mL) was filled with 0.30 g resin, activated by washing with HCl. The resin was acidified with 3 mL HNO_3 before sample loading to prevent any loss of Sr. Subsequently, the sample solution was loaded onto the column. Rb was eluted with 5 mL of 8M HNO_3, after which the Sr was collected in purified water washes (Table S4). The Sr solution was then evaporated and purified again through extraction separation. Finally, the $^{87}Sr/^{86}Sr$ isotope ratios were determined using MC ICP-MS.

Isotope Analysis Using MC ICP-MS

Strontium isotope ratio determinations were carried out using a Nu II multi-collector ICP-MS instrument (Nu Instruments, Ametek Inc., Wrexham, UK) fitted to an Aridus II™ Desolvating Nebulizer System (Teledyne Cetac, Omaha, NE, USA) by the procedure of Zuliani et al. (2020) [32]. All samples were run in a standard-sample-standard bracketing sequence using standard Sr isotopic solution (NIST SRM 987: *Strontium carbonate*; $^{87}Sr/^{86}Sr_{certified} = 0.71034 \pm 0.00026$; National Institute of Standards and Technology, Gaithersburg, MD, USA).

2.3. Multi-Elemental Analysis Using EDXRF

The multi-elemental composition of milk, including Sr stable isotope ratio, was performed using freeze-dried and homogenised milk samples. Energy-dispersive X-ray fluorescence spectrometry was used to determine the following elements: calcium (Ca),

chloride (Cl), potassium (K), phosphorus (P), sulphur (S), bromide (Br), rubidium (Rb), and strontium (Sr). Each milk sample (0.5–1.0 g) was pressed into a pellet using a hydraulic press. As primary excitation sources, the annular radioisotope excitation sources of Fe-55 (10 mCi) and Cd-109 (20 mCi) from Isotope Products Laboratories (Valencia, CA, USA) were used. The emitted fluorescence radiation was measured using an energy dispersive X-ray spectrometer composed of a Si(Li) detector (Canberra Industries, Meriden, CT, USA), a spectroscopy amplifier (M2024, Canberra Industries, Meriden, CT, USA), ADC (M8075, Canberra Industries, Meriden, CT, USA) and PC based MCA (S-100, Canberra Industries, Meriden, CT, USA). 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 [33,34].

Sample preparation and the analytical procedure were critically tested and evaluated according to uncertainty, accuracy, and limits of detection (LOD) in our previous investigation [35].

2.4. 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 ‰ using Equation (1) [36]:

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

where superscripts i and j denote the highest and the lowest atomic mass number of element E, and R_P and R_{Ref} 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 three hours. Measurements were performed using a Multiflow system (IsoPrime, Cheadle Hulme, Manchester, UK) connected to a continuous flow IRMS (GV Instruments, Manchester, UK). Analyses were calibrated against two internal laboratory reference materials: Snow water ($\delta^{18}O = -19.73 \pm 0.02$‰) and seawater ($\delta^{18}O = -0.34 \pm 0.02$‰). For independent control, laboratory reference material Milli-Q water was used as control material ($\delta^{18}O = -9.12 \pm 0.04$‰). 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, $\delta^{18}O = 0 \pm 0.02$ ‰).

Further, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ ratios were determined in casein samples. Milk fat was removed by centrifugation (Type Centric 322 A, TEHTNICA, Železniki, Slovenia, 10 min at 3200 rpm), and casein by precipitation from the skimmed milk by acidification at pH 4.3 with 2M HCl (Carlo Erba, Val de Reuil, Italy) followed by centrifugation for 10 min at 3200 rpm. The precipitate was rinsed twice with Milli-Q water (Millipore, Burlington, MA, USA), followed by acetone and petroleum ether (Carlo Erba, Val de Reuil, Italy) and freeze-dried [37].

The freeze-dried casein sample was transferred to a tin capsule, closed with tweezers and placed into the autosampler of the elemental analyser. For $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ determination, 10 mg of casein samples were analysed simultaneously using the IsoPrime 100-Vario PYRO Cube (OH/CNS Pyrolyser/Elemental Analyser) (IsoPrime, Cheadle, Hulme, UK). The results were calibrated 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-S-1 ($\delta^{34}S = -0.3$‰), IAEA-S-2 ($\delta^{34}S = +22.49 \pm 0.16$‰). 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 ($\delta^{13}C = -26.98 \pm 0.13‰$, $\delta^{15}N = +5.94 \pm 0.08‰$, $\delta^{34}S = +6.32 \pm 0.8‰$).

2.5. Statistical Analysis

All samples were prepared in triplicate, and the data are presented as mean with standard deviation (SD) of triplicate independent experiments. Statistical analysis was performed using the XLSTAT software package (Addinsoft, New York, NY, USA). Simple statistical analyses were carried out, including an analysis of variance (ANOVA) with the Mann–Whitney (MW) and Kruskal–Wallis (KW) tests, since the data are not normally distributed. Furthermore, to determine the key factors responsible for differentiation of the region of the geographical origin of milk, a discriminant analysis (DA) was used. Moreover, orthogonal partial least squares discriminant analysis (OPLS-DA) was introduced for pairwise comparisons among two overlapping geological groups using the SIMCA® software package (Umetrics, Umea, Sweden).

3. Results and Discussion

3.1. Strontium Isotope Ratio of Authentic Slovenian Milk

The first values for the $^{87}Sr/^{86}Sr$ ratio in Slovenian milk samples ($n = 77$) are presented in Table 1. Slovenia is a relatively small country covering a mere 20,273 km^2 but boasts great diversity in complex geology, relief, hydrological systems, and vegetation. Unfortunately, this diversity was not observed to the same extent in the analysed milk samples.

The $^{87}Sr/^{86}Sr$ ratios in the milk samples collected from farms at different locations showed a moderate degree of variation, spanning from 0.708 to 0.713. When comparing the $^{87}Sr/^{86}Sr$ ratios between samples collected during summer and winter seasons of 2014 (Table 1), a certain degree of variability for some samples was observed; however, the differences were not statistically significant (Mann–Whitney; $p = 0.9623$). Further, no statistical difference was observed in $^{87}Sr/^{86}Sr$ ratios according to the year of production (Mann–Whitney; $p = 0.1318$). The same conclusion may be drawn from the concentrations of Sr in the milk samples. On the other hand, the reported $\delta^{13}C$ and $\delta^{15}N$ data of Slovenian milk reflect intra-annual changes in diet [8].

The Kruskal–Wallis test indicates that only four parameters are significantly related to the geological region ($p < 0.001$): $^{87}Sr/^{86}Sr$ ratios, $\delta^{13}C_{cas}$, $\delta^{15}N_{cas}$ and Br.

Table 1. Sample ID, latitude, longitude, season and year of sampling together with the content of Sr determined by ICP-MS and $^{87}Sr/^{86}Sr$ ratios in authentic milk samples. SD values are the standard deviations between samples from different farms at the same or nearby locality.

Location ID	Latitude	Longitude	Season	Year	Sr (mg/kg)	$^{87}Sr/^{86}Sr \pm SD$
C1	45.70862	13.87428	summer	2014	2.09	0.70900 ± 0.00011
C1	45.70862	13.87428	winter	2014	2.44	0.70935 ± 0.00011
C2	45.60928	13.93719	summer	2014	1.05	0.70880 ± 0.00012
C2	45.60928	13.93719	winter	2014	2.60	0.70852 ± 0.00015
C2	45.60928	13.93719	winter	2015	2.26	0.70898 ± 0.00019
C3	45.55083	14.06222	summer	2014	1.71	0.70886 ± 0.00010
C3	45.55083	14.06222	winter	2014	1.16	0.70925 ± 0.00018
C3	45.55083	14.06222	winter	2015	2.02	0.70880 ± 0.00019
C4	45.77504	14.21382	winter	2014	1.09	0.70913 ± 0.00010
C4	45.77504	14.21382	winter	2015	1.79	0.70918 ± 0.00012
C5	45.91761	14.23516	summer	2014	0.94	0.70867 ± 0.00012
C5	45.91761	14.23516	winter	2014	1.65	0.71026 ± 0.00014
J1	46.30065	13.94305	summer	2014	1.00	0.70915 ± 0.00015
J2	45.83072	14.92945	summer	2014	1.25	0.70970 ± 0.00010
J2	45.83072	14.92945	winter	2014	1.26	0.70991 ± 0.00013
J2	45.83072	14.92945	winter	2015	1.21	0.70932 ± 0.00012
J3	45.97324	14.41981	summer	2014	1.79	0.70875 ± 0.00015
J3	45.97324	14.41981	winter	2014	1.41	0.70918 ± 0.00012

Table 1. *Cont.*

Location ID	Latitude	Longitude	Season	Year	Sr (mg/kg)	$^{87}Sr/^{86}Sr \pm SD$
J4	45.85574	15.15377	summer	2014	1.16	0.70936 ± 0.00015
J4	45.85574	15.15377	winter	2014	1.32	0.70939 ± 0.00015
J5	45.46142	15.25357	summer	2014	1.51	0.70940 ± 0.00010
J5	45.46142	15.25357	winter	2014	2.13	0.70954 ± 0.00012
J6	45.97639	14.61882	winter	2014	3.23	0.70961 ± 0.00010
N1	46.68544	15.70966	summer	2014	2.51	0.70933 ± 0.00014
N1	46.68544	15.70966	winter	2014	2.20	0.70962 ± 0.00022
N2	46.22219	14.60712	summer	2014	1.50	0.70923 ± 0.00017
N2	46.22219	14.60712	winter	2014	1.37	0.70945 ± 0.00012
N3	46.25371	15.44393	summer	2014	2.40	0.70959 ± 0.00021
N3	46.25371	15.44393	winter	2014	2.53	0.70960 ± 0.00015
N4	46.23378	15.63860	summer	2014	2.96	0.70959 ± 0.00015
N4	46.23378	15.63860	winter	2014	2.59	0.70955 ± 0.00015
T1	46.55463	15.64563	winter	2014	3.32	0.70950 ± 0.00017
T2	46.18696	13.75652	summer	2014	1.54	0.71043 ± 0.00011
T2	46.18696	13.75652	winter	2014	1.21	0.70956 ± 0.00012
T3	46.04773	14.21534	winter	2014	1.50	0.70940 ± 0.00019
T3	46.04773	14.21534	winter	2015	3.41	0.70928 ± 0.00017
T4	45.83366	14.63623	winter	2014	1.06	0.70981 ± 0.00011
T5	46.18810	15.01356	summer	2014	3.61	0.70924 ± 0.00018
T5	46.18810	15.01356	winter	2014	3.14	0.70929 ± 0.00019
P1	46.53564	15.26751	summer	2014	2.16	0.71008 ± 0.00015
P1	46.53564	15.26751	winter	2014	2.48	0.70975 ± 0.00013
P2	46.59800	15.16536	winter	2014	2.14	0.70993 ± 0.00014
P4	46.50779	15.07791	winter	2014	2.06	0.71041 ± 0.00010
P5	46.33926	14.95994	summer	2014	3.99	0.70811 ± 0.00014
P5	46.33926	14.95994	winter	2014	2.14	0.70866 ± 0.00015
P6	46.42414	15.01712	winter	2014	3.56	0.70963 ± 0.00015
Q1	46.30849	14.89704	summer	2014	1.28	0.71001 ± 0.00012
Q1	46.30849	14.89704	summer	2014	1.56	0.70979 ± 0.00011
Q1	46.30849	14.89704	winter	2014	1.80	0.70966 ± 0.00012
Q2	46.54691	14.91991	winter	2014	1.95	0.71095 ± 0.00011
Q3	46.58922	15.02460	summer	2014	2.07	0.71171 ± 0.00018
Q3	46.58922	15.02460	winter	2014	1.93	0.71087 ± 0.00014
Q4	46.28804	15.03957	winter	2014	1.91	0.71044 ± 0.00015
Q4	46.28804	15.03957	winter	2015	1.92	0.70902 ± 0.00012
Q6	46.01295	15.29799	winter	2014	2.17	0.70925 ± 0.00017
Q7	46.33665	15.42204	summer	2014	1.95	0.71064 ± 0.00015
Q7	46.33665	15.42204	winter	2014	1.86	0.71062 ± 0.00011
Q8	46.39199	15.57278	winter	2014	2.88	0.70945 ± 0.00015
Q9	45.90793	15.59578	summer	2014	1.86	0.71007 ± 0.00015
Q9	45.90793	15.59578	winter	2014	1.82	0.71005 ± 0.00015
Q10	46.42006	15.86960	summer	2014	2.52	0.71086 ± 0.00023
Q10	46.42006	15.86960	winter	2014	2.04	0.71181 ± 0.00023
Q10	46.42006	15.86960	winter	2014	2.18	0.71086 ± 0.00013
Q10	46.42006	15.86960	winter	2014	2.31	0.71154 ± 0.00021
Q10	46.42006	15.86960	winter	2015	1.83	0.71010 ± 0.00013
Q11	46.67660	15.99125	summer	2014	1.98	0.71173 ± 0.00020
Q11	46.67660	15.99125	winter	2014	1.87	0.71113 ± 0.00020
Q12	46.64402	16.04111	summer	2014	2.00	0.71146 ± 0.00021
Q12	46.64402	16.04111	winter	2014	2.53	0.71119 ± 0.00025
Q13	46.65485	16.16190	summer	2014	3.22	0.71126 ± 0.00018
Q13	46.65485	16.16190	winter	2014	3.15	0.71271 ± 0.00025
Q13	46.65485	16.16190	winter	2015	2.52	0.71233 ± 0.00019
Q14	46.51955	16.19726	summer	2014	2.33	0.71141 ± 0.00019
Q15	46.28095	15.07375	winter	2014	1.58	0.70969 ± 0.00016
Q16	46.52806	15.77623	summer	2014	2.19	0.71181 ± 0.00010
Q16	46.52806	15.77623	winter	2014	2.19	0.71122 ± 0.00019
Q17	46.80051	16.22926	winter	2014	2.79	0.71201 ± 0.00020

The relationship between $^{87}Sr/^{86}Sr$ ratios in the milk samples and rock type at each sampling location was also explored. The type and age of the soil were obtained from the geological map provided by the Geological Survey of Slovenia [31] (Figure 1). The $^{87}Sr/^{86}Sr$ isotope ratios in milk samples studied are in line with the isotopic values predicted for Slovenia, according to Hoogewerff et al. (2019) [38]. By their model, the soil $^{87}Sr/^{86}Sr$ ratios of most of Slovenia's central and western parts should be in the range of 0.708 to 0.709. The $^{87}Sr/^{86}Sr$ ratios should be higher in the north-eastern part, ranging from 0.710 to 0.712. The values found in milk in the present study are in agreement with the modelled values.

Moreover, this information is in line with the bedrock composition and age. Indeed, most of the Slovenian territory is covered by tertiary and quaternary dolomites, limestones and alluvial deposits such as sandstones and claystones. There is a slight difference between milk samples from locations with quaternary alluvial deposits with alumo-silicate rocks with $^{87}Sr/^{86}Sr$ ratios ranging between 0.710 and 0.712, and locations with limestone and dolomite bedrock with $^{87}Sr/^{86}Sr$ ratios in the range from 0.708 to 0.710. On closer examination of the regional Slovenian milk samples, the overlap highlights the similarity between the geological and pedological characteristics of originating regions (Figure 3).

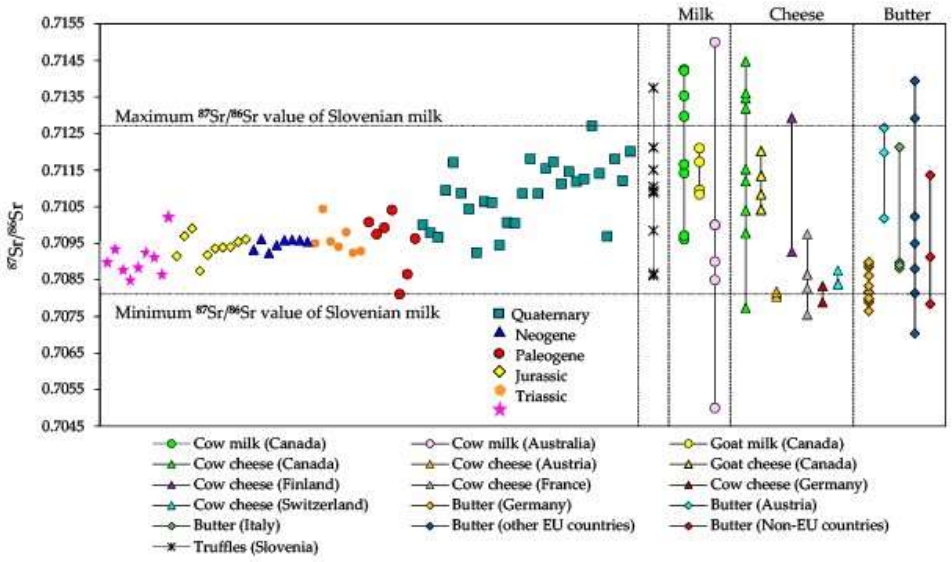

Figure 3. The $^{87}Sr/^{86}Sr$ isotope ratios in various dairy products from different countries, as reported in the literature. Horizontal dashed lines define the limits of the $^{87}Sr/^{86}Sr$ values measured in Slovenian milk of different geological regional origins, as indicated. References used for various dairy products worldwide: butter [24], cheese [27,30], and milk [29,30]. Dots on the vertical lines refer to the results obtained from the literature, whereas lines indicate the span of values. The $^{87}Sr/^{86}Sr$ isotope ratios in Slovenian truffles are also presented [39].

The data were compared with the Slovenian truffles, which have $^{87}Sr/^{86}Sr$ ratios ranging from 0.710 to 0.713 [39]. The values correspond to Slovenian milk samples except for the highest $^{87}Sr/^{86}Sr$ ratio of 0.71375 determined in truffles from Bloke, a karst plateau. When comparing the Sr isotopic ratios in dairy products originated from other countries with Slovenian milk, the span of the $^{87}Sr/^{86}Sr$ expressed in lower values has been recorded for cheese from Germany and Switzerland [24] and New Zealand [29].

In contrast, the $^{87}Sr/^{86}Sr$ ratios in milk and cheese from Quebec vary with a wide range of values, from 0.70961 up to a maximum of 0.71447, indicating a relative enrichment

with radiogenic isotope ^{87}Sr in Proterozoic and during the Paleozoic carbonate intrusive and limestone rocks composing the St. Lawrence Platform [30,40]. The large variability of Sr ratios in dairy products reflects the vast diversity of underlying bedrock and soils formed from them. Therefore, the widely scalable results of Sr ratios reflect the substantial heterogeneity of the geological background of its origin.

A specific pattern among samples was observed when comparing the Sr isotopic and elemental signatures in Slovenian milk samples based on geology (Figure 4).

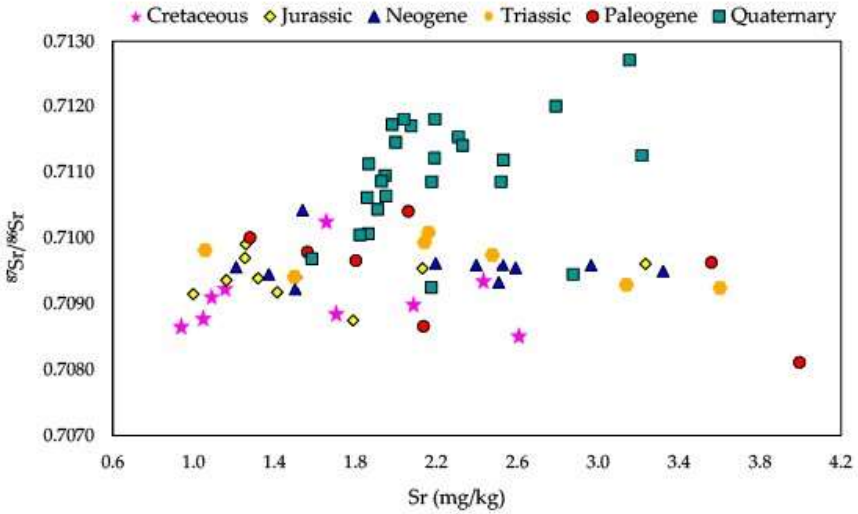

Figure 4. $^{87}Sr/^{86}Sr$ ratios versus Sr concentrations in milk from different geological regions.

Although several samples overlap, two trends can be identified: the first with high Sr concentration and high $^{87}Sr/^{86}Sr$ ratios (>0.7110) mainly from areas with quaternary alluvial deposits with alumo-silicate rocks and the second one related to lower $^{87}Sr/^{86}Sr$ ratios (<0.7090) at carbonate dominated areas. The overlapping values can be explained by: (i) different weathering rates of specific minerals in the rocks and soils, movement of water and sediments in a grazing area can influence Sr and Rb contents in milk samples leading potentially to different $^{87}Sr/^{86}Sr$ isotope ratios [27,41], (ii) the consumption of imported plants, particularly those enriched with high Ca and Sr content, can significantly alter the $^{87}Sr/^{86}Sr$ signatures in dairy products, even when consumed in small amounts. Thus, a consideration of total dietary intake is necessary when interpreting $^{87}Sr/^{86}Sr$ results.

The second source of Sr in milk is related to the drinking water supply. In Slovenia, most of the drinking water originates from groundwater and especially in the karst regions of the Sava River watershed, where river water represents the primary source of groundwater [42,43]. Therefore, we compared the $^{87}Sr/^{86}Sr$ ratios of milk with unpublished data of $^{87}Sr/^{86}Sr$ in the Sava, Ljubljanica, Pivka, Kamniška Bistrica and Logaščica rivers and rivulets and those determined in some mineral and spring bottled waters [32]. For comparison, we selected locations that lie close to the rivers for which the $^{87}Sr/^{86}Sr$ ratios are available. A good correlation between milk and groundwater data was observed (Figure 5), indicating that groundwater can represent an important source of Sr.

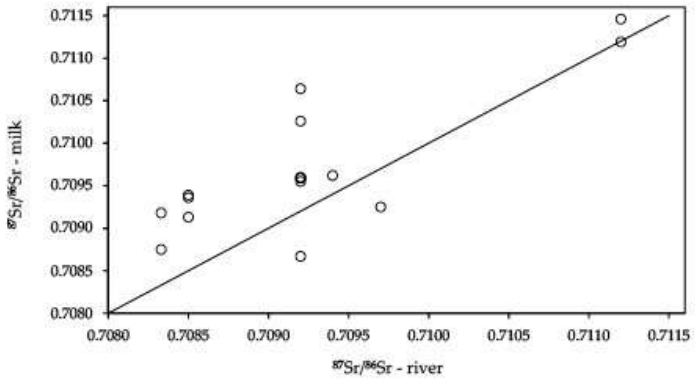

Figure 5. Relationship between $^{87}Sr/^{86}Sr$ ratios in rivers and milk. The line represents the 1:1 ratio indicating overlapping of the data.

However, it is interesting to note that most of the $^{87}Sr/^{86}Sr$ values for milk are higher than their corresponding river samples. One of the possible explanations could be the use of agricultural lime for soil improvement in fertile areas present mainly in the eastern part of Slovenia. This part is also known for its intensive agricultural practices where some field areas in specific locations are used to produce fodder plants for feeding livestock. It has been reported that the application of agricultural lime to low-calcareous soils can significantly lower the $^{87}Sr/^{86}Sr$ ratio of the watershed [44]. In these areas, maise silage is detected in more than 80% of the milk samples.

Given that the cow's body is up to 70% of water, the $^{87}Sr/^{86}Sr$ analysis of local drinking water might be helpful. Livestock in the Pannonian region is fed on the locally produced food, which also confirms the result of milk from Radenci ($^{87}Sr/^{86}Sr = 0.71119$), matching the $^{87}Sr/^{86}Sr$ ratio of the mineral water from the source Radenci (0.71120). This finding aligns with the investigation performed in the Parmigiano Reggiano milk and cheese production area [45]. In her study, the $^{87}Sr/^{86}Sr$ isotope ratio on water, whole milk, and diet samples allowed the construction of a linear relationship with multiple independent variables, from which the $^{87}Sr/^{86}Sr$ ratio of the milk is mainly correlated with the $^{87}Sr/^{86}Sr$ ratio of the hay. Thus, results indicate milk samples reflect the $^{87}Sr/^{86}Sr$ ratio of the feed linked to the soil and water. This is also in agreement with Stevenson et al. (2015) [30], in which the authors demonstrated a good correlation between the Sr isotopic composition of milk, cheese, and the bedrock geology of the dairy farm locations.

3.2. *Discriminant Analysis*

In the next step, we check if the $^{87}Sr/^{86}Sr$ ratio can increase the differentiation of Slovenian milk samples according to the geological region using different statistical approaches. In our statistical evaluation, stable isotope and elemental composition in milk samples were also included. The data are presented in Table S6, while the detailed description of these parameters according to geographical origin is described in Potočnik et al. [8].

Sixty-three milk samples of four different geological regions (1—Cretaceous: Carbonate Rocks and Flysch, $n = 8$; 2—Jurassic-Triassic: Carbonate Rocks, n = 15; 3—Neogene: Carbonate Rocks, Paleogene: Deposits, $n = 17$; 6—Quaternary: Deposits, $n = 23$) and twenty-two parameters including $^{87}Sr/^{86}Sr$, $\delta^{18}O_w$, $\delta^{13}C_{cas}$, $\delta^{15}N_{cas}$, $\delta^{34}S_{cas}$, Mn, Fe, Cu, Rb, Sr, Ca, K, Cl, S, P, Zn, Br, 1/Sr, Rb/Sr, Ca/Sr and K/Rb were processed by DA. In Figure 6, DA modelling results were shown as a discriminant function score plot (a) and a discriminant loadings plot (b).

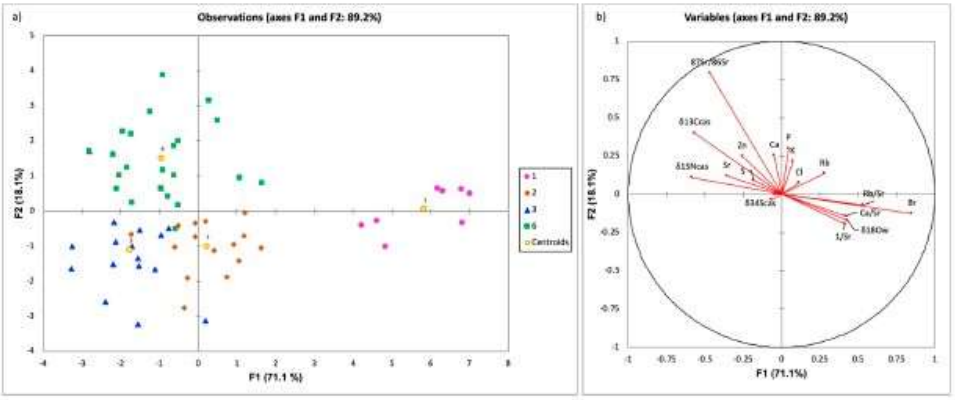

Figure 6. Discriminant function score plot (a) and a discriminant loadings plot (b) for milk samples collected in 2014 on four different geological origins (1—Cretaceous: Carbonate Rocks and Flysch, n = 8; 2—Jurassic-Triassic: Carbonate Rocks, n = 15; 3—Neogene: Carbonate Rocks, Paleogene: Deposits, n = 17; 6—Quaternary: Deposits, n = 24).

In the functional score plot, each group (centroid) is 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. The first two discriminant functions accumulated 89.2% of the total variability. Two groups (groups 1 and 6) show a good tendency of separation among each other and from groups 2 and 3, which overlap slightly. Group 1 (Cretaceous: Carbonate Rocks and Flysch) is positioned in the right part of DA graph and is associated with the vectors of Br, 1/Sr and $\delta^{18}O_w$. The mean values of these parameters in the centroid are the highest and the most influential for the separation. Inspection of the mentioned parameters with KW test reveals that they are significant for separating group 1 from the rest. A substantial amount of Br indicates that geologically is associated with a marine basement rocks origin. Higher $\delta^{18}O_w$ are also typical for coastal regions. Further, group 6 positioned in the upper right part of the plot a is associated with vectors $^{87}Sr/^{86}Sr$, $\delta^{13}C_{cas}$ and $\delta^{15}N_{cas}$ and according to KW test significant for discrimination among groups 1, 3 and 6. This group is located in the eastern part of Slovenia, located in Quaternary deposits, and it is also related to intensive milk production with higher content of corn in cow feed. Group 3, located in the lower right part of biplot a, is associated with Sr vectors, and inspection by KW and ANOVA tests reveal that both are significant for separation. Groups 2 and 3 are located in the lower part of biplot a, and here, $\delta^{15}N_{cas}$ and Br are significant for discrimination between both groups. The prediction ability was the highest for the Quaternary group (91.3%) and the lowest for group 3 (Neogene + Paleogene; 41.2%), with an overall prediction of 63.5%.

Further, OPLS-DA tests for pairwise comparisons among two overlapping geological groups (2—Jurassic + Triassic, 3—Neogene + Paleogene: Figure 7) was calculated similarly to in the study performed by Chung et al. (2020) [46]. This model had an explanatory power of 94% (F1) for variation in the X variables and displayed high quality, goodness of fit, and predictability. It was found that the separation of these two groups is governed by $\delta^{15}N_{cas}$ values govern, concentrations of Br and Rb/Sr ratio, indicating that not only geologic parameters are important for the separation, but also the way of cow feed and milk production—intensive with more corn silage or grass silage representative of the Jurassic + Triassic group.

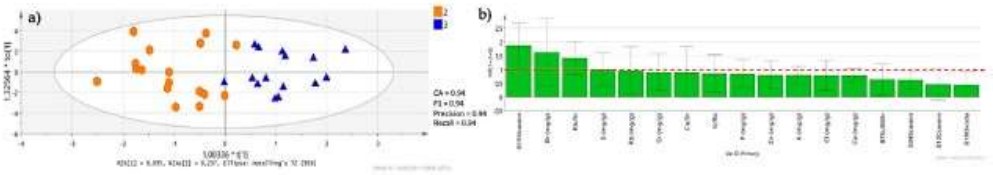

Figure 7. OPLS-DA score plots and VIP values in the pairwise comparison between three different geological regions derived from all isotopic and elemental composition data of milk samples. (**a**) The ellipse on the score plot represents the 95% confidence interval. (**b**) The red-dotted line indicates criteria used to identify the variables for model development.

4. Conclusions

In this study, we investigated the feasibility of the Sr isotope ratio analysis, combined with multivariate statistical analysis to discriminate milk samples from Slovenia based on their provenance. The $^{87}Sr/^{86}Sr$ ratios in milk samples were determined using an optimised method, which showed sufficient precision and accuracy to detect variations in Sr isotopic compositions between milk samples. Although Slovenia covers a relatively small area, its geology, geography and climate vary substantially. Large regional variability of $^{87}Sr/^{86}Sr$ ratios in Slovenian milk was observed, overlapping with other regions' values. Thus, a complete separation of the regions based solely on the $^{87}Sr/^{86}Sr$ ratio of the milk was not possible. However, it was found that a combination of Sr isotopic profiling coupled to multivariate analysis is a promising tool for characterising milk according to geological origin. The milk produced in the Quaternary areas had high Sr content and higher $^{87}Sr/^{86}Sr$ values and differed from those produced in carbonate dominated areas with lower $^{87}Sr/^{86}Sr$ values.

In conclusion, the $^{87}Sr/^{86}Sr$ ratio can distinguish between different dairy areas as long as geolithological differences characterise these areas. In cases of a similar geological environment, combining elemental concentrations and isotope ratios, both light and heavy isotopes, might be advantageous. However, this approach is limited in the case of Slovenian milk. The close distance between macro-regions in Slovenia and the variations in climate affecting these regions make discrimination between milk samples of different origins more difficult, particularly when milk samples originate from locations positioned close to a zone between two or more regions and thus share a similar isotopic signature.

Further, it has been confirmed that the cow's diet and geologic parameters are important for the separation. Indeed, our study shows the correlation between the isotope ratio of strontium in milk and possible source of drinking water, in which diverse sources of strontium from the environment are reflected. However, to better understand the influence of different factors, i.e., water, feed and supplements, on the Sr isotope ratio in the milk samples, future research should investigate the $^{87}Sr/^{86}Sr$ ratio utilising paired samples of feed, water, and soil originating from the same location as the milk. In the perspective of food traceability, the database of the $^{87}Sr/^{86}Sr$ values in soils and waters in Slovenia could be also beneficial for future studies of local foods, where it can be used as a reference map to identify the authenticity of particular food product, or whether there are any unexpected isotopic variations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10081729/s1: The Supplementary Materials describes the optimization and validation of the analytical method for $^{87}Sr/^{86}Sr$ isotope ratio determination in milk; Table S1: Microwave-assisted acid digestion program used for pre-treatment of milk sample; Table S2: Comparison of the Sr concentrations obtained after microwave digestion of freeze-dried milk samples (mean ± standard deviation; n = 3); Table S3: Determined Sr concentrations after pre-treatment in certified reference materials, NIST SRM 8435 and IAEA-153 (mean ± standard deviation; n = 3); Table S4: The efficiency of Rb removal; Table S5: $^{87}Sr/^{86}Sr$ isotope ratios for IAEA-153 milk sample; Table S6: The whole dataset of authentic milk sample analysis including the description of the location, season and year

of production, geological background, $^{87}Sr/^{86}Sr$ ratios, stable isotope ration of oxygen ($^{18}O/^{16}O$) in milk water and $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ in casein, elemental analysis in the freeze-dried samples determined with XRF [12,14,20,25,47–55].

Author Contributions: Conceptualization, S.H.G., T.Z. and N.O.; methodology, S.H.G., T.Z. and R.F.; validation, S.H.G. and T.Z.; formal analysis, S.H.G., M.N. and R.F.; investigation, S.H.G., N.O. and T.Z.; resources, N.O.; data curation, S.H.G., L.S. and M.N.; writing—original draft preparation, S.H.G.; writing—review and editing, T.Z., N.O. and R.F.; visualization, S.H.G. and L.S.; supervision, N.O.; funding acquisition, N.O. All authors have read and agreed to the published version of the manuscript.

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Chapter 4

Milk Authentication: Stable Isotope Composition of Hydrogen and Oxygen in Milks and Their Constituents

This chapter summarizes the paper entitled "Milk Authentication: Stable Isotope Composition of Hydrogen and Oxygen in Milks and Their Constituents" by Staša Hamzić Gregorčič, Doris Potočnik, Federica Camin and Nives Ogrinc. The paper was published in the *Molecules* journal, 2020. This paper belongs to the Special Issue Isotopic Techniques for Food Science.

The objective of this research was to determine the variability of oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$) isotopes in Slovenian milk samples and its major constituents: water, casein, and lactose. Authentic cow milk samples (n = 319) were collected between 2012 and 2015 during summer and winter. Given the interest in detecting commercial fraud of milk and dairy products, cow, sheep and goat milk were collected from farms located in the Mediterranean region (Brkini, Vipava), Dinaric (Karst) and Alpine region (Bovec) from May to June of 2012 and 2013. For pretesting the internal standardisation method, paired cow milk and drinking water samples from Alpine (Selnica ob Dravi, Črna na Koroškem), Mediterranean (Ajdovščina) and Pannonian (Ormož) regions were collected in May 2017. The isotopic composition of oxygen and hydrogen of milk and water was analysed using an IRMS coupled to a Multiflow Bio equilibration unit. The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ and measurements of casein and lactose fractions were performed at the Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach in San Michele all' Adige, Italy. Lactose and casein fractions were analysed using TC/EA pyrolyser coupled to a DELTA XP IRMS.

Delta ^{18}O values in milk depend on the sources of drinking water, metabolism and isotopic fractionation during milk synthesis. The study showed that by using the stable isotope composition of oxygen in milk water, it is possible to differentiate milk from Slovenia according to the season and animal species, and regional discrimination is limited. The average $\delta^{18}O$ in drinking water, which is mainly drawn from local groundwater sources, can be attributed to the mean annual precipitation. Milk water was enriched in ^{18}O compared to groundwater (GW), which opens up the possibility of detecting the addition of water. Based on our experiment, it was found that by determining $\delta^{18}O_{milk}$ and $\delta^{18}O_{GW}$ values it is possible to detect $> 15\%$ of added water. In addition, the $\delta^{2}H$ and $\delta^{18}O$ values of water in cow milk correlate more with geo-climatic characteristics of the area of origin than with dietary values. Further, $\delta^{2}H_{casein}$ and $\delta^{18}O_{casein}$ provide a more consistent isotopic

signature, as there were no significant statistical differences according to the season and region of milk production, allowing the determination of milk origin. Animal species also differ in their body composition, suggesting that diet and physiology exert a strong control over the isotopic composition of animal's body water and, consequently, milk. Delta ^{18}O water values were higher in sheep and goat milk when compared to cow milk.

Finally, it was possible to detect milk adulteration with water using the $\delta^{18}O$ values of lactose as an internal standard. The analysis of the $\delta^{18}O$ of the bulk milk water and the $\delta^{18}O$ of lactose extracted from the same milk through the use of different amount of water drunk by cows showed that the two parameters are closely related with each other with differences that can vary according to different amount of added water to milk. Although this method could potentially detect even lower amounts of added water (> 7 %), larger sample sizes from different regions is needed to test this hypothesis, and the limit of detection depends on the $\delta^{18}O$ of the added water source. Once this method is validated on an international scale, it could become a reference method for determining the adulteration of milk with water.

In this paper, I was responsible for the sample collection, storage and preparation. I performed isolation of casein and lactose fractions from raw milk. I also collected and analysed the data and prepared the manuscript. Preliminary results of the research were presented at the 5th MS Food Day, organised by the Mass Spectrometry section of the Italian Chemical Society, in Bologna, Italy (October 11-13, 2017).

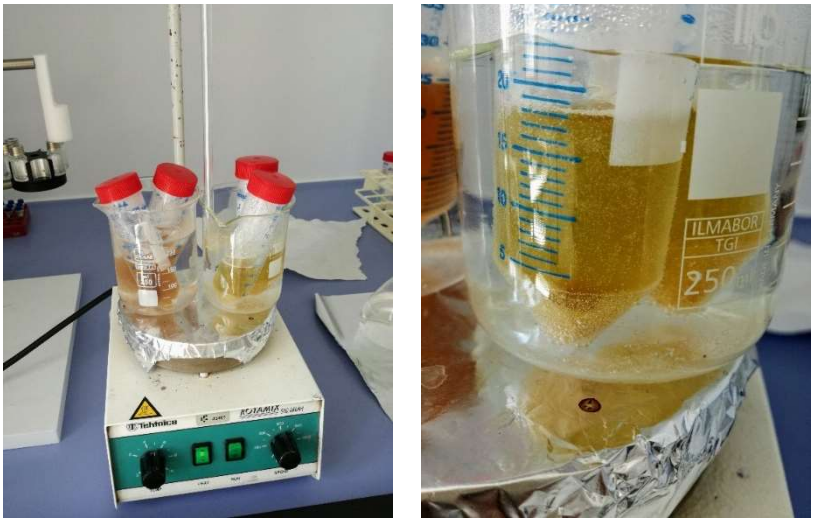

Figure 4.1: Isolation of lactose. (Photo: S.H.G., IJS Reactor Centre, 2017).

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Milk Authentication: Stable Isotope Composition of Hydrogen and Oxygen in Milks and Their Constituents

Staša Hamzić Gregorčič [1,2], Doris Potočnik [1,2], Federica Camin [3,4] and Nives Ogrinc [1,2,*]

1 Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia; stasa.gregorcic@ijs.si (S.H.G.); doris.potocnik@ijs.si (D.P.)
2 Jožef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia
3 Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, via Mach 1, 38010 San Michele all'Adige, Italy; federica.camin@fmach.it
4 Center Agriculture Food Environment (C3A), University of Trento, via Mach 1, 38010 San Michele all'Adige (TN), Italy
* Correspondence: nives.ogrinc@ijs.si; Tel.: +386-1-588-5387

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Abstract: This paper summarises the isotopic characteristics, i.e., oxygen and hydrogen isotopes, of Slovenian milk and its major constituents: water, casein, and lactose. In parallel, the stable oxygen isotope ratios of cow, sheep, and goat's milk were compared. Oxygen stable isotope ratios in milk water show seasonal variability and are also ^{18}O enriched in relation to animal drinking water. The $\delta^{18}O_{water}$ values were higher in sheep and goat's milk when compared to cow milk, reflecting the isotopic composition of drinking water source and the effect of differences in the animal's thermoregulatory physiologies. The relationship between $\delta^{18}O_{milk}$ and $\delta^{18}O_{lactose}$ is an indication that even at lower amounts (>7%) of added water to milk can be determined. This procedure once validated on an international scale could become a reference method for the determination of milk adulteration with water.

Keywords: milk; adulteration; water addition; oxygen stable isotopes; lactose

1. Introduction

After the melamine milk powder incident in China in 2008 the adulteration of milk and dairy products highlighted the need for greater transparency in the food chain, guarantees surrounding food quality and safety and the development of methods for determining the authenticity of dairy products [1]. Although milk is a frequent target for fraud [2], available knowledge and data about methods for the prevention or mitigation of the fraud issue is still limited. In order to assure the authenticity of milk, one requires a deep understanding of the characteristics of authentic milk. In response, scientists have developed new analytical techniques and strategies [3,4], which will assist milk producers and suppliers in the detection and prevention of milk fraud.

When milk is diluted with water, its nutritional value decreases and in addition chemicals are added to compensate the density and the colour after dilution, thus posing a potential risk to human health [2]. Further, there are very strong economic arguments of minimizing the allowed amount of added water to milk, since the price of milk is based on milk solids contents. The processing of milk also provides an opportunity for producers to add water beyond the acceptable limits in preserved milk, which is illegal. Since it is not compulsory to state the amount of added water on the label, some companies take advantage of this legal loophole.

Several methods to detect adulterants in milk exists including measurement of freezing point depression, electrical admittance spectroscopy, single-frequency conductance measurements, digital image chromatography, ultraviolet (UV) visible light spectroscopy, and enzyme linked immunosorbent assay [5–7]. Determining the milk water content is typically performed using traditional methods such as by measuring changes in freezing point of the milk or changes in the refraction of light through the whey component of milk after precipitation and removal of the casein and fat components using either acetic acid or copper sulfate. Current methods can be classed as direct contact methods, which are not reliable for making continuous measurements. Other methods involve separating the water from milk solids and then quantifying the amount of water by weight or volume—these techniques, although accurate, are time-consuming and expensive. Among modern techniques near infrared (NIR) spectroscopy has proved to be a fast non-destructive method for food safety evaluation and control [8–11], and can be also used to detect water and its content in milk [10]. The main drawback is that the milk has a near-infrared absorption spectrum similar to that of the water [9]. Time-domain nuclear magnetic resonance (TD-NMR) method has been used for quantification of fat and water content in cheese [12] and to identify several adulterants in milk such as water, whey, synthetic milk, synthetic urine and hydrogen peroxide [13]. Although the method is widely used in dairy studies, it has some restrictions, especially in samples with either low water or low-fat concentration (<5% *v*/*v*).

The use of stable isotopes of light elements is an approach of a grown interest in terms to discover possible commercial fraud [14]. Several studies have demonstrated that stable oxygen isotope ratios ($\delta^{18}O$ values) has been successfully applied to detect illegal watering of different types of food matrices such as wine [15], fruit juices [16], and concentrated spirits [17]. $\delta^{2}H$ and $\delta^{18}O$ values in water can provide key information on water origins (e.g., local precipitation, groundwater), climate (ambient temperatures during condensation and precipitation) and the degree of evapotranspiration [18–20]. The relationship between $\delta^{2}H$ and $\delta^{18}O$ values in the hydrosphere throughout the continents known as the meteoric water line (MWL; $\delta^{2}H = 8\ \delta^{18}O + 10$) was first defined by Craig [21]. Besides the 'latitude' effect, there is a 'continental' effect due to the distance from the sea, related to the vapour masses moving over continents leading to the lower $\delta^{2}H$ and $\delta^{18}O$ values in precipitation (mean decrease of −2.8‰/1000 km from the coast). Moreover, different altitudes inland also lead to decrease in $\delta^{2}H$ and $\delta^{18}O$ values in precipitation since at higher altitude there is isotopically lighter vapour. Finally, another variation in $\delta^{2}H$ and $\delta^{18}O$ values can occur due to seasonal trends; during summer the enrichment in ^{2}H and ^{18}O in precipitation, especially inland, occurs.

The sources of H and O in animals are drinking water, food, food water and in case of O also molecular O_2 [22]. Groundwater the main source of animal drinking water has an isotopic composition depending on geographical factors such as altitude, latitude and distance from the sea, but not on the season. In plants, the main components of feed, the isotopic composition of water are positive relative to those of the corresponding soil water. Furthermore, the $\delta^{18}O$ values in plants reflect evaporative enrichment transpiring leaves and isotopic exchange between plant water and organic molecules [23,24]. The average $\delta^{18}O$ value of the body water of most domestic animals is about 3 ± 1‰ more positive than that of the drinking water [25]. Consequently, the enrichment in ^{2}H and ^{18}O was observed also in milk where the metabolism, and isotopic fractionation during milk synthesis cause additional isotopic fractionation. Overall, the isotopic composition of milk depends on species, drinking, and respiration rates [25], season, farm conditions, breed, and the physiological condition of the animal [26,27]. Dairy animal species with different thermoregulatory physiology should have different water isotope fractionation in body fluids, which is related to evaporation, as vapour is more depleted in heavy isotopes than other body fluids [28,29]. Further, goat milk has a higher proportion of calcium compared to cow milk, which is linked to the higher metabolic rate of the smaller animal [30]. Likewise, according to Bryant and Froelich [30] and Podlesak et al. [31], body surface area relative to body mass makes a mammal prone to water loss via evaporation. A relationship between $\delta^{18}O$ in milk water and the season was reported by Kornexl et al. [32], due to seasonal changes in the $\delta^{18}O$

of forage plants, as well as in the body of the animal, linked to evapotranspiration. δ^2H and $\delta^{18}O$ stable isotopes in milk were also used to detect its geographical origin, due to the relationship between the isotopic signature of milk and that of the drinking water of regions located at different latitudes and/or altitudes [33,34]. More recently, the exchange of H and O between organic molecules and animal's body water due to metabolism and biosynthesis were studied. The results suggested that H isotopes carry a signature related to dietary habits of the animal, while O isotopic signature reflects more animal's physiological water balance [35].

Our paper introduces the concept of using δ^2H and $\delta^{18}O$ measurements in the milk and its constituents as a natural isotopic toolbox to provide information about the sources of water in milk and to detect possible adulteration of milk with water. Thus, the main objectives of our study were to: (1) identify the differences in the $\delta^{18}O$ value in milk according to the season, region and animal species; (2) identify the correlations between $\delta^{18}O$ values in milk and drinking water; and (3) to test the use of $\delta^{18}O$ values in lactose as an internal standard for the detection of water addition.

2. Results and Discussion

2.1. Stable Isotope Composition of Milk and Casein: Year, Season, Region and Species Variability

The $\delta^{18}O_{milk}$, $\delta^{18}O_{casein}$ and $\delta^{2}H_{casein}$ values of raw cow's milk from farms in four geographical regions in Slovenia: Alpine, Dinaric, Pannonian, and the Mediterranean, broken down according to season and year of production are presented in Table S1 (Supplementary Material). The $\delta^{18}O_{milk}$ values in collected cow milk samples (n = 319), produced between 2012 and 2015, ranged from −9.2‰ to −0.04‰ (Figure 1a–d). The $\delta^{18}O_{casein}$ values ranged from 8.8‰ to 14.6‰, and the $\delta^{2}H_{casein}$ values were from −150‰ to −100‰.

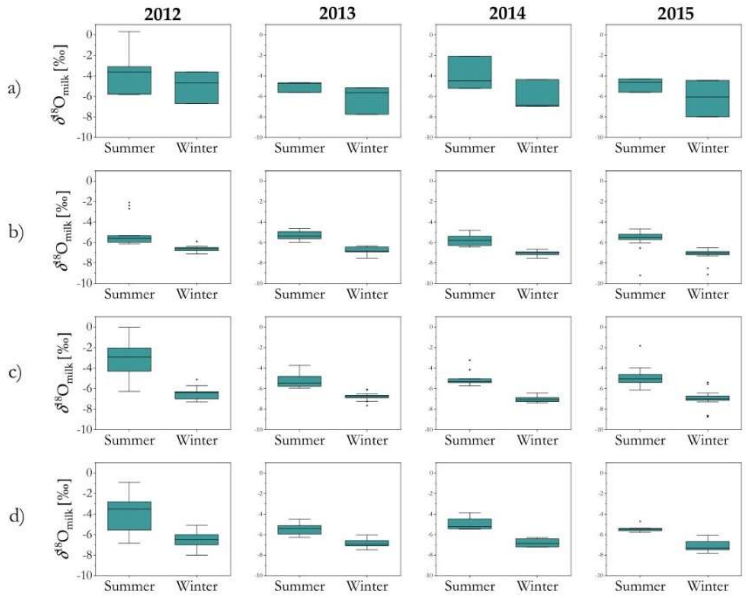

Figure 1. Box plots of the $\delta^{18}O_{milk}$ values in cow milk collected from four macro-regions in Slovenia: (**a**) the Mediterranean, (**b**) Pannonian, (**c**) Alpine, (**d**) Dinaric region during summer and winter in 2012–2015.

After applying an ANOVA test, significant differences ($p < 0.05$) in the $\delta^{18}O_{milk}$ values according to region, year, and season were observed. The $\delta^{18}O_{milk}$ values were higher in summer and in 2012 compared to 2013, 2014 and 2015. The results of the Tukey contrasts test ($p < 0.05$) indicate that the $\delta^{18}O_{milk}$ values were the highest in the Mediterranean region, which out of the four regions has the mildest climate. Our findings are consistent with previous studies that show a seasonal variation in the $\delta^{18}O$ values in milk water with higher ^{18}O content in the summer milk [32,34,36,37]. This increase results from the high evapotranspiration rate in fresh plant feed and animals during the summer. The use of water isotopes as an indicator of the geographical origin of milk is, however, only useful if the type of feed is known (i.e., fresh grass vs silage) [35], which unfortunately was not the case in our study.

From Figure 2, it is evident that the casein was ^{18}O-enriched by approximately 17‰ relative to the milk water, and both $\delta^{2}H_{casein}$ and $\delta^{18}O_{casein}$ values were consistent, although their values varied slightly from region to region in 2014 (Figure 2; Table S1). No regional differences in $\delta^{2}H_{casein}$ and $\delta^{18}O_{casein}$ values were observed in 2013. Conversely in 2014, both the Mediterranean ($\delta^{18}O_{casein}$ = 12.8 ± 1.3‰) and Pannonian ($\delta^{18}O_{casein}$ = 12.2 ± 1.3‰) regions differ significantly from the Alpine ($\delta^{18}O_{casein}$ = 11.4 ± 0.8‰) and Dinaric ($\delta^{18}O_{casein}$ = 11.2 ± 0.7‰) ones (Figure 2). The highest $\delta^{18}O_{casein}$ values were in milk produced at lower altitudes closer to the coast where the climate is dry and hot (Mediterranean region). Also, there were no significant regional differences in the average $\delta^{2}H_{casein}$ values. It is interesting to note, that winter samples from the Dinaric region had a higher mean value $\delta^{2}H_{casein}$ (−127‰) compared to the summer samples (−134‰).

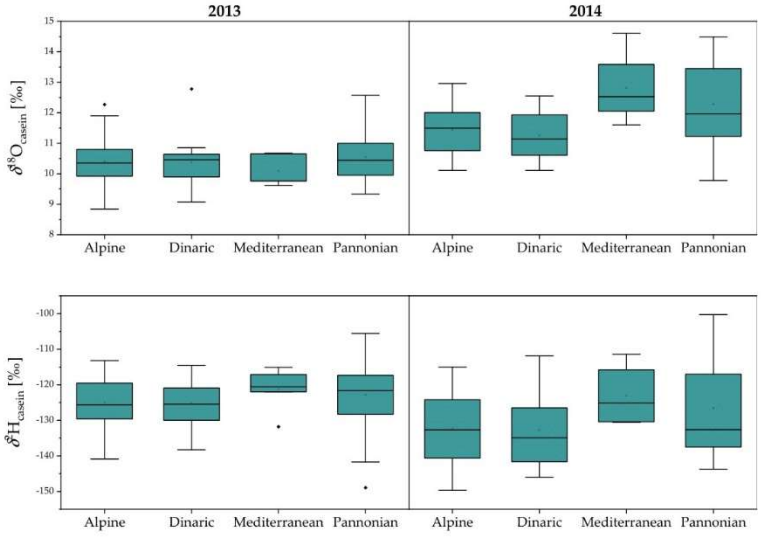

Figure 2. Box plots of $\delta^{2}H_{casein}$ and $\delta^{18}O_{casein}$ values in cow milk collected from four macro-regions in Slovenia in 2013 and 2014.

No correlation was observed between $\delta^{2}H_{casein}$ and $\delta^{18}O_{casein}$ values (Figure 3), which supports the finding from previous studies that 30% of the H and 70% of the O in milk protein derives from the local water, with the remaining fraction originating from the diet. Also, it is necessary to consider possible sources of variation related to isotopic fractionation in the animal's body water [29]. Similarly, the $\delta^{2}H_{casein}$ values are influenced by the continuous exchange of ^{2}H between the animal's body water and drinking water in a specific location over time [30,31]. Thus, compared to the $\delta^{18}O_{milk}$ values,

$\delta^{18}O_{casein}$ values provide a more consistent isotopic signature with which to determine the authenticity and origin of the milk.

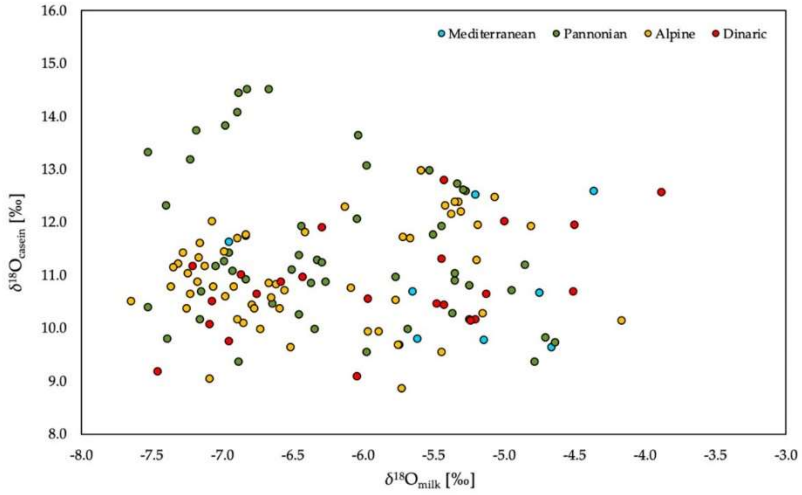

Figure 3. The relationship between $\delta^{18}O_{milk}$ and $\delta^{18}O_{casein}$ values in milk in relation to the region of production.

Given the interest in detecting commercial fraud of milk and dairy products, we determined the $\delta^{18}O_{milk}$ values in cow, sheep and goat milk collected from farms located in Mediterranean region (Brkini, Vipava), Dinaric (Karst) and Alpine region (Bovec) from May to June in 2012 and 2013. The $\delta^{18}O_{milk}$ values in goat, sheep and cow ranged from −3.6 to 2.4‰, from −5.6 to 1.2‰ and from −6.6 to −2.6‰, respectively.

It is evident from Figure 4 that $\delta^{18}O_{milk}$ values in goat (average values: $\delta^{18}O_{milk}$ = −0.9 ± 2.1‰ and $\delta^{18}O_{milk}$ = −1.8 ± 1.0‰, in 2012 and 2013, respectively) and sheep milk (average values: $\delta^{18}O_{milk}$ = −2.4 ± 1.6‰ in 2012 and $\delta^{18}O_{milk}$ = −3.1 ± 1.6‰ in 2013) are higher than the values in cow milk (average values: $\delta^{18}O_{milk}$ = −3.0 ± 0.5‰ in 2012; $\delta^{18}O_{milk}$ = −5.0 ± 0.7‰ in 2013). First, such differences could be related to the source of drinking water. Comparing to cows that predominantly drink groundwater, the sources of drinking water for goats and sheep are also rainwater and grazing on fresh pasture herbage that is enriched in ^{18}O. Another explanation for the isotopic difference is animal physiology and diet. Bryant and Froelich [30] proposed that herbivore oxygen isotope composition in water body depends principally on body size. Total water flux (amount of water into and out of animals each day) also scale with body size but can be also influenced by dietary inputs and environmental temperature. Larger animals might on average be less capable to conserving water compared to smaller animals, however difference in water conservation depends also on water consumption. For example, goat drink water every few days, while cows must drink water every day [29]. Thus, it is expected that goat with lower water turnover rate have higher $\delta^{18}O_{milk}$ values. Finally, because sweat, urine, and fecal water have higher $\delta^{18}O$ values than water vapor, animals that pant to lose heat (goat, sheep), have high urinary salt concentrations, and have low fecal water contents, should have a higher $\delta^{18}O_{milk}$ values than animals that lose more of their water as liquid (cow) [27,29].

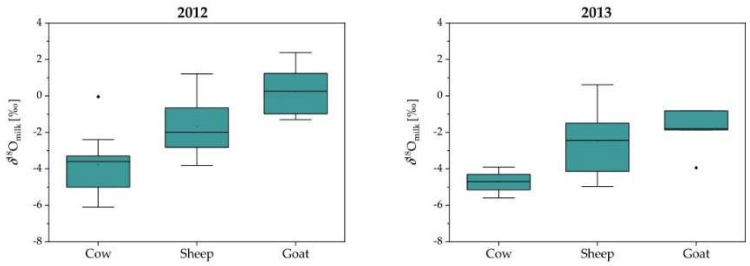

Figure 4. Box plot of the $\delta^{18}O_{milk}$ values of different species (cow, sheep, and goat), collected from farms located in Mediterranean (Brkini, Vipava), Dinaric (Karst) and Alpine region (Bovec) in May and June in 2012 and 2013.

Further, $\delta^{18}O_{milk}$ values in all three species are higher in 2012 comparing to 2013. One of the reasons could be unusual weather conditions in May and June in 2012 with extremely high temperatures (average: 14.1 and 20.6 °C, respectively) comparing to 2013 (average: 13.5 and 18.2 °C, respectively) that can influence the source of water as well as activity level and body temperature regulation [35]. Rapid metabolism and more intense respiration also likely cause evaporative ^{18}O-enrichment in body water. Lower $\delta^{18}O_{milk}$ values were also observed in the Alpine region connected to higher altitude, lower temperatures and higher amount of precipitation.

2.2. Stable Isotope Composition of Oxygen in Milk and Groundwater: Detection of Dilution with Water

Overall, $\delta^{18}O_{milk}$ values in milk depend on the sources of drinking water, metabolism, and isotopic fractionation during milk synthesis. In most cases, drinking water is taken from local groundwater (GW) sources, which reflects the isotopic composition of the mean annual precipitation [18]. For example, Liu et al. [38] found that $\delta^{2}H$ and $\delta^{18}O$ values in goat milk water were identical to that in drinking water. The $\delta^{2}H$ and $\delta^{18}O$ values of the water in cow milk correlate with geo-climatic characteristics of the area of origin, rather than dietary values [26]. Our data shows an ^{18}O-enrichment of raw cow milk ranging from 1.0 to 6.6‰ relative to that in the drinking water dependent on the season. Garbaras et al. [39] report a variation from 1–8‰ in the $\delta^{18}O$ values between cow milk water and the drinking water. Ehtesham et al. [40] report an ^{18}O-enrichment of approximately 4‰ in milk water compared to farm water, but no significant correlation between the two variables was found. Kornexl et al. [32] report an ^{18}O-enrichment of 2–6‰ in milk water compared to ground water and other water sources.

There is usually no significant seasonal changes in $\delta^{18}O$ values in groundwater ($\delta^{18}O_{GW}$) due to its mean age typically covering decades to centuries. The distribution of the $\delta^{18}O_{GW}$ values together with the mean recharge rates in the whole Slovenia is presented by Mezga et al. [41]. The $\delta^{18}O_{GW}$ values of groundwater reported in our study ranged between −9.1‰ (Pannonian region) and −6.7‰, (Mediterranean region) with an average standard deviation within one year of 0.5‰.

The monthly distribution of $\delta^{18}O_{milk}$ values, together with $\delta^{18}O_{GW}$ values throughout the year 2012, is shown in Figure 5. The box plot of $\delta^{18}O_{milk}$ values determined in June and December in 2013, 2014 and 2015 are also presented (Figure 5). Significant seasonal variations in the $\delta^{18}O_{milk}$ values were observed, with higher values in summer days and lower values during winter. These findings support the fact that the water uptake by the cattle during the summer (at least in part) is from ingestion of fresh plants with water enriched in ^{18}O as a consequence of evapotranspiration in leaves [23]. As previously discussed, body water is also strongly affected by temperature, which is related to the primary function of water in the thermoregulation of an animal's body temperature [27]. A relationship between $\delta^{18}O_{milk}$ and the season due to seasonal changes in the $\delta^{18}O$ of forage plants, as well as in the body of the animal, linked to evapotranspiration was also reported by other studies [32,34,36,39,40].

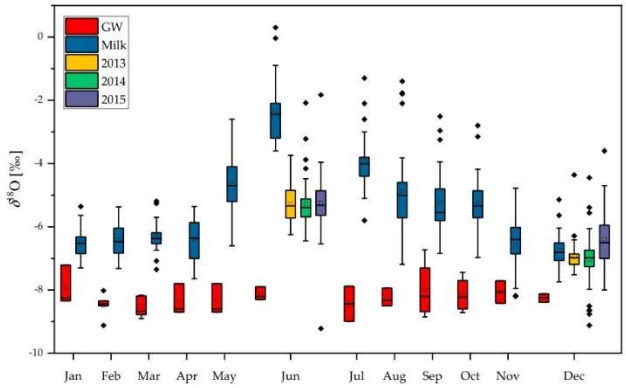

Figure 5. Seasonal variability in $\delta^{18}O_{milk}$ and $\delta^{18}O_{GW}$ values in 2012. For comparison, the box plot of $\delta^{18}O_{milk}$ values determined in June and December relative to the year (2013–2015) are presented on the graph. Data presented are taken for all regions in Slovenia.

The difference between the $\delta^{18}O_{milk}$ and $\delta^{18}O_{GW}$ values indicated that based on the isotopic composition of oxygen, it is possible to detect the addition of water to milk, i.e., with a greater certainty during the summer period (Figure 5). A simple experiment was performed to evaluate the capability to detect milk dilution with water. For this experiment samples of milk and GW from four locations covering three different regions were collected in May 2017: Mediterranean (Ajdovščina), Alpine (Črna na Koroškem, Selnica ob Dravi) and Pannonian (Ormož). The $\delta^{18}O_{milk}$ values were the following −7.4‰, −6.0‰, −6.5‰ and −5.6‰, while $\delta^{18}O_{GW}$ values were −9.3‰, −9.5‰, −10.0‰ and −10.2‰, respectively. A serial of dilution of a raw (authentic) cow milk with drinking water in the following volume percentages: 0%, 1%, 3%, 5%, 7%, 10%, 15%, 20% and 30% was performed.

The results presented in Figure 6 show that diluting milk with varying amounts of water decreases the $\delta^{18}O_{milk}$ values. The correlation coefficient between $\delta^{18}O_{milk}$ values and added water was high ($R^2 \geq 0.89$). Taking 2σ from determination of $\delta^{18}O_{milk}$ as a maximum acceptable difference between $\delta^{18}O_{milk}$ values in authentic and diluted milk the addition of >15% of water can be detected.

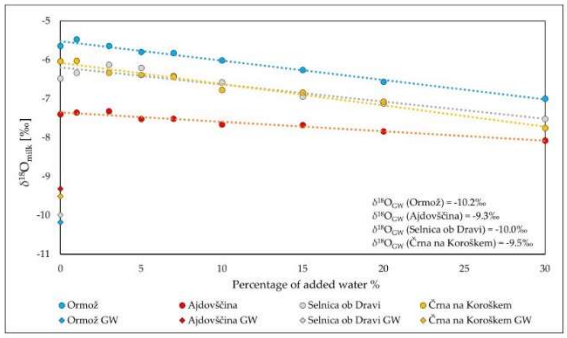

Figure 6. The relationship between $\delta^{18}O_{milk}$ values and percentage of added water to authentic milk samples from different locations with different $\delta^{18}O_{GW}$. Measured $\delta^{18}O_{milk}$ are significantly ($p < 0.05$) related to the $\delta^{18}O_{GW}$ in a regression analysis. The correlation coefficients between $\delta^{18}O_{milk}$ values and added water were 0.98, 0.89, 0.98 and 0.96 for Ormož, Selnica ob Dravi, Črna na Koroškem and Ajdovščina, respectively.

This experiment also shows that $\delta^{18}O_{milk}$ values in raw milk and groundwater can provide a reference to detect adulteration and supports the findings of Lin et al. [42] who studied raw and manufactured milk from Taiwan. The method is more efficient than making cryoscopic measurements, especially when sodium chloride (NaCl) is added, which is a common practice, together with water to milk. The addition of NaCl can decrease the freezing point of water in the milk, which means that the dilution of water with cryoscopic method cannot be detected.

2.3. Lactose as an Internal Standard

Further, we check if it is possible to improve the detection of water addition using lactose and $\delta^{18}O_{lactose}$ values as internal standard, since there is a close relationship between lactose synthesis and the amount of water drawn into milk [43]. Based on the findings from the European project [44], $\delta^{18}O_{lactose}$ values are less affected by the season and relatively insensitive to changes in the cow's diet. The difference for organically bound oxygen in lactose between regions is less pronounced than for oxygen of water, as oxygen-containing lactose is produced continuously over a longer time and therefore scrambling or exchange may occur. Also, $\delta^{18}O_{lactose}$ values are enriched by approximately 25‰ relative to the cattle feeding water (Figure 7). This increase is related to the plant cellulose breakdown by the cattle and the incorporation of its glucose oxygen into lactose during synthesis. By adding water, the $\delta^{18}O_{milk}$ changes accordingly, whereas the value of lactose does not change. It means that if $\delta^{18}O_{lactose}$ and $\delta^{18}O_{milk}$ are correlated, addition of water eliminates this correlation and can be detected. Thus, it seems that $\delta^{18}O_{lactose}$ values could be used as internal standard to detect possible adulteration with water.

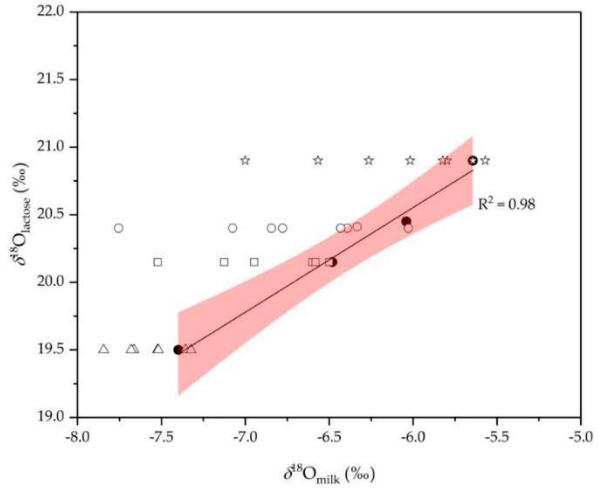

Figure 7. Relationship between $\delta^{18}O_{milk}$ and $\delta^{18}O_{lactose}$ values of authentic samples together with the regression line and 95% of confidence levels ($R^2 = 0.98$). The data for diluted milk from Ajdovščina, Črna na Koroškem, Selnica pri Dravi in Ormož are also included. From right to left, points show the $\delta^{18}O_{milk}$ when adding 3%, 5%, 7%, 10%, 20% and 30% of water to milk.

To test this hypothesis, we prepared a series of diluted milk samples and determined their $\delta^{18}O_{lactose}$ values. Authentic milk samples were collected from the same locations as the first experiment covering the typical $\delta^{18}O_{GW}$ values in Slovenia, to which 0%, 3%, 5%, 7%, 10%, 20% and 30% of water was added. The $\delta^{18}O_{lactose}$ values for authentic samples ranged from 19.3‰ to 20.8‰. The lowest $\delta^{18}O$ values of lactose were determined for Ajdovščina and the highest for Ormož. These data are comparable

with the data obtained in other EU countries, for example in France ($\delta^{18}O_{lactose}$ = 21.0 ± 1.8‰; n = 25), UK ($\delta^{18}O_{lactose}$ = 21.3 ± 1.1‰; n = 36), Italy ($\delta^{18}O_{lactose}$ = 16.8 ± 3.1‰; n = 55), and Spain ($\delta^{18}O_{lactose}$ = 19.1 ± 2.1‰; n = 50), as reported in the final report of the European project [44].

The authenticity of the milk was evaluated by comparing the $\delta^{18}O_{lactose}$ values with the corresponding $\delta^{18}O_{milk}$ values of authentic and diluted milk samples. The results are presented in Figure 7 indicating a good correlation between $\delta^{18}O_{lactose}$ values with the corresponding $\delta^{18}O_{milk}$ of authentic samples (R^2 = 0.98). For diluted samples, $\delta^{18}O_{milk}$ is not more correlated with $\delta^{18}O_{lactose}$ and falls in most of the cases outside the 95% confidence level of the regression line. Although the number of results is limited and this must be interpreted with care, it appears that it is possible to detect the adulteration with water even at lower amounts of added water (>7%). However, it should be pointed out that more research is needed on this topic especially since lactose, as the internal standard, may have its drawbacks in some cases. For example, higher measurement uncertainty is expected for milk with low lactose content.

3. Materials and Methods

3.1. Sampling and Sample Preparation

Authentic cow milk samples (n = 319) were collected directly from farms located in the four Slovenian macro-regions: Alpine, Dinaric, Pannonian, and the Mediterranean (Figure 8). The cow milk samples were obtained in summer (June) and winter (December) from 2013 to 2015. In 2012, samples of cow milk were collected monthly from January to December. In parallel, samples of groundwater (GW) were also collected. In addition, samples of goat (n = 15) and sheep milk (n = 22) were collected systematically during May, June and July in 2012 and 2013 from Bovec (Alpine), Karst (Dinaric), Vipava and Brkini (Mediterranean), and central Slovenian region (Dinaric). All samples were frozen and stored at −20 °C prior to analysis.

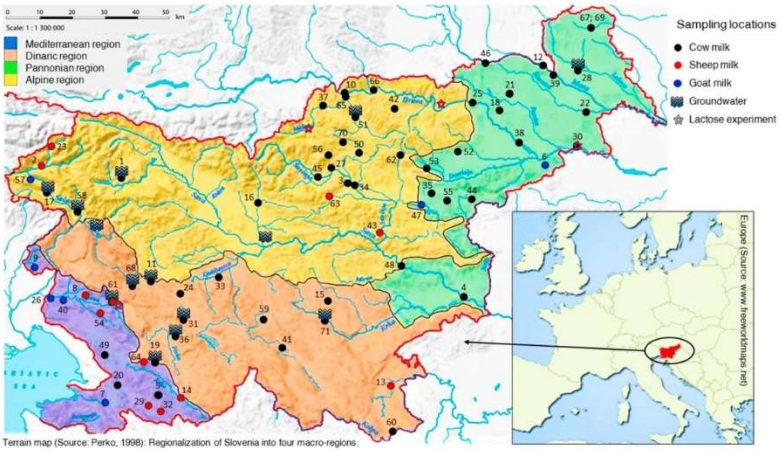

Figure 8. Macroregion division in Slovenia (after Perko, 1998) showing sampling locations of raw milk from different species covering geographical macro-regions (as indicated). The numbers correspond to the numbers of locations presented in Tables S1 and S2 (Supplement material). The locations used in experiments are also presented.

For pretesting the internal standardisation method, paired cow milk and drinking water samples from Alpine (Selnica ob Dravi, Črna na Koroškem), Mediterranean (Ajdovščina) and Pannonian

(Ormož), were collected in May 2017. Samples were delivered with ice packs and then immediately registered and stored in the fridge upon receipt. All samples were stored at 4 °C for a period not exceeding 24 h.

3.2. Isolation of Casein

Analytical preparation of milk samples was carried out according to the standard procedure [21]. Fat was removed from milk sample of 25 mL by centrifugation (Type Centric 322 A, TEHTNICA, Železniki, 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, Loop, France) followed by subsequent centrifugation (10 min at 3000× *g*). The precipitate was rinsed once with pure water (Milli-Q system, Millipore Sigma, Burlington, MA, USA) and once with petroleum ether:ether (2:1) (both Merck, Darmstadt, Germany). After the centrifugation, sample was heated in a water bath (40 °C) until solvent was completely removed, and then freeze-dried. In parallel, the supernatant fractions and the washing water were combined and used for the next step—isolation of lactose.

3.3. Water Addition Experiment

Two different experiments with water addition were prepared. First, we prepared a series of authentic raw cow milk samples (V = 25 mL) from four locations covering three different regions: Mediterranean (Ajdovščina), Alpine (Črna na Koroškem, Selnica ob Dravi) and Pannonian (Ormož). diluted with different proportions of drinking water (0, 1, 3, 5, 7, 10, 15, 20 and 30% *v*/*v*). In this samples $\delta^{18}O_{GW}$ and $\delta^{18}O_{milk}$ values were determined. In the second experiment, we also prepared a series of authentic cow milk samples (V = 25 mL) from the same locations diluted with different proportions of drinking water (0, 3, 5, 7, 10, 20, and 30% *v*/*v*). In this experiment lactose was isolated and $\delta^{18}O_{milk}$ values determined.

3.4. Isolation of Lactose

Lactose was obtained by heating the whey (supernatant) in a water bath (80 °C, 10 min) followed by filtration (Whatman 589/1, Sigma-Aldrich, St. Louis, MI, USA) and washing of the residue with not more than 5 mL of Milli-Q water [32]. The filtrate was then freeze-dried. Four replicates of each sample were prepared.

3.5. Determination of Stable Hydrogen and Oxygen Isotope Ratios

The determination of the stable of hydrogen and oxygen isotope ratios were performed using IRMS and expressed in the δ-notation in ‰ according to Equation (1) [45]:

$$\delta^{i}E = (R(^{i}E/^{j}E)_{sample}/R(^{i}E/^{j}E)_{standard}) - 1 \quad (1)$$

where E is the element (H, O), R is the isotope ratio between the heavier "i" and the lighter "j" isotope ($^{2}H/^{1}H$, $^{18}O/^{16}O$) in the sample and relevant internationally recognised reference standard. The delta values are multiplied by 1000 and expressed in units "per mil" (‰).The $\delta^{2}H$ and $\delta^{18}O$ were reported relative to the Vienna-Standard Mean Ocean Water (V-SMOW) standard [45].

The $\delta^{18}O_{milk}$ and $\delta^{18}O_{GW}$ values were determined directly in milk and water after equilibration with reference gas CO_2/He (5% CO_2) at 40 °C for 6 h. Measurements were performed on a continuous flow IRMS (GV Instruments ltd, Manchester, UK) connected with MultiFlow Bio preparation system (IsoPrime, GV Instruments ltd, Manchester, UK). The results for milk water were normalised against the following laboratory standards: W-3869 (seawater $\delta^{18}O_{VSMOW\text{-}SLAP}$ = 0.36 ± 0.04‰) and W-3871 (snow water; $\delta^{18}O_{VSMOW\text{-}SLAP}$ = −19.73 ± 0.02‰). An independent laboratory reference material W-3870 (Mili-Q water, $\delta^{18}O_{VSMOW\text{-}SLAP}$ = −9.12 ± 0.04‰) was analysed periodically throughout the sequence as a control to ensure the quality of the results. The laboratory standards used are calibrated against certified reference materials: NIST 8535a- (Vienna Standard Mean Ocean

Water 2; IAEA-VSMOW2) (water; $\delta^{18}O_{VSMOW\text{-}SLAP}$ = 0.00 ± 0.02‰), RM 8537a- (Standard Light Antarctic Precipitation water; IAEA-SLAP2) ($\delta^{18}O_{VSMOW\text{-}SLAP}$ = −55.50 ± 0.02‰) and NIST RM 8536 (Greenland Ice Sheet Precipitation water; GISP) ($\delta^{18}O_{VSMOW\text{-}SLAP}$ = −24.76 ± 0.09‰). For each set of measurement, laboratory reference materials (W-3869 and W-3871) for normalization were measured four times; two times at the beginning of the batch, and two times at the end of the batch, while the control material (W-3870; MiliQ water) was measured six times; at the beginning, in the middle and at the end of the batch. Measurements precision was 0.1‰ for $\delta^{18}O$ and 1‰ for $\delta^{2}H$.

The $^{2}H/^{1}H$ and $^{18}O/^{16}O$ and measurements of lactose and casein were performed at the Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach in San Michele all' Adige, Italy. The $\delta^{2}H$ and $\delta^{18}O$ values of lactose ($\delta^{2}H_{lactose}$, $\delta^{18}O_{lactose}$) and casein ($\delta^{2}H_{casein}$, $\delta^{18}O_{casein}$) were determined by transferring of freeze-dried samples, respectively, into a silver capsule and analysing the sample simultaneously using TC/EA pyrolyser (Thermo Finnigan, Waltham, MA, USA) coupled to a DELTA XP isotope ratio-mass spectrometer, IRMS (Thermo Scientific, Waltham, MA, USA). For normalisation of the results, two internal laboratory reference materials were applied: Caribou Hoof Standard (CBS) and Kudu Horn Standard (KHS). The sample weight was 0.2 mg and 0.4 mg for lactose and casein, respectively. The results for lactose and casein were calibrated against the following international reference materials: CBS keratin (Caribou Hoof Standard; $\delta^{2}H_{VSMOW\text{-}SLAP}$ = −157.0 ± 0.9‰, $\delta^{18}O_{VSMOW\text{-}SLAP}$ = +3.8 ± 0.3‰) and KHS keratin (Kudu Horn Standard; $\delta^{2}H_{VSMOW\text{-}SLAP}$ = −35.3 ± 1.1‰, $\delta^{18}O_{VSMOW\text{-}SLAP}$ = +20.3 ± 0.3‰). Measurements precision was ±0.2‰ for $\delta^{18}O$ and ±1‰ for $\delta^{2}H$.

Data quality control charts were systematically recorded throughout the study period. To ensure the validity and comparability of the isotope results, the laboratory regularly participates in the Food analysis using Isotopic Techniques-Proficiency Testing Scheme FIT-PTS organized by EUROFINS (Nantes, France) three times per year. In this scheme, water and casein are also included.

3.6. Statistical Analysis

The data was processed using the statistical software package OriginPro 2018 (OriginLab, MicroCal Inc., Harrisburg, PE, USA), and Microsoft Excel (Microsoft Office Professional Plus 2019, Microsoft Corporation, Redmond, WA, USA). The existence of differences was verified through representation of variables within numeric data with box-plots (which graphically display summary of a data set: median, minimum, and maximum) or through regression analysis at a confidence level of 95%. One-way ANOVA was performed to determine the significant temporal (season, year) and spatial difference of variables. 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.

4. Conclusions

This study demonstrated that by using the stable isotope composition of oxygen in milk water it is possible to discriminate milk from Slovenia according to the season and animal species, while regional discrimination is limited. The compositional differences in animal species indicate that diet and physiology have a strong control on animal isotope composition of body water and consequently also to milk. Seasonal variation in $\delta^{18}O_{milk}$ values are controlled by evaporation processes. Actually, the "evaporation effect" may be related directly to the animal physiology as well as to ingestion of fresh grass with water enriched in ^{18}O as a consequence of evapotranspiration in leaves. No significant statistical differences in $\delta^{2}H_{casein}$ and $\delta^{18}O_{casein}$ values according to the season and region of milk production was observed indicating that these two parameters provide more consistent isotopic signature with which to access the authenticity and origin of the milk. Further, the milk water is remarkably enriched in ^{18}O compared to groundwater providing a possibility to detect addition of water. Based on our experiment it was found that >15% of added water can be detected by determining $\delta^{18}O_{milk}$ and $\delta^{18}O_{GW}$ values. The method using $\delta^{18}O_{lactose}$ values as an internal standard was shown

to be even more promising in improving the detection of the illegal watering of milk (>7%). A further improvement of this approach could be made in the future by analyzing higher number of samples originating from different countries.

Supplementary Materials: The following are available online. Table S1: Geographical information of the sampling location together with $\delta^{18}O_{milk}$, $\delta^{18}O_{casein}$ and $\delta^{2}H_{casein}$ values during the summer and winter in the year period from 2012 to 2015; Table S2: Data collection of the $\delta^{18}O$ values of milk of different dairy species: sheep and goat. For comparison, samples were collected during summer season from May to June in 2012 and 2013.

Author Contributions: Conceptualization, S.H.G. and N.O.; Methodology, D.P., F.C.; Validation, S.H.G., D.P. and F.C.; Formal analysis, S.H.G. and D.P.; Investigation, S.H.G. and N.O.; Resources, N.O.; Data curation, S.H.G.; Writing—original draft preparation, S.H.G.; Writing—review and editing, N.O.; Visualization, S.H.G.; Supervision, N.O. All authors have read and agreed to the published version of the manuscript.

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Chapter 5

Can We Discover Truffle's True Identity?

This chapter summarizes the paper entitled "Can We Discover Truffle's True Identity?" by Staša Hamzić Gregorčič, Lidija Strojnik, Doris Potočnik, Katarina Vogel-Mikuš, Marta Jagodic, Federica Camin, Tea Zuliani and Nives Ogrinc. The paper was published in the *Molecules* journal, 2020. This paper belongs to the Special Issue Isotopic Techniques for Food Science. This study was part of a research-oriented RealMED project, which aimed to improve local economy and ensure the authenticity and quality of Mediterranean products, such as black Iberian pig meat products from Portugal and Span, Italian and Slovenian truffles, Moroccan argan oil and Tunisian mountain lamb.

The truffle market is growing faster with significant growth rates over the last few years. Slovenia sold 829 tonnes of mushrooms and truffles in 2020, which is more than the year before (246 tonnes). Although in Slovenia it is legally regulated who can hunt truffles, the matter of permits has not yet come to life in practice. Truffles have become popular with many food lovers, mainly because of their strong taste and aroma, which are key factors in gaining their attention. Because of the aroma, their price can range from a few hundred dollars to several thousands of dollars per kilogram. The most valued are the varieties grown in Europe (mainly in Croatia, France, Hungary, Italy, Slovenia and Spain), which represent 85 % of the world market. Due to differences in the prices of truffle species, there is a high probability of food fraud, which cannot always be detected by traditional methods. Thanks to the database and developed techniques, it is possible to determine the geographical origin or identification of the species of fungi.

The purpose of this study was to create a database to see if it could be used to identify the origin and the species of truffles and thus prevent truffle fraud. A multi-element and stable isotope ratio approach was used to characterise truffles (n = 58) from eight different countries (Bosnia and Herzegovina, China, Croatia, Italy, North Macedonia, Poland, Spain as well as Slovenia) and multivariate statistical analysis was used to classify truffles according to species and geographical origin. Elements concentrations (Al, As, Ba, Ca, Cd, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Rb, Sr, V, and Zn) were determined using ICP-MS. For elemental screening (Ca, Fe, Mn, Cu, and Zn) in gleba and peridium parts of ascocarps, micro-XRF imaging of freeze-dried samples was performed. $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ were determined simultaneously using an IsoPrime-100 Vario PYRO Cube (OH/CNS) pyrolizer/Elemental Analyzer, whereas $\delta^{18}O$ and $\delta^{2}H$ were analysed using TC/EA pyrolyser coupled to a DELTA XP IRMS. The $^{87}Sr/^{86}Sr$ isotope ratio values in truffles samples were determined using the Nu II multicollector ICP-MS instrument.

Despite the fact that Slovenian truffles shared some similar characteristics with samples from other countries, the differences in the concentrations of elements show that individual species of truffles can selectively respond to nutrients from a certain type of soil under

environmental and soil conditions. Cross-validation resulted in a 77 % correct classification rate for determining the geographic origin and a 74 % correct classification rate for species discrimination. The critical parameters for distinguishing the geographical origin were Sr, Ba, V, Pb, Ni, Cr, Ba/Ca and Sr/Ca ratios, while the values of δ^{18}O and δ^{13}C are the most important. The key variables that distinguish *T. magnatum* from other species are the levels of V and Zn and δ^{15}N values. *Tuber aestivum* can be separated based on Ni, Cr, Mn, Mg, As, and Cu content. The details on the composition of truffles vary from species to species and from region to region, due to differences in the isotopic composition of water molecules that vary with altitude, longitude and distance from the sea. In this context, these details can make an significant contribution to truffle authentication studies. This study also suggests broadening the scope to include stable isotopes of Sr. Finally, the methods and approaches developed for truffles can be easily adapted to other food products to confirm their authenticity.

In this paper, I was responsible for the sample preparation, pre-treatment and analysis. For the Sr isotope ratio analysis, I applied the method optimised for milk with minor modifications. I also collected and, in part, analysed the data and jointly prepared the manuscript.

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Can We Discover Truffle's True Identity?

Staša Hamzić Gregorčič [1,2], Lidija Strojnik [1,2], Doris Potočnik [1,2], Katarina Vogel-Mikuš [3], Marta Jagodic [1,2], Federica Camin [4,5], Tea Zuliani [1,2] and Nives Ogrinc [1,2,*]

1 Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia; stasa.gregorcic@ijs.si (S.H.G.); lidija.strojnik@ijs.si (L.S.); doris.potocnik@ijs.si (D.P.); marta.jagodic@ijs.si (M.J.); tea.zuliani@ijs.si (T.Z.)
2 Jožef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia
3 Department of Biology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia; Katarina.VogelMikus@bf.uni-lj.si
4 Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, 38010 San Michele all'Adige, Italy; federica.camin@unitn.it
5 Center Agriculture Food Environment (C3A), University of Trento, via Mach 1, 38010 San Michele all'Adige (TN), Italy
* Correspondence: nives.ogrinc@ijs.si; Tel.: +386-1-588-5387

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Abstract: This study used elemental and stable isotope composition to characterize Slovenian truffles and used multi-variate statistical analysis to classify truffles according to species and geographical origin. Despite the fact that the Slovenian truffles shared some similar characteristics with the samples originating from other countries, differences in the element concentrations suggest that respective truffle species may respond selectively to nutrients from a certain soil type under environmental and soil conditions. Cross-validation resulted in a 77% correct classification rate for determining the geographical origin and a 74% correct classification rate to discriminate between species. The critical parameters for geographical origin discriminations were Sr, Ba, V, Pb, Ni, Cr, Ba/Ca and Sr/Ca ratios, while from stable isotopes $\delta^{18}O$ and $\delta^{13}C$ values are the most important. The key variables that distinguish *T. magnatum* from other species are the levels of V and Zn and $\delta^{15}N$ values. *Tuber aestivum* can be separated based on the levels of Ni, Cr, Mn, Mg, As, and Cu. This preliminary study indicates the possibility to differentiate truffles according to their variety and geographical origin and suggests widening the scope to include stable strontium isotopes.

Keywords: *Tuber*; species; stable isotopes; elemental composition; multivariate discriminant analysis; geographical origin

1. Introduction

Truffles (*Tuber* spp.) belong to the ectomycorrhizal fungi (EMF) that undergo a complex life cycle in association with various forest species. They are among the most prized ingredients in the culinary world and can fetch hundreds to thousands of Euros per kilogram, depending upon the species and size [1]. Europe accounts for 85% of the world export market, where the most sought-after black and white truffles grow in France, Italy, Croatia, Slovenia, and Hungary. Among the different species of truffle, only three are commercially important: the white truffle (*Tuber magnatum* Pico), the black truffle (*Tuber melanosporum* Vittad.), and the summer truffle (*Tuber aestivum*). *Tuber magnatum* is the most valuable species, but its spread is limited to the limestone-rich floodlands of Italy and the Balkan peninsula, whereas *T. aestivum* is the most widely spread truffle species in Europe [2]. In Slovenia, truffles are located in areas of high ecological value and biodiversity due its geographic location combined with diverse relief structure, complex geology, substantial water resources and

modified Mediterranean, continental and mountainous climates [3]. A combination of these parameters contributes to a considerably rich mycodiversity in the country and experts estimate that there are 16 species of truffle in Slovenian forests. These include the white truffles *T. magnatum*, *T. borchii* and *T. asa*, and the black truffles *T. aestivum* Vitt., *T. ubicatum*, *T. melanosporum* [4].

High truffle prices have led to several forms of adulteration. For example, 15 per cent of French truffles tested in 2012 were the more common, cheaper truffle species originating mainly from China. *Tuber borchii* can be visually confused with *T. magnatum* and sold as the latter. Another fraudulent practice involves the use of unripe fruiting bodies (ascocarps) of cheaper species in processed foods [5]. The extent of the fraud means that there is an urgent need to protect the truffle market and establish clear information on the truffle products' link to its country of origin. Despite a large number of published studies on the ecology, genetics and cultivation of truffles [6–8], few studies have attempted to validate truffle authenticity. Of these, most used molecular approaches [9–11] and the analysis of volatile organic compounds in order to determine the authenticity and adulteration of truffles and truffle containing products [12–15].

Although stable isotopes and elemental fingerprinting has become increasingly important in establishing the authenticity and geographical origin of food products [16], to date, it has not been applied to truffles [17]. Studies where the stable isotope techniques have been applied were orientated towards investigating carbon isotope fractionation during sucrose decomposition [18], the ecophysiological relationship between truffles, soil and host plants [19], or assessing the mycorrhizal versus the saprophytic status of fungi using the natural abundance of carbon and nitrogen stable isotopes [20]. Habitats with different nutrient inputs and plant communities can show significant differences in overall carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios [21–24]. Geographical differentiation involving stable isotope composition have been performed for other fungal species. Based on the $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{34}S/^{32}S$ ratios in mushroom (*Agaricus bisporus*), it was possible to differentiate between specimens from six regions in Korea [25], while Puscas et al. [26] were able to distinguish samples from different regions of Transylvania using carbon isotope ratios of bulk fungi ($^{13}C/^{12}C$) and the hydrogen and oxygen isotope ratios ($^{2}H/^{1}H$, $^{18}O/^{16}O$) in water extracted from the samples. It was also possible to obtain supplementary information about the geographical origin of truffles from the $^{87}Sr/^{86}Sr$ ratios, since almost the entire life cycle of the ascocarps takes place underground in the presence of the host tree. Importantly, the $^{87}Sr/^{86}Sr$ isotopic fingerprint remains unaltered up to the end product, even after processing, and hence provides an unique and well-established geographical tracer for several types of plant food product, such as rice [27], vegetables [28–31], cereals and mushrooms [32,33].

To date, only two studies have looked at the elemental composition of *T. magnatum* in order to assess potential differences in their assimilation and accumulation abilities [34,35]. *Tuber magnatum* appears to be the more competitive of the different varieties, being able to more efficiently assimilate/accumulate Cu, K, Na, P, and Zn. At the same time, *T. brumale* was more successful in accumulating/assimilating sulphur. Segneanu et al. [35] investigated antioxidant activity, total organic carbon as well as the levels of As, Cu, Pb, Zn, Mn, Fe and Ni in *T. magnatum* and *T. melanosporum*. Their results also show that *T. melanosporum* contains a high amount of C and Fe than *T. magnatum* Pico, while there was no difference in the levels of the other elements between two truffle species.

The accurate discrimination of the geographical origin of truffles remains a critical issue because of unknown influence of genetic and environmental variations that affect their elemental and stable isotope composition. To overcome this lack of information, we performed a study to (i) characterise Slovenian truffle species for elemental and stable isotope composition; and (ii) explore the possibility of differentiating truffles according to species and geographical origin using multivariate statistical analysis. This study is part of the REALMed project (https://realmedproject.weebly.com/).

2. Results and Discussion

The present study gives insight into the elemental and isotopic composition of commonly cultivated *Tuber* species in Europe, while Chinese samples were collected from the local market. In this study, 58 samples of truffles from Slovenia (n = 33; *T. aestivum*, *T. brumale*, *T. magnatum*), Italy (n = 6; *T. aestivum*, *T. magnatum*, *T. melanosporum*), Croatia (n = 3; *T. aestivum*, *T. brumale*, *T. macrosporum*), Poland (n = 3; *T. aestivum*), Bosnia and Herzegovina (n = 2; *T. aestivum*), Spain (n = 3; *T. melanosporum*), North Macedonia (n = 5; *T. aestivum*, *T. mesentericum*), and China (n = 3; *T. indicum*) were considered.

A summary of geo-environmental, climatic and host tree species information for *Tuber* species is presented in Table S1 (Supplementary Material). The elemental profiles and isotopic composition of light elements of truffles are presented in Tables S2 and S3, respectively (Supplementary Material). The most commonly evaluated elements in fungi were analysed, including Ca, Cd, Cu, Fe, Hg, K, P and Pb. Also. Al, As, Ba, Co, Cr, Cs, Mg, Mn, Na, Ni, Rb, S, Sr, V, and Zn, which are rarely evaluated, were determined. The data were then used to create a complete overview of elements for the average, minimum and maximum ranges of elements in different truffle species of wide geographical origin (Table 1). Furthermore, in total 33 different variables were included in multivariate statistical analysis to differentiate truffles according to the species and geographical origin.

2.1. Elemental Composition

The mean concentrations of each element varies from species to species, which appears to be related to their geographical origin and growing conditions, such as soil characteristics, water availability and climate [36]. The results are presented in Table 1.

Thus, elemental composition can serve as a fingerprint for truffles, providing a useful marker for geographical classification. For this reason, samples were sorted into different groups based on species affiliation and geographical origin.

Furthermore, the mobilization and redistribution of elements in truffle tissues are also important and will have a large impact on their heterogeneity [37]. The distribution of elements in truffles (*T. aestivum*) was recorded on the BL6b beamline, SLRI (Synchrotron light research institute, Thailand, Project proposal 3457). The results show that the microelements (Mn, Fe, Cu and Zn) and Ca accumulate mainly in the melanised layer of the truffle surface, while K accumulates in the core (Figure 1). Thus, the interpretation of our results is oriented mainly in the peridial layer of the fruiting bodies.

Taking into account that forests are complex systems, where large fluxes of essential and trace elements are balanced over time [36], we studied the possible differences or similarities in elemental composition relating to the geographical origin of samples of *T. aestivum* from Slovenia and abroad. The mean contents of elements determined in the peridial layer of investigated species decreased in the following order: K > P > Ca > S > Mg > Al > Fe > Zn > Na > Cu > Mn > Rb > Ba > Cd > Sr > Cr > V > Ni > Pb > As > Co > Cs > Hg.

After applying an ANOVA test, significant differences ($p < 0.05$) in the levels of Na, Mg, S, Cu, and Ba between one or more countries were observed (Figure 2). The Croatian truffle samples differ from those of other countries by containing higher levels of Mg and Ba, and lower amounts of Na and Cu. High S and Sr contents are characteristic of the samples from Poland.

In Slovenia, six main regions were identified: Sežana, Bloke, Rajndol, Spodnje Blato, Žlebič and Slovenian Istria. Truffles from Sežana exhibit higher concentrations of Al, Ca, V, Cr, Mn, Fe, Co, Ni, Pb, As and Sr, while concentrations of Mg, Rb, Cd and Hg were the highest at Bloke. On the other hand, the lowest concentrations of element were mainly observed at Rajndol and Žlebič. In Slovenian Istria higher concentrations of Na, K and Zn were observed, while concentrations of Ca and Cd are lower compared to other locations. The main reason for this difference is probably geological background and soil depth. For example, Sežana is located on calcaric flysch with eutric brown soil, while Žlebič and Rajndol are located on marine carbonate and clastic rocks.

Table 1. Minimum (Min), maximum (Max), and mean concentration (Mean) ± standard deviation (SD) of elements in the peridial layer of the fruiting bodies of different truffle species. All elements are expressed in mg/kg.

Element	*Tuber magnatum* (*n* = 4)			*Tuber melanosporum* (*n* = 5)			*Tuber mesentericum* (*n* = 4)			*Tuber aestivum* (*n* = 39)			*Tuber brumale* (*n* = 2)			*Tuber indicum* (*n* = 3)			*Tuber macrosporum* (*n* = 1)
	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	
Al	1103 ± 424	715	1557	233 ± 116	113	382	511 ± 699	143	1559	498 ± 435	29.0	1711	1376 ± 59	1335	1418	59.6 ± 41.1	22.4	104	1961
As	0.20 ± 0.05	0.16	0.26	0.07 ± 0.05	0.03	0.14	0.19 ± 0.20	0.04	0.49	0.20 ± 0.17	0.03	0.72	0.28 ± 0.06	0.23	0.32	0.05 ± 0.01	0.04	0.06	0.28
Ba	8.21 ± 5.87	3.91	16.90	2.13 ± 0.91	1.08	3.08	6.41 ± 4.55	3.56	13.2	6.84 ± 2.96	1.20	15.2	11.5 ± 9.0	5.09	17.8	1.99 ± 0.69	1.26	2.63	21.7
Ca	1594 ± 642	1220	2551	3489 ± 1120	2527	5418	2217 ± 1068	1380	3681	2831 ± 820	668	5135	2653 ± 2365	980	4325	987 ± 274	747	1285	3868
Cd	2.17 ± 1.00	0.77	2.97	2.35 ± 1.75	0.49	4.03	5.49 ± 3.01	1.36	8.50	6.25 ± 3.35	1.78	15.4	9.36 ± 4.46	6.20	12.5	1.30 ± 1.08	0.42	2.51	7.90
Co	0.40 ± 0.13	0.32	0.54	0.17 ± 0.14	0.04	0.37	0.17 ± 0.17	0.07	0.41	0.17 ± 0.12	0.03	0.48	0.32 ± 0.05	0.29	0.36	0.10 ± 0.04	0.07	0.15	0.36
Cr	3.38 ± 1.72	1.82	5.22	1.18 ± 0.29	0.84	1.52	1.46 ± 0.93	0.69	2.80	2.24 ± 1.21	0.44	5.13	3.31 ± 1.68	2.13	4.50	0.87 ± 0.54	0.51	1.50	2.62
Cs	0.12 ± 0.04	0.08	0.16	0.04 ± 0.01	0.02	0.05	0.06 ± 0.07	0.02	0.17	0.07 ± 0.06	0.01	0.23	0.14 ± 0.00	0.13	0.14	0.01 ± 0.00	0.01	0.02	0.19
Cu	61.9 ± 29.1	25.5	94.2	122 ± 119	32.6	270	51.9 ± 8.7	43.3	63.5	46.6 ± 19.0	22.0	116	88.5 ± 80.1	31.9	145	27.5 ± 16.3	17.1	46.4	29.8
Fe	322 ± 84	228	387	156 ± 67	84.2	222	367 ± 492	107	1104	325 ± 284	27.0	1215	646 ± 261	462	831	50.7 ± 30.7	24.5	84.4	589
Hg	0.04 ± 0.00	0.04	0.05	0.05 ± 0.02	0.03	0.08	0.09 ± 0.04	0.06	0.14	0.07 ± 0.04	0.01	0.20	0.03 ± 0.02	0.02	0.05	0.06 ± 0.01	0.05	0.07	0.03
K	31,612 ± 3926	28,203	35,844	20,289 ± 2086	18,194	23,097	21,689 ± 3772	17,194	25,824	21,364 ± 3510	15,464	32,571	26,207 ± 7426	20,956	31,458	23,676 ± 6690	17,051	30,429	23,702
Mg	980 ± 173	813	1167	567 ± 188	360	849	1147 ± 244	947	1503	1090 ± 319	521	1911	1544 ± 777	994	2093	706 ± 52	646	740	2268
Mn	20.0 ± 4.9	15.1	24.6	10.1 ± 4.2	5.36	15.5	20.2 ± 15.3	9.94	43.0	18.2 ± 13.9	5.42	87.3	24.5 ± 12.0	16.0	33.0	8.81 ± 2.80	6.80	12.0	20.8
Na	178 ± 38	138	213	72.0 ± 33.2	27.0	111.0	194 ± 34	168	245	106 ± 44	43.1	227	381 ± 334	145	617	57.7 ± 16.2	41.1	73.5	354
Ni	2.68 ± 1.29	1.36	3.93	0.39 ± 0.18	0.15	0.58	0.79 ± 0.47	0.39	1.46	0.93 ± 0.81	0.23	3.91	1.55 ± 0.28	1.35	1.76	0.36 ± 0.13	0.27	0.51	2.18
P	8209 ± 1400	7091	10252	6124 ± 596	5561	7003	6095 ± 1353	4210	7330	4319 ± 1342	2067	8285	8485 ± 2398	6790	####	7453 ± 2336	6027	10149	6965
Pb	0.59 ± 0.36	0.25	1.08	0.18 ± 0.09	0.08	0.27	0.72 ± 0.67	0.22	1.69	0.48 ± 0.43	0.07	2.33	0.67 ± 0.04	0.65	0.70	0.15 ± 0.16	0.04	0.33	1.01
Rb	10.0 ± 4.4	5.48	14.5	4.95 ± 3.59	1.81	9.18	11.0 ± 5.4	4.88	15.6	9.00 ± 6.38	1.24	28.2	17.8 ± 11.1	10.0	25.7	1.51 ± 0.30	1.16	1.70	16.7
S	3202 ± 1772	1897	5808	4297 ± 1467	2552	6630	1933 ± 285	1752	2353	2014 ± 572	1251	3677	3007 ± 2307	1376	4638	2821 ± 775	2114	3649	1230
Sr	6.12 ± 3.17	3.45	10.7	3.52 ± 2.55	0.81	7.34	2.91 ± 1.43	1.59	4.66	4.43 ± 4.98	0.58	23.8	9.41 ± 9.11	2.98	15.9	3.70 ± 1.25	2.42	4.92	12.0
V	2.28 ± 0.87	1.48	3.21	0.50 ± 0.24	0.25	0.80	0.92 ± 1.18	0.23	2.69	1.20 ± 1.15	0.07	4.95	2.59 ± 0.61	2.16	3.02	0.17 ± 0.09	0.11	0.27	3.37
Zn	346 ± 51	275	390	100 ± 13	83.1	117	156 ± 45	100	196	136 ± 37	55.0	269	184 ± 58	143	225	75.8 ± 25.4	60.7	105	298

The content of individual elements in certain ectomycorrhizal fungal species is known to vary [38–40], while element contents in the fruiting bodies are species-dependent, which is consistent with findings in this study. For example, Ambrosio et al. [41] showed that the concentration of Cu, Zn, Sr, and Sb in Porchini mushrooms is very similar to that measured in soil layers, especially at the surface, while certain elements such as Cr and Ni had different distributions. Soil moisture may also have a significant effect on nutrient uptake, since the water phase is enriched in the more soluble Ca, Sr, Mg, Na, and K ions, whereas Al, Fe, and Ba, being less soluble, are enriched in the soil compartment. In addition, the elemental composition in truffles depend also on an accumulation and and assimilation capacity of the respective truffle species, which is also controlled by the host plant demand.

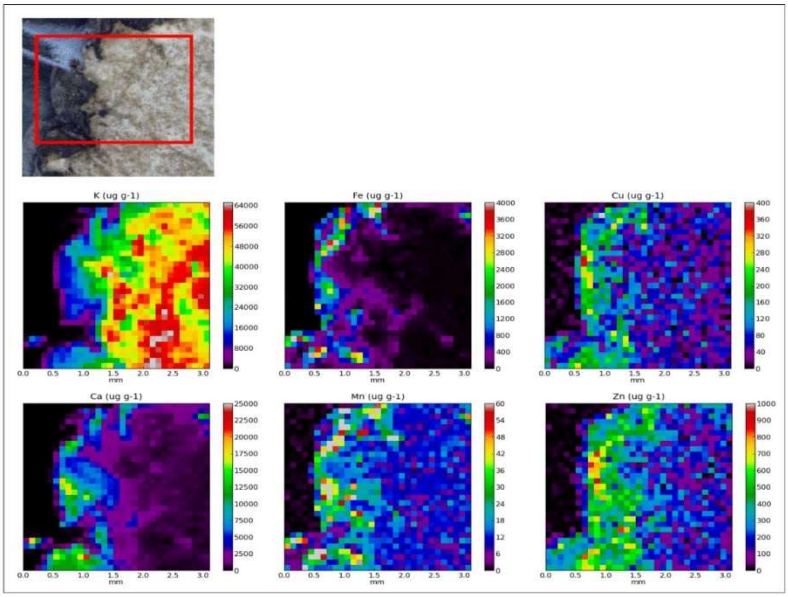


Figure 1. XRF quantitative analysis and Micro-XRF analysis of *T. aestivum* recorded using the BL6b, SLRI, Polychromatic Beam (Synchrotron Light Research Institute) with 100 µm lateral resolution.

The ANOVA test also revealed significant differences in the amounts of Na, Mg, Al, P, S, Cr, Zn, and Ba in different species (Figure 3). The amounts of Na, Mg, Zn, Ba and S are significantly higher in *T. magnatum*, while the concentrations of P and S are significantly lower in *T. aestivum*.

The Zn content of *T. magnatum* is twice that of the other truffle species, which is consistent with the results obtained for *T. magnatum* and *T. brumale* from Serbia [34]. In their study, *T. magnatum* appeared to be more competitive, being able to more efficiently assimilate/accumulate Cu, K, Na, P, and Zn. At the same time, *T. brumale* was more successful in assimilating/accumulating S. In the case of Cr, a significant difference was only observed between *T. melanosporum* and *T. magnatum*, with lower amounts in *T. melanosporum*. Except for Mg, P, Hg, and Sr, *T. indicum* had overall lower contents of elements.

There have been numerous studies over past twenty years, particularly in Europe, examining the presence of heavy metals in ectomycorrhizal fungi and the results show heterogeneous behavior between species [42–45]. Ectomycorrhizal fungi tend to accumulate toxic elements such as Cd, As, Pb, and Hg, which may originate from both natural and anthropogenic sources [46,47]. The toxic effect of

these elements seems to affect enzymes. The determination of the concentration of toxic elements in the truffle ascocarps is also essential for dietary intake studies. The range of toxic elements (As, Cd, Cr, Hg, Ni, and Pb) present in truffle samples tested in this study was lower than the permissible range for fungi (<0.5–5 mg/kg), indicating that their consumption is safe provided that it is occasional [48].

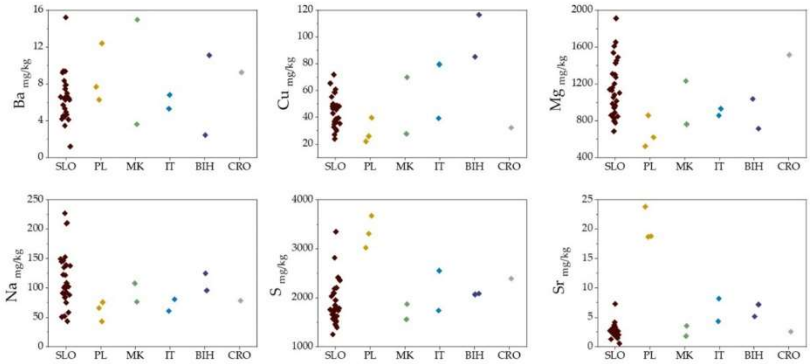


Figure 2. Column scatter plots of element contents in *T. aestivum* from different geographical regions: SLO (Slovenia), PL (Poland), MK (North Macedonia), IT (Italy), BIH (Bosnia and Herzegovina), CRO (Croatia).

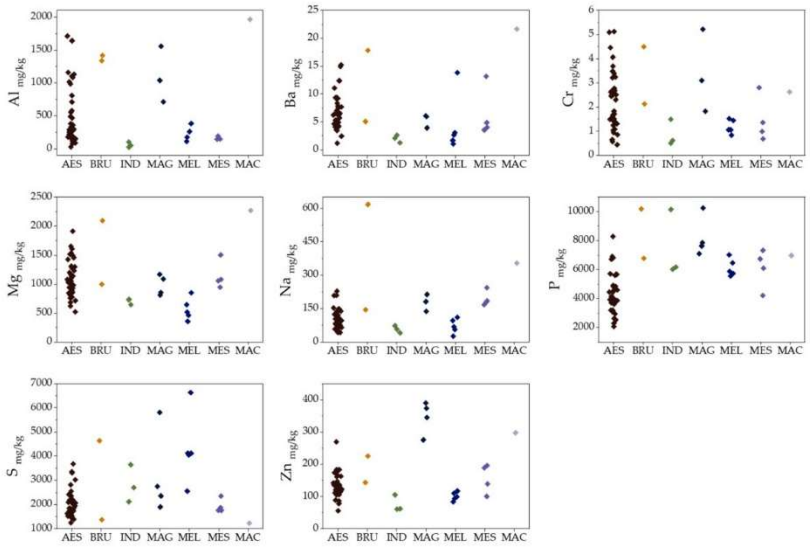


Figure 3. Column scatter plots of element contents in different truffle species: AES (*T. aestivum*), BRU (*T. brumale*), IND (*T. indicum*), MAG (*T. magnatum*), MEL (*T. melanosporum*), MES (*T. mesentericum*), and MAC (*T. macrosporum*).

2.2. Stable Isotope Ratios of Light Elements

Table S3 gives the data for the stable isotope ratios of light elements. In this study, a wide range of $\delta^{15}N$ values (1.8‰ to 19.6‰) are observed, while $\delta^{13}C$ values were from −28.5‰ to −23.8‰. *Tuber magnatum* and *T. melanosporum* had the highest $\delta^{15}N$ and $\delta^{13}C$ values, respectively, while *T. aestivum* had the lowest. Figure 4 shows the relationship between $\delta^{15}N$ and $\delta^{13}C$. These data are comparable with the literature data for EMF fungi [24,49,50]. A statistically significant difference in the $\delta^{15}N$ values is found between different species and geographical location, while $\delta^{13}C$ values showed no significant difference. The isotopic index $\Delta_{CN} = \delta^{13}C - \delta^{15}N$, which allows the assignment of a mycorrhizal or a saprotrophic strategy for sporophore differentiation, ranged from −47.6‰ to −29.0‰, and suggests that *Tuber* does not exhibit a saprotrophic strategy. The limit between saprotrophic and symbiotic strategies is 24‰ [21]. This observation agrees with the findings of previous studies [49–51], but contradicts what the authors stated in handbooks related to truffle cultivation [52].

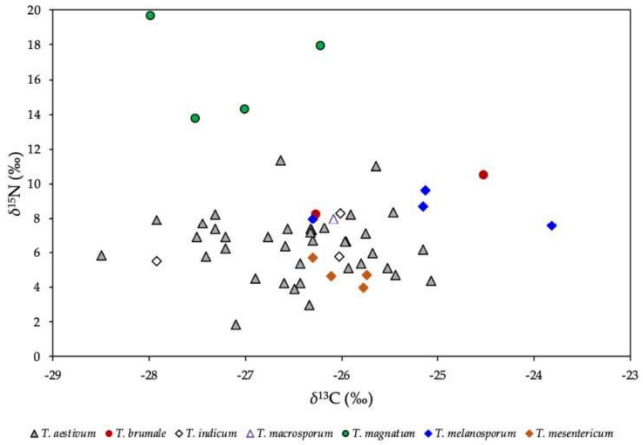


Figure 4. Relationship between $\delta^{15}N$ and $\delta^{13}C$ values in *Tuber* species.

Although the molecular mechanisms for nutrient exchange between mycorrhizal symbionts are not clearly understood, the occurrence of mycorrhizal fungi is primarily controlled by the accessibility of primary nutrients; plants provide carbon, while nitrogen is derived from the soil, since ectomycorrhizas can uptake, reduce and metabolise nitrate [53]. The large variation in $\delta^{15}N$ values, therefore, cannot be explained by taxonomic variation but rather by the nutrient source ($\delta^{15}N_{inorganic} < \delta^{15}N_{organic}$) and soil depth of N acquisition ($\delta^{15}N_{shallow} < \delta^{15}N_{deep}$) [22]. Also, the relative dependence of organic N pools appear to vary according to the location with latitude and altitude of mycorrhizal origin [54]. Another possible explanation for higher $\delta^{15}N$ values is isotopic fractionation, which can occur during the transport of nitrogenous compounds from ectomycorrhizal fungi to their host plants [49].

As already mentioned, EMF obtain their carbon from living trees. Glucose and other monosaccharides are transferred from trees to the EMFs through the ectomycorrhizae. By this way, more than half of the photosynthates of a seedling and up to 21% of the photosynthates of a mature tree are allocated to their symbiotic partner. Trees growing together share almost 40% of the total carbon [55]. The diversity of tree species concerning their water and carbon fluxes in a mixed forest ecosystem is reflected in the carbon isotope composition of the photosynthetic assimilated organic matter [56]. The $\delta^{13}C$ of organic matter is influenced by many environmental factors, including light intensity, atmospheric CO_2 levels and water availability [57]. Furthermore, mycorrhizal fungi are

enriched in ^{13}C compared to their host trees, which is consistent with fungi receiving up to 20% of the total carbon fixed by their host trees [58,59]. In summary, forest ecosystems are driven by their complex settings [60], thus making it not possible to discriminate geographical origin among truffles based on their carbon isotope signatures. The $\delta^{15}N$ and $\delta^{13}C$ values in Slovenian samples are comparable to other samples and ranged from 4.2‰ to 19.6‰ and from −28.5‰ to −24.5‰, respectively. The highest $\delta^{15}N$ and the lowest $\delta^{13}C$ values were also observed in *T. magnatum* from Slovenian Istria, while the highest $\delta^{13}C$ value was observed in Dinaric region (Marija Snežna). The $\delta^{13}C$ value of −26.6‰ observed in Snežna jama is indeed enriched in ^{13}C compared to the plant material available at this location ranging from −32.8‰ to −27.0‰ [61].

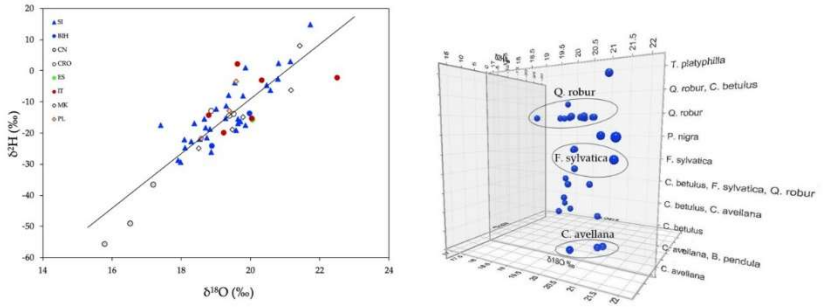
A broad range of $\delta^{34}S$ values (−15.4‰ to +11.3‰) was also observed (Table S3). The lowest and the highest $\delta^{34}S$ values were recorded in the Italian samples, which is consistent with the high heterogeneity of Italian forest ecosystems with high fungal biodiversity [62]. Typically, 95% of the forest soil sulphur is in organically bound forms such as the ester sulphate, which is synthesised by soil microorganisms, and carbon-bonded S [63,64], where plants take up sulphur primarily as the sulphate anion, SO_4^{2-} [65]. The incorporation of sulphur into the fungi is, therefore, influenced by root access to SO_4^{2-} [66]. Since there is little or no fractionation of the sulphur isotopes in plant metabolism, plants will have $\delta^{34}S$ values reflective of those in rainwater, which in turn will influence the isotopic ratio of sulphur in the truffle. However, sulphur not only has multiple biological roles, but it is also a key component of volatile substances that add to the unique truffle aroma such as dimethyl sulphide, which is the predominant aroma compound in black truffle [67]. Recently, two new sulphur compounds were identified in the aroma of black truffle, 1-(methylthio)propane and 1-(methylthio)-1-propene, while a key compound responsible for white truffle aroma is bis(methylthio)methane [14,67]. To date, no indication of the range of $\delta^{34}S$ values in the aroma compounds of truffles has been reported in the literature. Thus, we believe that such a high range in $\delta^{34}S$ values and especially very low $\delta^{34}S$ value of −15.4‰ found in *T. magnatum* from Perugia could be related to sulphur metabolic pathway in truffles that needs to be further investigated.

Delta ^2H values range from −56.0‰ to 14.8‰ (mean = −15.8‰ ± 13.0‰), and $\delta^{18}O$ values from 15.8‰ to 22.5‰ (mean = 19.4‰ ± 1.3‰). The lowest δ^2H (mean = −47.4‰ ± 9.7‰) and $\delta^{18}O$ (mean = 16.5‰ ± 0.7‰) values were recorded in samples from China. In Slovenian truffles, higher δ^2H and $\delta^{18}O$ values were observed in Sežana due to the mild and dry climate comparing to other regions. Figure 5a reveals how the isotopic ratios of these two elements are tightly linked, providing information about the composition of environmental water. The observed slope is similar to a slope of eight ($\delta^2H = 8 \times \delta^{18}O + 10$) in the meteoric water relationship [68], indicating that both isotopes originate from local meteoric water and that metabolism and biosynthesis are of minor importance. Hydrogen and oxygen fixed in the same tissues may be a mixture of atoms derived from body water and water-tissue fractionation during biosynthesis.

Trees can develop deep root systems, the activity of which can be traced using the stable isotopes of water (δ^2H and $\delta^{18}O$). Sources from which trees take up water (soil water at different depths, fog, dew, and groundwater) tend to have different isotopic compositions due to evaporative fractionation and the rainout effect and as a result of carbon fluxes in mixed forest ecosystems through the assimilation of CO_2 during photosynthesis [69,70]. It is thought that isotopic fractionation does not occur during water uptake by plants, which means it is possible to identify the source of the water, i.e., rainwater or groundwater. Interestingly, Barbeta et al. [71] recently raised the possibility of fractionation during root-soil interactions, taking into account the effect of different soil and plant root characteristics on the exchange of water, carbon and the atmosphere. The authors observed that *Quercus robur* used deeper soil water with more negative δ^2H and $\delta^{18}O$ values than *Fagus sylvatica*, which typically has a shallower root system. It is interesting to also note that truffle samples associated with *Quercus robur* have lower δ^2H and $\delta^{18}O$ values comparing to truffles associated to *Fagus sylvatica* (Figure 5b). Water access also depends on soil porosity. Thus, δ^2H and $\delta^{18}O$ values of the truffles will record the geographic information associated with a specific location. In theory, the δ^2H and $\delta^{18}O$ signatures in truffles could

be affected not only by changes in transpiration but also by the photosynthetic reactions occurring in the host trees [69].

Considering these findings, it is likely that isotopic signatures in truffle ascocarps depend on the dominance of tree species in mixed forests. *T. aestivum* is mainly associated with the *Quercus* spp., *Carpinus betulus*, *Betula pendula*, *Fagus sylvatica*, and *Corylus avellana* trees (Table S1, Supplementary Material), thereby showing different δ^2H and δ^{18}O isotopic signatures (Figure 5b). Collectively, a combination of precipitation and temperature affects the functioning of the forest ecosystem, profoundly changing the environment in which truffles grow.



(a) (b)

Figure 5. (**a**) A plot of the relationship between δ^2H and δ^{18}O of truffle samples from eight countries: SLO (Slovenia), BIH (Bosnia and Herzegovina), CN (China), CRO (Croatia), ES (Spain), IT (Italy), MK (North Macedonia), PL (Poland). A line drawn through the data in the plot shows that the data are strongly linked together ($y = 8.1x - 171.8$; $r^2 = 0.75$, $p < 0.001$); (**b**) comparison of natural isotopic abundance (δ^2H and δ^{18}O) of *T. aestivum* in association with host tree species in mixed forest systems (Table S1, Supplementary Material).

2.3. Strontium Stable Isotope Ratios

Isotope application of heavier elements such as Sr can provide additional information on geographical origin since plants inherit the isotopic signature from their geological and pedological environment [72]. In order to improve the geographical discrimination of truffle samples, we analysed ^{87}Sr/^{86}Sr values and combined them with Sr levels and Rb/Sr, Sr/Ca, Ba/Ca, and Mg/Ca molar ratios (Table 2).

A wide range of Rb/Sr, Sr/Ca, Ba/Ca, and Mg/Ca ratios of the truffles were observed (Table 2). The highest Sr/Ca and Ba/Ca ratios were determined in *T. magnatum* from Lukini (Slovenian Istria) compared to the rest of samples, regardless of geographical origin and species affiliation. The highest Mg/Ca ratios were also observed in Slovenian samples *T. brumale* from Marija Snežna, followed by *T. magnatum* from Lukini and *T. aestivum* from Rajndol. A high Mg/Ca ratio is related to dolomite weathering, while a low Mg/Ca ratio typically less than 0.1 corresponds to calcite weathering conditions. The bedrock in Slovenia is primarily composed of Mesozoic carbonates (limestone and dolomite) and siliclastic sediments exposed near the surface, especially in areas with high topographic relief. The majority of dolomite bedrock is found in the Dinaric karst region. The siliclastic sediments are primarily Late Paleozoic, the limestone rocks are primarily Triassic, and the dolomites are primarily Jurassic in age. As is seen, most Slovenian locations are located in the Dinaric karst region, where dolomite prevails.

Table 2. Summary of the $^{87}Sr/^{86}Sr$ rato data for truffle species of different geographical origin, associated with local soil geology (Table S1, Supplementary Material), concentrations of Sr and molar ratios of Rb/Sr, Sr/Ca, Ba/Ca and Mg/Ca.

Species	Country	Location	Sr $_{mg/kg}$	Rb/Sr	Sr/Ca	Ba/Ca	Mg/Ca	$^{87}Sr/^{86}Sr$
	SLO	Meja	3.13	0.0019	0.0006	0.0009	0.50	0.71212
	SLO	Pluska	2.28	0.0020	0.0005	0.0006	0.94	0.71088
	SLO	Spodnje Blato	2.66	0.0049	0.0004	0.0006	0.78	0.71094
	SLO	Žlebič	1.45	0.0039	0.0005	0.0008	0.95	0.70985
	SLO	Bloke	2.67	0.0052	0.0005	0.0009	1.05	0.71375
TUBAES	SLO	Sežana	7.32	0.0019	0.0007	0.0009	0.87	0.70862
	SLO	Rajndol	1.75	0.0076	0.0004	0.0010	1.19	0.71151
	PL	n.d.	18.8	0.0003	0.0025	0.0011	0.41	0.70896
	IT	Perugia	4.35	0.0020	0.0006	0.0005	0.41	0.70894
	CRO	n.d.	2.54	0.0067	0.0005	0.0012	1.13	0.71102
	BIH	Šipovo	7.19	0.0010	0.0011	0.0011	0.56	0.70975
TUBBRU	SLO	Marija Snežna	2.98	0.0088	0.0014	0.0015	1.67	0.70868
TUBIND	CN	n.d.	4.92	0.0002	0.0018	0.0006	0.94	0.70953
TUBMAG	SLO	Lukini	10.7	0.0014	0.0040	0.0040	1.55	0.71105
TUBMEL	ES	Cantavieja	7.34	0.0013	0.0006	0.0007	0.26	0.71219

The proportion of ^{87}Sr to total Sr increases at a rate dependent on the available Rb in soil minerals. Accordingly, geological regions rich in Rb relative to Sr will have a high $^{87}Sr/^{86}Sr$ ratio, while regions with low Rb relative to Sr will retain low $^{87}Sr/^{86}Sr$ ratios for long periods of geological time. It was found that the Rb and Sr concentrations in Slovenian truffles were in the range from 1.16 to 25.7 mg/kg and 1.45 to 18.8 mg/kg, respectively (Table 2, Table S2). The highest Rb/Sr concentration ratio was observed in samples from Marija Snežna. The $^{87}Sr/^{86}Sr$ isotope ratio values in Slovenian truffles ranged from 0.70862 to 0.71375. Most of the samples were in the range from 0.710 to 0.713. The lowest $^{87}Sr/^{86}Sr$ values were determined in Sežana and Marija Snežna, 0.70862 and 0.70868, respectively. The highest $^{87}Sr/^{86}Sr$ ratio was determined in truffles from Bloke, a karst plateau, composed mainly from limestone and dolomite [73]. It is notable that truffles from Žlebič had lower $^{87}Sr/^{86}Sr$ isotopic ratios compared to truffles from Bloke and Rajndol. The central parts of Slovenia are mainly covered in Quaternary terrestrial deposits (gravel and sand), potentially contributing to high $^{87}Sr/^{86}Sr$ isotopic ratios of truffles from Spodnje Blato, Pluska, and Meja. Comparing the $^{87}Sr/^{86}Sr$ ratios in the analyzed Slovenian truffles with the predictions of the $^{87}Sr/^{86}Sr$ ratios in the bioavailable fraction of the soil [74], it is evident that in general there is a certain degree of correlation between truffles and soil. The exceptions were the truffles from Bloke that had much higher isotope ratio than the predicted value of the soil. However, it should be noted that the soil analyzed by Hoogewerff et al. [74] is farmland which is often treated with lime and fertilizer, which can alter strontium composition [75], whereas the truffles presumably are collected in forests that receive no or minimal treatment. Thus the baseline maps made using farmland soil samples may not reflect the strontium isotopic composition of forests in a given area.

From the foreign truffle samples, the highest $^{87}Sr/^{86}Sr$ ratio of 0.71219 was determined in truffles form Cantavieja, Spain. Truffles from Perugia, Italy and Poland had low $^{87}Sr/^{86}Sr$ ratios. The geological deposits of Umbri, a where Perugia (500 m above sealevel) is located, consists largely of limestone (formed in the ocean) that has the $^{87}Sr/^{86}Sr$ ratio of the ocean at the time of formation [76,77]. In Poland, *T. aestivum* are dominant in the southern part, where the lithology comprises Jurassic and Cretaceous limestone and marlstone on rendzic leptosols [78], having intermeditate Sr isotopic values, 0.706–0.709 [79,80]. The relief of the Šipovo region is mostly formed from the Lower to Middle Devonian sedimentary material composed of lime rocks and dolomite [81]. Truffles *T. indicum* are native to southern China, in the Sichuan and Yunnan provinces where terrain is dominated by metamorphic or igneous rocks [82]. This could explain the lowest Rb/Sr ratio in the Chinese truffles tested.

The large variability of Sr ratios in truffles reflects the wide diversity of the local biogeochemistry of the environment in which truffles grow. This could be useful for the assignment of the geographic origin of truffles. For example, for truffles coming from different regions within the same climate zone (and therefore having similar δ^2H and $\delta^{18}O$ values), supplementary information about the $^{87}Sr/^{86}Sr$

isotope ratio may contribute an additional level of geographical resolution, provided that different lithologies exist within a certain region. As the $^{87}Sr/^{86}Sr$ ratio plays a significant role in authenticating the geographical origin of environmental and food matrices, it is important to identify where the Sr fingerprint in truffles comes from. Therefore, for a statistical approach to the geographical origin of truffles, a large dataset of precise and accurate $^{87}Sr/^{86}Sr$ values is needed in order to evaluate the indicator variability range of both the truffle and the soils and to build classification models. Despite the small sample size of the present study, the determined $^{87}Sr/^{86}Sr$ values indicate, as expected, that truffles reflect the local geochemistry of the environment in which they grow. Although it is difficult to determine quantitatively which Sr source exerts dominant control over $^{87}Sr/^{86}Sr$ in truffles, the $^{87}Sr/^{86}Sr$ of truffles appeared at the first glance to be controlled by the carbonate fraction of soil, which makes Sr most useful for determination proveniance in areas without limestone. It should also be pointed out that this approach is associated with uncertainties and site-specific challenges, since the mycorrhizal relationships with trees and mineral weathering are equally complex, often involving varying combinations of Sr sources and isotopic signatures, thus making determining the truffle's geographical origin a yet greater challenge.

2.4. Geographical Discrimination of Truffle Samples

Multivariate discriminant analysis (DA) was used to classify truffle samples based on elemental compositon and isotopic values. Stable isotopes and elemental fingerprinting were then used to determine the geographical origin of truffles collected from seven countries: Slovenia (n = 31), Italy (n = 6), North Macedonia (n = 5), Croatia (n = 3), Poland (n = 3), China (n = 3), and Spain (n = 2). The statistical method was used to check the two- and three-dimensional charts to test if the groups to which the observations belong are distinct and to show the properties of the groups using explanatory variables.

A confusion matrix was also constructed to describe the classification performance. This method can be used to create a predictive framework. After optimization of the model, including 33 variables, five variables were excluded: Mg/Ca, Cd, Mn, $^{87}Sr/^{86}Sr$ and Rb/Sr. Therefore, the analysis was performed using 28 variables belonging to the origin classes: SLO (Slovenia), IT (Italy), MK (North Macedonia), CRO (Croatia), PL (Poland), CN (China), and ES (Spain). Figure 6 shows a two-dimensional chart with centroids and confidence circles at a significance level of 5%. Cross-validation resulted in 77% correct classification.

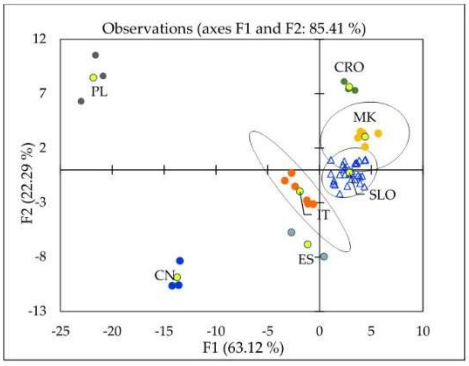


Figure 6. Projection of discriminant analysis (DA) of truffle samples regarding their geographical origin: CN (China), CRO (Croatia), ES (Spain), IT (Italy), MK (North Macedonia), PL (Poland), SLO (Slovenia). Yellow markers refer to centroids.

The geographical discrimination of truffle samples is also presented in Orange Visual Programming by using linear projection. A different colour represents each origin class, and the score was computed as follows: for each data instance, the method finds the ten nearest neighbours in the projected 2D space, that is, on the combination of attribute pairs. It then checks how many of them have the same colour. The total score of the projection is then the average number of same-coloured neighbours. Computation for continuous colours is similar, except that the coefficient of determination is used to measure the local homogeneity of the projection. The model gives the same discrimination result as obtained by the XLSTAT software, but more importantly, it gives also an excellent graphical projection of the importance of each variable (Figure 7).

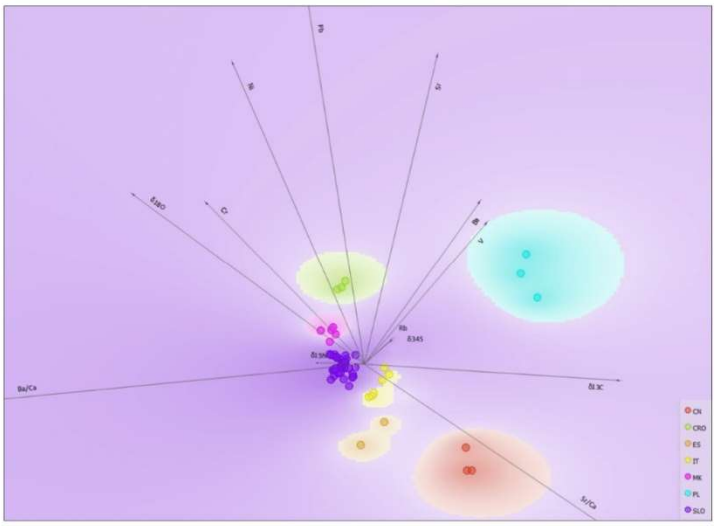


Figure 7. Projection of unit vectors for discriminant analysis (DA) of truffle samples regarding their geographical origin: CN (China), CRO (Croatia), ES (Spain), IT (Italy), MK (North Macedonia), PL (Poland), SLO (Slovenia).

Projections of unit vectors, that is, their corresponding anchors, that are very short compared to the others indicate that their associated attribute is not very informative for a particular classification task. The most important anchors (variables) that separate Polish truffles from other countries truffles are Sr, δ^{13}C, Ba, and V. These parameters could be related to different forest ecosystems and soil properties that exhibit higher concentrations of Sr and low concentrations of Ba and V. The δ^{13}C values are also higher. Samples from Croatia and North Macedonia are similar, but both groups are well separated from the other countries based on their Pb, Sr, Ni, Cr content and δ^{18}O values. Chromium and δ^{18}O are also important for distinguishing between these two groups. In particular, δ^{18}O could be related to different climatic conditions, since the North Macedonia truffles were collected at altitude (>2000 m). Climatic conditions are also an important factor that separates Slovenian truffles from the Italian, Spanish and Chinese truffles. However, between these groups, separation is possible based on the Ba/Ca ratio and Sr/Ca, indicating different soil properties and geology. The last anchor separates Italy, Spain and China. Although other variables are less important, they still contribute to the overall good discrimination and must be included in the model, while Cd and Mn should be excluded. The reason why these two elements do not contribute to the good discrimination of the groups remains unclear. For example, Cd concentrations are highly variable in the samples and as Cd is volatile, it may be that

high Cd samples are from areas with more airborne pollution, which does not really correlate with any specific geographic origin.

2.5. Species Discrimination of Truffle Samples

A model was also developed for discriminating truffles based on species. The analysis included 16 different variables belonging to the six origin classes: TUBAES (*T. aestivum*), TUBBRU (*T. brumale*), TUBMAG (*T. magnatum*), TUBMEL (*T. melanosporum*), TUBMES (*T. mesentericum*), and TUBIND (*T. indicum*). Certain species were collected only from one country; therefore, to eliminate of the effect of the geographical origin, several parameters were excluded, since their signature relates to geological and pedological environment (δ^{34}S, Ba, Ba/Ca, Cs, δ^{18}O, δ^{2}H, Sr, Mg/Ca, Rb, δ^{13}C, and Sr/Ca, ^{87}Sr/^{86}Sr and Rb/Sr), or pollutants (Pb, Al, Cd, and Hg). Figure 8 shows a two-dimensional chart with centroids and confidence circles at a significance level of 5%. Cross-validation resulted in a 74% correct classification.

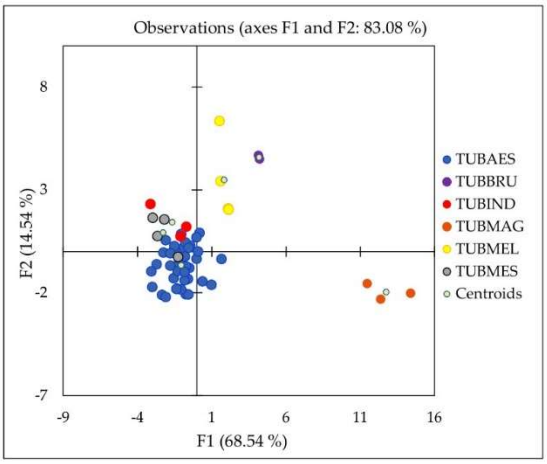


Figure 8. Projection of discriminant analysis (DA) of different truffle species: TUBAES (*T. aestivum*), TUBBRU (*T. brumale*), TUBIND (*T. indicum*), TUBMAG (*T. magnatum*), TUBMEL (*T. melanosporum*), and TUBMES (*T. mesentericum*).

This is only the preliminary study with a limited number of samples present in certain classes. Nevertheless, the discrimination of some specific species such as for *T. magnatum* (100% correct classification) and *T. aestivum* (87% correct classification) gives satisfactory results. The key variables that distinguish *T. magnatum* are δ^{15}N, V and Zn. The Zn content of *T. magnatum* was twice that of the other truffle species (Figure 3), which supports Popović-Djordjević et al.'s [34] finding that *T. magnatum* can assimilate/accumulate Zn. Unfortunately, in their study, V was not determined. High δ^{15}N values determined in *T. magnatum* could be related to differences in the source of organic N or the depth of N acquisition. *Tuber brumale* and *T. melanosporum* can be distinguished based on δ^{15}N values and the levels of Na, Cu, S and P.

Also, these two species exhibit higher δ^{15}N values compared to other species, but not as high as *T. magnatum*. These values can be explained by different nutrient sources, although isotopic fractionation during the transport of nitrogenous compounds from truffles to their host plants cannot be excluded. *Tuber brumale* can also assimilate/accumulate Na, Cu, S and P [34]. The assimilation of S is likely related to sulphur metabolism in truffles, which appears to be active in ascocarps of *T. melanosporum*, especially the part related to the production of volatile substances [83]. *Tuber aestivum*,

T. mesentericum and *T. indicum* are separated from other species based on the levels of Ni, Cr, Mn, Mg, As, and Cu, which are related to regional soil properties. However, to characterise different truffle species more precisely, the analysis of a higher number of countrywide representative samples is needed. In addition, the differentiation according to the species could be further improved if other parameters such as volatile organic compounds, esters, amino acid, organic acids were included using appropriate chemometric tools.

3. Materials and Methods

3.1. Sample Collection

Samples (n = 58) of truffle species (*Tuber aestivum* (40), *Tuber melanosporum* (5), *Tuber mesentericum* (3), *Tuber brumale* (2), *Tuber indicum* (3), *Tuber macrosporum* (1), and *Tuber magnatum* (4)) were collected directly from their natural and cultivated habitats from seven countries—SLO (Slovenia), IT (Italy), MK (North Macedonia), CRO (Croatia), BIH (Bosnia & Herzegovina), PL (Poland), CN (China), and ES (Spain)—during harvest season (from August 2018 to February 2019) (Figure 9). The details of harvesting dates are included in Table S1. To discriminate between species and origin multi-elemental composition and stable isotope ratio analyses were performed. Further, fifteen samples were selected for determining the natural variation of $^{87}Sr/^{86}Sr$ isotope ratios of the truffles.

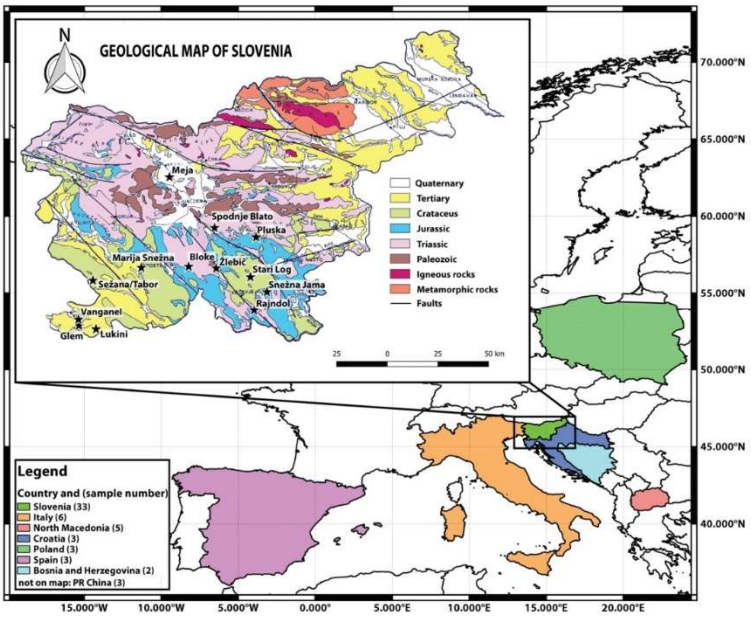


Figure 9. Geologic map of Slovenia (GeoSZ, 2013) with sample sites from the dataset (Table S1, Supplementary Material) marked as black stars. In the background are colored European countries with marked natural sites where truffles were collected: Italy, North Macedonia, Croatia, Poland, Spain, and Bosnia and Herzegovina. Only samples from China were purchased on the market.

3.2. Reagents, Standards, Calibration Solutions and Samples

Ultrapure water (18.2 MΩ cm) was obtained using a Milli-Q Element System (Merck Millipore, Watertown, MA, USA). Nitric acid (HNO_3, Suprapur 65%), hydrogen peroxide (H_2O_2, Emsure ISO 30%), and hydrofluoric acid (HF, Suprapur 40%) were purchased from Merck (Darmstadt, Germany). All polypropylene materials were cleaned by soaking them in 10% (*v*/*v*) HNO_3 solution, thoroughly rinsed with MilliQ water and dried before use.

An inductively coupled plasma (ICP) multi-element standard solution XXI (10 mg/L) containing the following elements: Al, As, Ba, Ca, Cd, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Rb, Sr, V, and Zn, was obtained from Merck (Darmstadt, Germany). A standard solution of Hg (10 mg/L) was prepared separately. A series of ICP single-element standards (1000 mg/L) of P, S, Sr, and Rb, were also obtained from Merck (Darmstadt, Germany). Strontium and Rb standards were used only after a chromatographic extraction procedure. Since no suitable certified and standard reference material is available for fungi, the accuracy of the sample pretreatment method was assessed using the two certified reference materials: tomato leaves (NIST 1573a) and peach leaves (NIST 1547), both acquired from the National Institute of Standards and Technology, NIST (Gaithersburg, MD, USA).

The accuracy of the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ determination was checked with the following international and laboratory reference materials: ammonium sulfates IAEA-N-1 ($\delta^{15}N$ = +0.43‰ ± 0.07‰) and IAEA-N-2 ($\delta^{15}N$ = +20.41‰ ± 0.16‰), barium sulfate NBS 127 (+21.12‰ ± 0.22‰), casein OAS (Sercon; $\delta^{13}C$ = −26.98‰ ± 0.13‰, $\delta^{15}N$ = +5.94‰ ± 0.08‰, $\delta^{34}S$ = +6.32‰ ± 0.8‰), and casein IAEA-CRP ($\delta^{13}C$ = −20.3‰ ± 0.09‰, $\delta^{15}N$ = +5.62‰ ± 0.19‰, $\delta^{34}S$ = +4.18‰ ± 0.74‰). The 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‰ standards were used for $\delta^{2}H$, and $\delta^{18}O$ determination.

For Sr-matrix separation, a Sr-specific resin (TrisKem International, Bruz, France) was used. For $^{87}Sr/^{86}Sr$ isotope ratio analysis, all samples were analysed in a sample-standard bracketing sequence with a Sr isotopic standard solution of NIST SRM 987 $SrCO_3$ (strontium carbonate, $^{87}Sr/^{86}Sr$ = 0.71034 ± 0.00026, NIST, Gaithersburg, MD, USA).

3.3. Sample Pretreatment Method

Soil residues were removed from the truffles either by mechanical cleaning of the fruiting body or by washing with Milli-Q water. After the cleaning, the fruiting bodies were cut into 1–2 mm thick slices using a ceramic knife and then freeze-dried. The analysis was performed using the peridium. After lyophilisation, the samples were ground and homogenised before analysis.

Each freeze-dried sample (0.10 g) was weighed directly into a teflon microwave digestion vessel, to which was added 2 mL of HNO_3. The sample was then digested using an UltraWAVE™ microwave system (Single Reaction Chamber Microwave Digestion System, Milestone, Soristone, Italy). The program was as follows: a 20-minute temperature increase to 240 °C, held for 15 min at 100 bar and then allowed to cool to room temp. Two replicates were prepared for each sample. Certified reference materials and blank samples were also prepared.

Each solution was quantitatively transferred into a 10 mL polyethylene graduated vials and filled up to the mark with Milli-Q water. In order to remove any residuals, samples were filtered through Millex-HV syringe filters (0.45 µm, Millipore hydrophilic PVDF filter membrane; Merck Millipore Ltd., Tulagreen, Ireland) and stored at 4 °C until analyses. After each mineralisation cycle, a cleaning cycle was performed with 2 mL of HNO_3:H_2O (1:1, *v*/*v*) to eliminate cross-contamination.

3.4. Analytical Procedure and Instrumentation

Multi-elemental (ICP-MS) analysis and stable isotope determination ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, $^{87}Sr/^{86}Sr$) was performed at the Department of Environmental Sciences, Jožef Stefan Institute in Ljubljana,

while the determination of $\delta^{18}O$ and $\delta^{2}H$ values was obtained at the Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach in San Michele all'Adige, Italy.

3.4.1. Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

Measurements were performed on an Agilent 8800 triple quard instrument (ICP-QQQ, Agilent Technologies, California, USA). Twenty-three elements (Na, Mg, Al, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Rb, Sr, Cd, Cs, Ba, Hg, and Pb) were determined using a multi-element method. Multi-element standards for external calibration was prepared using the ICP standard solution XXI and single-element standards (P and S), which were diluted with MQ water with the addition of HNO_3 (5%, *v*/*v*). Two external calibration curves were prepared. A six-point calibration covered the range between 0 and 10 ng/g for Hg, while nine-point calibration curve covered the range between 0 and 250 ng/g for other elements investigated.

All measurements were made under strict quality control procedures. Blank samples and reference materials were run together with the samples daily. The limits of detection (LOD) were calculated as three times the standard deviation of blank noise. The mean values of the replicate sample measurement were used for data analysis. Levels of Al, Ni, and Pb in some samples were below LOD. However, elements were not excluded if a significant number of samples were concentrated markedly above the LOD level as they may be characteristic of provenance. The LODs for Na, Mg, Al, P, S, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cr, Zn, As, Rb, Sr, Ni, Cd, Cs, Ba, Hg, and Pb was 5.5, 1, 3.5, 5.5, 5, 20, 6, 0.013, 0.05, 0.02, 0.7, 0.005, 0.1, 0.2, 0.75, 0.007, 0.01, 0.08, 0.0025, 0.003, 0.06, 0.004, and 0.025 mg/kg of truffle sample, respectively.

3.4.2. Quantitative-XRF Analysis

Micro-XRF imaging of freeze-dried tuber hand cross-sections ($1 \times 1 \times 0.1$ cm) was performed at the Synchrotron Light Research Institute, Thailand at the BL6b beamline. The BL6b utilized continuous synchrotron radiation from the bending magnet with energy range from 2–8 keV and the beam size of 100 μm at the sample position using polycapillary half-lens as the X-ray optics. To perform the measurements, the sample was placed on a three-degrees of freedom high precision motorized stage in air. The XRF signal was collected by an AMPTEK single-element Si (PIN) solid-state detector with thin Be window and energy resolution of 150 eV at 5.9 keV [84].

The tuber cross-sections were mounted between two layers of a 4 μm Mylar foil stretched on an Al frame and raster scanned with a 100 μm polychromatic beam and a 100 μm step-size. The X-ray fluorescence spectra obtained in each pixel were batch fitted by PyMCA [85]. Due to the complexity of polychromatic synchrotron excitation, quantification was performed according to Zidar et al., (2016) [86]. In short, the average intensity was calculated from the intensities of specific X-ray emission lines (Ca-Ka, Fe-Ka, Mn-Ka, Cu-Ka and Zn-Ka) obtained after fitting of XRF spectra by PYMCA in each pixel and assigned to the total Ca, Fe, Mn, Cu or Zn concertation measured in particular cross-section by energy dispersive X-ray fluorescence spectrometry (EDXRF) [87]. Prior to the EDXRF measurement, each particular cross-section was homogenised and pressed to a pellet with a diameter of one centimetre. The EDXRF was performed using an energy dispersive X-ray spectrometer, equipped with a Cd-109 radioisotope source and a Si(Li) detector (Canberra, 157 Meriden, USA). The XRF measurements were performed in air. The analysis of the XRF spectra was performed according to Nečemer et al. [87], and quantification according to Kump et al. [88]. Quality assurance for the element analyses was performed using standard reference materials: NIST SRM 1573a (tomato leaves as a homogenised powder) and CRM 129 (hay powder) were both analysed in the form of pressed pellets.

3.5. Stable Isotope Ratio Analysis of Light Elements

The stable isotope ratios measurements were performed using isotope ratio mass spectrometry (IRMS) and expressed in the δ-notation in ‰ according to Equation (1) [89]:

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

where superscripts i and j denotes the higher and the lower atomic mass number of element E, R_p and R_{Ref} represent the ratios between the havier 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. The δ^2H and δ^{18}O were reported relative to the V-SMOW (Vienna-Standard Mean Ocean Water) standard, δ^{13}C values were reported relative to the V-PDB (Vienna-Pee Dee Belemnite) standard, while δ^{15}N and δ^{34}S were reported relative to AIR and the V-CDT (for Vienna Cañon Diablo Troilite) standard, respectively [89].

The δ^{13}C, δ^{15}N and δ^{34}S values in freeze-dried samples were determined simultaneously using an IsoPrime–100 Vario PYRO Cube (OH/CNS) Pyrolizer/Elemental Analyzer (IsoPrime, Cheadle Hulme, UK). Approximately 4 mg of the sample and 4 mg of tungsten oxide (WO_3) were weighted into a tin capsule, sealed and placed into the automatic sampler of the elemental analyser. Each sample was measured in triplicate, and the average values was considered. The results were normalized against the following international and laboratory reference materials: IAEA-N-1 and IAEA-N-2 for nitrogen; IAEA-CRP-2013 and Casein OAS B2155 Sercon for carbon and suphfur, respectively.

The ^{2}H/^{1}H and ^{18}O/^{16}O values were determined by transferring 0.2 mg of dry truffles into a silver capsule and analysing the sample simultaneously using TC/EA pyrolyser (Thermo Finnigan) coupled to a DELTA XP isotope ratio-mass spectrometer, IRMS (Thermo Scientific). For normalisation of the results, two internal laboratory reference materials were applied: CBS (Caribou Hoof Standard) and KHS (Kudu Horn Standard). Measurements precision was 0.2‰ for δ^{13}C and δ^{15}N, 0.3‰ for δ^{34}S and δ^{18}O and 1‰ for δ^2H.

3.6. Stable Isotope Ratio Analysis of Heavy Elements

Sr isotope ratio determinations were performed using the Nu II multicollector ICP-MS instrument (Nu Instruments, Ametek Inc., United Kingdom). After the microwave digestion, samples were preconcentrated by evaporation to near dryness. The residuals were dissolved in 1 mL of 8 M HNO_3 and the Sr separated from the matrix using a Sr-specific resin. For this, a column was filled with 0.30 g of the resin, which was activated by performing several washing and elution cycles (Table S4, Supplementary Material).

3.7. Statistical Analysis

Statistical analysis included one-way ANOVA and Duncan's test. Probability (p) values of less than 0.05 were used to indicate a significance level. If a 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 non-normally distributed data, a one-way analysis of variance by ranks (Kruskal–Wallis test) was performed.

Further, to identify those parameters that can discriminate truffles according to the geographical origin and/or variety, a multivariate discriminant analysis (DA) was used. The data were evaluated using the statistical software packages XLSTAT (Addinsoft, NY, USA), Orange Visual Programming (University of Ljubljana, Ljubljana, Slovenia) and OriginPro 2018 (OriginLab Corporation, Northampton, MA, USA).

4. Conclusions

A wide variability of the element concentrations within and among truffle species and locations/countries was observed. Despite the fact that the Slovenian truffles shared some similar

characteristics with the samples originating from other countries, differences in the element concentrations suggest that the respective truffle species may respond selectively to nutrients from a certain soil type under environmental and soil conditions. The heterogeneity of geographical and/or environmental factors influencing the element composition of truffles, therefore, enable the possibility to discriminate between geographical origin/species. It was found that a combination of elemental and isotopic profiling coupled to multivariate analysis is a promising tool for characterizing truffles according to geographical origin and species. However, the classification model performance must be improved by increasing the size of the dataset and include other natural tracers such as strontium isotopes ratios, since $^{87}Sr/^{86}Sr$ ratios differ according to the natural geology of the region of production and makes Sr most useful for determination proveniance in areas with and without limestone. Future research should also investigate the maturation stage, water source and availability, in association with specific host tree species that can influence elemental and stable isotope composition of truffles. It is expected that the proposed approach will aid in protecting consumers and truffle producers from fraudulent labeling regarding species affiliation and geographical origin.

Supplementary Materials: The following are available online, Table S1: The summary on geo-environmental information of sampling locations and date of harvesting for Tuber species (n = 58) including latitude, longitude, altitude, climatic conditions, soil geology, geological age and host tree. The data for temperature and amount of precipitation were obtained by the Slovenian Environmental Agency of the Ministry of the Environment and Spatial Planning of the Republic of Slovenia; Table S2: The content of elements (mg/kg) in the peridial layer of fruiting bodies of *Tuber* species (n = 58); Table S3: Natural isotopic abundances of light elements (per mil, ‰) in peridium of the fruiting bodies of Tuber species (n = 58); Table S4: Protocol for effective Sr-matrix separation from the truffle samples.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available from the authors.

Chapter 6

Conclusions

Protecting local produce is not easy, as determining the geographical origin of agricultural produce is challenging and depends on various conditions, e.g. fertilisation, botanical origin, history of the field, climatic conditions during cultivation, location, and soil composition. Therefore, the goal in food authentication has been to develop appropriate analytical methods and tools to identify unique markers that can be used to identify the origin of foods that can resolve questions of authenticity. The aim was to answer specific questions with the results obtained from elemental and isotopic analysis.

In order to assess the strength of the stable oxygen isotope ratio of milk lactose as a possible internal standard for the dectection of adulteration of milk with water, authentic milk samples collected from four dairy farms (Selnica ob Dravi, Črna na Koroškem, Ajdovščina and Ormož) were diluted by the addition of different amounts of water and analysed. In the next step, the study was designed to investigate the possibility of using Sr isotope analysis in combination with multi-element and stable light isotopic compositions to determine the provenance of milk and truffles in Slovenia. For the first time, we screened a set of milk and truffle samples for the determination of Sr isotopes. Using authentic milk samples collected from 43 dairy farms and truffles collected from 7 natural habitats, Sr isotopes representing the pool of bioavailable isotopes in Slovenia were obtained from 77 samples of milk and 33 samples of truffles (*T. aestivum*, *T. brumale*, *T. magnatum*). Questions of intra- and inter-sample variation were addressed before Sr isotope data were organized by soil type, seasonal variation and location in the case for milk, and by soil type and species affiliation in the case for truffles. These parameters were considered throughout the analysis as possible explanations for the variation (or lack thereof) in the observed Sr isotope ratios.

The main findings of this thesis are summarized below, while a discussion of the limitations of this research and future avenues of study is included in the next chapter.

1) Food isotope research can be improved by careful study design and the adoption of standardised sample preparation procedures. There is no universal method for determining the authenticity of foods or their origin. The choice of the appropriate method depends on the matrix to be analysed and must take into account the growing conditions of the plant and the production process of the final product, therefore the choice of the sample preparation procedure plays an important role in determining the origin of foods. In this research, special attention was paid to the decomposition of organic matter in milk samples. We evaluated two sample pre-treatment methods, namely microwave-assisted acid digestion and dry ashing, from the point of view of efficiency of decomposition of organic matter, applicability and limitations. Due to sample loss, higher risk of contamination, and the time-consuming nature of dry ashing, microwave digestion was chosen for our purposes.

2) The results, although promising, cannot ignore possible interannual or annual variability in the isotopic composition of Sr in milk.

3) A preliminary database for Sr isotopic fingerprints of milk samples was established. Such a database can be very important, as it could provide researchers with more knowledge and could simplify determining the origin of milk in the future. Despite the uncertainties regarding the factors of variability within and between milk samples, much can still be learned from Sr isotopic values. As expected, the soil in Slovenia is very heterogeneous and the results showed significant variability of $^{87}Sr/^{86}Sr$ isotopic values between milk samples.

4) Complete separation of regions based only on the $^{87}Sr/^{86}Sr$ ratio in milk was not possible. However, the combination of Sr isotopic profiling coupled with multivariate analysis was found to be a promising tool for milk characterisation according to geological origin. Milk produced in the Quaternary areas had high Sr content and higher $^{87}Sr/^{86}Sr$ values and differed from that produced in carbonate-dominated areas with lower $^{87}Sr/^{86}Sr$ values.

5) Isotope values of Sr in milk samples originate from the cattle diet (feed and drinking water), reflecting geological characteristics of a given area. Discriminant analysis, including the elemental composition and stable isotopes of light elements, showed that the $^{87}Sr/^{86}Sr$ ratio together with $\delta^{13}C_{casein}$ and $\delta^{15}N_{casein}$ values has the main discriminating power to distinguish the Quaternary group from others. The Cretaceous group (carbonate rocks and flysch) was related to the Br content, 1/Sr and $\delta^{18}O_{water}$ values. The overall predictive ability was found to be 63.5 %. Pairwise comparisons using OPLS-DA confirmed that diet and geological parameters are important for separation.

6) Differences in element concentrations indicate that the truffle species in question may selectively respond to nutrients from a specific soil type under environmental and soil conditions. The heterogeneity of geographical and/or environmental factors that influence the elemental and isotopic composition of truffles makes it possible to differentiate truffles according to origin (77 % correct classification) and species (74 % correct classification).

7) A correlation was observed between the $^{87}Sr/^{86}Sr$ ratios measured in truffles and predicted Sr isotopic values in European soils.

8) In the case of adulteration of milk with water, the results showed that even a small addition of water (> 15 %) can be effectively detected.

Chapter 7

Future Perspectives

If one of the goals is to enhance traceability systems in the food industry and the effectiveness of protection of local foods and their origin, then a key implication of this research is the importance of the robust method, as most of the existing analytical methods of food traceability are quite prone to seasonal fluctuations or specific (sometimes even intensive) farming practices. On the other hand, $^{87}Sr/^{86}Sr$ analysis can be valuable for distinguishing uncertain samples or as a confirmatory tool. The findings in this study can serve as a starting point for the development of efficient and robust models based on large datasets, which need to be further validated to evaluate their effectiveness as tools for geographic authentication of milk and truffles. A greater awareness of the unknowns and assumptions associated with the use of Sr isotopic data will serve both to improve the research questions we can currently address and to guide the way forward for future research.

When using Sr isotopes as a geographical tracer, it is important to obtain a reliable fingerprint to assess the origin of Slovenian milk, taking into account both the elemental composition and the isotopic pattern of milk and dairy products, as well as investigating the connection between the composition of the milk and the geographically related composition of soil, feed and drinking water. The standard deviations of milk samples collected from different dairy farms at the same or nearby site were greater than 0.00010, and the differences in $^{87}Sr/^{86}Sr$ ratios appear to reflect the heterogeneity of surrounding soils and differences in cattle diet. Analysis of the $^{87}Sr/^{86}Sr$ can be a very useful tool in food provenance, but its success depends on the precise problem to be addressed and the question to be answered. Heterogeneous geology in individual regions in Slovenia can be a problem for the differentiation of samples originating from these regions due to large variations of Sr fingerprints within these individual regions and consequently the results between regions overlap. In this case, an adequate database of authentic milk samples is needed to ensure that these samples will represent the entire Sr isotopic variability of the area. Given the good correlation between $^{87}Sr/^{86}Sr$ ratios of groundwater and milk, and to better characterise the groups in the data set, future efforts should focus on collecting the soils, feed and local water and combining them with existing data on $^{87}Sr/^{86}Sr$ isotope ratios in milk.

As already mentioned, Sr isotopes are strongly related to the geological characteristics of the soil. Nevertheless, relying solely on $^{87}Sr/^{86}Sr$ values to determine the specific geographic origin of milk may not be reliable because feeding patterns (e.g. imported feed) can cause overlapping and confounding isotopic variations, even in milk. The complexity of animal diet presents a challenge for determining whether milk is produced locally. It is therefore important to temper interpretation with dietary isotope proxies such as carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and sulphur ($\delta^{34}S$). Results that deviate significantly from the

majority of the Sr isotopic values in the data set should also be considered, as such data may contain valuable information that is part of the study.

Although there are obvious limitations in distinguishing the origin of different foods on a global scale, the Sr isotope tracing technique remains a powerful and reliable tool for determining the geographic origin of foods when combined with detailed knowledge of geological and soil characteristics. Limited Sr isotope data are currently available for milk and dairy products, so it is crucial to collect more information on this topic. Further investigations are highly recommended to fill this knowledge gap and improve the understanding of its applicability for traceability purposes. Different approaches should be evaluated simultaneously and then optimized as necessary, as studies using Sr isotopes are usually performed on a smaller scale, i.e. locally. Without a large amount of data, such comparisons between different regions and/or countries can be risky. One way to fill this gap is to develop a Sr isoscape baseline for the Slovenian territory, which could provide a useful reflection of the underlying geology of the area to inform food researchers in the coming years. This could also be useful in forensic, archaeological and anthropological applications. Furthermore, the impact of anthropogenic activities such as agricultural practices involving the use of fertilizers and the impact of sea spray in coastal areas have in some cases been difficult to determine based on the $^{87}Sr/^{86}Sr$ signature alone. These influences can be distinguished when considered in combination with elemental composition data sets. For example, most researchers use information on the composition of major and minor elements to authenticate foods, so the next step should also include the use of rare earth elements in combination with $^{87}Sr/^{86}Sr$ isotope ratios to investigate whether they might be regionally specific.

Similarly, for a correct classification of the geographical origin of ectomycorrhizal fungi, it would be necessary to include a large set of Slovenian truffles and different types of truffles. By comparing with literature data, we confirmed the correlation between $^{87}Sr/^{86}Sr$ ratios measured in truffles and predicted Sr isotopic values in European soils. For actual work, the measured Sr isotopic data of local soils can be used as a reference map for the entire distribution of $^{87}Sr/^{86}Sr$ values in Slovenian truffles. As a result, in order to confirm a certain origin and identify an unknown origin, it is necessary to establish reference databases of isotopic ratios of water and soil for different locations in Slovenia. Given that some habitats in Slovenia share similar geology and climate, it is possible that truffles will have an identical isotopic signature. It is therefore important to identify area-specific markers in order to differentiate between truffle samples and thereby improve their potential for authenticity and traceability of geographical origin and/or counterfeit detection. Despite the promising preliminary results of the study, the insufficient sample size does not allow the use of multivariate analysis, so the Sr isotopic values of truffles were not included in the statistical analysis. However, the observed variability in elemental composition suggests that the combination of Sr isotopic values with other natural tracers, such as Ba/Ca, Mg/Ca and Sr/Ca ratios, could further increase the possibilities for distinguishing the origin of truffles based on the composition of truffles in a given area.

As truffles undergo a complex life cycle in association with various forest species, future research should also investigate the maturation stage, water source and availability, in association with specific host tree species, which may affect the elemental and stable isotopic composition of truffles. It is believed that the proposed approach will help protect consumers and truffle producers from fraudulent practices.

Given the ever-growing field of application and the continuous development of newer generations of mass spectrometers with better high resolving power and mass accuracy, the future use of Sr isotopes in food science will surely grow over time. In addition to the $^{87}Sr/^{86}Sr$ isotope ratio, boron and lead isotope analysis by MC-ICP-MS could also be useful for provenance studies. Lead is continuously released from natural and anthropogenic

sources into the atmosphere and deposited on the Earth's surface through dust and precipitation. During atmospheric circulation, plants absorb some lead in a form available for bioaccumulation, so the different contributions of Pb are expressed in plants. On the other hand, boron absorption in plants depends on soil properties. By combining isotopes of lead, boron and strontium, it would be possible to gain insight into the atmosphere, soil and geology from which the food was grown/produced. The use of lead and boron isotopes, also in combination with Sr isotopes, as tools in provenance and authentication studies has been successfully demonstrated on tea, coffee and wine [240], [242], [373].

Furthermore, it is recommended to use CRMs to verify the performance of instruments, validate analytical procedures including sample digestion and Sr separation, calculate measurement uncertainties as well as for monitoring the measurement trueness and assessing the quality of Sr isotope ratio measurements. NIST SRM 987 is usually used to correct the mass bias, however, several studies show that matrix matching and analyte separation is mandatory when external mass bias correction is performed. Since no matrix CRM for $^{87}Sr/^{86}Sr$ isotope analysis of specific food commodity is currently available, this calls for the possible development of Sr isotope standards for food studies. An interlaboratory comparison study on already existing CRMs with similar matrix composition to that of the milk or truffles should be performed which could serve as an example for future food studies and is strongly recommended to be repeated on different matrices.

In order to evaluate the power of the analysis of the ratio of lactose stable isotopes in milk as a potential internal standard for detecting the adulteration of milk with water, analysed authentic milk samples covering three different regions in Slovenia were tested. The $\delta^{18}O$ values alone may not be sufficient to detect adulteration of milk with water, hence the oxygen isotope ratio was determined in lactose extracted from the corresponding milk samples. For authentic milk samples, a mean difference of +0.1 ‰ (range: +1.1 ‰ to -0.9 ‰) was measured with more negative differences, indicating the addition of water. Thus, there is a general consensus that in authentic milk the difference between the $\delta^{18}O$ values of milk and lactose in it should not exceed 1 ‰. For some samples, even a small addition of water can be effectively detected, while for others large amounts of water can be added before they are detected. By simulating authentic milk samples and adulterations, significant improvements can be achieved using multivariate methods, as they are more effective in detecting adulteration even at a lower level.

Appendix A

Method Optimisation

Table A1: Microwave-assisted acid digestion programme used for pre-treatment of milk samples.

Step	**Time**	**Ramp °C/min**	**T final**	**Hold time**	**Power**
1	00:20:00	11°C/min	240°C	30 min	1500 W

Table A2: Operating conditions for the Nu MC-ICP-MS and 7900x ICP-MS at the JSI used for the optimization of the method in milk analysis.

SPS-SW1 was analysed at the beginning and end of each analytical sequence for quality control. Good agreement between certified and determined Rb and Sr concentrations was obtained.

MC-ICP-MS	
RF power	1300 W
Plasma gas flow	13 L min^{-1}
Auxiliary gas flow	0.80 L min^{-1}
Nebuliser gas pressure	38 psi
Sampler cone	Ni, aperture diameter 0.9 mm
Skimmer cone	Ni, aperture diameter 0.7 mm
Integration time	10 s
Number of cycles	30
Number of blocks	2
Mass assignment to Faraday cup detectors	H7 88
	H5 87
	H3 86
	H1 85
	L1 84
	L3 83

ICP-MS	
Nebulizer	Micromist
Spray chamber	Scott double-pass
RF Power	1550 W
Plasma gas flow	15 L min^{-1}
Carrier gas	1.05 L min^{-1}
Makeup gas	0.15 L min^{-1}
Sampling depth	7.5 mm
Sample uptake rate	0.3 mL min^{-1}
Sampling and skimer cones	Nickel
Cell gas (flow rate) / Elements	He Mode (4.3 mL He min^{-1}): ^{88}Sr, ^{85}Rb
Internal standard	^{103}Rh

Supplementary Materials

The provenance of Slovenian milk using $^{87}Sr/^{86}Sr$ isotope ratios

Staša Hamzić Gregorčič[1,2], Nives Ogrinc[1,2], Russell Frew[3], Marijan Nečemer[2], Janja Vrzel[4], Lidija Strojnik[1,2] and Tea Zuliani[1,2,*]

[1] Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, Ljubljana, Slovenia; stasa.gregorcic@ijs.si (S.H.G.); nives.ogrinc@ijs.si (N.O.); lidija.strojnik@ijs.si (L.S.); tea.zuliani@ijs.si (T.Z.)
[2] Jožef Stefan International Postgraduate School, Jamova 39, Ljubljana, Slovenia; S.H.G.; N.O.; marijan.necemer@ijs.si (M.N.); L.S.; T.Z.
[3] Department of Chemistry, University of Otago, PO Box 56, Dunedin, New Zealand
[4] JR-AquaConSol GmbH, Graz, Austria
* Correspondence: tea.zuliani@ijs.si; Tel.: +386 1 477 3542

This supplement material describes the optimisation and validation of an analytical method for $^{87}Sr/^{86}Sr$ isotope ratio determination in milk. The protocol includes: reagents and standard solutions, sample pre-treatment, decomposition of the sample matrix, Sr-matrix separation and method validation.

1. Reagents and standard solutions

Hydrochloric acid (30% (w/w) HCl, Suprapur) and hydrogen peroxide (30% (w/w) H_2O_2) were obtained from Merck Ltd. (Darmstadt, Germany). Nitric acid (68-70% (w/w) HNO_3, Superpure) was obtained from Carlo Erba Reagents Srl (Milan, Italy). For elemental screening, the standard solutions containing Sr and Rb were prepared by dilution of the single-element ICP standards (1000 mg/L, Certipur, Merck, Darmstadt, Germany). Rhodium (Rh), as an internal standard, was prepared by dilution of the single-element ICP standard (1000 mg/L, Certipur, Merck, Darmstadt, Germany). SPS-SW1 Surface-water-trace elements, was obtained from Spectrapure Standards (Oslo, Norway). Ultrapure water with a resistivity of 18.2 MΩ cm was obtained from a Milli-Q Element System (Merck Millipore, Watertown, MA, USA) and used throughout this work for the preparation of standard solutions and diluting concentrated acids and samples. For separation of Sr from the sample matrix, Sr-selective resin (100-150 μm, TrisKem International, Bruz, France) was used. For the quality control of the pre-treatment techniques, namely the total Sr and Rb concentrations, CRMs NIST RM 8435 (whole milk powder; National Institute of Standards and Technology, Gaithersburg, USA) with declared fat content 21.3% and IAEA-153 (milk powder; International Atomic Energy Agency, Vienna Austria) were used. The fat content of IAEA-153 is not declared. This reference material was analysed for interlaboratory comparison to validate the optimised method and determine its $^{87}Sr/^{86}Sr$ isotope ratio value.

2. Sample pre-treatment

As no matrix certified reference material (CRM) for Sr isotope analysis exists, the preliminary experiments were carried out using a sample of whole milk powder labelled PM (whole milk powder, 26% fat content, Pomurske Mlekarne, Murska Sobota) that was purchased on the local market in Slovenia.

Before the sample pre-treatment step, all glass and plastic wares were soaked in 10% (v/v) analytical grade HNO_3 overnight, rinsed with Milli-Q water and dried in the oven. Samples were pretreated using microwave-assisted acid digestion system UltraWAVE (Single Reaction Chamber Microwave Digestion System; Milestone, Soristone, Italy). Freeze-dried and homogenised milk sample (0.30 g) was weighed into Teflon (PTFE) tube, and 6 ml of concentrated HNO_3 was added. The tubes

were closed and subjected to microwave digestion. Samples were pretreated using the program, as shown in Table S1. Further, the resulting solutions were quantitatively transferred into 30 ml polyethylene (PE) graduated vials and filled up to 15 ml with Milli-Q water. Samples were stored at 4 °C until further processing. After each mineralisation cycle, a cleaning cycle was performed with 6 ml of diluted HNO_3 (1:1, v/v) to eliminate cross-contamination.

Table S1. Microwave-assisted acid digestion program used for pre-treatment of milk sample.

Step	Time	Ramp °C/min	T final	Hold time	Power
1	00:20:00	11°C/min	240°C	30 min	1500 W

After mineralisation, sample solutions were evaporated to near dryness on a sand bath at T ≤ 90°C (IKA-C-MAG HP 10, IKA-Werke GmbH & Co., Staufen im Breisgau, Germany). Residual sample materials were redissolved in concentrated HNO_3 and H_2O_2 to destroy any residual organic matter. If organic matter was still present, the evaporation of sample was repeated until the complete destruction of the organic matter. Samples were then dissolved in 8M HNO_3 for subsequent Sr/matrix separation. The Sr selective resin (0.30 g) was weighed directly into 2 ml columns. The columns were rinsed with Milli-Q water, activated with 6M HCl and preconditioned with 8M HNO_3. Measurements of Sr and Rb concentrations in mineralised milk samples and the Sr fraction were performed by ICP-MS (7900x, Agilent Technologies Inc., Tokyo, Japan) to follow the mass balance. For the quality control of the measurements, SPS-SW1 was analysed at the beginning and end of each analytical sequence. Good agreement between certified and determine Rb and Sr concentrations were obtained.

3. Decomposition of the sample matrix

The method was optimised in terms of completeness of mineralisation, chemical recovery of Sr isolated from the sample matrix, minimal contamination, and turnaround time. The blanks of analyte-free media were prepared using the same materials and reagents as for the samples. To monitor the percentage recovery of target analytes and the effectiveness of matrix destruction, total concentrations of Sr were measured by ICP-MS after sample pre-treatment and after the Sr separation procedure (Table S2). The non-digested residual organic matter can strongly affect Sr/Rb separation efficiency on the column. Thus, Sr recovery after Sr/matrix separation step gives us information about the effectiveness of the matrix destruction. The experiments were carried out on a sample of whole milk powder (PM – Pomurske mlekarne), which was previously analysed, and its Sr concentration was estimated to be 2.90 ± 0.08 mg/kg.

The method was optimised based on the recovery of Sr after the Sr/matrix separation using varying sample size (0.30 and 0.70 g) and acid digestion volumes (4 and 6 ml) and acid mixtures (HNO_3 and/or a mixture of HNO_3 and H_2O_2) (Table S2).

Table S2. Comparison of the Sr concentrations obtained after microwave digestion of freeze-dried milk samples (mean ± standard deviation; n = 3).

Sample weight [g]	Reagents	Sr concentration after pretreatment [mg/kg]	Sr concentration after Sr-matrix separation [mg/kg]	Sr recovery [%]
0.70	4 ml HNO_3	2.88 ± 0.08	2.10 ± 0.78	72.5 ± 7.2
0.70	6 ml HNO_3	2.90 ± 0.10	2.27 ± 0.66	78.5 ± 10.0
0.70	5 ml HNO_3 + 1 ml H_2O_2	2.89 ± 0.08	2.04 ± 0.49	70.7 ± 0.8
0.30	4 ml HNO_3 + 2 ml H_2O_2	2.94 ± 0.13	2.59 ± 0.23	88.6 ± 6.7
0.30	6 ml HNO_3	2.98 ± 0.07	2.91 ± 0.28	97.9 ± 1.6

Given the results of the microwave method, all tested samples and reagents quantities gave similar Sr concentrations. Therefore, it can be concluded that total Sr recovery from the sample was obtained. As for accurate isotope determination, 100% recovery is desirable to avoid possible isotope fractionation, and the method with the highest recovery was selected. Low Sr recoveries after the separation (~75%) were found for the sample size of 0.70 g, most probably due to the presence of larger quantities of residual organic matter. Based on this observation, the optimal sample size to digest was

found to be 0.30 g with 6 ml HNO_3, which achieved high Sr recovery (~98%) and was used for further analysis.

To evaluate the accuracy and precision of pre-treatment techniques, NIST SRM 8435 and IAEA-153 were analysed for Sr concentration. The results found for the CRMs were compared with their corresponding certified values and are reported in Table S3. At a 95% confidence level, good agreement with the certified values was found in all cases (Student's *t*-test, $p < 0.05$). The relative standard deviation (RSD) for three replicates of each standard ranges from 0.55 to 1.92%. The Sr concentrations in method blanks from three independent analyses were 0.0026 ± 0.0006 mg/kg.

Table S3. Determined Sr concentrations after pre-treatment in certified reference materials, NIST SRM 8435 and IAEA-153 (mean ± standard deviation; n = 3).

CRM	Sample weight [g]	Pretreatment method	Certified Sr [mg/kg]	Determined Sr [mg/kg]	Sr recovery [%]
NIST 8435	0.30	microwave	4.35 ± 0.50	4.12 ± 0.26	94.6 ± 6.0
IAEA-153	0.30	microwave	4.09 ± 0.34[a]	3.99 ± 0.05	97.3 ± 1.8

[a] Values are informative according to the certificate (based on dry weight).

4. Sr-matrix separation

Regarding the Sr and Rb content in milk samples, optimising the procedure for efficient Sr loading and Rb removal has been performed. Before the loading of the sample onto the Sr selective resin, pretreated samples were evaporated to near dryness and redissolved in 1 ml of 8M HNO_3 to obtain concentrations ranging between 300 – 700 ng Sr/ml before being loaded onto the column and 30 – 70 ng Sr/ml after the column, which is the optimal concentration range for Sr isotope ratio determination by MC-ICP-MS.

Strontium is strongly retained on the Sr resin with the increasing HNO_3 concentration of the sample and eluting solution [25]. While 3M HNO_3 has been shown to adequately separate Sr from Rb during chromatographic extraction separation from biological, environmental, and nuclear waste samples [47], 8M HNO_3 was shown to maximise the separation of other matrix elements such as Ba and Ca [48,49]. As milk contains high concentrations of these elements, 8M HNO_3 was therefore selected. The samples were loaded on the resin in 8M HNO_3, and the resin was rinsed with 8M HNO_3 to maximise the elution of Rb and other possible interferences. Given the high Rb/Sr ratio in milk samples, it was difficult to remove all Rb in a single separation step, as is the case with biological tissues [50] and geological materials [51]. Therefore, after the first separation, the solution was evaporated again to near dryness and redissolved in 1 ml of 8M HNO_3 and submitted for the second separation on a freshly prepared column. The Rb removal was monitored by measuring its concentration before and after the chromatographic extraction procedure by ICP-MS. For this experiment, five different freeze-dried milk samples with different Rb/Sr ratios were analysed. The results are presented in Table S4.

Table S4. The efficiency of Rb removal.

Sample	Before separation Rb [ng/mL]	Rb/Sr ratio	After 1st separation Rb [ng/mL]	After 2nd separation Rb [ng/mL]
Milk 1	2216	8.1	0.103	< 0.005
Milk 2	1297	4.1	0.042	< 0.005
Milk 3	8402	28.0	0.278	< 0.005
Milk 4	5455	17.9	0.115	< 0.005
Milk 5	4858	16.1	0.257	< 0.005

As far as the efficiency of the Sr elution from the resin is concerned, Sr can be eluted from the column with either Milli-Q water or diluted HNO_3. In the present study, Milli-Q water [12,14,20] and

0.05M HNO3 [11,52,53] were used to determine the appropriate eluent. Better Sr yield was achieved by using Milli-Q water.

With a smaller resin size (0.20 g), it is possible that a small amount of strontium was flushed away from the column, which resulted in lower recovery compared to 0.30 g resin. The lowest extraction efficiency was observed using 0.70 g resin, probably due to the inadequate quantity of mobile phase. The optimal size of the resin was found to be 0.30 g and was used in further experiments (Figure S1).

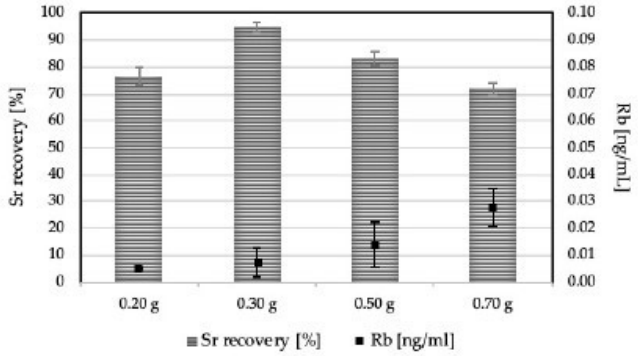

Figure S1. Extraction efficiency using different amounts of the Sr-Spec resin.

5. Method validation

Unfortunately, no matrix-matched certified reference materials are available for the $^{87}Sr/^{86}Sr$ isotope ratio determination in milk. Consequently, the optimised method's repeatability, precision, and trueness were tested by analysis of the candidate reference material, IAEA-153.

Eight independent replicates were prepared in two days (4 each day). One sample from each day was measured three times. All measurements were carried out under optimised experimental parameters. Estimation of the measurement uncertainty for the Sr isotope ratio was done according to the guide to the expression of measurement uncertainty JCGM 100:28 and ISO GUM [54,55], applying the uncertainty propagation law without using second-order covariances. Combined uncertainty included contributions from the ion intensity measurements, mass bias corrections, and contributions from certified values of relevant ratios. The results are presented in Table S5 and were found to be comparable.

Table S5. $^{87}Sr/^{86}Sr$ isotope ratios[1] for IAEA-153 milk sample.

Laboratory	$^{87}Sr/^{86}Sr$	Average	SD
JSI	0.70831		
	0.70838		
	0.70819		
	0.70825	0.70829	0.00008
	0.70835		
	0.70823		
	0.70828		
	0.70824	0.70828	0.00005
	0.70829		
	0.70824		

	0.70830	0.70828	0.00003
	0.70835		
	0.70824		
	0.70830	0.70829	0.00005
JSI value +/- U (k=2)		*0.70828*	*0.00015*
UO	0.708288		
	0.708294		
	0.708286	0.708289	0.000004

[1] Strontium isotope ratio determinations were carried at JSI out using a Nu II multi-collector ICP-MS instrument (Nu Instruments, Ametek Inc., UK) fitted to an Aridus IITM Desolvating Nebulizer System (Teledyne Cetac, Omaha, Nebraska, USA). At UO, determinations were conducted using a Nu Plasma HR MC-ICP-MS (Nu Instruments, Ametek Inc., UK) operating in low mass resolution mode coupled with a DSN-100 (Ametek, USA) desolvating sample introduction system.

The measurement uncertainty was larger than the standard deviations since the NIST 987 is the main contributor. The trueness was tested by comparing the $^{87}Sr/^{86}Sr$ isotope ratio independently obtained from the Department of Chemistry at the University of Otago in New Zealand (UO). UO prepared and measured three independently prepared samples. Based on the results, it can be concluded that the optimised method described above is fit-for-purpose for the determination of the $^{87}Sr/^{86}Sr$ isotope ratios of milk.

Table S6. The whole dataset of authentic milk sample analysis including the description of location, season and year of production, geological background, stable isotope ratio of oxygen ($^{18}O/^{16}O$) in milk water and $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ in casein, elemental analysis in the freeze dry samples determined with XRF.

Basic data											XRF											
Location ID	Location	Latitude	Longitude	Season	Year	Geology	δ $^{18}O_w$ ‰	δ $^{13}C_{cas}$ ‰	δ $^{15}N_{cas}$ ‰	δ $^{34}S_{cas}$ ‰	Ca (mg/g)	K (mg/g)	Cl (mg/g)	S (mg/g)	P (mg/g)	Zn (mg/g)	Br (mg/g)	Rb (mg/g)	Sr (mg/g)	Ca/Sr	K/Rb
C1	Sežana	45.70862	13.87428	summer	2014	Cretaceous: Carbonate Rocks and Flysch	-4.5	-26.5	3.6	1.5	7.62	10.7	8.59	2.23	9.25	24.6	29.9	10.4	2.23	3.42	1.03
C1	Sežana	45.70862	13.87428	winter	2014	Cretaceous: Carbonate Rocks and Flysch	-6.9	-26.0	3.8	1.0	8.80	11.9	8.91	2.60	8.05	27.4	27.9	8.48	2.07	4.25	1.40
C2	Kozina	45.68928	13.93719	summer	2014	Cretaceous: Carbonate Rocks and Flysch	-2.1	-26.8	5.8	6.4	8.93	10.4	5.88	2.54	8.84	31.0	26.3	19.6	1.40	6.38	0.53
C2	Kozina	45.68928	13.93719	winter	2014	Cretaceous: Carbonate Rocks and Flysch	-4.4	-23.6	4.9	4.9	9.21	11.4	7.12	2.54	7.86	30.3	24.2	10.5	2.77	3.32	1.09
C2	Kozina	45.68928	13.93719	winter	2015	Cretaceous: Carbonate Rocks and Flysch	-4.5	-18.9	5.6	5.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C3	Bekinj	45.55083	14.06222	summer	2014	Cretaceous: Carbonate Rocks and Flysch	-5.2	-23.7	4.6	5.1	6.86	8.96	5.42	2.04	9.25	28.2	19.6	14.4	1.46	4.70	0.62
C3	Bekinj	45.55083	14.06222	winter	2014	Cretaceous: Carbonate Rocks and Flysch	-7.0	-26.1	5.2	5.9	8.80	12.5	6.80	2.45	8.18	22.5	24.6	28.3	0.94	9.38	0.44
C3	Bekinj	45.55083	14.06222	winter	2015	Cretaceous: Carbonate Rocks and Flysch	-6.1	-22.2	5.5	7.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C4	Postojna	45.77504	14.21382	summer	2014	Cretaceous: Carbonate Rocks and Flysch	-3.9	-25.9	5.1	5.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C4	Postojna	45.77504	14.21382	winter	2014	Cretaceous: Carbonate Rocks and Flysch	-6.3	-20.5	5.8	4.3	7.46	8.8	7.87	2.51	8.86	28.1	17.1	25.1	1.12	6.86	0.35
C4	Postojna	45.77504	14.21382	winter	2015	Cretaceous: Carbonate Rocks and Flysch	-6.1	-24.4	5.3	3.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C5	Logatec	45.91761	14.23516	summer	2014	Cretaceous: Carbonate Rocks and Flysch	-4.5	-24.9	4.6	4.4	9.40	14.3	9.74	2.76	9.25	36.3	28.3	37.3	1.50	6.27	0.38
C5	Logatec	45.91761	14.23516	winter	2014	Cretaceous: Carbonate Rocks and Flysch	-6.8	-22.7	5.1	5.4	8.20	10.8	6.88	2.50	8.83	31.2	28.0	13.0	2.32	3.53	0.83
C6	Šmihel	45.95110	13.73780	winter	2014	Cretaceous: Carbonate Rocks and Flysch	-6.8	-24.3	5.8	4.4	7.98	9.68	6.71	2.72	8.67	29.9	13.2	13.2	2.40	3.33	0.73
J1	Bohinj	46.38065	13.94305	summer	2014	Jurassic: Carbonate Rocks	n.d.	-24.1	3.8	5.0	7.79	10.2	7.42	2.24	7.95	27.5	13.9	27.0	1.39	5.60	0.38
J1	Bohinj	46.38065	13.94305	winter	2014	Jurassic: Carbonate Rocks	-7.1	-24.3	3.9	4.5	7.55	10.4	7.26	2.25	7.84	28.4	15.2	17.1	0.95	7.94	0.61
J2	Žužemberk	45.83072	14.92945	summer	2014	Jurassic: Carbonate Rocks	-5.0	-22.8	5.2	5.9	8.04	9.99	6.50	2.21	7.57	26.1	18.1	17.2	1.21	6.64	0.58
J2	Žužemberk	45.83072	14.92945	winter	2014	Jurassic: Carbonate Rocks	-6.9	n.d.	n.d.	n.d.	8.83	11.1	7.41	2.74	8.41	32.4	16.8	16.7	1.32	6.69	0.66
J2	Žužemberk	45.83072	14.92945	winter	2015	Jurassic: Carbonate Rocks	-7.8	-21.3	5.7	4.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
J3	Podpeč	45.97324	14.41981	summer	2014	Jurassic: Carbonate Rocks	-5.4	-20.2	5.1	5.5	8.22	9.53	6.19	2.47	7.63	32.1	12.7	12.5	5.03	2.71	0.76
J3	Podpeč	45.97324	14.41981	winter	2014	Jurassic: Carbonate Rocks	-6.4	-19.6	5.2	5.4	9.79	11.5	7.86	2.93	9.41	37.7	12.0	10.0	1.56	6.28	1.15
J4	Kamenje	45.85574	15.15377	summer	2014	Jurassic: Carbonate Rocks	-5.5	-21.1	6.0	4.8	7.38	10.7	7.13	2.35	6.57	29.4	17.2	16.7	1.23	6.00	0.64
J4	Kamenje	45.85574	15.15377	winter	2014	Jurassic: Carbonate Rocks	-7.2	-19.5	5.4	4.9	7.85	10.5	6.88	2.49	8.09	30.2	24.2	16.0	2.15	3.65	0.66
J5	Vinica	45.48142	15.25357	summer	2014	Jurassic: Carbonate Rocks	-5.4	-22.6	5.5	6.7	7.36	10.6	7.22	2.30	7.72	28.7	23.4	18.1	1.24	5.94	0.59
J5	Vinica	45.48142	15.25357	winter	2014	Jurassic: Carbonate Rocks	-6.9	-20.0	5.4	4.7	8.14	9.70	5.80	2.51	8.72	34.0	9.73	12.7	3.26	2.50	0.76
J6	Šmarje pri Jelšah	45.95639	14.61882	winter	2014	Jurassic: Carbonate Rocks	-7.2	-19.2	5.9	3.3	8.49	11.2	7.64	2.72	8.09	31.1	10.3	12.8	3.06	2.77	0.88
N1	Šentilj	46.68544	15.70966	summer	2014	Neogene: Carbonate Rocks	-5.5	-24.0	6.6	4.8	7.84	9.45	6.39	2.56	8.00	24.6	11.7	14.4	2.96	2.65	0.66
N1	Šentilj	46.68544	15.70966	winter	2014	Neogene: Carbonate Rocks	-7.1	-20.1	6.1	3.9	8.71	10.6	7.32	2.80	8.50	31.9	10.7	17.1	3.23	2.70	0.62
N2	Kamnik	46.22219	14.60712	summer	2014	Neogene: Deposits	-5.3	-22.4	5.8	4.2	6.93	9.79	6.43	2.05	6.44	21.3	12.6	10.9	1.90	3.65	0.90
N2	Kamnik	46.22219	14.60712	winter	2014	Neogene: Deposits	-6.6	-20.9	5.4	4.5	7.94	9.79	5.85	2.10	6.75	24.6	8.99	10.0	0.89	8.83	0.98
N3	Ponikva	46.25371	15.44393	summer	2014	Neogene: Deposits	-5.4	-22.9	6.0	3.3	7.63	9.99	6.63	2.31	7.69	28.3	9.43	16.0	2.57	2.97	0.62
N3	Ponikva	46.25371	15.44393	winter	2014	Neogene: Deposits	-7.0	-19.8	6.2	3.5	8.78	11.3	7.83	3.10	9.55	32.6	10.6	13.1	2.47	3.55	0.88
N4	Rogaška Slatina	46.23078	15.63860	summer	2014	Neogene: Deposits	-5.3	-22.7	5.9	2.9	8.34	11.2	7.17	2.55	8.24	33.6	9.93	14.9	2.50	3.34	0.75
N4	Rogaška Slatina	46.23078	15.63860	winter	2014	Neogene: Deposits	-7.5	-24.4	6.0	4.7	8.76	11.2	7.69	3.08	8.72	30.4	9.92	12.6	2.99	2.93	0.89
T1	Maribor	46.55463	15.64363	winter	2014	Triassic: Carbonate Rocks	-7.2	-19.7	6.0	1.3	8.11	10.9	7.34	2.62	8.05	32.4	11.0	11.5	3.24	2.50	0.95
T2	Tolmin	46.18696	13.75632	summer	2014	Triassic: Carbonate Rocks	-5.2	-25.7	3.4	4.7	10.1	12.9	9.05	2.76	7.30	27.9	15.7	20.9	1.65	8.12	0.62
T2	Tolmin	46.18696	13.75632	winter	2014	Triassic: Carbonate Rocks	-6.4	-24.1	4.2	5.1	7.64	10.1	7.22	2.66	8.34	30.3	17.9	17.1	1.80	4.24	0.59
T3	Planina	46.04773	14.21534	winter	2014	Triassic: Carbonate Rocks	-7.3	-20.5	5.7	3.5	7.31	9.83	6.82	2.15	7.20	31.0	19.7	14.1	1.36	5.38	0.70
T3	Planina	46.04773	14.21534	winter	2015	Triassic: Carbonate Rocks	-8.7	-20.4	6.1	2.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
T4	Velike Lašče	45.83366	14.63623	summer	2014	Triassic: Carbonate Rocks	-5.2	-24.0	5.1	5.1	9.12	11.5	7.25	2.39	8.53	29.1	22.0	11.9	2.60	3.51	0.97
T4	Velike Lašče	45.83366	14.63623	winter	2014	Triassic: Carbonate Rocks	-7.2	-22.6	4.8	5.5	8.50	9.80	6.32	2.54	8.24	30.1	19.3	13.2	1.49	5.70	0.74
T5	Vrhe	46.18810	15.01356	summer	2014	Triassic: Carbonate Rocks	-5.6	-25.4	5.1	3.7	9.01	11.6	7.68	2.37	7.80	30.6	12.6	15.6	4.75	1.90	0.74
T5	Vrhe	46.18810	15.01356	winter	2014	Triassic: Carbonate Rocks	-7.3	-22.5	5.6	2.3	8.63	11.6	7.82	2.66	9.72	27.5	11.8	17.1	3.54	2.44	0.68
P1	Ilirska na Pohorju	46.53564	15.26751	summer	2014	Paleogene: Deposits	-5.1	-22.4	6.1	4.9	9.59	13.2	8.57	2.80	8.84	34.8	14.5	19.4	2.61	3.67	0.72
P1	Ilirska na Pohorju	46.53564	15.26751	winter	2014	Paleogene: Deposits	-7.2	-20.3	6.2	4.7	8.10	10.8	7.25	2.54	7.78	29.6	9.37	16.7	1.63	4.97	0.65
P2	Vuzenica	46.58800	15.16836	summer	2014	Paleogene: Deposits	-5.3	-23.5	6.6	7.0	8.48	11.7	7.66	2.31	7.47	31.4	11.3	17.1	2.83	3.00	0.68
P2	Vuzenica	46.58800	15.16836	winter	2014	Paleogene: Deposits	-7.2	-21.0	6.1	5.3	9.00	11.3	7.99	3.04	9.04	30.2	9.44	14.7	1.59	3.47	0.77
P3	Vitanje	46.58151	15.25933	summer	2014	Paleogene: Deposits	-5.0	-23.4	5.8	4.0	8.51	10.6	6.88	2.36	7.80	28.7	11.3	17.4	2.16	3.94	0.61
P3	Vitanje	46.58151	15.25933	winter	2014	Paleogene: Deposits	-7.0	-21.0	6.3	3.1	9.11	11.1	8.06	3.27	9.66	28.5	13.6	17.1	2.02	4.51	0.65
P4	Slovenj Gradec	46.50779	15.07791	summer	2014	Paleogene: Deposits	-5.7	-22.7	5.7	4.4	7.91	11.6	7.29	2.48	7.86	27.2	13.0	35.5	2.23	3.35	0.33
P4	Slovenj Gradec	46.50779	15.07791	winter	2014	Paleogene: Deposits	-7.2	-21.7	5.8	4.7	8.03	10.4	7.01	2.66	7.95	27.2	10.9	17.1	2.00	4.02	0.61
P5	Mozirje	46.33926	14.95894	summer	2014	Paleogene: Deposits	-4.2	-25.5	6.7	6.5	7.80	10.2	6.54	2.14	9.25	36.3	14.5	10.1	4.18	1.87	1.01
P5	Mozirje	46.33926	14.95894	winter	2014	Paleogene: Deposits	-6.6	-21.1	5.6	3.9	8.64	10.8	7.64	2.83	7.74	34.1	12.9	17.1	2.15	4.02	0.63
P6	Zavodnje	46.42414	15.01712	winter	2014	Paleogene: Igneous Rocks	-7.1	-21.0	6.1	4.6	8.93	11.1	7.76	3.25	9.62	31.2	12.3	13.0	3.57	2.50	0.85
Q1	Savinjska dolina	46.38848	14.89704	summer	2014	Quaternary: Deposits	-5.4	n.d.	n.d.	n.d.	8.64	11.2	7.44	2.73	9.20	32.4	17.4	14.6	2.02	4.58	0.77
Q1	Savinjska dolina	46.38848	14.89704	summer	2014	Quaternary: Deposits	-5.7	-23.3	5.6	2.9	7.75	10.7	7.51	2.25	7.09	29.4	15.4	14.5	1.97	3.93	0.74
Q1	Savinjska dolina	46.38848	14.89704	winter	2014	Quaternary: Deposits	-7.3	-20.7	5.6	4.1	8.37	10.7	7.19	2.84	8.42	26.4	14.7	17.1	1.79	4.68	0.63
Q2	Prevalje	46.54691	14.91991	summer	2014	Quaternary: Deposits	-5.3	-24.6	5.8	3.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Q2	Prevalje	46.54691	14.91991	winter	2014	Quaternary: Deposits	-7.4	-21.4	4.5	4.2	8.29	11.2	8.00	2.92	8.31	29.9	11.1	17.1	1.83	4.53	0.65
Q3	Dravograd	46.58922	15.02460	summer	2014	Quaternary: Deposits	-5.2	-24.2	5.9	4.8	8.22	11.7	7.89	2.48	9.12	34.7	12.8	28.1	4.43	1.86	0.42
Q3	Dravograd	46.58922	15.02460	winter	2014	Quaternary: Deposits	-7.3	-21.7	5.8	4.6	9.10	11.6	8.74	3.57	10.2	31.4	11.6	19.2	2.92	3.12	0.60
Q4	Braslovče	46.28804	15.03457	summer	2014	Quaternary: Deposits	-7.1	-20.1	5.7	4.2	8.11	10.3	6.41	2.01	7.62	30.7	12.6	11.7	2.04	3.88	0.88
Q4	Braslovče	46.28804	15.03457	winter	2015	Quaternary: Deposits	-8.8	-19.3	6.1	3.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Q5	Škale	46.38925	15.09451	winter	2014	Quaternary: Deposits	-6.9	-20.3	6.0	4.5	8.56	10.8	7.33	2.90	8.37	32.6	14.1	12.3	2.29	3.74	0.88
Q6	Sevnica	46.01295	15.29799	winter	2014	Quaternary: Deposits	-7.0	-20.2	5.1	4.9	8.22	10.3	6.78	2.43	7.74	27.9	13.1	11.1	1.95	4.22	0.93
Q7	Revenske Konjice	46.33665	15.42204	summer	2014	Quaternary: Deposits	-5.4	-21.5	5.7	4.9	8.42	10.0	6.42	2.53	7.56	28.1	10.6	22.1	1.30	6.48	0.45
Q7	Revenske Konjice	46.33665	15.42204	winter	2014	Quaternary: Deposits	-7.0	-19.2	6.1	4.6	10.4	13.5	8.85	3.58	11.3	35.9	14.5	22.5	2.68	3.88	0.60
Q8	Slovenska Bistrica	46.38199	15.57278	winter	2014	Quaternary: Deposits	-7.4	n.d.	n.d.	n.d.	8.44	10.6	7.81	3.20	9.09	25.9	13.1	11.7	2.78	3.84	0.91
Q9	Breiice	45.90793	15.59578	summer	2014	Quaternary: Deposits	-5.2	-24.2	5.8	3.6	8.96	11.9	7.57	2.92	9.30	33.7	17.2	23.7	2.46	3.84	0.97
Q9	Breiice	45.90793	15.59578	winter	2014	Quaternary: Deposits	-7.4	-19.5	5.4	3.8	8.93	11.4	7.65	2.90	8.21	31.8	13.6	12.2	2.54	3.52	0.93
Q10	Ptuj 1	46.42006	15.86860	summer	2014	Quaternary: Deposits	-5.6	-19.8	5.9	3.8	7.82	10.7	8.51	2.26	7.79	27.3	14.8	13.7	2.63	2.97	0.78
Q10	Ptuj	46.42006	15.86860	winter	2014	Quaternary: Deposits	-7.0	-19.3	5.8	3.9	8.42	11.0	7.17	2.52	7.95	31.2	10.8	13.9	2.48	3.40	0.79
Q10	Ptuj	46.42006	15.86860	winter	2014	Quaternary: Deposits	-6.9	-18.5	6.2	2.8	7.52	9.75	5.81	2.06	6.30	29.1	10.8	11.5	2.10	3.58	0.85
Q10	Ptuj 2	46.42006	15.86860	winter	2014	Quaternary: Deposits	-6.9	-18.4	6.8	4.6	8.37	10.5	6.43	2.53	8.86	34.3	7.95	11.9	2.33	3.59	0.88
Q10	Ptuj	46.42006	15.86860	winter	2015	Quaternary: Deposits	-6.9	-18.6	5.7	3.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Q11	Gornja Radgona	46.67660	15.99125	summer	2014	Quaternary: Deposits	-6.0	-20.1	5.7	4.2	8.94	11	7.21	2.71	9.24	36.1	12.5	13.3	5.30	2.71	0.83
Q11	Gornja Radgona	46.67660	15.99125	winter	2014	Quaternary: Deposits	-7.2	-19.1	6.2	4.8	8.38	10.9	6.49	2.50	8.91	32.4	8.54	13.6	3.23	2.59	0.80
Q12	Radenci	46.64402	16.04111	summer	2014	Quaternary: Deposits	-6.3	-20.8	6.1	4.5	8.17	10.4	6.60	2.25	7.29	33.0	11.2	17.1	3.60	2.27	0.61
Q12	Radenci	46.64402	16.04111	winter	2014	Quaternary: Deposits	-6.7	-19.4	6.1	4.6	8.33	10.4	6.33	2.24	7.67	28.4	11.4	9.71	2.49	3.35	1.07
Q13	Murska Sobota	46.65485	16.16590	summer	2014	Quaternary: Deposits	-6.3	-20.6	6.2	5.1	8.76	10.9	7.56	2.48	8.80	32.2	13.8	17.1	3.60	2.43	0.64
Q13	Murska Sobota	46.65485	16.16590	winter	2014	Quaternary: Deposits	-7.0	-19.8	5.7	5.5	9.73	12.1	7.69	2.66	9.79	34.4	10.5	14.7	3.57	2.73	0.82
Q13	Murska Sobota	46.65485	16.16590	winter	2015	Quaternary: Deposits	-6.9	-19.4	7.7	7.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Q14	Ljutomer	46.51955	16.19726	summer	2014	Quaternary: Deposits	-6.0	-20.9	6.4	4.6	8.81	11	6.86	2.49	9.21	34.3	12.9	17.1	3.19	2.76	0.64
Q14	Ljutomer	46.51955	16.19726	winter	2014	Quaternary: Deposits	-6.9	-18.9	6.2	5.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Q15	Polzela	46.28095	15.07375	winter	2014	Quaternary: Deposits	-6.9	-19.6	5.6	4.1	8.56	11.1	7.52	2.95	9.26	28.4	11.5	9.8	1.57	5.45	1.13
Q16	Kozina	45.52806	15.77623	summer	2014	Quaternary: Deposits	-4.8	n.d.	n.d.	n.d.	7.42	10.9	7.27	2.30	7.02	26.9	9.16	21.5	2.69	2.76	0.51
Q16	Kozina	45.52806	15.77623	winter	2014	Quaternary: Deposits	-7.2	-21.7	5.2	3.7	8.89	11.0	6.83	2.69	8.55	32.5	9.20	17.1	1.98	4.49	0.64
Q17	Zahodno Goriško	46.88051	16.22926	summer	2014	Quaternary: Deposits	-6.3	-21.5	6.3	4.5	8.37	10.5	6.86	2.19	7.33	30.3	9.04	17.1	2.81	2.88	0.61
Q17	Vzhodno Goriško	46.88051	16.22926	winter	2014	Quaternary: Deposits	-6.8	-19.5	6.7	5.1	7.97	10.0	6.14	2.29	7.83	34.5	9.07	14.3	2.49	3.20	0.70

Appendix B

Milk Authentication

Table S1. Geographical information of the sampling location together with $\delta^{18}O_{milk}$, $\delta^{18}O_{casein}$ and $\delta^{2}H_{casein}$ values during the summer and winter in the year period from 2012 to 2015.

							$\delta^{18}O_{milk,water}$ (‰)								$\delta^{18}O_{casein}$ (‰)				$\delta^{2}H_{casein}$ (‰)			
Sam. no.	Sample location	Geo -region	Latitude	Longitude	Distance from the coast (km)	Altitude (m)	2012		2013		2014		2015		2013		2014		2013		2014	
							sum	win	sum	win	sum	win	sum	win	sum	win	sum	win	sum	win	sum	win
1	Bohinj	Alpine	46°17'36.4"N	13°54'40.5"E	64	592	n.d.	n.d.	-5.2	-7.6	n.d.	-7.1	-4.0	n.d.	11.3	10.5	10.3	10.8	-134	-114	-134	-135
2	Tolmin	Alpine	46°11'08.9"N	13°43'52.5"E	48	201	n.d.	n.d.	n.d.	-6.1	-3.2	-6.4	-1.8	n.d.	n.d.	12.3	12.0	11.8	n.d.	-122	-124	-126
3	Kamnik	Alpine	46°13'01.5"N	14°37'04.8"E	91	308	-1.9	-5.7	-5.8	-6.1	-5.3	-6.6	-5.6	-6.5	9.7	10.7	12.2	10.7	-124	-138	-141	-129
4	Ribnica na Pohorju	Alpine	46°32'09.1"N	15°16'05.5"E	153	600	-2.2	-6.4	-4.8	-6.8	-5.1	-7.2	-5.3	-7.2	11.9	10.1	12.5	10.9	-124	-128	-122	-119
5	Mozirje	Alpine	46°20'21.3"N	14°57'40.5"E	121	340	-1.1	-5.1	-3.7	-6.7	-4.2	-6.6	-4.9	-6.8	n.d.	n.d.	10.1	10.3	n.d.	n.d.	-146	-128
6	Sav. dolina	Alpine	46°15'57.3"N	15°07'13.4"E	127	276	n.d.	n.d.	-5.4	n.d.	-5.4	-7.3	-5.7	-7.0	9.5	n.d.	12.3	n.d.	-130	n.d.	-145	n.d.
7	Vitanje	Alpine	46°22'48.7"N	15°17'41.8"E	144	449	n.d.	-6.5	-5.2	-6.7	-5.0	-7.0	-4.1	-6.7	10.3	10.8	n.d.	10.6	-116	-123	n.d.	-127
8	Ponikva	Alpine	46°15'14.1"N	15°26'32.4"E	147	338	n.d.	n.d.	-5.0	n.d.	-5.4	-7.0	-5.0	-6.6	n.d.	n.d.	12.1	11.4	n.d.	n.d.	-134	-115
9	Prevalje	Alpine	46°32'56.5"N	14°55'19.1"E	134	322	n.d.	n.d.	-5.9	n.d.	-5.3	-7.4	-4.6	-7.2	9.9	n.d.	12.4	10.8	-141	n.d.	-150	-122
10	Slovenj Gradec	Alpine	46°30'28.0"N	15°04'37.2"E	138	413	n.d.	-7.1	-5.6	-7.3	-5.7	-7.2	-4.9	-7.2	n.d.	10.4	11.7	11.3	n.d.	-115	-140	-122
11	Vrh	Alpine	46°34'30.5"N	15°02'05.0"E	141	346	n.d.	n.d.	-5.7	n.d.	-5.6	-7.3	-5.3	-7.3	8.8	n.d.	13.0	11.2	-113	n.d.	-145	-134
12	Dravog.	Alpine	46°35'23.5"N	15°01'21.8"E	141	390	n.d.	-7.3	-5.8	-7.2	-5.2	-7.3	-5.1	-7.0	10.5	11.0	11.9	11.4	-118	-124	-148	-133
13	Vuzenica	Alpine	46°35'53.8"N	15°09'54.4"E	150	365	n.d.	n.d.	-6.0	n.d.	-5.3	-7.2	-4.6	-7.0	9.9	n.d.	12.4	10.6	-124	n.d.	-143	-132
14	Polzela	Alpine	46°16'50.4"N	15°04'29.9"E	124	292	n.d.	-5.9	n.d.	-6.9	n.d.	-6.9	-5.4	-7.0	n.d.	10.1	n.d.	11.7	n.d.	-133	n.d.	-124
15	Zavodnje	Alpine	46°25'29.8"N	15°01'02.9"E	129	642	n.d.	-7.0	n.d.	-6.8	n.d.	-7.1	-5.1	-6.8	n.d.	11.7	n.d.	12.0	n.d.	-112	n.d.	-120
16	Šmarje pri Jelšah	Alpine	46°14'08.6"N	15°31'30.9"E	153	234	n.d.	-6.6	n.d.	-6.8	n.d.	-7.2	-5.8	-6.7	n.d.	10.4	n.d.	11.6	n.d.	-121	n.d.	-124
17	Škale	Alpine	46°23'28.1"N	15°05'29.9"E	132	456	n.d.	-6.7	n.d.	-6.6	n.d.	-6.9	-5.0	-6.6	n.d.	10.8	n.d.	10.8	n.d.	-139	n.d.	-133
18	Planina	Alpine	46°06'17.2"N	15°24'17.4"E	140	561	n.d.	-7.3	n.d.	-6.5	n.d.	-7.3	-5.4	-8.7	n.d.	9.6	n.d.	11.1	n.d.	-129	n.d.	-135
19	Braslovče	Alpine	46°17'18.8"N	15°02'20.8"E	123	307	n.d.	-7.3	n.d.	-6.8	n.d.	-7.1	-5.1	-8.8	n.d.	10.4	n.d.	11.2	n.d.	-127	n.d.	-139
20	Šmihel	Alpine	46°09'32.1"N	15°13'27.8"E	129	428	n.d.	n.d.	n.d.	n.d.	n.d.	-6.8	-5.8	-8.6	n.d.	n.d.	n.d.	11.7	n.d.	n.d.	n.d.	-127
21	Vinica	Dinaric	45°27'41.5"N	15°15'12.6"E	60	184	-3.3	-6.5	-5.5	-7.1	-5.4	-6.9	-5.5	-7.3	10.4	10.1	10.4	11.0	-130	-127	-138	-139
22	Velike Lašče	Dinaric	45°50'05.3"N	14°38'18.6"E	62	526	n.d.	n.d.	n.d.	n.d.	-5.2	-7.2	-5.4	-7.3	n.d.	n.d.	10.1	11.1	n.d.	n.d.	-146	-142
23	Kamenje	Dinaric	45°51'08.3"N	15°08'58.5"E	82	387	-3.6	-8.0	-6.3	-7.0	-5.5	-7.2	-5.6	-7.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24	Logatec	Dinaric	45°55'01.6"N	14°13'32.7"E	48	476	-2.8	-5.8	-4.5	-7.0	-4.5	-6.8	n.d.	n.d.	10.7	10.9	11.9	10.6	-131	-126	-135	-132
25	Žužem.	Dinaric	45°49'53.4"N	14°55'41.3"E	70	209	-3.2	-6.8	-6.0	-7.5	-5.0	-6.9	-4.7	-7.8	10.5	9.2	12.0	n.d.	-115	-138	-120	n.d.
26	Podpeč	Dinaric	45°58'22.9"N	14°24'56.5"E	62	292	-3.4	-5.1	-5.1	-7.1	-5.4	-6.4	-5.8	-6.7	10.6	10.5	11.3	10.9	-126	-130	-127	-142
27	Postojna	Dinaric	45°46'29.4"N	14°12'55.7"E	39	556	-2.2	-6.3	-5.4	-6.0	-3.9	-6.2	-5.4	-6.1	12.8	9.1	12.6	11.9	-117	-120	-128	-112
28	Ribnica	Dinaric	45°45'6.97"N	14°42'59.64"E	79	492	-2.2	-7.5	-5.2	-7.0	n.d.	n.d.	n.d.	n.d.	10.1	9.7	n.d.	n.d.	-112	-120	n.d.	n.d.
29	Brkini	Mediterranean	45°34'17.8"N	14°02'35.8"E	20	588	-3.1	-4.7	-4.7	-5.6	-5.2	-7.0	-4.3	-6.1	10.7	10.7	12.5	11.6	-123	-114	-130	-120
30	Kozina	Mediterranean	45°36'30.0"N	13°55'43.9"E	11	493	-3.3	-3.6	-5.6	-5.1	-2.1	-4.4	-5.6	-4.5	9.8	9.8	14.6	12.6	-132	-112	-131	-111
31	Sežana	Mediterranean	45°42'09.1"N	13°51'06.5"E	10	360	-3.6	-6.7	-4.7	-7.7	-4.5	-6.9	-4.6	-8.0	9.6	n.d.	n.d.	n.d.	-121	n.d.	n.d.	n.d.

32	Godovič	Mediterranean	46°0'10.01"N	14°1'50.02"E	41	595	n.d.	-6.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
33	Vipava	Mediterranean	45°50'51.21"N	13°57'44.48"E	24	108	n.d.	-5.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
34	Zadlog	Mediterranean	45°57'11"N	14°00'59"E	34	715	0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
35	Brežice	Pannonian	45°55'17.1"N	15°35'50.1"E	149	162	-2.5	-7.0	-4.6	-6.5	-5.2	-7.4	-6.0	-7.1	9.7	10.2	10.8	9.8	-118	-131	-137	-134
36	Korena	Pannonian	46°31'27.1"N	15°46'20.1"E	188	350	-2.1	-5.9	-4.9	-6.3	-4.8	-7.2	-5.2	-7.1	11.2	10.0	n.d.	10.2	-123	-134	n.d.	-140
37	Ptuj	Pannonian	46°25'18.3"N	15°52'05.4"E	186	232	-2.7	-6.4	-5.5	-6.8	-5.6	-7.0	-5.7	-6.9	n.d.	n.d.	n.d.	11.4	n.d.	n.d.	n.d.	-120
38	Ptuj	Pannonian	46°25'18.3"N	15°52'05.4"E	186	232	-2.4	-6.6	-5.7	-6.4	-5.3	-6.9	-5.7	-6.9	9.7	10.8	12.7	n.d.	-129	-122	-133	n.d.
39	Ptuj	Pannonian	46°25'18.3"N	15°52'05.4"E	186	232	n.d.	-6.5	-5.3	-7.4	-6.5	-6.9	-6.0	-7.3	11.0	12.3	11.4	14.1	-116	-116	-144	-101
40	Rogaška Slatina	Pannonian	46°14'09.8"N	15°38'08.9"E	159	224	n.d.	n.d.	-5.5	n.d.	-5.3	-7.5	-5.5	-6.7	n.d.	n.d.	11.9	13.3	n.d.	n.d.	-141	-114
41	Slo. Konjice	Pannonian	46°20'16.1"N	15°25'23.6"E	152	322	n.d.	n.d.	-5.8	n.d.	-5.5	-7.0	-5.7	-6.5	10.9	n.d.	13.0	11.2	-117	n.d.	-140	-119
42	Z.Goričko	Pannonian	46°49'27.6"N	16°05'09.1"E	222	270	n.d.	-6.5	-5.4	-6.9	-6.3	-7.0	-5.1	-7.1	10.3	11.1	11.3	n.d.	-142	-130	-130	n.d.
43	Ljutomer	Pannonian	46°32'09.9"N	16°11'54.8"E	214	175	n.d.	-6.4	-4.9	-6.9	-6.0	-6.9	-5.2	-7.2	10.7	9.4	13.0	14.4	-130	-116	-133	-100
44	Murska Sobota	Pannonian	46°39'29.4"N	16°09'47.5"E	218	189	n.d.	-6.8	-5.3	-6.5	-6.3	-7.0	-5.2	-6.9	12.6	11.1	10.9	13.8	-105	-122	-133	-104
45	Radenci	Pannonian	46°38'35.2"N	16°02'20.1"E	209	202	n.d.	-6.5	-4.7	-6.6	-6.3	-6.7	-4.9	n.d.	9.8	10.4	11.2	14.5	-115	-123	-137	-107
46	Ormož	Pannonian	46°25'10.7"N	16°08'46.2"E	203	218	n.d.	-6.7	-4.8	-7.5	-6.4	n.d.	n.d.	n.d.	9.3	10.4	11.9	n.d.	-114	-131	-134	n.d.
47	V Goričko	Pannonian	46°49'57.4"N	16°17'04.8"E	235	242	n.d.	-6.8	-5.3	-6.8	-6.0	-6.8	-5.6	-7.2	10.9	10.9	13.6	14.5	-113	-120	-121	-104
48	Gornja Radgona	Pannonian	46°40'38.7"N	15°59'23.5"E	207	206	n.d.	-6.5	-5.2	n.d.	-6.0	-7.2	-5.5	-7.0	10.2	11.6	12.0	13.7	-128	-110	-136	-122
49	Gornja Radgona	Pannonian	46°40'38.7"N	15°59'23.5"E	207	206	-6.0	-6.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
50	Sevnica	Pannonian	46°00'42.5"N	15°18'32.5"E	126	183	n.d.	-6.0	n.d.	-7.1	n.d.	-7.0	-5.6	-9.1	n.d.	9.0	n.d.	11.2	n.d.	-122	n.d.	-142
51	Maribor	Pannonian	46°32'50.5"N	15°38'42.8"E	176	262	n.d.	n.d.	n.d.	n.d.	n.d.	-7.2	-6.5	n.d.	n.d.	n.d.	n.d.	13.2	n.d.	n.d.	n.d.	-115
52	Šentilj	Pannonian	46°41'17.99"N	15°43'3.00"E	190	292	n.d.	-7.0	-6.0	-6.7	-5.5	-7.1	-5.2	-7.0	9.5	10.6	11.8	10.7	-149	-121	-138	-127
53	Slovenska Bistrica	Pannonian	46°23'34.01"N	15°34'27.98"E	162	275	n.d.	-6.8	n.d.	-6.7	n.d.	-7.4	-5.3	-7.0	n.d.	10.0	n.d.	n.d.	n.d.	-123	n.d.	n.d.
54	Lenart	Pannonian	46°33'28.76"N	15°48'49.79"E	190	264	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-7.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S2. Data collection of the $\delta^{18}O$ values of milk of different dairy species: sheep and goat. For comparison, samples were collected during summer season from May to June in 2012 and 2013.

Location	Date sampling	Latitude	Longitude	Altitude (m)	Distance from the coast (km)	Species	$\delta^{18}O_{milk}$ (‰)
Branik	17.5.2012	45°51'4.32"N	13°47'30.48"E	87	19	sheep	-1.9
Ajdovščina	22.5.2012	45°53'9.64"N	13°54'34.06"E	106	25	sheep	-2.8
Gradac	29.5.2012	45°38'49.99"N	15°18'51.01"E	150	72	sheep	-1.6
Ilirska Bistrica	29.5.2012	45°34'43.61"N	14°18'23.47"E	421	26	sheep	1.2
Čičarija	30.5.2012	45°29'10"N	14°06'00"E	1028	25	sheep	0.1
Vremščica	23.5.2012	45°41′16″N	14°03′45″E	1027	22	sheep	-5.2
Vremščica	23.5.2012	45°41′16″N	14°03′45″E	1027	22	sheep	-5.6
Bovec	22.5.2012	46°20'15"N	13°33'10.01"E	454	64	sheep	-3.7
Log pod Mangartom	22.5.2012	46°24′21.27″N	13°36′11.29″E	644	69	sheep	-3.3
Soča	22.5.2012	46°20'36"N	13°39'23"E	492	63	sheep	-3.8
Bovec	22.5.2012	46°20'15"N	13°33'10.01"E	454	64	sheep	-2.1
Vransko	14.5.2012	46°14'38"N	14°57'5"E	340	116	sheep	-2.0
Senožete	28.5.2012	46°6′58.04″N	15°11′41.43″E	441	115	sheep	-2.8
Branik	8.6.2012	45°51'4.32"N	13°47'30.48"E	87	19	sheep	0.1
Ilirska Bistrica	15.6.2012	45°34'43.61"N	14°18'23.47"E	421	26	sheep	-0.7
Ilirska Bistrica	15.6.2012	45°34'43.61"N	14°18'23.47"E	421	26	sheep	-2.4
Ajdovščina	7.6.2012	45°53'9.64"N	13°54'34.06"E	106	25	sheep	-1.1
Soča	7.6.2012	46°20'36"N	13°39'23"E	492	63	sheep	-3.6
Bovec	12.6.2012	46°20'15"N	13°33'10.01"E	454	64	sheep	-3.6
Bovec	7.6.2012	46°20'15"N	13°33'10.01"E	454	64	sheep	-2.8
Ilirska Bistrica	15.6.2012	45°34'43.61"N	14°18'23.47"E	421	26	sheep	-0.7
Ilirska Bistrica	15.6.2012	45°34'43.61"N	14°18'23.47"E	421	26	sheep	-2.4
Ajdovščina	7.6.2012	45°53'9.64"N	13°54'34.06"E	106	25	sheep	-1.1
Soča	7.6.2012	46°20'36"N	13°39'23"E	492	63	sheep	-3.6
Bovec	12.6.2012	46°20'15"N	13°33'10.01"E	454	64	sheep	-3.6
Bovec	7.6.2012	46°20'15"N	13°33'10.01"E	454	64	sheep	-2.8
Dobrovo	24.5.2012	45°59'47"N	13°31'35"E	123	24	goat	-1.0
Renče	24.5.2012	45°53'24"N	13°40'07"E	51	13	goat	0.3

Miren	19.6.2012	45°53'44.02"N	13°36'27"E	50	13	goat	-2.0
Srpenica	26.5.2012	46°17'38"N	13°30'4"E	364	61	goat	-1.3
Miren	14.5.2012	45°53'44.02"N	13°36'27"E	50	13	goat	0.5
Miren	11.5.2012	45°53'44.02"N	13°36'27"E	50	13	goat	1.2
Cirkulane	23.5.2012	46°20'26″N	15°59'45″E	229	166	goat	-2.4
Laško	28.5.2012	46°09'16.67"N	15°14'7.98"E	260	116	goat	-5.3
Črni Kal	21.6.2012	45°33'16.18"N	13°52'9.79"E	253	8	goat	2.4
Srpenica	7.6.2012	46°17'38"N	13°30'4"E	364	61	goat	-0.4
Miren	19.6.2012	45°53'44.02"N	13°36'27"E	50	13	goat	-2.0
Bovec	13.5.2013	46°20'15"N	13°33'10.01"E	454	64	sheep	-4.4
Log pod Mangartom	13.5.2013	46°24'21.27″N	13°36'11.29″E	644	69	sheep	-4.7
Bovec	13.5.2013	46°20'15"N	13°33'10.01"E	454	64	sheep	-2.6
Bovec	13.5.2013	46°20'15"N	13°33'10.01"E	454	64	sheep	-4.2
Soča	13.5.2013	46°20'36"N	13°39'23"E	492	63	sheep	-4.1
Ajdovščina	13.5.2013	45°53'9.64"N	13°54'34.06"E	106	25	sheep	-2.6
Branik	13.5.2013	45°51'4.32"N	13°47'30.48"E	87	19	sheep	-2.9
Ajdovščina	13.5.2013	45°53'9.64"N	13°54'34.06"E	106	25	sheep	-3.6
Bovec	13.5.2013	46°20'15"N	13°33'10.01"E	454	64	sheep	-2.3
Črni Kal	22.5.2013	45°33'16.18"N	13°52'9.79"E	253	8	sheep	0.6
Čičarija	31.5.2013	45°29'10"N	14°06'00"E	1028	25	sheep	-5.0
Ilirska Bistrica	17.5.2013	45°34'43.61"N	14°18'23.47"E	421	26	sheep	-1.5
Srpenica	13.5.2013	46°17'38"N	13°30'4"E	364	61	goat	-1.6
Bovec	13.5.2013	46°20'15"N	13°33'10.01"E	454	64	goat	-0.8
Bovec	13.5.2013	46°20'15"N	13°33'10.01"E	454	64	goat	-0.8
Miren	13.5.2013	45°53'44.02"N	13°36'27"E	50	13	goat	-1.8
Renče	13.5.2013	45°53'24"N	13°40'07"E	51	13	goat	-1.8
Dobrovo	13.5.2013	45°59'47"N	13°31'35"E	123	24	goat	-1.9
Dobrovo	13.5.2013	45°59'47"N	13°31'35"E	123	24	goat	-3.9

Appendix C

Characterisation of Truffles

Table S1. The summary on geo-environmental information of sampling locations and date of harvesting for *Tuber* species (n = 58) including latitude, longitude, altitude, climatic conditions, soil geology, geological age and host tree. The data for temperature and amount of precipitation were obtained by the Slovenian Environmental Agency of the Ministry of the Environment and Spatial Planning of the Republic of Slovenia. (n.d. – no data available)

Species	Harvest time	Country	Location	Latitude	Longitude	Altitude [m]	Annual mean T [°C]	Annual mean precipitation [mm]	Soil geology	Geological age	Host tree
TUBAES	14.11.2018	BIH	Šipovo (Location 1)	44.2837	17.0866	526	n.d.	n.d.	flysch; clastic-carbonate flysch	Jurassic-Cretaceous; late Cretaceous	*Corylus avellana, Carpinus betulus, Quercus robur*
TUBAES	14.11.2018	BIH	Šipovo (Location 2)	44.2837	17.0866	526	n.d.	n.d.	flysch; clastic-carbonate flysch	Jurassic-Cretaceous; late Cretaceous	*Corylus avellana, Carpinus betulus, Quercus robur*
TUBAES	07.11.2018	CRO	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	shallow-marine carbonate rocks	Cretaceous (Mesozoic)	n.d.
TUBAES	xx.02.2019	IT	Perugia	43.1105	12.3910	493	n.d.	n.d.	porous or fissured limestone; calcareous sandstones	Tertiary	n.d.
TUBAES	xx.02.2019	IT	Perugia	43.1105	12.3910	493	n.d.	n.d.	porous or fissured limestone; calcareous sandstones	Tertiary	n.d.
TUBAES	14.11.2018	MK	Bistra Mountain	41.6232	20.7340	2163	n.d.	n.d.	limestone	Paleozoic	*Fagus sylvatica*
TUBAES	10.11.2018	MK	Korab	41.8150	20.5676	2764	n.d.	n.d.	shale; limestone; gypsum rocks	Paleozoic; Permo Triassic	*Fagus sylvatica*
TUBAES	xx.10.2018	PL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	rendzinas on marlstone, limestone, gypsum	Tertiary-Quaternary (Cenozoic)	*Carpinus betulus*
TUBAES	xx.10.2018	PL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	rendzinas on marlstone, limestone, gypsum	Tertiary-Quaternary (Cenozoic)	*Corylus avellana*
TUBAES	xx.10.2018	PL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	rendzinas on marlstone, limestone, gypsum	Tertiary-Quaternary (Cenozoic)	*Quercus robur*
TUBAES	20.09.2018	SLO	Bloke	45.7731	14.5094	729	9.1	1403	shallow-marine predominantly carbonate rocks	Jurassic-Triassic (Mesozoic)	*Corylus avellana, Betula pendula*
TUBAES	20.09.2018	SLO	Bloke	45.7731	14.5094	729	9.1	1403	shallow-marine predominantly carbonate rocks	Jurassic-Triassic (Mesozoic)	*Corylus avellana, Betula pendula*
TUBAES	26.09.2018	SLO	Bloke	45.7731	14.5094	729	9.1	1403	shallow-marine predominantly carbonate rocks	Jurassic-Triassic (Mesozoic)	*Corylus avellana, Betula pendula*
TUBAES	26.09.2018	SLO	Bloke	45.7731	14.5094	729	9.1	1403	shallow-marine predominantly carbonate rocks	Jurassic-Triassic (Mesozoic)	*Corylus avellana, Betula pendula*
TUBAES	03.11.2018	SLO	Meja	46.1913	14.3765	265	10.7	1410	terrestrial deposits	Quaternary (Cenozoic)	*Quercus robur, Carpinus betulus*
TUBAES	13.10.2018	SLO	Pluska	45.9120	14.9603	320	11.8	1106	rendzinas and brown soils on limestone and dolomite	Jurassic (Mesozoic); Jurassic-Triassic (Mesozoic)	*Carpinus betulus, Fagus sylvatica, Picea abies*
TUBAES	30.08.2018	SLO	Rajndol	45.5678	14.9475	510	10.0	1485	shallow-marine predominantly carbonate rocks	Jurassic (Mesozoic); Jurassic-Triassic (Mesozoic)	*Corylus avellana, Carpinus betulus*
TUBAES	30.08.2018	SLO	Rajndol	45.5678	14.9475	510	10.0	1485	shallow-marine predominantly carbonate rocks	Jurassic (Mesozoic); Jurassic-Triassic (Mesozoic)	*Carpinus betulus*
TUBAES	03.11.2018	SLO	Sežana	45.7034	13.8706	360	10.5	1698	eutric brown soils, calcaric on flysch	Cretaceous (Mesozoic)	*Quercus pubescens, Corylus avellana*
TUBAES	03.11.2018	SLO	Sežana	45.7034	13.8706	360	10.5	1698	eutric brown soils, calcaric on flysch	Cretaceous (Mesozoic)	*Tilia platyphilla, Ostrya carpinifolia*
TUBAES	16.10.2018	SLO	Sežana	45.7034	13.8706	360	10.5	1698	eutric brown soils, calcaric on flysch	Cretaceous (Mesozoic)	*Quercus pubescens, Corylus avellana*
TUBAES	27.08.2018	SLO	Sežana	45.7034	13.8706	360	10.5	1698	eutric brown soils, calcaric on flysch	Cretaceous (Mesozoic)	*Pinus nigra, Quercus pubescens*
TUBAES	27.08.2018	SLO	Sežana	45.7034	13.8706	360	10.5	1698	eutric brown soils, calcaric on flysch	Cretaceous (Mesozoic)	*Pinus nigra, Quercus pubescens*
TUBAES	28.08.2018	SLO	Sežana	45.7034	13.8706	360	10.5	1698	eutric brown soils, calcaric on flysch	Cretaceous (Mesozoic)	*Quercus pubescens, Corylus avellana*
TUBAES	06.10.2018	SLO	Snežna jama	45.6541	15.0270	875	8.3	1330	rendzic leptosol on carbonate bedrock	Cretaceous (Mesozoic)	*Fagus sylvatica, Abies alba*
TUBAES	03.11.2018	SLO	Spodnje Blato	45.9554	14.6817	338	11.3	1332	shallow-marine predominantly carbonate rocks	Quaternary (Cenozoic); Jurassic-Triassic (Mesozoic)	*Carpinus betulus, Fagus sylvatica, Quercus robur*
TUBAES	13.10.2018	SLO	Spodnje Blato	45.9554	14.6817	338	11.3	1332	brown soils on limestone and dolomite	Quaternary (Cenozoic); Jurassic-Triassic (Mesozoic)	*Quercus robur*
TUBAES	13.10.2018	SLO	Spodnje Blato	45.9554	14.6817	338	11.3	1332	brown soils on limestone and dolomite	Quaternary (Cenozoic); Jurassic-Triassic (Mesozoic)	*Carpinus betulus, Fagus sylvatica, Quercus robur*
TUBAES	13.10.2018	SLO	Stari Log	45.7254	14.9223	400	10.0	1485	brown soils on limestone and dolomite	Cretaceous (Mesozoic)	*Corylus avellana, Carpinus betulus*
TUBAES	06.10.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Corylus avellana, Carpinus betulus*
TUBAES	09.09.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	09.09.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	19.08.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	19.08.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	19.08.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	19.08.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	30.08.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	30.08.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBAES	30.08.2018	SLO	Žlebič	45.7660	14.6931	512	7.9	1380	shallow-marine carbonate and clastic rocks	Carboniferous (Paleozoic); Triassic (Mesozoic)	*Quercus robur, Carpinus betulus*
TUBBRU	19.11.2018	CRO	Krčenik - Donji Miholjac	45.7494	17.9695	97	n.d.	n.d.	clastic deposits	Holocene	*Carpinus betulus, Quercus robur, Corylus avellana*
TUBBRU	27.10.2018	SLO	Marija Snežna	45.7661	14.1932	467	10.8	1426	brown soils/rendzina on limestone and dolomite	Jurassic-Triassic (Mesozoic)	*Tilia platyphilla*
TUBIND	10.01.2019	CN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	igneous crystal rocks or metamoprhic rocks/cambisols	Quaternary (Cenozoic)	n.d.
TUBIND	10.01.2019	CN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	igneous crystal rocks or metamoprhic rocks/cambisols	Quaternary (Cenozoic)	n.d.
TUBIND	10.01.2019	CN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	igneous crystal rocks or metamoprhic rocks/cambisols	Quaternary (Cenozoic)	n.d.
TUBMAC	19.11.2018	CRO	Krčenik - Donji Miholjac	45.7494	17.9695	97	n.d.	n.d.	clastic deposits	Holocene	*Carpinus betulus, Quercus robur, Corylus avellana*
TUBMAG	01.01.2019	IT	Perugia	43.1105	12.3910	493	n.d.	n.d.	shallow-marine predominantly carbonate rocks	Jurassic-Triassic (Mesozoic)	n.d.
TUBMAG	15.12.2018	SLO	Glem	45.4887	13.7829	302	14.8	873	flysch and other deep-marine rocks	Paleogene (Cenozoic)	n.d.
TUBMAG	15.12.2018	SLO	Lukini	45.4722	13.8961	321	14.8	873	flysch and other deep-marine rocks	Paleogene (Cenozoic)	n.d.
TUBMAG	15.12.2018	SLO	Vanganel	45.5171	13.7780	34	14.8	873	flysch and other deep-marine rocks	Paleogene (Cenozoic)	*Populus tremula, Quercus* spp.
TUBMEL	18.01.2019	ES	Cantavieja	40.5256	-0.4059	1290	n.d.	n.d.	leptic cambisol; limestone, calcareous soils on shales	Cretaceous (Mesozoic)	n.d.
TUBMEL	15.01.2019	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TUBMEL	20.01.2019	ES	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TUBMEL	xx.02.2019	IT	Perugia	43.1105	12.3910	493	n.d.	n.d.	porous or fissured limestone; calcareous sandstones	Tertiary	n.d.
TUBMEL	xx.02.2019	IT	Perugia	43.1105	12.3910	493	n.d.	n.d.	porous or fissured limestone; calcareous sandstones	Tertiary	n.d.
TUBMES	xx.02.2019	IT	Perugia	43.1105	12.3910	493	n.d.	n.d.	porous or fissured limestone; calcareous sandstones	Tertiary	n.d.
TUBMES	xx.12.2018	MK	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TUBMES	09.11.2018	MK	Šar Mountains	42.0844	20.8332	2748	n.d.	n.d.	limestone rocks; stony ground	n.d.	*Fagus sylvatica*
TUBMES	09.11.2018	MK	Šar Mountains	42.0844	20.8332	2748	n.d.	n.d.	limestone rocks; stony ground	n.d.	*Fagus sylvatica*

Table S2. The content of elements (mg/kg) in the peridial layer of fruiting bodies of *Tuber* species (n = 58). (n.d. – no data available)

Species	Country	Location	Al	As	Ba	Ca	Cd	Co	Cr	Cs	Cu	Fe	Hg	K	Mg	Mn	Na	Ni	P	Pb	Rb	S	Sr	V	Zn
TUBAES	BIH	Šipovo (Location 1)	1158	0.43	11.1	3048	10.6	469	4.46	0.17	116	632	0.11	20078	1035	26.5	125	3.91	5703	0.55	6.79	2087	7.19	2.52	174
TUBAES	BIH	Šipovo (Location 2)	157	0.29	2.44	2843	1.80	115	0.85	0.08	85.2	130	0.10	17492	714	8.33	95.7	0.91	3867	0.13	2.77	2067	5.13	0.34	123
TUBAES	CRO	n.d.	264	0.13	9.24	2210	9.41	77.1	2.64	0.03	32.1	159	0.05	24739	1515	30.8	78.3	0.57	4051	0.54	16.7	2389	2.54	0.61	269
TUBAES	IT	Perugia	362	0.09	6.81	2740	6.00	120	1.08	0.04	79.4	258	0.05	16820	930	10.8	60.8	0.54	6801	0.13	1.24	2552	8.21	0.75	132
TUBAES	IT	Perugia	170	0.05	5.29	3406	3.54	70	1.24	0.03	39.4	118	0.12	18239	855	8.31	80.9	0.34	3747	0.20	8.68	1739	4.35	0.41	183
TUBAES	MK	Bistra Mountain	181	0.08	3.61	1548	2.62	61.0	0.65	0.02	27.7	130	0.07	24203	761	14.8	108	0.25	6915	0.72	5.84	1560	1.82	0.38	137
TUBAES	MK	Korab	1128	0.72	14.9	2591	5.61	342	2.52	0.12	69.9	546	0.06	22204	1230	31.9	76.4	1.34	5674	0.69	2.66	1872	3.54	2.09	114
TUBAES	PL	n.d.	273	0.13	7.67	4804	5.83	133	0.57	0.03	22.0	223	0.04	16792	619	13.9	65.9	0.37	4596	0.45	1.93	3026	23.8	0.85	120
TUBAES	PL	n.d.	88	0.08	6.27	3424	3.27	54.2	0.44	0.01	25.9	60.0	0.03	18923	521	5.42	43.1	0.57	3610	0.25	3.62	3310	18.7	0.31	118
TUBAES	PL	n.d.	808	0.23	12.4	3443	4.28	201	1.40	0.08	39.7	444	0.10	22741	858	16.0	75.6	1.14	5610	0.92	4.77	3677	18.8	1.72	184
TUBAES	SLO	Bloke	552	0.25	4.65	2536	7.95	153	1.61	0.05	31.0	411	0.08	23547	1652	16.0	139	0.94	4469	0.80	14.0	1808	2.20	2.20	127
TUBAES	SLO	Bloke	291	0.09	9.23	2503	12.4	116	0.95	0.03	48.3	198	0.20	22426	1131	15.6	91.6	0.44	4571	0.22	4.52	1658	2.71	0.54	180
TUBAES	SLO	Bloke	1640	0.57	7.45	2522	9.70	413	3.40	0.16	23.9	1215	0.03	17641	1608	29.6	98.8	2.03	2306	0.88	13.4	1778	2.67	4.95	55.0
TUBAES	SLO	Bloke	1081	0.39	6.67	2685	11.3	291	2.68	0.11	30.1	656	0.06	21775	1911	30.0	108	1.52	3879	0.69	28.2	2204	2.75	3.70	78.1
TUBAES	SLO	Meja	578	0.21	7.88	2576	6.49	114	1.51	0.07	48.6	429	0.13	16305	775	17.9	75.2	0.59	2067	0.78	5.79	3350	3.13	1.08	182
TUBAES	SLO	Pluska	332	0.16	4.52	2290	15.4	171	1.69	0.05	43.0	214	0.06	18935	1307	12.8	90.8	0.86	4903	0.30	4.54	1572	2.28	0.91	178
TUBAES	SLO	Rajndol	142	0.06	4.67	1702	5.04	63.7	1.31	0.02	45.8	100	0.05	24811	1300	10.6	210	0.41	3922	0.16	8.21	1528	1.75	0.39	136
TUBAES	SLO	Rajndol	179	0.07	6.97	1979	6.89	65.9	1.49	0.03	47.2	119	0.05	26212	1426	10.7	211	0.53	3804	0.14	12.9	1625	1.75	0.43	115
TUBAES	SLO	Sežana	492	0.28	4.46	3612	3.02	220	3.49	0.11	35.0	344	0.05	17242	685	18.4	52.0	1.28	3072	0.41	6.05	1537	2.76	2.13	105
TUBAES	SLO	Sežana	168	0.10	3.46	2689	3.77	155	4.07	0.03	27.0	121	0.07	15464	798	7.40	227	0.63	3192	0.24	9.03	2818	2.80	0.56	161
TUBAES	SLO	Sežana	1103	0.61	4.93	3757	4.00	322	5.13	0.23	38.8	719	0.06	21423	961	26.7	92.6	2.34	3691	0.66	6.60	1507	3.35	3.34	119
TUBAES	SLO	Sežana	1711	0.52	6.26	3132	3.72	482	5.09	0.21	37.4	498	0.01	18931	1081	36.3	153	2.88	4465	1.28	27.6	1628	3.72	3.56	142
TUBAES	SLO	Sežana	1015	0.27	5.32	2634	3.40	268	2.66	0.14	36.7	665	0.08	20531	827	21.9	83.5	1.57	6711	0.93	21.3	2183	3.43	2.03	88.7
TUBAES	SLO	Sežana	3710	1.77	15.2	5135	5.18	1014	8.79	0.71	61.0	1215	0.12	22455	2718	87.3	139	6.90	5657	2.33	13.9	1951	7.32	9.66	163
TUBAES	SLO	Snežna jama	563	0.21	6.38	3123	13.0	196	2.51	0.06	32.7	397	0.04	18102	1053	19.6	135	0.81	4468	0.57	7.29	2101	2.26	1.08	147
TUBAES	SLO	Spodnje Blato	467	0.17	6.68	3056	1.78	276	2.78	0.06	36.3	304	0.09	18666	1272	22.1	109	0.35	2642	0.29	12.8	1430	2.21	1.02	135
TUBAES	SLO	Spodnje Blato	276	0.10	6.42	3164	2.05	113	1.40	0.04	39.4	154	0.08	24370	1490	15.5	102	2.14	3931	0.16	12.8	1739	2.66	0.52	135
TUBAES	SLO	Spodnje Blato	296	0.10	6.51	2905	2.28	198	2.30	0.04	48.7	180	0.04	22997	1454	18.0	43.3	0.27	2530	0.21	14.3	1395	2.44	0.55	128
TUBAES	SLO	Stari Log	710	0.22	7.81	3447	5.29	205	1.83	0.07	37.7	340	0.13	21547	886	19.4	122	0.89	3972	0.46	9.15	1459	4.18	1.59	109
TUBAES	SLO	Žlebič	256	0.09	9.37	3577	6.51	105	2.48	0.04	58.4	170	0.09	22810	1201	15.8	84.6	0.59	3716	0.32	6.16	1850	3.33	0.54	135
TUBAES	SLO	Žlebič	291	0.08	6.51	3030	7.84	92.3	2.44	0.04	58.8	192	0.05	20263	940	11.4	103	0.51	4160	0.33	9.24	1726	2.60	0.57	121
TUBAES	SLO	Žlebič	216	0.07	4.11	1470	4.56	69.5	2.62	0.02	39.5	166	0.07	20928	846	8.73	58.3	0.27	3197	0.17	5.54	2416	1.45	0.36	111
TUBAES	SLO	Žlebič	215	0.07	8.33	3054	7.41	57.2	3.69	0.03	71.8	120	0.10	21961	1537	14.8	100	0.36	4847	0.18	6.91	2090	2.87	0.48	167
TUBAES	SLO	Žlebič	81.1	0.03	4.18	1850	8.76	47.4	1.71	0.01	55.2	55.0	0.06	25471	857	8.65	50.6	0.35	4750	0.13	3.68	2033	1.28	0.21	139
TUBAES	SLO	Žlebič	29.0	0.03	1.20	668	9.02	36.1	1.03	0.01	35.4	27.0	0.06	32571	1100	5.72	138	1.00	8285	0.07	5.26	2357	0.58	0.07	133
TUBAES	SLO	Žlebič	984	0.25	6.58	2658	8.57	313	3.22	0.10	65.4	559	0.07	20738	1013	16.8	149	1.14	3729	0.88	14.0	1757	2.73	1.54	86.1
TUBAES	SLO	Žlebič	116	0.05	5.69	2650	7.69	29.9	1.29	0.02	50.1	63.0	0.05	27086	1139	9.23	146	0.25	4849	0.08	5.32	1735	2.28	0.24	135
TUBAES	SLO	Žlebič	180	0.06	6.26	2870	4.86	41.7	3.25	0.02	48.2	117	0.04	20410	981	6.92	88.3	0.33	2946	0.13	3.88	1786	2.03	0.43	106
TUBAES	SLO	Žlebič	379	0.13	9.38	3069	2.76	76.1	1.67	0.05	46.6	210	0.12	25332	1159	8.04	123	0.23	3142	0.51	3.67	1251	3.14	0.65	139
TUBBRU	CRO	Krčenik - Donji Miholjac	1418	0.23	17.8	4325	6.20	291	2.13	0.13	31.9	831	0.05	20956	2093	16.0	617	1.35	6790	0.70	10.0	1376	15.9	2.16	225
TUBBRU	SLO	Marija Snežna	1335	0.32	5.09	980	12.5	356	4.50	0.14	145	462	0.02	31458	994	33.0	145	1.76	10181	0.65	25.7	4638	2.98	3.02	143
TUBIND	CN	n.d.	104	0.04	2.63	1285	0.96	154	1.50	0.02	19.1	84.4	0.08	17051	731	12.0	58.4	0.51	6027	0.33	1.16	3649	4.92	0.27	60.7
TUBIND	CN	n.d.	52.6	0.06	2.08	930	2.51	73.4	0.62	0.01	46.4	43.2	0.05	30429	646	7.61	73.5	0.27	10149	0.08	1.65	2114	3.77	0.13	105
TUBIND	CN	n.d.	22.4	0.04	1.26	747	0.42	77.9	0.51	0.01	17.1	24.5	0.06	23548	740	6.8	41.1	0.31	6183	0.04	1.70	2701	2.42	0.11	61.6
TUBMAC	CRO	Krčenik - Donji Miholjac	1961	0.28	21.7	3868	7.90	360	2.62	0.19	29.8	589	0.03	23702	2268	20.8	354	2.18	6965	1.01	16.7	1230	12.0	3.37	298
TUBMAG	IT	Perugia	715	0.16	3.91	1220	0.77	330	1.82	0.08	72.7	387	0.04	34061	1087	15.1	213	1.36	10252	0.25	6.98	2354	4.95	1.48	345
TUBMAG	SLO	Glem	1038	0.16	5.97	2551	2.97	318	3.10	0.12	25.5	228	0.04	28339	813	16.4	138	2.75	7091	0.44	5.48	2749	5.36	2.15	275
TUBMAG	SLO	Lukini	3238	0.21	16.9	1238	2.78	958	8.41	0.34	55.2	1847	0.05	28203	1167	23.9	501	6.79	7860	1.08	14.5	1897	10.7	6.35	373
TUBMAG	SLO	Vanganel	1557	0.26	6.09	1366	2.15	543	5.22	0.16	94.2	352	0.04	35844	853	24.6	182	3.93	7632	0.57	12.8	5808	3.45	3.21	390
TUBMEL	ES	Cantavieja	3595	1.94	13.8	5418	2.82	946	1.06	0.41	40.4	2162	0.05	17820	849	56.3	111	3.15	7003	1.81	9.18	2552	7.34	7.26	83.1
TUBMEL	ES	n.d.	260	0.14	1.69	3037	0.49	143	0.84	0.04	32.9	204	0.03	23097	645	10.1	97.1	0.44	5730	0.27	1.81	4122	4.71	0.57	117
TUBMEL	ES	n.d.	113	0.03	1.08	3096	0.53	39.0	1.45	0.05	32.6	84.2	0.05	18194	515	5.36	27.0	0.15	5561	0.08	8.49	4048	0.81	0.25	93.5
TUBMEL	IT	Perugia	382	0.08	3.08	2527	3.90	369	1.52	0.04	270	222	0.08	20447	464	15.5	56.6	0.58	5870	0.25	2.15	4132	2.39	0.80	109
TUBMEL	IT	Perugia	178	0.04	2.69	3366	4.03	134	1.06	0.02	234	116	0.03	19418	360	9.39	68.5	0.39	6456	0.12	3.13	6630	2.36	0.38	98.9
TUBMES	IT	Perugia	148	0.04	3.56	2342	5.55	99.4	0.69	0.02	43.3	118	0.14	17194	1058	9.94	168	0.39	6101	0.35	4.88	1752	3.46	0.35	139
TUBMES	MK	n.d.	1559	0.49	13.2	3681	1.36	413	2.80	0.17	48.0	1104	0.12	20240	947	43.0	180	1.46	4210	1.7	15.3	1872	4.66	2.69	100
TUBMES	MK	Šar Mountains	192	0.14	4.87	1467	6.54	68.3	0.98	0.02	63.5	137	0.06	23499	1503	14.7	245	0.53	7330	0.59	15.6	2353	1.92	0.40	196
TUBMES	MK	Šar Mountains	143	0.11	4.03	1380	8.50	83.2	1.36	0.02	52.8	107	0.06	25824	1080	13.3	184	0.76	6739	0.22	8.07	1757	1.59	0.23	189

Table S3. Natural isotopic abundances of light elements (per mil, ‰) in *peridium* of the fruiting bodies of *Tuber* species ($n = 58$). (n.d. – no data available)

Species	Country	Location	$\delta^{2}H$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{18}O$	$\delta^{34}S$
TUBAES	BIH	Šipovo (Location 1)	n.d.	-26.6	11.3	n.d.	5.6
TUBAES	BIH	Šipovo (Location 2)	-24.3	-27.3	8.2	18.9	6.3
TUBAES	CRO	n.d.	-14.1	-27.4	5.7	19.5	7.5
TUBAES	IT	Perugia	-3.4	-27.9	7.9	20.3	0.2
TUBAES	IT	Perugia	-20.3	-25.1	4.4	19.2	10.8
TUBAES	MK	Bistra Mountain	7.8	-26.4	4.2	21.4	7.1
TUBAES	MK	Korab	-25.0	-25.6	11.0	18.5	7.4
TUBAES	PL	n.d.	-12.9	-26.3	3.0	19.4	4.9
TUBAES	PL	n.d.	-3.5	-27.1	1.8	19.6	5.2
TUBAES	PL	n.d.	-21.8	-25.4	4.7	18.6	5.1
TUBAES	SLO	Bloke	-28.9	-28.5	5.8	17.9	7.2
TUBAES	SLO	Bloke	-22.1	-26.2	7.4	18.5	6.2
TUBAES	SLO	Bloke	-17.0	-27.5	6.8	18.4	7.6
TUBAES	SLO	Bloke	-17.5	-27.2	6.2	19.9	8.1
TUBAES	SLO	Meja	-8.1	-26.5	3.9	19.8	8.7
TUBAES	SLO	Pluska	0.8	-26.6	4.2	19.9	4.9
TUBAES	SLO	Rajndol	-15.5	-25.7	5.9	19.6	6.2
TUBAES	SLO	Rajndol	-15.6	-25.5	8.3	18.7	6.5
TUBAES	SLO	Sežana	-6.3	-25.8	5.4	20.6	7.3
TUBAES	SLO	Sežana	-2.7	-25.2	6.2	20.8	8.7
TUBAES	SLO	Sežana	-4.8	-26.4	5.3	20.5	6.2
TUBAES	SLO	Sežana	2.2	-26.8	6.9	20.8	7.0
TUBAES	SLO	Sežana	14.8	-25.9	5.1	21.7	6.8
TUBAES	SLO	Sežana	-7.5	-26.3	6.8	20.2	6.9
TUBAES	SLO	Snežna jama	-18.8	-26.6	6.4	18.8	6.8
TUBAES	SLO	Spodnje Blato	-11.3	-27.5	7.7	19.3	5.9
TUBAES	SLO	Spodnje Blato	-22.3	-27.2	6.9	18.1	5.8
TUBAES	SLO	Spodnje Blato	-21.6	-27.3	7.3	18.7	5.6
TUBAES	SLO	Stari Log	-12.4	-25.8	7.0	19.0	6.5
TUBAES	SLO	Žlebič	-22.8	-26.3	6.7	18.3	5.6
TUBAES	SLO	Žlebič	-8.0	-25.5	5.1	19.4	5.4
TUBAES	SLO	Žlebič	-16.4	-26.3	7.3	19.7	6.2
TUBAES	SLO	Žlebič	-17.0	-26.3	7.3	19.6	7.9
TUBAES	SLO	Žlebič	-17.5	-26.0	6.6	17.4	6.3
TUBAES	SLO	Žlebič	-15.3	-26.0	6.6	19.3	6.5
TUBAES	SLO	Žlebič	-29.3	-26.3	7.1	18.0	6.9
TUBAES	SLO	Žlebič	-24.9	-25.9	8.2	18.1	6.9
TUBAES	SLO	Žlebič	-26.2	-26.6	7.4	18.9	7.0
TUBAES	SLO	Žlebič	-18.3	-26.9	4.5	18.7	6.7
TUBBRU	CRO	Krčenik - Donji Miholjac	-13.1	-26.3	8.2	18.9	-0.9
TUBBRU	SLO	Marija Snežna	2.9	-24.5	10.5	21.1	8.8
TUBIND	CN	n.d.	-56.0	-27.9	5.5	15.8	6.2
TUBIND	CN	n.d.	-36.9	-26.0	5.7	17.2	6.2
TUBIND	CN	n.d.	-49.3	-26.0	8.2	16.5	6.6
TUBMAC	CRO	Krčenik - Donji Miholjac	-14.8	-26.1	7.9	19.4	-0.7
TUBMAG	IT	Perugia	-15.5	-27.5	13.7	20.0	-15.4
TUBMAG	SLO	Glem	-19.1	-26.2	17.9	19.6	4.4
TUBMAG	SLO	Lukini	-5.4	-28.0	19.6	18.5	-3.2
TUBMAG	SLO	Vanganel	-4.0	-27.0	14.2	19.6	7.4
TUBMEL	ES	Cantavieja	-16.4	-28.2	7.4	21.4	7.8
TUBMEL	ES	n.d.	-16.0	-26.3	7.9	20.1	3.1
TUBMEL	ES	n.d.	-13.8	-25.1	9.6	20.0	11.2
TUBMEL	IT	Perugia	1.9	-25.2	8.7	19.6	11.2
TUBMEL	IT	Perugia	-2.5	-23.8	7.5	22.5	11.0
TUBMES	IT	Perugia	-14.6	-25.8	3.9	18.8	11.0
TUBMES	MK	n.d.	-19.0	-25.7	4.7	19.5	8.4
TUBMES	MK	Šar Mountains	-14.9	-26.1	4.6	19.8	7.5
TUBMES	MK	Šar Mountains	-6.3	-26.3	5.6	21.2	7.4

Appendix D

Comment on the paper

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Comment

The Need to Consider Geochemistry When Interpreting Sr-Isotopes. Comment on Gregorčič et al. The Provenance of Slovenian Milk Using $^{87}Sr/^{86}Sr$ Isotope Ratios. *Foods* 2021, *10*, 1729

Micha Horacek [1,2]

[1] Department of Lithospheric Research, Vienna University, 1090 Vienna, Austria; untertrias@gmail.com
[2] HBLFA Francisco-Josephinum-BLT Wieselburg, 3250 Wieselburg, Austria

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I was very interested in the investigation of the $^{87}Sr/^{86}Sr$ ratio of Slovenian milk by Gregorcic et al. (2021) [1]. As Slovenia is a very geologically diverse country, the differentiation of geographic origin by strontium isotopes would be a very important result, as it should be transferable to different food commodities, as this proxy usually isn't significantly influenced by plant or animal metabolisms. However, classification of the milk samples, based on the geological age of the bedrock, was done very impractically and in an unfortunate way. Also, some interpretations do not seem probable.

Generally, the geological age of the bedrock does not necessarily indicate a certain $^{87}Sr/^{86}Sr$ ratio, and furthermore, the $^{87}Sr/^{86}Sr$ ratio is far more influenced by the type of bedrock (the kind of rock, e.g., carbonate, clay, basalt, granite, etc.) than the age of bedrock.

Sample Grouping

Based on the geological situation of Slovenia, as shown by Gregorcic et al. (2021) [1], a far more logical grouping of the samples would be as follows: samples from north-eastern Slovenia (including Paleogene, Neogene, and Quaternary samples, all dominated by clastic (siliciclastic) deposits, according to [1]), and carbonate-dominated areas of all other regions. Taking into account additional isotopic parameters (e.g., H, O, C), a further subdivision of the carbonate areas into central (alpine?) Slovenia (including the Triassic and Jurassic samples) and coastal Slovenia (Cretaceous samples) seems reasonable, to evaluate potential climatically and topographically induced differences. It seems that later on, such a grouping was done anyway for statistical evaluation, but to me, it would be much more logical (because defined by regions) to have such a grouping from the start. Consequently, statistical evaluation of the differentiation of the initial grouping solely according to $^{87}Sr/^{86}Sr$ ratio does not seem to have been successful, as this isn't even shown. Such an evaluation was done only in combination with further isotopic parameters analyzed.

$^{87}Sr/^{86}Sr$ in Milk

As already stated, instead of geological age, the type of bedrock (carbonate, siliciclastic, metamorphic, and magmatic rock) is the dominant influence on the $^{87}Sr/^{86}Sr$ ratio of soil (Figure 1). Marine (Phanerozoic) carbonates show $^{87}Sr/^{86}Sr$ ratios within a very well-defined range (within 0.7068–0.7092 [2], Figure 2). Siliciclastic rocks usually possess notably higher $^{87}Sr/^{86}Sr$ ratios with respect to carbonates, whereas igneous/magmatic/metamorphic rocks can be either depleted (basaltic rocks) or also enriched (acidic-magmatic and metamorphic rocks) [2–4]. Still, although marine carbonates are that restricted in $^{87}Sr/^{86}Sr$ ratio variations, often the soil in carbonate bedrock areas possesses higher $^{87}Sr/^{86}Sr$ ratios than the marine carbonate bedrock range. This is because during erosion and soil formation, carbonate is often/usually removed by chemical erosion (dissolution), and the "eroded"

material is then removed by the dissolving water. In this way, siliciclastic impurities and intervals in the carbonate succession, and aeolian sediments (e.g., loess) are enriched in the soil covering the carbonate bedrock; thus, the soil (and consequently the plants as well as animals feeding on these plants) can possess higher $^{87}Sr/^{86}Sr$ ratios than the bedrock. This is also the case in Gregorcic et al. (2021) [1] (Figure 2). Gregorcic et al. (2021) [1] explain that elevated $^{87}Sr/^{86}Sr$ ratios in milk with respect to ambient river water (their Figure 5) could be due to the potential addition of lime for soil improvement. However, this explanation is invalid in the present case, as the referenced article [5] documents a lowering of the $^{87}Sr/^{86}Sr$ ratio due to lime (most probably marine (calcite) carbonate) addition to low-/non-calcareous soils. The data of Gregorcic et al. (2021) [1], however, document an increase in the $^{87}Sr/^{86}Sr$ ratio of most probably calcareous soils (evidenced by the $^{87}Sr/^{86}Sr$ ratios; an identification of the data in the Figure is not possible). Thus, as stated above, an increase in the $^{87}Sr/^{86}Sr$ ratio of the milk with respect to the ambient bedrock geology and river water being due to the influence of siliciclastic material/sediments is the more probable explanation. This result supports the interpretation that the $^{87}Sr/^{86}Sr$ ratio of milk is dominantly influenced by the feed instead of the water (as the latter (in carbonate bedrock) is usually dominated by the carbonate bedrock $^{87}Sr/^{86}Sr$ ratio due to the dissolved carbonate).

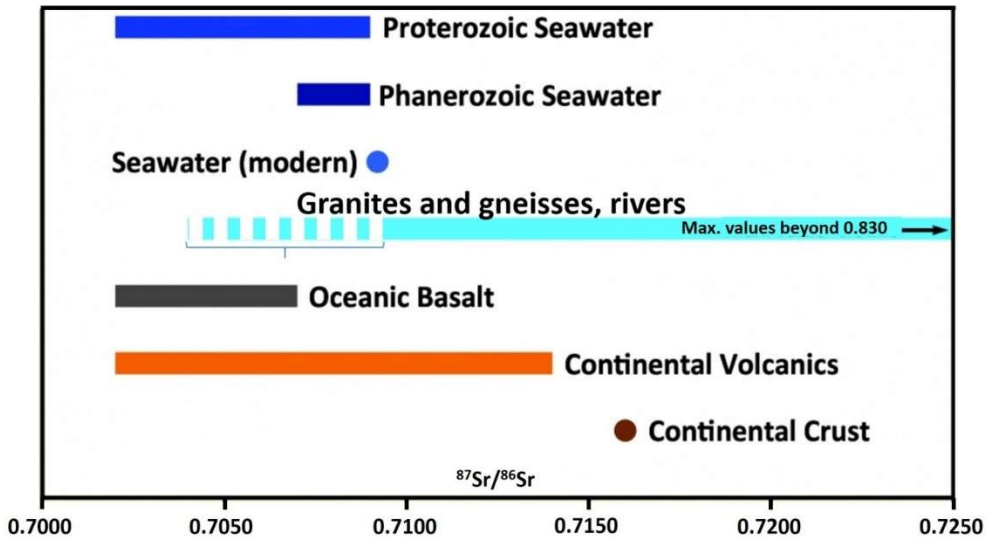

Figure 1. $^{87}Sr/^{86}Sr$ ratios of different rock and water types. Seawater $^{87}Sr/^{86}Sr$ ratios are the result of interplay of Sr transfer from oceanic basalts and transport of Sr from the continents (erosion of granites and gneisses) via rivers into the sea. Marine carbonates incorporate the $^{87}Sr/^{86}Sr$ ratio of the ambient seawater and thus have the same ratio. Point "Continental Crust" indicates average value. Bracket marks the isotope interval where rivers and gneisses $^{87}Sr/^{86}Sr$ ratios are, if dominantly influenced by oceanic basalts or marine carbonates. Data accumulated after [2–4].

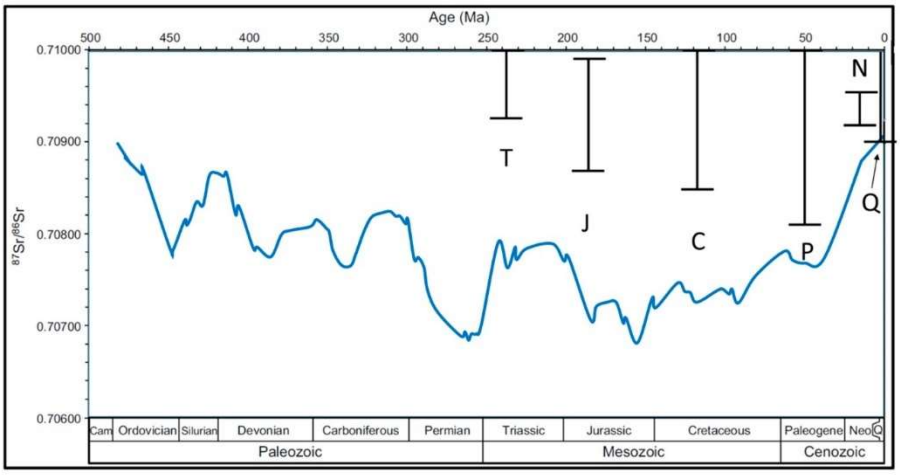

Figure 2. Seawater $^{87}Sr/^{86}Sr$ curve modified after [2], with the curve giving 1 Ma averaged increments. Whiskers show the milk isotope data by Gregorcic et al. (2021) [1]. T: Triassic, J: Jurassic, C: Cretaceous, P: Paleogene, N: Neogene, Q: Quaternary. Missing upper whiskers indicate milk $^{87}Sr/^{86}Sr$ ratios beyond the scale.

To explain the two trends identified in the milk $^{87}Sr/^{86}Sr$ ratio and Sr concentration data, Gregorcic et al. (2021) [1], (Figure 3) outline two processes: "(i) different weathering rates of specific minerals in the rocks and soils; movement of water and sediments in a grazing area can influence Sr and Rb content of milk samples, potentially leading to different $^{87}Sr/^{86}Sr$ isotope ratios; (ii) the consumption of imported plants, particularly those enriched with high Ca and Sr content, . . . ". However, the most plausible explanation for these trends is in fact as follows: Trend 1 (no change in $^{87}Sr/^{86}Sr$ ratio with increasing Sr concentrations, Figure 3) indicates different amounts of marine Sr in the carbonate bedrock, which can most likely be explained by carbonate mineralogy and chemistry. Synchronously formed marine carbonates can possess highly variable Sr concentrations, but with the same $^{87}Sr/^{86}Sr$ ratio (e.g., [6] supplementary materials). Trend 2 (increase in $^{87}Sr/^{86}Sr$ ratio with increasing Sr concentrations, Figure 3) documents a mixing line from (+/− marine carbonate) low $^{87}Sr/^{86}Sr$ ratio towards high $^{87}Sr/^{86}Sr$ ratio, with increasing amounts/influence of siliciclastics, the latter in the present case most likely coming from areas with Precambrian and Palaeozoic metamorphic and igneous bedrock (north-eastern Slovenia). Of course, imported feed, or feed coming from a locality with significantly different geology, can potentially play a significant role in changing the $^{87}Sr/^{86}Sr$ ratio of milk (e.g., with respect to summer/winter), as imported feed can potentially overprint the local $^{87}Sr/^{86}Sr$ ratio (and modify the Sr concentration). However, in the data presented by Gregorcic et al. (2021) [1], differing $^{87}Sr/^{86}Sr$ ratios between summer and winter values could be documented for only a few of the farms sampled (e.g., C5, Q4). Thus, this explanation by Gregorcic et al. (2021) [1] cannot satisfactorily explain the two identified trends.

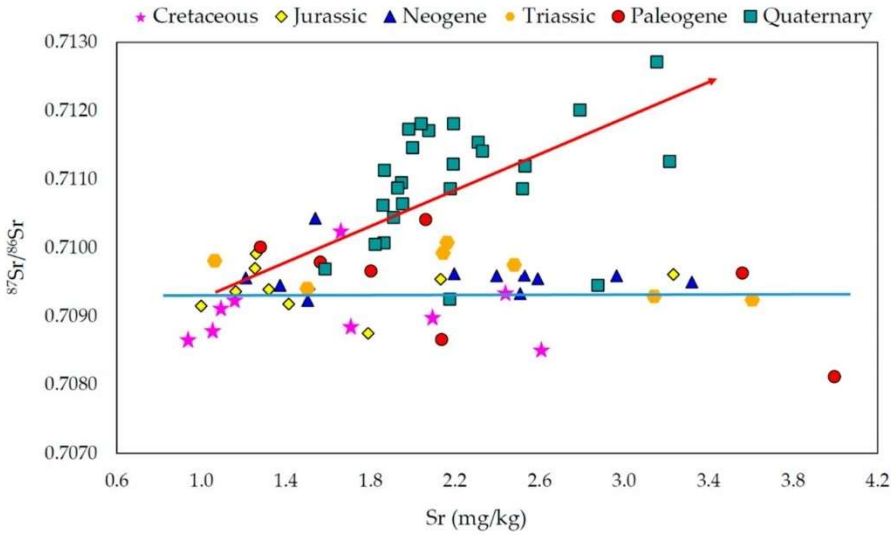

Figure 3. $^{87}Sr/^{86}Sr$ ratios of milk reported by Gregorcic et al. (2021) [1] versus Sr concentrations. Blue line approximates Trend 1: increasing Sr concentrations with constant $^{87}Sr/^{86}Sr$ ratio. Red arrow indicates Trend 2: increasing Sr concentrations with increasing $^{87}Sr/^{86}Sr$ ratios.

Application of $^{87}Sr/^{86}Sr$ ratio for determination and control of geographic origin can be a very potent tool, depending on the exact question intended to be answered. Areas with homogenous bedrock geology and variations in $^{87}Sr/^{86}Sr$ ratio are very well suited, whereas heterogeneous bedrock geological settings are a challenge for this method, as outliers and heterogeneous values within individual areas are to be expected. One must keep in mind that the highest $^{87}Sr/^{86}Sr$ ratio for Slovenian truffles [1,7] was reported from central Slovenia dominated by Triassic and Jurassic carbonate bedrock.

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Reply

Reply to Horacek, M. The Need to Consider Geochemistry When Interpreting Sr-Isotopes. Comment on "Gregorčič et al. The Provenance of Slovenian Milk Using $^{87}Sr/^{86}Sr$ Isotope Ratios. *Foods* 2021, *10*, 1729"

Staša Hamzić Gregorčič [1,2], Nives Ogrinc [1,2], Russell Frew [3], Marijan Nečemer [2], Lidija Strojnik [1,2] and Tea Zuliani [1,2,*]

[1] Department of Environmental Sciences, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia; stasa.gregorcic@ijs.si (S.H.G.); nives.ogrinc@ijs.si (N.O.); lidija.strojnik@ijs.si (L.S.)
[2] Jožef Stefan International Postgraduate School, Jamova 39, 1000 Ljubljana, Slovenia; marijan.necemer@ijs.si
[3] Department of Chemistry, University of Otago, P.O. Box 56, Dunedin 9016, New Zealand; russell.frew@otago.ac.nz
* Correspondence: tea.zuliani@ijs.si; Tel.: +386-1-477-354

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We appreciate Dr Horacek's interest in our paper and his feedback [1]. Indeed, we feel that we have already addressed his comments in our paper. For example, we are aware that the bedrock type has a greater influence on the $^{87}Sr/^{86}Sr$ ratios in soil than the age of the bedrock. However, our data agree with rock type and age (see [2] p. 8). For example, we write that the *"relationship between $^{87}Sr/^{86}Sr$ ratios in the milk samples and rock type at each sampling location was also explored"*. We also state how rock types and ages were obtained from the geological map provided by the Geological Survey of Slovenia [3]. Additionally, we point out that the $^{87}Sr/^{86}Sr$ isotope ratios in the milk samples agree with predicted values for Slovenia, as determined by Hoogewerff et al. [4], and refer to rock type, for example, when we write, " ... *this information is in line with the bedrock composition and age"* and " ... *most of the Slovenian territory is covered by tertiary and quaternary dolomites, limestones and alluvial deposits such as sandstones and claystones"*. Our statistical analysis reveals the differences and similarities between the rock types (Figure 6 in the original paper), so in the end, little disagreement exists between Dr Horacek's comment and our paper.

We also go on to state how there "is a slight difference between milk samples from locations with quaternary alluvial deposits with alumo-silicate rocks with $^{87}Sr/^{86}Sr$ ratios ranging between 0.710 and 0.712, and locations with limestone and dolomite bedrock with $^{87}Sr/^{86}Sr$ ratios in the range from 0.708 to 0.710". However, because of Slovenia's complex geology, we decided to present the data and statistical evaluation using the age of the bedrock since, in this way, it allowed us to differentiate alpine carbonate from coastal carbonate areas. In addition, since only samples from soils above quaternary rocks differ significantly from other samples ($p < 0.0001$), we left this group separate. Furthermore, Dr Horacek is right in his assessment that statistical analysis based on the strontium ratio alone would not distinguish between the groups identified in the original paper, apart perhaps from the quaternary samples in the northeastern part of Slovenia. However, that is exactly what the statistical analysis in the original paper shows.

Regarding the two trends observed in Figure 4 [2] (Figure 3 in the Comment [1]), we explain their relevance as follows: *"Although several samples overlap, two trends can be identified: the first with high Sr concentration and high $^{87}Sr/^{86}Sr$ ratios (>0.7110) mainly from areas with quaternary alluvial deposits with alumo-silicate rocks and the second one related to lower $^{87}Sr/^{86}Sr$ ratios (<0.7090) in carbonate dominated areas"*. Perhaps a more extensive explanation would be that the first trend represents a mixing line from marine carbonate with low $^{87}Sr/^{86}Sr$ towards high $^{87}Sr/^{86}Sr$ influenced by siliciclastics, in the present case

most probably coming from areas with Precambrian and Palaeozoic metamorphic and igneous bedrock (north-eastern Slovenia). In contrast, the second trend indicates different amounts of marine Sr in the carbonate bedrock.

As I am sure Dr. Horacek would agree, the relationship between strontium in the rivers and strontium in milk is complex. In our paper, we did not explicitly discuss the higher $^{87}Sr/^{86}Sr$ values in milk with respect to water but only pointed out the difference that has been noticed. However, we want to stress that the data could not be explained based only on geology, but many factors, such as farming practice in the area, are important. Indeed, additional explanation can be added to support our statements with respect to the relationship between elevated $^{87}Sr/^{86}Sr$ values in milk concerning ambient river water as follows: It is known that the addition of agricultural lime to the soil will lower the strontium ratio of the soil and the river water as the readily dissolvable agricultural lime will dominate runoff from the fields. However, suppose the lime is primarily applied to a field with cash crops for the largest return of investment to the farmer, and not to the hay field and grassland where the cows obtain the majority of their food. In that case, one could imagine a situation where strontium in the surface environment is very heterogeneously distributed, with higher ratios in the milk (less affected by agricultural lime) than in the rivers (more affected by agricultural lime). Thus, besides geology, detailed knowledge of land use is also important.

Finally, like Dr Horacek, we believe that $^{87}Sr/^{86}Sr$ ratios can be a powerful tool for determining the geographical origin of food originating from countries with more homogenous geology, while any interpretation based on $^{87}Sr/^{86}Sr$ ratios can be challenging for countries with heterogeneous geology, such as Slovenia and many other EU countries. This complexity is why we believe that geographical origin determination can be more powerful if $^{87}Sr/^{86}Sr$ data are combined with stable isotopes of light elements and elemental composition, which is the main conclusion of our paper.

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Biography

Staša Hamzić Gregorčič

Date of birth: 07/09/1982 | **Email address:** stasa.gregorcic@gmail.com | **Address:** Maribor, Slovenia (Home)

ABOUT ME

Passionate about science and art.

I am also deaf and I read lips. I successfully overcame this my 'disadvantage' through education with preseverance, motivation and hard work. In a way, being different is not what disables people. What disables people is society's attitude towards difference. Nowayads, I do not see my deafness as an obstacle, but as a proof of my character and strength.

EDUCATION AND TRAINING

2016 – CURRENT Ljubljana, Slovenia
DOCTORAL CANDIDATE International Postgraduate School Jožef Stefan

Foodomics, Organic analysis, Environmental colloid chemistry, Chemistry of Environmental Systems, Food traceability and authenticity, speciation of elements in biological systems and environment, metrology

Field of study Ecotechnologies | **Thesis** Tracing origin of food using stable isotopes of light and heavier elements

2013 – 2015 Maribor, Slovenia
MASTERS OF SCIENCE IN BIOCHEMICAL ENGINEERING Faculty of Chemistry and Chemical Engineering, University of Maribor

Genomics in Biomedical technology, Biochemistry and Molecular Biology, Industrial Microbiology, Environmental biotechnology, Enzyme technologies, Active pharmaceutical ingredients, Bioseparation processes and Biocatalysis

Field of study Biochemical engineering | **Level in EQF** EQF level 7 |

Thesis Methylation analysis of tumor suppressor genes in head and neck cancer

2007 – 2013 Maribor, Slovenia
BACHELOR DEGREE IN CHEMICAL ENGINEERING Faculty of Chemistry and Chemical Engineering, University of Maribor

Organic chemistry, Physical chemistry, Analytical chemistry, Industrial analysis, chemometric methods

Field of study Chemical engineering | **Level in EQF** EQF level 6 |

Thesis Determination of magnesium in nodularises ferrosilicon magnesium FeSiMg 25-5-1 BY ICP-OES

WORK EXPERIENCE

2021 – CURRENT Ljubljana, Slovenia
AS&T SPECIALIST II NOVARTIS PHARMACEUTICAL MANUFACTURING LLC

- ensuring that all activities are in compliance with cGxP, and with data integrity
- routinely assess testing monographs and proactively sustain compliance of all activities with current official regulations, pharmacopoeias, GOP and SOP
- responsible for preparing analytical documentation in alignment with registration dossiers in timely manner
- review and approval of analytical documentation
- responsible for collaborating in optimizations and technological improvements, both in internal and external teams, ensuring continuous improvements
- support quality incidents (deviation, OOS, audits etc.)
- fulfill the responsibilities at the right time and correctly in conformance with methods, procedures and work flow

2016 – 2021 Ljubljana, Slovenia
RESEARCHER JOŽEF STEFAN INSTITUTE

- preparation of PhD
- research and development analytics
- participation in collaborative researches in the field of health, food and environmental sciences
- additional training (webinars, seminars,workshops)
- participation in national and international conferences

2015 – 2015 Maribor, Slovenia
PROJECT RESEARCHER UNIVERSITY MEDICAL CENTRE

Methylation analysis of tumor suppressor genes in head and neck cancer using MS-MLPA technique. Tumor tissue analysis – the presence of human papillomavirus (HPV) DNA in head and neck cancer patients. Quantitative gene expression profiling in normal and cancer tissues.

2011 – 2015 Ruše, Slovenia
ANALYST - RESEARCHER TDR LEGURE D.O.O.

Research, method development and validation; routine sample analyses and preparation of final laboratory reports for external clients (Vitiva d.d., Talum d.d., Ikema d.o.o., NLZOH, Albaugh TKI d.d., SykoFin d.o.o., Naz Met (Poland), Treibacher Industrie AG (Austria), Japan, China).

PUBLICATIONS

Paper: Reply to Horacek, M. The Need to Consider Geochemistry When Interpreting Sr-Isotopes. Comment on "Gregorčič et al. The Provenance of Slovenian Milk Using 87Sr/86Sr Isotope Ratios. Foods 2021, 10, 1729

Gregorčič, S.H., Ogrinc, N., Frew, R., Nečemer, M., Strojnik, L., Zuliani, T. Reply to Horacek, M. The Need to Consider Geochemistry When Interpreting Sr-Isotopes. Comment on "Gregorčič et al. The Provenance of Slovenian Milk Using 87Sr/86Sr Isotope Ratios. *Foods* 2021, *10*, 1729". Foods 2022, 11, 581.

Paper: The Provenance of Slovenian Milk Using 87Sr/86Sr Isotope Ratios

S. Hamzić Gregorčič, N. Ogrinc, R. Frew, M. Nečemer, L. Strojnik, T. Zuliani, "The Provenance of Slovenian Milk Using $^{87}Sr/^{86}Sr$ Isotope Ratios," *Foods*, special issue *Food Origin with Isotope* Fingerprints, vol. 10, no. 8, 1729, 2021.

Paper: Can we discover truffle's true identity?

S. Hamzić Gregorčič, L. Strojnik, D. Potočnik, K. Vogel-Mikuš, M. Jagodic, F. Camin, T. Zuliani, N. Ogrinc, "Can we discover truffle's true identity?," *Molecules,* special issue *Isotopic techniques for Food Science*, vol. 25, no. 9, pp. 2217-1 – 2217-22, 2020.

Paper: Milk authentication: Stable isotope composition of hydrogen and oxygen in milks and their constituents

S. Hamzić Gregorčič, D. Potočnik, F. Camin, N. Ogrinc, "Milk authentication: Stable isotope composition of hydrogen and oxygen in milks and their constituents," *Molecules,* special issue *Isotopic techniques for Food Science*, vol. 25, no. 17, pp. 4000-1 – 4000-14, 2020.

Conference paper: Characterization of truffles (Tuber sp.) in Slovenia using stable isotope approach and elemental composition

B. Krajnc, M. Nečemer, F. Camin, K. Vogel-Mikuš, S. Hamzić-Gregorčič, L. Strojnik, N. Ogrinc, "Characterization of truffles (*Tuber* sp.) in Slovenia using stable isotope approach and elemental composition," in *Programme and book of abstracts*, Portorož, Slovenia: 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality, 2019.

Conference paper: Provenance of milk samples from different regions in Slovenia: Strontium stable isotope ratios

S. Hamzić Gregorčič, N. Ogrinc, T. Zuliani, "Provenance of milk samples from different regions in Slovenia: Strontium stable isotope ratios," in *Book of abstracts: science of the future how to up-tod-date with your research!*, Planica, Slovenia: 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day, 2019.

Conference paper: Strontium isotope ratio used as provenance indicator for milk samples from different regions in Slovenia

S. Hamzić Gregorčič, T. Zuliani, N. Ogrinc, "Strontium isotope ratio used as provenance indicator for milk samples from different regions in Slovenia," in *Book of abstracts*, Pau, France: European Winter Conference on Plasma Spectrochemistry, 2019.

Conference paper: Characterization of Slovenian truffles using elemental composition and stable isotope approach

N. Ogrinc, B. Krajnc, M. Nečemer, F. Camin, S. Hamzić Gregorčič, L. Strojnik, "Characterization of Slovenian truffles using elemental composition and stable isotope approach," Antwerp, Belgium: MASSTWIN Workshop on Mass spectrometry in support of the environment, food, and health interaction and disease, 2018.

Conference paper: Characterization of slovenian truffles using stable isotope approach and elemental composition

B. Krajnc, M. Nečemer, F. Camin, S. Hamzić Gregorčič, L. Strojnik, N. Ogrinc, "Characterization of slovenian truffles using stable isotope approach and elemental composition," in *Book of abstracts*, Messina, Italy: 2nd Isotope Ratio MS Day, 2018.

Conference paper: Optimisation of the method for Sr isolation from the matrix for reliable determination of 87Sr/86Sr isotope ratio by MC-ICP-MS in milk

S. Hamzić Gregorčič, N. Ogrinc, T. Zuliani, "Optimisation of the method for Sr isolation from the matrix for reliable determination of $^{87}Sr/^{86}Sr$ isotope ratio by MC-ICP-MS in milk," in *Proceedings*, Piran, Slovenia: 10th Jožef Stefan International Postgraduate School Students' Conference and 12th Young Researchers' Day, 2018.

Conference paper: Stable oxygen composition of slovenian milk and possible use of lactose as an internal standard to detect milk adulteration with water

S. Hamzić Gregorčič, D. Potočnik, F. Camin, N. Ogrinc, "Stable oxygen composition of slovenian milk and possible use of lactose as an internal standard to detect milk adulteration with water," in *Book of abstracts*, Bologna, Italy: 5th MS Food Day, 2017.

HONOURS AND AWARDS

Zois scholarship for oustanding achievements

Award for the best poster presentation, 11th MPŠ & CMBO conference, Piran, Slovenia (11.05.2018)