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Selective Methods to Investigate Authenticity and Geographical Origin of Mediterranean Food Products

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Abstract

The Mediterranean diet is promoted as one of the healthiest and closely linked to socioecological practices, knowledge and traditions, promoting sustainable food production, and linking geographical origin with food quality and ecosystem services. Consumer adherence to this dietary pattern drives increased consumption of authentic "premium" foods, such as Iberian pig meat and dry-cured ham from Portugal and Spain, argan oil from Morocco, "*Djebel*" lamb from Tunisia and truffles from Italy and Slovenia, i.e., food products that respond to current ethical, environmental and socially sustainable demands. Geographical indication and appellation of origin can increase traditional food products competitiveness, but the high-value recognition of these products can also lead to economically motivated product adulteration. It is therefore imperative to protect the high added value of these unique food products by ensuring their quality, authenticity, provenance and sustainable production systems. In this review, we provide a critical evaluation of the analytical methods that are currently used for the determination of provenance and authenticity of these Mediterranean products as well as possible strategies for improving the throughput and affordability of the methods discussed.

Keywords: geographical origin; stable isotope ratios; elemental profiles; molecular characterization; authenticity; traceability

Introduction

The concept of food "authenticity" refers to its genuineness, and intactness, implying that the food complies with its label description. It is a term that also encompasses features, such as the origin (specific, geographic or genetic), production management system (conventional, organic, traditional practices, free-range) and processing technology. The term "traceability" refers to the ability to track any food, feed, food-producing animal or substance that will be used for consumption, through all stages of production, processing, and distribution (Regulation (EC) No 178/2002) [1]. Worldwide, consumer demand for food quality and distinctiveness is growing, as is concern over issues of food authenticity, traceability, safety, nutrition, and sustainable production. The awareness of traditional cultivation and processing practice provides consumers with the perception that food is authentic, safe and has a high intrinsic quality. A major concern is that the link between food and territory has been largely lost over time due to changes in food production and marketing strategies, along with the consumer exposure to external supply through travels and media [2]. Food adulteration is potentially harmful to human health and so food safety and quality control constitute an important issue in food chemistry and related subjects. For this reason, the main players in the food chain, regulatory authorities, food processor, retailers and consumers, are very interested in the certification of food authenticity.

In Europe, geographical origin is one of the main authenticity issues concerning food, as stressed in a recent publication [3]. The Council Regulation (EC) No 509/2006 [4] protects consumers through a system of effective and impartial controls that define, within the Common Market, the safeguard of the 'Protected Designation of Origin' (PDO), 'Protected Geographical Indications' (PGI) and 'Traditional Specialties Guaranteed' (TSG). More recently, Regulation (EU) No 1151/2012 introduced an optional second tier of quality systems based on the quality terms "mountain product" and "product of island farming" [5], while at the same time, meeting the producers' requirement only objective and precise controls can protect the authenticity of food products on the market. Such regulation is also of economic importance to many stakeholders allowing them recognition and a premium price. The use of standards and certifications can act as a warranty of quality to gain the trust and confidence of consumers since food products must respond to current ethical, environmental and socially sustainable claims. Several traditional Mediterranean food products can benefit from these measures since their intrinsic value exceeds nutritional quality. Such intrinsic values are usually associated with peculiar microclimates and soil properties (e.g. the terroir), unique agricultural systems of production, varieties or races. This is often reflected in higher content of antioxidants and healthy fats and has specific organoleptic characteristics associated with consumers' preferences [6]. Beyond their nutritional values, the distinctiveness of these food products is linked to the highest quality associated with traditional sustainable production methods which, in turn, boost local economies and cultural and natural heritage protection.

The Mediterranean is also the region where a body of national definitions and rules has been created and developed for recognising and protecting these geographical indications. The first "controlled designation of origin" appeared in France, and the approach then spread around vineyard designations throughout the countries on the northern shores of the Mediterranean followed by the European Union and was finally recognised at the international level (TRIPS Agreement). At that level, the Mediterranean countries seem to be amongst the most dynamic in this field; a large number of geographical indications have been registered there (in France, Italy, Spain and Turkey), and policies to support these measures have been developed and strengthened, particularly in Morocco, Tunisia, Jordan and Lebanon. Thus, it is not surprising that most of food authentication studies came from Mediterranean countries [7].

Several national and international projects have been proposed to promote Mediterranean traditional food products. One of these is the recently funded REALMed project “Pursuing authenticity and valorization of Mediterranean traditional products” (ARIMNET 2 call 2016) that focuses on premium Mediterranean products, typical of local cultures of various countries, for example, Moroccan argan oil, Portuguese and Spanish black Iberian pig, Italian and Slovenian truffles and Tunisian mountain lamb (Fig. 1). A characteristic feature of these projects is that each premium product is linked intimately to its socioecological context. Thus, any attempt of production broadening is likely to overcome both the geographic boundaries and the fraud limits.

[Figure 1 near here]

False use of geographical indications by unauthorised parties is detrimental to consumers and legitimate producers. The three most important features that consumers appreciate, as reported in Bryla (2015) [8], are in fact: traditionality, linked both to history and common diet of a place; territoriality, linked with the geographic origin; and quality, linked to health issues. Nevertheless, from a commercial and legal point of view, regulatory authorities are requested to continuously update the analytical methods and conditions allowed to validate the authenticity of a certain product as this may support law enforcement actions [7]. It is an analytically challenging problem that is currently the focus of much attention within Europe and worldwide [2]. A variety of analytical techniques, for the verification of food authenticity and provenance, have been developed and tested. All of them have strengths and weaknesses, however classifying them is a useful way to point out the current state of the art. Chromatographic analysis such as gas (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS), have emerged as useful food authentication tools since they provide rapid and reliable separation of chemically similar compounds in complex food matrices [7]. These chromatographic techniques are usually used for determining the authenticity of high-quality products adulterated with inexpensive or sub-standard ingredients as in the case of argan oil [9]. The main drawback is the cost of equipment that is generally unsuitable for point-of-use testing. High resolution, particularly, needs skilled operators and a well-controlled environment. Test methods must be developed, optimised and validated for each specific application, and in many cases need extensive sample preparation and clean-up. A noticeable class of compounds that need to be mentioned in food authenticity are volatile organic compounds. There are several techniques for identification of aroma compounds including Gas Chromatography Olfactometry (GCO), Headspace Gas Chromatography Time of Flight Mass spectrometry (HS GC/TOF-MS), proton-transfer-reaction mass spectrometry (PTR-MS), and Headspace–Solid Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS). These methods can be used for the characterization and identification of aroma compound in food products [10], possible adulteration [11] or even geographical origin determination [12]. HS-SPME-GC-MS can be undisputedly considered as an environmentally friendly technique, offering a good compromise between selectivity and sensitivity, cost, and easiness of use, albeit the quantitative analysis is challenging.

Stable isotope and elemental fingerprinting as well as DNA-based genetic methods have become increasingly important in establishing authenticity and geographical origin of food products [7,13]. The basis of the stable isotope approach lies in the transfer of isotopic signals of the light elements (H, C, N, O and S)[1] from water, soil, and atmosphere to plant

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[1]Isotope data are expressed with the conventional δ-notation using the general formula:
$\delta^{i}E = (R(^{i}E/^{j}E)_{sample} / R(^{i}E/^{j}E)_{standard}) - 1$

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 recognized reference standard. The delta values are multiplied by 1000 and expressed in units "per mil" (‰). For hydrogen and oxygen Vienna Standard Mean and animal tissue. The physiological and ecological frame of such isotopic imprinting along the food web has been deeply investigated and is now reasonably well understood. The use of some heavier stable isotopes such as strontium (Sr) can help to trace the geochemical fingerprinting of a particular region to its food products. Stable isotope approach is a successful tool for determining the geographical authenticity of numerous food products, although the instrumental costs are quite high and the speed of the analysis is moderate [2]. Similarly, element concentrations in plants and animals are also increasingly being used to control food origin and authenticity. These include macro-elements (e.g. sodium, calcium, potassium) and trace elements (such as copper, zinc, and selenium), rare earth elements (e.g. lanthanum, cerium, samarium) or other low-abundance elements like gold and iridium. The application could be even more effective when combined with the stable isotope approach [13].

DNA-based genetic methods have also been applied to identify species and variety and to verify food label claims objectively. DNA-based methodologies are characterized by short sample preparation, high sample throughput, good inter-laboratory reproducibility and low operating costs. Nevertheless, the main limit is in the molecular variability of the organisms and, therefore, a high level of resolution is required for organisms with low intraspecific polymorphism. Further not all food sample types have intact DNA that can be extracted. Highly processed meat products, stocks, soups and gelatins have very low amounts of viable DNA.

A common requirement in food authenticity and traceability studies is the need for a product reference database, which is a major drawback in terms of both time and costs. A

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.

large databank, comprising samples from a broad and representative range of geographical, seasonal, dietary and production conditions is needed [13]. To evaluate the authenticity of commercial samples, they must be characterized and then compared with those referenced in the databank and evaluated in terms of their fit within statistical limits [14].

This review aims to describe, for selected traditional Mediterranean food products, the state-of-the-art of analytical techniques used for assessing traceability and authenticity. Further, the review will be used as a starting point within the framework of the REALMed project to determine the best strategies to promote the selected commodities.

REALMed Leading Mediterranean Commodities

Meat Products from the Iberian Black Pig

The production of **Iberian pig**, a traditional breed of *Sus scrofa domesticus* dubbed *Sus ibericus*, is deeply bound to the Mediterranean ecosystem and is currently found in the central and southern parts of Portugal and Spain [15]. It is a rare case in the world of swine production adapted to an agro-silvopastoral setting. In traditional management, animals range freely in sparse oak forests, where the land (*montado* in Portugal; and *dehesa* in Spain) is particularly rich in natural food sources, such as acorns from the holm oak (*Quercus ilex* L.), gall oak (*Quercus lusitanica* Lam.) and cork oak (*Quercus suber* L.) [15]. The peculiar characteristics of the breed and productive system lead to high-quality meat products, with increasing importance and high turnover. However, this promising trend in the market of Iberian pig meat products has raised new problems of increasing importance: the imitation of the products and the increase of fraudulent practices in its production and commercialization.

Situation in Portugal

The *Presunto de Barrancos / Paleta de Barrancos* – (Barrancos' ham) (Commission Regulation (EC) No 2400/96 [16]), *Presunto do Alentejo / Paleta do Alentejo* - Alentejo's ham (Commission Regulation (EC) No 944/2008 [17]), *Carne de Porco Alentejano* - Alentejo's pork (Commission Regulation (EC) No 617/2003 [18]); and one PGI ham: *Presunto ou Paleta de Santana da Serra* – ham from Santana da Serra (Commission Regulation (EC) No 943/2008 [19]) are all products produced from adult pigs born, reared, fattened and finished under the *montanheira* system, and 100% *Porco Alentejano*. *Montanheira* refers to a peculiar feeding period, where the animals range free in the montado ecosystem, between October/November and January/February. During this period the pigs feed exclusively on grass and acorns, and are later slaughtered in a defined geographical area.

There are approximately 170 breeders rearing animals on an area of about 200 000 ha in the *Montanheira* system (feeding adult pigs with grass and acorns) and 948 000 ha of *Montado* (land with holm or cork oak) worth in total approximately 120 million euros [20]. The ACEPA – Complementary Business Grouping (ACE) of *Porco Alentejano,* made up of the ACPA and ANCPA (the two *Porco Alentejano* breeders association), is responsible for managing the Genealogical Portuguese Book of the *Porco Alentejano* pig (LGSRA).

Situation in Spain

Royal decree No 4/2014 [21] has been recently approved and sets the quality standards for Iberian-labelled meat, ham, and loin. It also establishes the criteria to be able to use the label "Ibérico" on pork products. It refers not only to pickling and salting – used to gradually reduce the moisture content to preserve the meat, but also the feeding conditions and breed purity. For example, regarding ham there are four distinct categories that refer to the animals diet and breed purity: (i) *jamón de bellota 100% Ibérico* – from pure Iberian pigs that have been fattened and finished under the *montanera* system; (ii) *jamón de bellota ibérico* – from mix-breeds fed using the dehesas but complemented with acorns and grass in a programmed way; (iii) *jamón de cebo de campo ibérico* – from mix-breeds living under intensive conditions, but herding in the dehesa and fed with cereals and legumes, and (iv) *jamón de cebo ibérico* – mix-breeds fed under an intensive regime with cereals and legumes.

Anti-Fraud Approach and Geographical Origin Determination

To date, the number of scientific papers, dealing with Iberian black pig products is limited. Of these, only a few investigate and develop methodologies regarding the traceability and the authentication of Iberian black pig meat products. Initial studies were conducted by Toro et al. [22,23], who proposed the use of molecular markers to identify founder animals and to estimate the co-ancestry of Iberian pigs, which is a recurrent problem since traditionally Iberian pigs have been crossed with Duroc pigs [24]. Other approaches for classifying pork include the use of near-infrared reflectance spectroscopy (NIRS) artificial neural network (ANN) [25–27].

Stable Isotope Ratio Analysis

The application of stable isotope ratio analysis for authenticating Iberian pig meat products has focused on discriminating between animals fed using different dietary regimes (e.g. [28–30]). González-Martín et al. [31] were able to distinguish swines of different breeds (Iberian vs white) with different diets (acorns or feed) by analyzing the carbon ($\delta^{13}C$) and sulfur ($\delta^{34}S$) isotope composition in the liver. Recio et al. [32] were able to distinguish between meat products from Iberian pigs raised traditionally or fattened from the $\delta^{13}C$ values of palmitic, stearic, oleic and linoleic acid methyl esters determined by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). The authors also proposed a stable carbon isotope value of oleic acid ($\delta^{13}C_{18:1}$) of −25.9‰ as the threshold value. Similarly, Delgado-Chavero et al. [33] combined the fatty acid (FA) profile and the $\delta^{13}C$ of

FA methyl esters and were able to classify animals according to the feeding system type, with a confidence level of 85% for the four feeding groups together (*Bellota, Recebo, Campo* and *Cebo*), and with a 91% confidence level when comparing only two categories (*Cebo* and *Bellota*).

Elemental Analysis

The elemental composition of Iberian pig remains to be thoroughly investigated; however, Galián et al. [34] characterized the mineral content of Chato Murciano pigs and the Chato Murciano breed crossed with Iberian pigs, whereas Castellano et al. [35] found differences between the mineral composition of the sow's milk and the suckling piglet's meat in different Iberian genotypes. Mineral analysis of fresh Iberian pork loin may be performed in a high-throughput way using NIRS [36]. All the aforementioned techniques have their limitations suggesting the need to combine several techniques — for example, evaluating the dietary regime (e.g., acorns versus alternative feeding sources) while determining the authenticity of Iberian black pig. Nevertheless, the limiting factor is the cost of routinely implementing this approach in a high throughput manner. In terms of affordability, NIRS techniques may represent an alternative for simultaneously assessing differences.

Molecular Techniques

Phylogenetic analysis of mitochondrial DNA (mtDNA) sequences of Iberian black pig have been used to distinguish between meat products from purebred Iberian pigs and those from crossbred or other breeds [37–39]. The Iberian and Majorcan Black pig were the only ones to display the European cytochrome B haplotypes, a feature that proves these pigs have not been crossed with either Chinese or European commercial populations [38]. Furthermore, Van Asch et al., [39] found that Iberian samples have a high frequency of a sub-cluster (E1c) of the

European haplogroup E1 with a small genetic distance (F_ST = 0.105) between *Alentejano* (Portugal) and Iberian pig breeds (Spain) as well as with Iberian and Central European wild boars (F_ST = 0.215). Óvilo et al. [40] used the amplified fragment length polymorphism (AFLP) technique for the characterization of highly inbred Iberian pig breed genotypes and the detection of strain-specific polymorphisms. Twelve different primer combinations were used on individual DNA samples from animals belonging to two black hairless Iberian pig strains (*Guadyerbas* and *Coronado*). The authors identified 26 amplification products as being strain-specific markers [40]. Although the DNA analysis is not very used for traceability of Iberian pig, some examples are available in the literature. The results obtained by Alves et al. [41] may be valuable to resolve the problems of Iberian and wild boar maternal origin determination, while other studies used genomic approach for authentication of the raw material of the Iberian pig meat products [42-44]. For example, Garcia et al. [43] detected up to 20% of ham samples with a genetic composition incompatible with current legislation - either because the Duroc genome was present in a percentage greater than that permitted, or because of the significant presence (>25%) of white coat pig genomes.

Argan oil

Argan oil production plays a key role in the environmental and social-economic context of Moroccan agriculture. It is produced from the kernels of the argan tree (*Argania spinosa* L.), a species endemic to Morocco and traditionally prepared by village women following a laborious seven-step (fruit picking, fruit peeling, nut cracking, kernel roasting, kernel grinding, dough malaxing, and oil collection) low-efficiency process. Argan oil is highly valuable as a food product, since it is rich in unsaturated fatty acids, polyphenols, sterols, and antioxidants, but can also be used in cosmetics. Argan oil production begins with peeling of the ripe fruit, manual cracking of the nuts with stones and selection of the appropriate kernels. Depending on the end-use, two methods for preparing the kernel for extraction, are generally used. For the cosmetic use, the "cosmetic grade", argan oil is extracted from raw kernels, while edible argan oil is extracted from roasted kernels. Kernel roasting gives the oil its specific organoleptic characteristics and improves the yield of oil extraction. Press extraction of the edible oil can be done either in a traditional (manual) or in a semi-mechanical way. The traditional technique involves roasting of kernels in clay containers and grinding the roasted kernels with a millstone until a brownish viscous dough is obtained. With the addition of water, the dough is kneaded for a certain time and then afterwards hand-pressed to obtain a cake and an emulsion from which the oil is separated by decantation. The traditional technique is time-consuming, gives low oil yield, and an end product with poor shelf-life [45]. In the semi-mechanical way, the kernels are roasted inside a rotating oven, and a mechanical press is used to extract the oil. The "mechanization" of the process not only improves the quality of the oil and extraction yield but also significantly reduces the time of production [45]. Officially, recognized types of argan oil are: virgin argan oil, extra-virgin argan oil, edible argan oil, cosmetic argan oil, beauty argan oil, and enriched argan oil. Cosmetic argan oil has become one of the major actors in the dermo-cosmetics industry during the last 15 years. Beauty argan oil is produced by cold pressing of the finely crushed kernels, while enriched argan oil is produced by distillation of cosmetic argan oil and can be supplemented by antioxidants to enhance its cosmetic potential. In the Moroccan tradition, argan oil is used as a medicine for conditions such as cardiovascular disease, rheumatology, nephrology, neurodegenerative diseases, and postmenopausal disorders. The health properties of argan oil have been the main focus of investigations in the last years (e.g. [46,47]).

Inevitably, the success of argan oil and its high price increase the risk of adulteration, often resulting in the blend of high and low-quality argan oil. Although analytical methods have now been designed to detect oil blending, protecting argan oil remains a prerequisite to protect oil prices and thus indirectly the environment and ecosystem.

Situation in Morocco

Argan tree is endemic in South-western Morocco, where its forests extend into the arid, semi-arid bioclimate, and cover an area of approximately 870 000 ha [48]. These represent the second most abundant tree species in the country with over twenty million trees and play a vital role in the ecosystem. It is perfectly adapted to the region's harsh environment, with the ability to survive extreme heat (over 50°C), drought and poor soil. The tree's roots grow deep into the ground in search of water, which helps to bind the soil and prevents erosion. The argan tree alone represents a symbol of the ecological and socio-economic life of the southwest of the country. It plays a major role in the fight against desertification and the preservation of ecological balances and biodiversity. It is a multipurpose tree (forest, fruit, and forage) and all its products are utilized; wood in the form of charcoal, kernels for the extraction of oil, leaves, fruit pulp and the residue of kernels (cake) serve as animal feed. The sustainable development of the argan forest, therefore, has been actively encouraged.

UNESCO recognized the importance of the argan tree in 1998 when the south-western region of Morocco became a Biosphere Reserve under UNESCO's Man and the Biosphere Program [49]. Legislation involving the argan forest and its use is based on three specific texts: the Dahir of the 4th March 1925 [50], the Codirectorial Order of the 1st May 1938 [51] and the Dahir of the 28th March 1951 [52]. Under the terms of this legislation, rights are granted to users, which are particularly extensive and are referred to as rights of enjoyment. The Dahir of March 4th, 1925, is about the protection and denomination of argan tree forests [50].

The incorporation of modern, mechanized aspects into the commercialization of argan oil has also played an essential role in stabilizing argan forests. The argan oil, produced by several Moroccan cooperatives, has become famous for its cosmetic virtues and has been exported at prices up to several hundred dollars per liter to Europe, Japan, and the United States. The total annual production of argan oil reaches approximately 4,000 tonnes per year [53]. In 2010, argan oil received the PGI recognition by the Moroccan Government. The name argan oil is now protected and can no longer be used to describe oil whose production does not comply with the specifications of the production and quality protocol. New methods of production and commercialization combined with traditional knowledge have not only reduced oil extraction time but have also made the process more efficient. Cooperatives, therefore, do not have to use as much fruit as before and can get more oil out of each tree, thus protecting a vital ecological and socio-economic resource.

Anti-Fraud Approach and Geographical Origin Determination

Argan oil is a relatively new product on the international market and is exported only by Morocco. The yield of oil extracted from the fruit is low at about 1.1% to 1.5% relative to the weight of the fruit and preparation time and to obtain 1 liter of oil it takes about 20 hours [54]. Consequently, the difference in price between argan oil and other virgin and refined vegetable oils can lead to adulteration with cheaper oils. The majority of available scientific papers on argan oil are mainly focused on its chemical characterization, in particular relating to the characteristics of the production processes and the determination of its effects on human health when applied as a cosmetic or when consumed as part of the Mediterranean diet. There has been an increasing number of studies looking at methods for determining its authenticity and typically involve the use of volatile compounds, fatty acid profile and phenolic composition. So far, however, the use of stable isotope signatures in bulk samples or individual fatty acids have not been applied.

Elemental Composition

Three studies have investigated the elemental composition of argan oil all using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) [55–57]. Samples of edible and cosmetic argan oil collected from different regions of Morocco and in different years showed little elemental variability, but the authors did propose this method as a way to differentiate argan oil from other vegetable oils (e.g. sunflower, olive, seeds, and soybeans).

Volatile Organic Compounds

Edible argan oil has a rich aroma and flavor and thus has a high culinary value as a seasoning and cooking oil. Previous studies on aroma characterization of commercial edible argan oils [58] revealed that pyrazines, aldehydes, ketones, hydrocarbons, alcohols, pyrroles, and furans were the main aroma compounds. Moreover, qualitative and quantitative differences in aroma profile were observed between commercial oils and were attributed to differences in the roasting step and extraction techniques used in their preparation. In a study about the effect of oil extraction methods on the quality of edible argan oil during storage, Matthäus et al. [59] found no change in the sensory characteristics of argan oil obtained by mechanical extraction after 20 weeks at 20°C; however, for oil obtained by traditional extraction, a “Roquefort cheese taste” developed after 12 weeks of storage.

To date, only two studies focused on the determination of volatile organic compounds of argan oil as possible methods for detecting adulteration. In particular, Bougrini et al. [60] used a combined e-nose and e-tongue technology to detect the adulteration of argan oils. Using this approach, the authors could determine the percentage of cheaper oils added to virgin argan oil. Alternatively, Kharbach et al. [61] used selected-ion flow-tube mass spectrometry fingerprinting from the volatile oil fraction combined with multivariate analysis to assess the geographical origin of argan oil as an alternative to chemical profiling (reference methods).

Fatty Acids, Phenols, Chemical Composition in General

Most studies on the authentication of argan oil refer to either chemical composition or the number of particular compounds or classes of compounds. For instance, two studies focus on changes in the triacylglycerol profile, determined using either UHPLC-PDA or HPLC light scattering [62,63], for detecting the addition of different vegetable oils and for quantifying the content of argan oil in different formulations. A study performed by Ait Aabd et al. [64], proved that a significant amount of variation in fatty acid (FA) composition is due to environmental factors. The study proposed that FA composition can be used to check the provenance of argan oil. Kharbach et al. [61] also used 'general' chemical profiling (including acidity, peroxide value, spectrophotometric indices, and the composition of fatty acids, tocopherols, and sterols) and report significant differences between argan oils from different geographical locations. Oussama et al. [65], used Mead InfraRed (MIR) spectroscopy combined with chemometrics for the classification and quantification of argan oil adulteration with sunflower or soybean oils. Whereas, Ourrach et al. [66] proposed 3,5-stigmastadiene, fatty acids alkyl esters, chlorophyll pigments and hydrocarbons as markers of the adulteration of argan oil with other edible oils. These markers were identified as the result of adulteration studies focused on the phenolic profile of extra virgin argan oil to detect the presence of other vegetable oils [67,68]. Other studies have looked at specific markers of argan oil, such as campesterol, coenzyme Q9 or ferulic acid, respectively [69–71]. Argan oil contains between 142 and 220 mg of phytosterols per 100 g of oil, in particular schottenol and spinasterol. It contains only traces of campesterol that is a phytosterol commonly found in vegetable oils. The authors proposed to use these compounds as markers to identify the adulteration of argan oil with less expensive oils and to assess its purity.

Molecular Techniques

To our knowledge there is no paper reporting DNA analysis on argan oil, however there are two studies where genomic approach was used to analyse the genetic variability of argan trees [72,73], which could represent the basis to identify potential biomarkers for authentification. Moreover, some papers describe DNA based technologies for the identification of plant oils [74,75] that may be an alternative or complementary platform to the traditional analytical methods to found adulteration in argan oil. In the review by Agrimonti and Marmiroli [75], the molecular tools to trace the varietal composition of virgin olive oil and to detect the adulterant oils from other botanical species are summarized.

Lamb-Goat Meat

Sheep (*Ovis aries* L.) were among the first animals to be domesticated, have played an important role in human life for thousands of years and are common symbols in culture and religion. The ancient Egyptian fertility god *Heryshaf* was depicted as a man with the head of a ram. In Chinese Buddhism, the ram was one of the animals that attended the birth of Buddha and one of the signs of the Chinese zodiac. Sheep and sheepherding also play important roles for the three Abrahamic religions, Judaism, Christianity and Islam [76]. In the Modern World, sheep and goats are widely distributed and adapted to a wide range of environments. The highest consumption of animal-source protein *per capita* comes from sheep and goat meat in regions related to different religions such as North Africa, Middle East and India [77]. The Mediterranean societies, especially the Southern ones, are among the largest consumers of lamb. Thus, the exigencies of the consumer on the characterization of production systems, locally adapted native genotypes, nutritional information, and sensorial analysis to target the preferences must be answered [77,78]. **Mountain lamb kid meat** is believed to have a superior quality that is related to the farming system. This reflects the capacity of these animals to adapt to a wide range of ecological conditions. The meat is not only appreciated as a food resource but is also important in social and religious ceremonies of South Mediterranean countries.

In Tunisia, sheep and goats are the two most important livestock species, and their production has been based mainly on the traditional rangeland management system. Until the eighties, about 70-90% of the local production of mutton and lamb came from sheep raised in natural grazing areas. Nowadays, small ruminant producers are compelled to practice the feedlot system of fattening lambs to increase the slaughter body weight, especially for the period of the increasing demand of lamb and goat meat corresponding to various religious observances [79]. However, in Tunisia like in other regions of the world, people believe that only sheep and beef produced on grassland and natural pasture is authentic meat and are often considered to be of superior quality [80,81] with additional market value [82]. Quality is now, more than ever, a fundamental concept of agricultural and food policies at both local and international level. It is also a major asset for economic and territorial development that satisfy consumer expectations.

Mountain "Djebel" Lamb

Djebel lamb is produced in the mountainous areas of the Northwest "Djebel" of Tunisia, the Kroumirie - Mogods (Djebel). This region is covered by 2900 km^2 of forests, estuaries, and mountains with relief varying between 300 m in the hills (Mogods) and 1200 m in the western Kroumirie. This area receives the highest amount of precipitation in Tunisia (on average over 1000 mm per year) allowing diverse vegetation with forest formations distributed over about 100,000 hectares conferring a unique Mediterranean landscape with the possibility of food production, tourism and leisure economy [83] and shelters over

200,000 heads of sheep and goat. The production system is close to that of the Iberian pig and is based on forest vegetation composed of oak trees and herbaceous plants. These locally adapted "populations" of small ruminants are a major source of livelihood and contribute to the sustenance of landless, smallholder and marginal farmers. The meat produced by such production system, with its culinary, socio-cultural, tacit knowledge of farmers, has unique characteristics that deserve better representation on the market. Most lamb consumption occurs during the religious festivity of "Aïd el Idha". During this period, the sale of mutton takes place in reserved places in large cities. The preliminary results of surveys, including both traders and customers, showed that consumers choose their "festival" animal according to the proximity or the reputation of the seller. About 10% of customers choose according to the origin of the animal they buy, and about 31% of customers ask about the provenance of the animals. The taste of the animal represents about 60% of the criteria of meat selection. In parallel with these consumer surveys, analytical tests to characterize Djebel lamb are currently in progress. For grazing lambs in Tunisian Northwest pastures, interesting results were recorded concerning meat quality of three local breeds [84]. The results show the higher meat quality of lambs reared on pasture compared to those on concentrate (feedlot). The former has a lower proportion of saturated FAs (44.5 vs 50.6%), a higher proportion of polyunsaturated ones ($C_{18:2}$, $C_{18:3}$ and CLA) and a higher lipid antioxidation power. Such results favor of Djebel lambs reared in mountains of Northwest. The effect of the intake of a forest product, e.g., oak acorn – before and after weaning – on the properties of lamb meat and FA composition [85] show that the polyunsaturated FA, C18:3n-3, was higher in lambs coming from mountains and receiving oak acorns during fattening than those reared in the feedlot. The former also has the highest sensory parameters (tenderness, juiciness and general acceptance).

Anti-Fraud Approach and Geographical Origin Determination

Many papers have been published on the quality of Tunisian lamb in relation to the breed, management system (grazing and feedlot), and slaughtering weight [86-87]. However, only three studies focus on the traceability of grazing lambs. The first paper describes the quality of Bahra kid' meat using chemical composition, fatty acid profile, antioxidation stability and other parameters [88], while the second paper focuses on the discrimination of pasture meat in the Tunisian Northwest from meat produced in conventional systems using visible reflectance spectroscopy [89]. The third paper deals with the quality and traceability of Djebel lamb meat [90].

Stable Isotope Ratio Analysis and Fatty Acids Composition

Mekki et al. [90] applied stable isotope composition in proteins (δ^{13}C, δ^{15}N, δ^{34}S, δ^{2}H and δ^{18}O) and in fat (δ^{13}C, δ^{2}H and δ^{18}O) in combination with the profile of FAs for tracing lamb production systems in four farms located in the North-West of Tunisia. The initial application of these analytical techniques on Djebel lamb provided promising results for both large-scale discrimination of north-west Tunisia, as an overall lamb-producing geographical region, and small-scale classification of regional farming systems. Based on the low δ^{34}S values in protein and the high δ^{15}N values, it was possible to distinguish between Amdoun herbaceous pasture farming system from other Tunisian production systems. However, to make the methodology more robust, a higher number of samples would be needed.

Molecular Techniques

In Tunisia, many papers report the quantitative genetic and phenotypic characterization of local sheep breeds (*Barbarine*, *Queue Fine de l'Ouest*, and *Noire De Thibar*) for developing genetic evaluation tools and elaborating genetic improvement programs [91]. However, studies using molecular techniques to determine the authenticity of Tunisian lamb are rare.

Truffles

Truffles are fruit bodies of hypogeous ascomycetous fungi that grow underground through a symbiotic relationship with the roots of specific host trees (e.g. oak, poplar, willow, hazel, and various shrubs). Truffle production in the Mediterranean area accounts for almost 85% of the world's export market. Economically speaking, the most interesting species belongs to the genus *Tuber*. The most sought-after truffles grow in France, Italy, Croatia, Slovenia, Spain, and Hungary.

Based on their color, truffles (*Tuber spp.*) are divided into two groups, white and black truffles. The *Tuber* genus has been estimated to contain from 180 to 230 species distributed worldwide [92]. Thirty of these species produce edible fruiting bodies (ascocarps) of high nutritional, sensorial and economic value because of their unique aromas and flavors [93,94]. Truffles are the world's most expensive fungi [95], and the global production of truffles, although amounting in the hundreds of tonnes, cannot meet the demand and keeps the price high [94]. The value of their retailed price is in the hundreds to thousands of € per kg, depending upon truffle species, characteristics of the season, dimension and appearance. In 2003, when the season was particularly bad for truffles (hot and dry), the average price for *Tuber magnatum* Pico (1788), the most expensive among truffles, was about 5000 €/kg, while the best items were sold for 8000 to 12000 €/kg [96]. The second most valued species the black truffle, *Tuber melanosporum* Vittadini (1831) can reach about the 2/3 of the *T. magnatum* price. Other commercially interesting species are, in descending economic order are: *Tuber brumale* Vittadini (1831) (1/5 to 1/3 the price of *T. magnatum*)*; Tuber borchii* Vittadini (1831); *Tuber aestivum* Chatin (1887) *and Tuber uncinatum* Chatin (1887), which are sometimes considered as the morphotype of the same species and sometimes as two different species [97,98] and can reach about 1/10 of the price of *T. magnatum, Tuber mesentericum* Vittadini (1931); and *Tuber macrosporum* Vittadini (1931) [96].

Tuber magnatum is mostly found in Italy [99,100] and in a small region of Slovenia [100,101], Croatia [102], Serbia and Hungary [103]. Some species of truffles are also farmed; e.g., *T. melanosporum* [104], *T. aestivum* [105] and to a lesser extent also *T. borchii* and *T. brumale.* However, despite repeated attempts, the most highly-priced truffle *T. magnatum*, has not yet been successfully cultivated [106].

The distribution of *Tuber* species depends on several factors: the spread and migration of the host trees, dispersion of underground spores, dispersion via mammals, climate conditions, and the existence of geographical barriers [107]. Besides this, certain soil parameters have to be met, for example, pH, C/N ratio, the percentage of organic matter, amount of calcium carbonate, nutrient availability, structure, and texture.

Rubini et al. [100] pointed out that natural production of truffles in the past century has been drastically declining due to many factors such as deforestation of the natural habitats of the *Tuber* species, poor forest management, unselective harvesting and the introduction of new or exotic plant species, which are unable to form a symbiotic relationship with the edible mushrooms. Moreover, European production is influenced by climatic change, negative effects being linked to increasing temperature and decreasing rainfall [108]. Production has decreased from 2000 tonns/year 100 years ago, to just 20 tonns/year today [109]. The decrease in the natural production combined with an increase in global demand and high prices makes truffles a target for fraud, especially when species are morphologically similar [110].

In terms of global export, 85% of truffles come from Europe, 10% from China and 5% from North Africa. High truffle prices have led to several forms of adulterations. Most commonly, in the case of cheap truffles (around 15 € per kg) originating mainly from China, aromas are added and sold on the market as visually similar to the European black truffle [96]. Other species, such as the desert truffles *Terfezia sp.* growing in the Mediterranean area, and especially abundant in Morocco, are illegally sold on the black market as *T. borchii* or even as *T. magnatum* [111]. In addition, truffles from less expensive European species are sold as the most prized species. For example, *T. borchii* can be visually confused with *T. magnatum*. Another known fraud practice for truffles in processed food is the use of unripe ascocarps of cheaper species. These ascocarps do not have spores; therefore, morphological classification is not possible [112,113]. The value of their retailed price is in the hundreds to thousands of € per kg, depending upon truffle species, characteristics of the season, dimension and appearance.

Situation in Slovenia

Truffle harvesting in Slovenia has a long tradition. One of the oldest quotations is found in the second edition of Flora Carniolica in 1772 [114], although truffles have been probably known since Roman times. However, in recent history, the harvesting of wild truffles (*Tuber sp.*) was illegal [115] until 2011 [116], and the majority of Slovenian truffles were sold illegally and marketed as originating from elsewhere. The situation has currently changed, and the truffle culture is in its revival. The whole production of truffles in Slovenia comes from harvesting wild truffles. The truffle harvest in Slovenia is based on an estimate of 40 collectors. Slovenian Istria has several collecting locations of *T. magnatum* (especially in the valley of Dragonja and Rižana rivers), while the black truffle species are spread over a large area of the country. The cultivation of truffles in Slovenia is still in its early phase.

Most literature relating to truffles in Slovenia reflects two major issues: (i) the assessment and determination of the number of species occurring in the country [96]; and (ii) the potential for cultivation and assessment of potential growing areas [117–122].

Situation in Italy

The history of truffles' collection in Italy also dates back to Roman times. In the 18th century, truffles from Piedmont were considered a delicacy in all the European Courts. In the same period Vittorio Pico, a doctor from Turin, took care of the classification of *T. magnatum* as a part of his doctoral thesis [123, 124]. Today Italy is the European country that boasts the presence of the highest number of species of wild edible truffles. There are eight species that can be collected and marketed in Italy, according to the National Law No 752/85 [125], and its subsequent modification Law No 162/91 [126], including *T. magnatum*, *T. melanosporum*, *T. aesti*vum, *T. uncinatum*, *T. brumale*, *T. brum*ale var. *moscha*tum De Ferry (1888), *T. borchii*., *T. macrosporum*, and *T. mesentericum*. The national production of truffles was about 95 tons per year in the period 1980-2008 [127]. The harvest of truffles is regulated by specific national and regional laws that govern the specific periods. Moreover, the maturation status of the truffles is taken into account to define the harvest periods.

The main truffle areas are Abruzzo, Marche, Molise, Piedmont, Emilia-Romagna, Umbria and Veneto regions. *T. magnatum* can be found in Piedmont and in the northern and central part of the Apennines. Nowadays, more than 70,000 people are licensed by local public administrations to harvest truffles. The number of harvesters has been increasing significantly since the 80s with the most of them located in the mid-Northern area of the Apennines and the Piedmont area of mid-Eastern Alps [128].

Anti-Fraud Approach and Geographical Origin Determination

In scientific literature, several studies consider or at least mention the possibility of determining the geographical origin of truffles. Most studies use molecular approaches and analysis of volatile compounds as more reliable methods than morphological determination.

Stable Isotope Ratio Analysis

At the moment, there is no study on the use of stable isotopes and elemental composition for determining the geographic origin of truffles, but some studies have focused on epigeous fungi (mushrooms). Those studies where stable isotope techniques have been applied in truffles were orientated towards either looking at carbon isotope fractionations during the decomposition of sucrose [128], at the ecophysiological relation between truffles, soil and host plants [129] or assessing the mycorrhizal versus saprophytic status of fungi using the natural abundance of carbon and nitrogen stable isotopes [130]. More recently, a few papers have been published on the application of stable carbon isotopes to determine the authenticity of truffle aromas. Sciarrone et al. [131] developed a method for determining δ^{13}C values in bis (methylthio) methane by Headspace Solid-Phase Microextraction Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (HS-SPME GC-C-IRMS). The determination of this parameter in authentic white truffles harvested in Italy led to values between −42.6‰ and −33.9‰. The same method was applied to the analysis of pasta, sauce, olive oil, cream, honey, and fresh cheese flavored with truffle aroma to determine their authenticity. Wernig et al. [132] found that the δ^{13}C values of 2,4-dithiapentane, a characteristic truffle odorant detected in most flavored oil samples, is not a useful marker for discriminating between natural and synthetic truffle flavors.

As regards mushrooms, particularly ascomycetes of genus other than *Tuber* or even basidiomycetes, due to similarities in their ecology and physiology, they provide case studies of potential application in the truffles' traceability. E.g., Ill-Min et al. [133] determined $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ to verify the regional traceability of Agaricus bisporus mushroom from six regions of Korea. They found that all four isotope ratios were significantly different among the six cultivation regions. The same results were obtained by Puscas et al. [134] that determined $\delta^{13}C$ on bulk fungi and $\delta^{2}H$ and $\delta^{18}O$ in the water extracted from the samples. In particular, they were able to distinguish samples from different Transilvania areas and, furthermore, found a link between the isotopic composition and the characteristics of the place of growth of the fungi (hilly or mountainous).

Elemental Analysis

The elemental composition of truffle has been addressed in only a few papers. Sawaya et al. [135] determined the chemical composition and nutritional quality of Saudi Arabian truffles: *Terfezia claveryi* and *Tirmania nivea*. Further, Segneanu et al. [136] determined the trace element content of *T. magnatum* and *T. melanosporum* using atomic absorption spectrometry. They found that *T. melanosporum* contained higher levels of Fe than *T. magnatum*. The level of the other elements was approximately the same.
As regards mushrooms, Giannaccini et al. [137], determined by ICP-OES 14 trace elements in *Boletus edulis* and *Macrolepiota procera* harvested in different areas of Tuscany region. A different elemental content was reported within-species and according to the growth site. Similarly, Nikkarinena and Mertanen [138] analysed 33 elements in mushrooms grown in two geochemically different regions in Finland by ICP MS. They confirmed the influence of the geochemical characteristics of the place of growth of the sample on the trace element concentrations. Therefore, they declared that this is a confirmation that the inclusion of geographically linked information in food composition databases would enhance their value and allow better utilization in applied studies.

Molecular Techniques

The majority of molecular studies are designed to either determine species or differentiate between morphologically similar ones, to make species determination easier and to prevent frauds. These methodologies are especially important when truffles have not yet developed the ascocarp, finding applications to test the inoculation material, which is sold to commercial trufferies, or to identify in the trufferies competitive mycorrhizae [139–144].

An attempt to differentiate geographic origin on the basis of genetic diversity (owing to evolutionary and adaptive processes driven by different environmental conditions) was made for *T. magnatum* by Frizzi et al. [145]. Later, Jeandroz et al. [146] developed the first comprehensive molecular phylogeny of the genus *Tuber* and analyzed its biogeography. The resulting molecular phylogeny divided the genus *Tube*r into five distinct clades. The *Puberulum*, *Melanosporum*, and *Rufum* groups were diversified in terms of species and geographical distribution. Alternatively, the *Aestivum* and *Excavatum* groups were less diversified and were located only in Europe or North Africa. Bonito et al. [137] performed similar phylogenetic work and found similar results.

Amicucci et al. [112] used a molecular identification approach to analyze food products containing fragments of some *Tuber* species. This method is useful when the morphological characteristics of truffle are difficult to interpret owing to the drastic treatments utilized in food preparation or the use of unripe fruit bodies (lack of spores). Furthermore, the method requires tiny amounts of sample and is amenable for degraded DNA. It will also have important applications in both the production and sale of such food products, in order to avoid fraud and reveal the possible presence of other fungal species. Séjalon-Delmas et al. [148] proposed a protocol with a single PCR step to detect the fraudulent presence of Chinese truffles or any other fungal species, either in a fresh batch of truffles and in canned truffles. Rizzello et al. [113] reported the application of molecular techniques to authenticate truffle species in commercial products. In particular, they obtained good quality DNA using a kit generally employed for DNA extraction from soil, and a new primer pair was developed to authenticate *T. magnatum* in commercial products.

So far, only one proteomic study of truffles has been performed. Islam et al. [149] functionally annotated the truffle proteome from the sequence of 2010 of *T. melanosporum* genome comprising 12771 putative nonredundant proteins. Using sequential BLAST search strategies, they identified homologues for 2587 proteins with 2486 (96.0%) fungal homologues (available from http://biolinfo. org/protannotator/blacktruffle.php). A combined 1D PAGE and high-accuracy LC-MS/MS proteomic study was employed to validate the results of functional annotation and identified 836 (6.5%) proteins.

Volatile Organic Compounds

The majority of studies relating to truffles concern volatile compounds. Some use an electronic nose to analyze the change in aroma composition during the ageing of truffles [150], while others use HS-SPME-GC–MS [151] and HS GC/TOF-MS [152] to characterize volatile compounds of truffles from different species. Aprea et al. [153] combined an electronic nose and PTR-MS, while Zampioglou and Kalomiros [154] showed that an intelligent odor-discriminating system based on a gas sensor array could contribute to the identification and classification of truffles based on their stage of maturation and place of origin. Vita et al. [155] were able to determine both the origin of fruiting bodies (Alba – Piedmont region versus San Miniato – Marche region) and the two biological phases of fruiting body formation in San Miniato truffles using PTR-TOF-MS signals of the volatile organic compounds. Moreover, Díaz et al. [151], reported the possibility of using the aroma composition to assess the geographical origin of truffles.

Volatile organic compounds can also be used to detect fraud in processed food containing truffles or truffle derivatives. These studies aim to distinguish between truffles of different species that are morphologically very similar but have very different aromas. Culleré et al. [156] used GC-O and HS-SPME-GC–MS to study the aromatic composition of black and summer truffles of *T. indicum* and *T. melanosporum*, respectively. They concluded that both analytical approaches, either in combination or separately, could be used as a way of screening frauds. D'Auria et al. [157,158] also used SPME-GC–MS to characterize the volatile profile of different species of truffles and false truffles (e.g. Basidiomycetes) from the Italian region of Basilicata. Finally, GC combined with different types of interfaces/devices and extraction methods has been used to characterize the key aroma compounds of *T. Magnatum*, *T. Uncinatum* [159], and of *T. melanosporum* [160,161]. Using GC–MS and an electronic nose, Pacioni et al. [162] checked the authenticity of Italian olive oil flavored with white and black truffles. The method was able to distinguish the aromas from the species of truffle declared on the label and confirmed the established malpractice of the use of bismethyl (dithio) methane when flavoring with black truffles. Similarly, Torregiani et al. [163] used an SPME-GC-MS approach to test the aroma profile of raw truffles, truffle sauces, and natural and artificial truffle, flavored oils made from or made to imitate *T. magn*atum, *T. melano*sporum, and *T. aestivum*.

Conclusions

This literature review reveals that among the studied Mediterranean food products, truffles were the most investigated, while little information is available on Tunisian Djebel lamb. The currently used methodologies for determining authenticity and origin are related to the chemical analysis of fatty acid profiles and sterol composition in argan oil, aroma compounds in truffles, and DNA and other molecular methods in Iberian pig meat. However, these analyses do not allow extensive verification of food geographical origin or their authenticity. No study combines the use of elemental composition and stable isotopes ratios in regards to authenticity, although their reliability in determining food traceability and authenticity has been proven for many products.

Moreover, novel approaches such as prediction mapping (e.g. isoscapes) may provide a cost-effective extension to the databank approach. The term “isoscape” derives from the words **iso**tope and land**scape**. An isoscape offers a spatially georeferenced representation of the distribution of isotopic compositions (generally of light elements). These are generated by incorporating isotopic data into geographic maps using a Geographic Information System (GIS). Ancillary variables other than isotopic observations and reliable on a larger spatial scale (lower data density for the spatial unit), are needed. For instance, ancillary data can be meteorological, geographical or geological ones, which drive the fractionation processes and can lead to a robust reconstruction of expected isotopic compositions of food products. The advantage of process-based modeling over statistical modeling is that the former requires a much smaller reference dataset, which means that it can be applied to those areas where isotopic information is scarce.

A composite methodological approach appears promising for future studies aimed to ensure geographical traceability and food authenticity, and this will be pursued within the REALMed project.

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The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in writing of the manuscript; or in the decision to publish the results.

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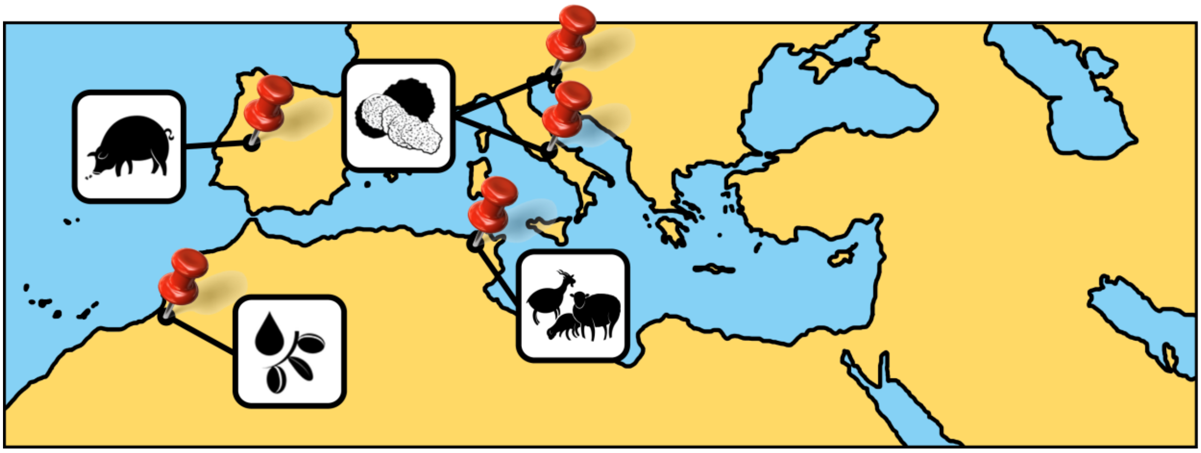

Figure1. A graphic representation of selected Mediterranean products: Meat products from the Iberian black pig from Spain and Portugal; Argan oil from Morocco; Mountain “Djebel” lamb meat from Tunisia; and Truffles from Slovenia and Italy.