Geographical Traceability of Tunisian Olive Oils Based on the Elemental and Stable Isotope Composition

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Fadwa Damak

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Fadwa Damak

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Prof. Mitsutoshi Nakajima: University of Tsukuba

Prof. Hiroko Isoda: University of Tsukuba

Prof. Masatomo Kobayashi: National Institute of Physical and Chemical Research (RIKEN)

Prof. Kenji Tamura: University of Tsukuba (Supervisor)

Summary

The protected geographical indications (GI) are powerful sustainability and marketing tools that have extensively been applied in the EU to protect, promote and valorize many foodstuffs originating from specific geographical areas. The success of this marketing strategy prompted many countries, including Tunisia, to establish the favourable conditions to create such lables starting from the legislative framework. But in addition to the existence of the legislative framework that regulates the activities related to GIs, in order for a foodstuff to register as a protected GI in the national legislation, the causal link showing how the characteristics of a particular region (geology, climate, etc…) affect a product in a way that other regions cannot, must be justified. The main objectives of this research are the establishment of a database and the development of a traceability methodolgy for the Tunisian olive oils based on the multielemental and stable isotopic composition – coupled to chemometrics - to ultimately contribute to the creation of Tunisian olive oil GIs. The causal link with the environment will constitute a big part of this research.

The first part of this study represents a pilot study conducted using the multielemental profiling approach by ICP-MS technique. Despite the long and established use of multielements in geographical traceability of olive oils in other countries, their validity as markers of the provenance factors (e.g. soil geochemical composition) is not proven, in contrast to the information available for stable isotopes whose variability in olive oils according to origin was correlated with climatic and geographical parameters. Therefore, the link between the olive oils elemental composition and the geochemical soil composition determined by XRF and LA-ICP-MS techniques was investigated. The results of this pilot study showed the predominant geochemical source of the elements in the olive oils based on their associations in a latent space according to Goldschmidt rule and their correspondence with the provenance soils composition. These results proved that a multielemental profiling approach is a valid and promising geographical traceability tool. However, the total concentration of the elements in the soils on which the olive trees are grown cannot predict the elemental composition of the derived olive oils. This will direct us towards exploring additional environmental factors that may impact the oils' multielemental profile. (The results of this pilot study have been published in Food Chemistry journal: Damak et al., 2019). Additionally, the results pointed out flaws of the used analytical method, which was the

microwave-assisted extraction of the elements, as it could not reach relatively low detection limits that allow measuring a wide range of elements. The reach of lower detection limits and inclusion of more elements can increase the effectiveness and robustness of the model by identifying and including elements with a higher discriminatory power.

The second part of this study deals with the optimization of the multielements extraction from the olive oils. Three methods are compared among which one was originally proposed in this research. The three methods are the microwave-assisted extraction, the ultrasound-assisted extraction and the combined microwave-assisted extraction of the elements and evaporation of the digestate. Results showed that despite reaching lower detection limits as compared to the microwave only, the combined microwave-assisted extraction and evaporation was characterized by very high relative standard deviations caused by the prolonged preparation time and contact with the environment resulting in increased contamination. The ultrasound-assisted extraction resulted in even lower detection limits and higher precision and was therefore selected as the preparation method of choice for the next part.

The next part of the thesis is focused on two aspects: (i) the creation of an extensive, first of its kind, national database of multielements in Tunisian olive oils from the main 11 producing regions with a view of confirming their inter-regional discriminatory power and (ii) the geochemical and edaphic characterisation of these 11 regions to deepen the understanding of the relationship between the elemental composition of olive oil and its environment. The results showed a good consensus between the geochemical (around 40 elements) and edaphic characteristics pointing out some geochemical processes; and that the regions with similar outcrop lithology presented similar soil chemical characteristics. Out of the 11 regions, only 2 regions presented distinct soil characteristics whereas the rest of the regions which were located above similar outcrop lithologies could hardly be distinguished. The same observations apply to olive oil multielemental composition. This fact highlights the limitation of the traceability approach based on the multielemetal profiling when similar driving factor (here is the geology) prevails in the regions to be discriminated. The study of the relationship between the olive oil elemental composition and bioavailable fraction of elements in soil revealed that Ti, Fe, Ni and Ba presented significant positive Spearman correlation coefficients. This is the first study addressing and reporting such significant correlations which show the effective link of olive oil inorganic composition to its environment and the elements that can establish strong and well-established traceability.

The last part of this research studies the isotopic composition of C and O using IRMS technique of olive oils from the same 11 regions and compares the performance of the isotopic and elemental profiling as a traceability tool for intra- and inter-country scales. The main climatic and geographical factors that determine the isotopic signature of Tunisian olive oils have been identified and significant Pearson correlations have been proven which will allow the future prediction of their isotopic composition based on regressions. Concerning the intra-country discriminatory power, both tracers performed similarly with difficulties encountered whenever the drivers are similar between the regions to be discriminated. As for the inter-country, multielements showed a better efficacy than stable isotopes to discriminate between Tunisian and Southern Italian oils. This is due to the geographical proximity of the two countries and the similar climatic conditions prevailing in southern Italy and Tunisia.

This research provided the first ever characterization of the Tunisian olive oils elemental and isotopic compositions from wide range of geographical areas spanning the country form North to South and from East to West and constituting the most extensive Tunisian database in the scientific litertaure. The multielemental fingerprinting approach showed excellent potential in the geographical traceability of Tunsian olive oils due to their proved link with the provenance soil geochemistry and their good discriminatroy power. Our results also showed that the combination of the elemental and isotopic fingerprinting approach increase the discriminatory power of the model. In conclusion, multielements and stabe isotopes of Tunisian olive oils are clearly linked to their provenance environment and a successful geographical traceability needs to take into consideration the geological and climatic characteristics of the regions from which the olive oils to be discriminated were derived. In future work, an integrated research should be carried out in the highly potential GI areas to materialize the objective of valorizing olive oil as a key bioresource in arid and semi-arid region such as Tunisia.

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General Introduction

Tunisia is a small country located on the southern shore of the Mediterranean sea that has seen its olive oil sector expanding throughout the years, decades and centuries since the 8th century BC. Olive cultivation in Tunisia dates back to the era of Carthaginians and Phoenicians and had known a prosperous phase during the Roman era during which Romans developed intensified irrigation techniques and new olive oil extraction process. The Tunisian olive orchard is spread over one third of the country's crop area with 95% of olive trees being cultivated under rain-fed conditions. There are approximately 60 million olive trees in Tunisia, 30% of which are distributed in the north and contributing by 18% of the total oil production, 45% in the centre contributing by 27% and 25% in the south contributing by 55%. The higher oil share in the south despite the lower olive trees number is due to the higher oil content in the southern olives (Olivae, 2017). The Tunisian olive orchard is dominated by two main cultivars: Chetoui in the north and Chemlali in the centre and the south but it is characterized by dozens of "secondary" cultivars many of them specific to delimited areas such as Oueslati in Kairouane and Chemchali in Gafsa (Rekik et al., 2008).

Since the overwhelming majority of olive farmers in Tunisia don't possess their own olive oil mill, their main task is the olive fruits growing and harvesting. Depending on the situation, some farmers decide to entrust the task of olive oil extraction to the local mills for a defined fee and they get their ready olive oil to sell in bulk in the local market. However, in most cases, the farmers sell the olive fruits to the mill that offers the best buying price.

Nowadays, Tunisia occupies a respectful place in the world olive oil figures and comes fourth after Spain, Italy and Greece in terms of production and second after Spain in terms of exportation. Yet, this key food product suffers from low international recognition since about 99% of Tunisian olive oil has traditionally been exported in bulk for a relatively low price (Kashiwagi, 2012).With growing international competitiveness, Tunisian authorities have aimed to increase the export of bottled olive oil by establishing the Fund for the Promotion of Packaged Olive Oil (FOPROHOC) in 2006 and developing new products based on the organic production certification, in order to enhance the recognition of Tunisian olive oil and to reach new markets such as Japan (Kashiwagi, 2012). Despite these efforts, exports of packaged olive oils are still far below the target of 20% (IOC, 2017). To solve this problem and to push Tunisia's olive oil producers to export less oil in bulk and more in bottled and branded products, the development of differentiated olive oil products, such as those utilizing geographical indication (GI) labels, is strongly promoted in order to introduce a product with

higher added value and in line with consumer preferences in a highly competitive market. According to Agreement on Trade Related Aspects of Intellectual Property Rights (TRIPS), GIs are "indications which identify a good as originating in a territory of a Member, or a region or locality in that territory, where a given quality, reputation or other characteristic of the good is essentially attributable to its geographical origin". In the same context, the World Intellectual Property Organization (WIPO), describes a GI as a "sign used on goods that have a specific geographical origin and possess qualities or a reputation that are due to that place of origin" (Monten, 2005).

In fact, food products carrying an origin label have shown an increased demand over the past decades and consumers are placing value on them as the origin is perceived as a guarantee of quality when it is associated with and reminiscent of excellent reputation and renowned "brand" (i.e. origin) name. This increasing demand is prompted and driven by factors such as the rising consciousness of food safety, the socio cultural status of consuming certain foods and renewed interest in gastronomic patrimony. Given the global competitive market characterized by declining foodstuff prices, this trend towards quality value-added products with a strong cultural and geographical link or territorial origin indication provides producers with the opportunity to transition from commodity markets into more profitable niche markets through differentiation based on geographical labelling. (Bramley et al., 2009).

Cei et al. (2018) stated that GIs have a link with the rural development which involves several aspects: i) support of the GI supply chain, ii) support to rural economic diversification, iii) the empowerment and activation of human resources and development of local social organization, and iv) the protection of the environment, amenities and local cultures. The value created by a GI on the market may be directly transferred to local actors which can improve local economic conditions. Simultaneously, typical products may encourage the inception of other types of activities in their area of origin such as tourism. In fact, the presence of local typical foods stimulates of touristic initiatives and programs and the distribution of local products in agritourism framework which encourages tourists to buy and consume these local products and consequently generates incomes for the area. The authors of the same study concluded that GIs are valuable tool to promote local development processes and to improve agricultural economic sustainability (Cei et al., 2018). In another study, Bajoub et al. (2016) stated that GIs can help obtaining added-value, premium price, enhanced competiveness in the traditional and emerging olive oil markets and providing socio-economic benefits to rural areas. (Bajoub et al., 2016). Therefore, and taking into consideration these benefits of the recognition of GI products, Tunisia is aiming at the high-

end market, seeking to enhance its competitiveness therein. In 2008, a study conducted by the Agency for the Promotion of Agricultural Investments (APIA) identified 21 areas eligible for obtaining a registered designation of origin also known as geographical indication (GI) qualification. This selection was based on the differentiation of the areas according to olive variety, soil, climate, and know-how, but it did not take into account the physicochemical characteristics of the oils themselves (Laroussi-Mezghani et al., 2015). However, the process of assessing the suitability of a product in a specific area to pursue a GI label should be based on robust scientific methodology (Bajoub et al., 2016). In order to register a GI product for protection, the terroir should be delimited, the natural and human factors of the terroir need to be defined and the uniqueness and typicality of that product need to be proven (Bajoub et al., 2016). The typicality will be our main concern in this study. The typicality can be evaluated by demonstrating that the qualitative and compositional profiles of olive oils produced within the potential GI area are similar and different as compared to those of olive oils produced outside that potential GI area. Hence, it is of absolute interest and priority to develop analytical methodologies capable of confirming the provenance of food in order to accomplish food valorisation objectives based on differentiation.

Several analytical strategies based on instrumental techniques have been used to deal with proving the typicality and the authenticity as broader subject matter.These methods can be classified into two groups depending on their operating principle: i) physico–chemical techniques, such as spectroscopy, spectrometry, chromatography, electronic nose, etc. and ii) molecular biological approaches, such as DNA-based methods. From another perspective, the analytical parameters, also called traceability markers or tracers, can be distinguished into direct and in-direct. Direct markers include the elemental composition (major, trace and rare earth elements (REEs)) and the stable-isotope ratio of light or bio (carbon, oxygen hydrogen, nitrogen) elements and heavy or geo elements (Sr, Pb, etc…). They are called direct because they can be directly linked to the same measurements in soil samples or precisely attributed to particular geographical origin. On the other hand, indirect markers are variables attributed to the raw or processed food and can be regarded as foodstuff fingerprint. These variables may allow discrimination from food products coming from a different geographic origin but cannot be directly associated to the production region. In fact, Even though discrimination of olive oils' origin is possible, none of these methods is capable of precisely identifying the geographical origin especially when olive oils are derived from the same cultivar. Only when provenance characteristics are taken into account (i.e. climatic and geological characteristics) can the origin be identified. According to Bertacchini et al. (2013), three methodological

approaches can be used to link the foodstuff to its region of origin. The first one is based mainly on measurements of the marker on representative set of samples of known and authentic origin to generate a database that can be processed by various multivariate statistical analyses to reveal a possible link or influence of the origin. The geographical, geological, and climatic reference information are or are not taken from bibliographic references or responsible agencies. The second methodology is based on systematic measurements of the marker in the foodstuff, similarly to the first approach, but in that case random samples of soil of provenance or climatic parameters of the environment are also taken and/or measured. The third methodology is based on a systematic and representative sampling of both the foodstuff and its environmental factors (soil, climate). The same authors declared that the choice of one methodology over another should be made based on the nature of the marker that will be used, the aims of the study and the research questions that need to be answered. However, the authors recommended building traceability models that can support the cause/effect relationship between the foodstuff and its environment (Bertacchinie et al., 2013).

Our attention was firstly oriented towards the inorganic composition of olive oils. In fact, and based on the hypothesis of Greenough that stated that the transfer of the elements from soil to the foodstuff is subjected to minor vaiations, numerous studies have demonstrated the effectiveness of multiements as tracers of the geographical origin. The objective of the first chapter will be then to investigate the validity of multielements as markers of the geographical provenance of olive oils in the geological and climatic context of Tunisia. This chapter will be presented in the form of a research article published in "*Food Chemistry*" journal (Damak et al., 2019). This pilot study shows that multielements are promising geographical markers of Tunisian olive oils at an interregional scale. Accordingly, the multielemental fingerprinting approach will be applied to build a database of Tunisian olive oils to help promote product proliferation and market differentiation of non-certified regional products towards origin-labelled products, which will constitute the content of an additional chapter (third chapter). The second chapter will actually deal with the comparative study on the performance of three analytical methods to quantify the elements in olive oils with a greater robustness. This study was inspired by the results of the pilot study and insights from the ongoing experimental work. The last chapter will deal with the isotopic characterization of olive oils from the major producing regions to compare the discriminatory power of these two markers.

Chapter 1

Pilot study on validity of multilements as markers of provenance and possible link with the soil of origin

1. Introduction

Olive oil production in Tunisia has increased due the development of modern orchards alongside intensification of traditional orchards and expansion into new crop-producing areas, making it the second-largest producer after the European Union (IOC, 2017). Tunisian olive orchards are characterized by a rich genotypic heritage comprised of seventy olive tree varieties, each having unique attributes (Laroussi-Mezghani et al., 2015).

In the previous decade, intensive research effort has been put into the development of methodological approaches for characterizing and proving the uniqueness of Tunisian olive oils according to their geographical origin and/or varietal origin. Most of the efforts toward selecting a suitable geographical marker focused on the molecular and biochemical composition and sensory profile of the oil. In olive oils, the latter two parameters are highly dependent on storage conditions (Gomez-Alonso, Mancebo-Campos, Desamparados-Salvador & Fregapane, 2007; El Haouhay et al., 2018), and as such their use as geographical markers could be unreliable. In addition, molecular markers are more appropriate for elucidating the olive varietal rather than the geographical origin (Adamo et al., 2012). An alternative and reliable marker of provenance is therefore needed.

Numerous studies have shown that elemental patterns, in combination with multivariate statistics, can be successfully used to identify the geographical origin of foodstuff in general (Ariyama, Nishida, Noda, Kadokura & Yasui, 2006; Greenough, Fryer & Mallory-Greenough, 2010) and of olive oils in specific geographical contexts (Jimenez, Velarte, Gomez & Castillo, 2004; Benincasa, Lewis, Perri & Sindona, 2007; Camin et al., 2010a; Camin et al., 2010b; Cabrera-Vique, Bouzas & Oliveras-Lopez, 2012; Beltrán, Sanchez-Astudillo, Aparicio & Garcia-Gonzalez, 2015). These studies have laid the foundation for testing the suitability of multielements as potential provenance markers within the geographical and geological context of Tunisia.

The suitability of multielements as geographical markers is essentially owed to the fact that the elemental profile of olive oil reflects its complex interaction with the environment,

including climate, mineralogy, the mobility and bioavailability of elements in soils, and physiological aspects typical of the species from which it is derived (Giaccio & Vicentini, 2008). Even though the composition of olive oil could be affected by cultivation practices, the extraction process, and environmental pollution, it has been suggested that the selection of elements that reflect the geogenic origin, rather than the anthropogenic origin, such as the alkaline metals Rubidium (Rb) and Cesium (Cs) which are easily mobilized in the soil (Kelly, Heaton & Hoogewerff, 2005), or Lithium (Li) and Rb which are not influenced by technological processing (Giaccio & Vicentini, 2008), can solve this issue. It is therefore necessary to consider the possible origins of the elements contained in the Tunisian olive oil.

Apart from being proven successful in the same context in other countries, elemental fingerprinting can provide valuable information regarding the quality of olive oils in relation to the maximum residue limit, which is a quality attribute defined by the International Olive Council fixing the maximum allowable concentrations of certain toxic heavy metals (IOC, 2016).

Although multielements (major, minor, trace, and rare earth elements) have been extensively used to relate olive oils to their provenance, information on how the natural factors of the provenance, including geochemical factors, correlate with the elemental profile of olive oil is very scarce (Beltrán et al. 2015). This is in contrast to the information available for other tracers, for instance the stable isotope ratios of light elements, whose variability in olive oils according to origin was correlated with climatic and geographical parameters (Chiocchini, Portarena, Ciolfi, Brugnoli & Lauteri, 2016).

In order to prove the utility of multielement fingerprint as a tool for authentication of origin, its relationship with the provenance environment should be proved. First, the sources of the elements in olive oil should be evaluated in terms of whether they depend on geochemical or anthropogenic factors. Second, the strength of the relationship between the geochemistry of the orchard soil and the elemental composition of the olive oil needs to be quantified.

The present work constitutes a pilot study aiming at identifying the origin of Tunisian olive oils using chemical markers. The objectives of the present study were to: characterize the elemental profile of Tunisian olive oils from four geographical origins; assess the quality of the oils based on the maximum residue limit criteria; verify the source of the elements in the Tunisian oils based on their associations; evaluate the viability of multielement analysis in

classifying and predicting the geographical origin of Tunisian olive oils; and study the link between the chemical compositions of olive oils and their provenance soils.

To the best of our knowledge, no previous study on Tunisian olive oil has used geochemical fingerprints (i.e., major and trace elements) to elucidate its geographical origin. The only available multielement data concerns olive oils obtained from trees irrigated with treated wastewater in one limited geographical area (Benincasa et al., 2012).

2. Materials and methods

2.1. Sampling areas

The olive oils used in this study originated from four producing regions, namely Sfax (2 sites) and Monastir (5 sites) in the mid-Eastern region, and Gafsa (5 sites) and Medenine (2 sites) in the South of Tunisia. These regions are considered important olive oil-producing areas, with considerable proportions of their production destined for exportation. The climate in these regions varies from arid to semi-arid Mediterranean. During the time period between 1991 and 2015, the average annual temperature and rainfall of the four regions were as follows: Gafsa 20.4 °C and 194.3 mm/year; Medenine 21.2 °C and 215.8 mm/year; Sfax 20 °C and 260 mm/year; Monastir 20.7 °C and 346.5 mm/year (World Bank Group, 2018). In terms of geological features, outcrops of the sampling areas are shown in Fig. 1. The samples from Gafsa were located on recent and actual (Holocene) sandy alluvial sediments and sand dune sediments. In Sfax, the first sampling site was located on old alluviums that are mostly aeolian sand-loam with limestone concretions and gypsum crusts (Middle-Late Pleistocene), and the second sampling site was located on Mio-Pliocene continental sediments made of conglomerates and sandy clays. The sampling sites of Monastir samples were located on old alluviums and Mio-Pliocene sediments similar to those of the Sfax sites. Medenine samples originated from a site lying above similar old alluviums and Villafranchian-lower Pleistocene conglomerates and carbonate crusts (Cohen, Schamel & Boyd-Kaygi, 1980; Sghari, 2014).

2.2. Olive and soil sampling

Twenty-one monovarietal olive fruit samples were hand-picked from three representative trees for each sample. These included autochthonous Tunisian varieties such as Chemleli Sfax, Chemleli Sahli, and Chemchali of Gafsa, as well as introduced foreign varieties like Spanish Arbosana, Italian Frangivento, and Greek Koroneiki (Table 1). The introduced varieties were mostly from Gafsa, where they are cultivated in new intensive systems more
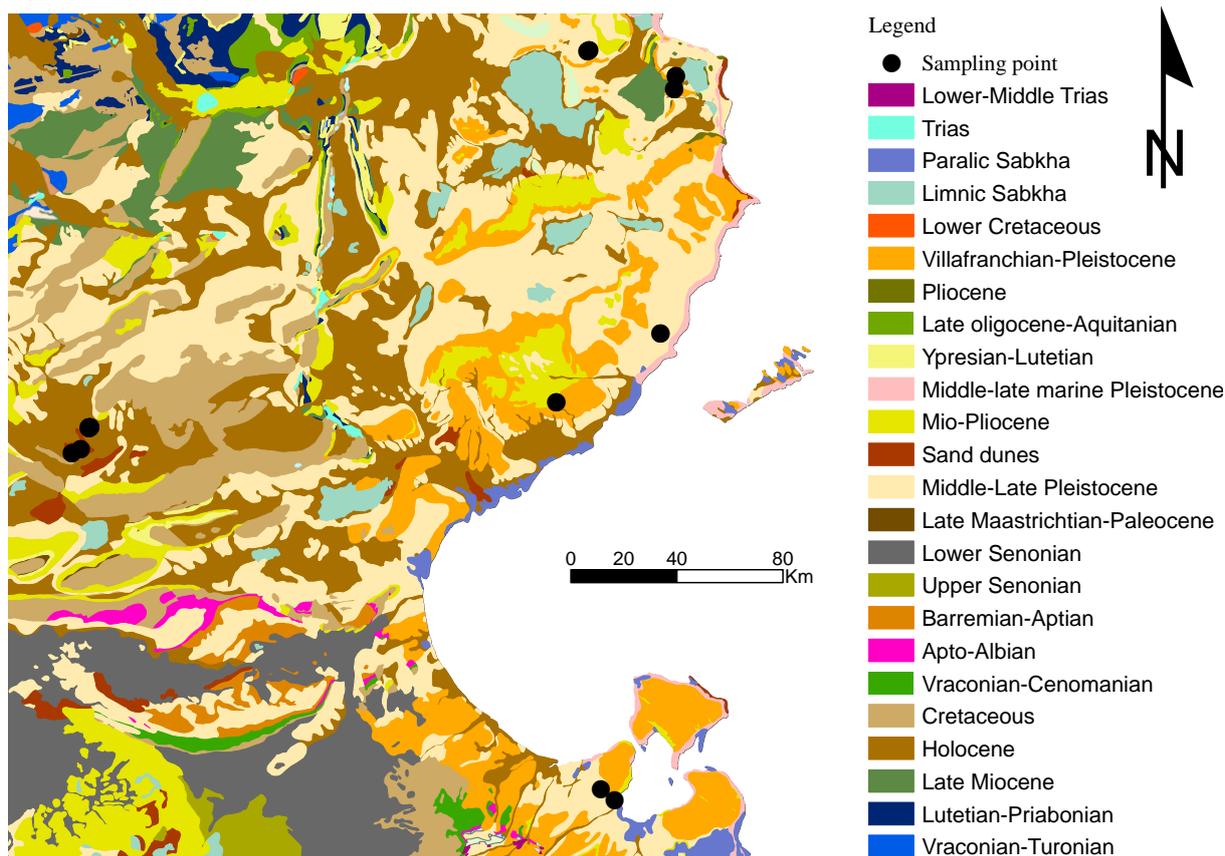
Figure 1. Geological map showing the location of the sampling points in Gafsa, Medenine, Sfax, and Monastir.

extensively than in other regions. The sampling followed additional criteria so as to sample paired olive fruit and soil samples from the same orchard, allowing evaluation of the relationship between provenance soil and olive oil composition. Top and subsoil samples were collected from two layers at depths of 0-30 and 30-60 cm using a hand auger. Soil samples were taken from under each sampled tree and then mixed in equal-weight proportions to form a representative sample of both depths at each sampling site. In total, twenty-eight soil samples have been prepared for subsequent analysis. Fig. 1 shows the locations of the fourteen total sampling sites recorded by a handheld GPS receiver (eTrex Legend HCx, GARMIN, UK). All samples were collected in polyethylene plastic bags and rapidly transported to the laboratory for preparation for the chemical analyses. Sampling was conducted from November to December 2015.

Table 1. Number of olive fruit samples and their corresponding soil samples collected from four regions: Gafsa, Monastir, Sfax and Medenine.

Origin	Gafsa	Monastir	Sfax	Medenine
Arbosana	3			
Chemchali	1			
Koroneiki	3	1		
Frangivento	1			
Chemleli Sahli		5		
Chemleli Sfax	3		2	
Zalmati				2
Total	11	6	2	2
Soils	10	10	4	4
Sampling sites	5	5	2	2

2.3. Sample preparation and analysis

2.3.1. Olive oil samples

2.3.1.1. Oil extraction

Each batch of olive oil was extracted in the Sfax Olive Institute from 1.5 kg of whole washed olive fruits by mechanical means alone. A laboratory-scale 2-phase oil mill was used following the standard methods employed in industrial olive oil mills. This method consists of crushing, malaxation for 30 minutes at ambient temperature, centrifugation without addition of water, and finally natural decantation. This process yields an oily phase and a very wet pomace phase. Olive oil samples were stored in amber glass bottles at 4 °C until their major and trace element analyses, which were performed at the Advanced Analysis Center, National Agriculture and Food Research Organization (NARO) in Japan.

2.3.1.2.Digestion

Mineralization of olive oil samples was carried out according to the method described by Llorent-Martinez, Fernandez-de Cordova, Ortega-Barrales, and Ruiz-Medina (2014), with minor modifications. The method consists of weighing 0.5 g of sample, vigorously shaking it and placing it directly into the digestion vessel, and adding 7 mL of 61% electronic-grade (EL) nitric acid (HNO_3; Cica-Merck Kanto Chemicals, Japan) and 1 mL of 30% atomic absorption spectrometry-grade hydrogen peroxide (H_2O_2; Wako Pure Chemical Industries, Japan) for ultra-trace analysis. The vessels were placed in a microwave digestion system (ETHOS 1600 Advanced Microwave Labstation, Milestone Inc., Italy). The program of the microwave consisted of a ramp of 15 minutes to reach 200 °C and 1000 W, where the system was maintained for an additional 15 minutes. After being cooled to room temperature, samples were transferred into DigiTUBES with an ultra-low leachable metal content and class A tolerance at the 25 mL graduation (DigiTUBES, SCP Science, Canada) and diluted to volume with ultrapure water (Milli-Q Integral 3, Nihon Millipore, Japan). Samples were filtered using a 0.20 μm pore size syringe filter (Captiva econofilter, Agilent Technology, USA). Vessels were cleaned using the same microwave operating program after each digestion batch and successively rinsed with Milli-Q water. The use of glassware was avoided to prevent the release of metals.

2.3.1.3. Quality control

Confidence in measurement results is important in food analysis, especially in the case of a geographic marker that will be used to verify authenticity. Evaluating the reliability of trace element measurement results in olive oil is hindered by the absence of a matrix-matching certified reference material. The accuracy of the method was therefore evaluated using the multielement oil standard S23-100Y of 100 ppm concentration (SPEXCertiPrep, USA). The limit of detection (LOD) and limit of quantification (LOQ) of each element were calculated as three and ten times the standard deviation of the average concentrations determined in 9 independent method blank samples, respectively. The method blank sample was prepared using Conostan 75 cSt blank oil (SCP Science, Canada) as a substitute for the olive oil in the extraction step. Each olive oil sample was digested and measured three times to ensure precision. Each digestion batch contained one method blank to monitor contamination. The influence of instrumental drift was corrected by using a 10 □g L^{-1} internal standard solution of Sc, Y, In, Tb, Ho, and Bi prepared from 10 mg L^{-1} CLISS-1 standard (SPEX CertiPrep, USA).

2.3.1.4. Calibration

External calibration curves were built using eight different mass concentrations. Standard solutions were prepared by diluting a multielement solution (XSTC-622B) containing Na, Mg, V, Fe, Mn, Zn, As, Rb, Sr, Ba, Pb in 10 mg L^{-1} concentration (SPEXCertiPrep, USA) in 5.0% w/w nitric acid matrix. The resultant concentration range for the elements was 0-50 µg L^{-1}.

2.3.1.5. ICP-MS measurements

The quantification of the elements was carried out by inductively coupled plasma-mass spectrometry (ICP-MS; Elan DRC-e, Perkin-Elmer SCIEX, Canada). Oil samples were introduced by means of a borosilicate glass nebulizer. The ICP torch was a standard Fassel-type torch with a ceramic injector. ICP-MS is known to suffer from unwanted polyatomic isobaric interference. Therefore, the elements were monitored in standard and CH_4 dynamic reaction cell (DRC) modes to check for and reduce polyatomic interference, and the appropriate isotopes were used. Instrument performance was checked by a midrange continuous calibration verification (5 µg L^{-1}) every ten samples.

The operating conditions and parameters of ICP-MS were as follows: Rf power 1100 W; plasma Ar flow rate 15 L min^{-1}; auxiliary Ar flow rate 1.3 L min^{-1}; nebulizer (carrier gas) flow rate 0.77 L min^{-1}; sampler and skimmer cones of nickel; lens voltage 7.5 V; analog stage voltage −1700 V; pulse stage voltage 950 V; discriminator threshold 70 V; quadrupole rod offset −1.5 V; dual detector; speed of peristaltic pump 20 rpm; 20 sweeps/reading; 3 replicates; dwell time 50 ms; peak hopping scan mode; CH4 reaction gas flow 0.6 L min^{-1} in DRC mode; rejection parameter *a* 0, and rejection parameter *q* 0.65. Monitored isotopes were ^{23}Na, ^{24}Mg, ^{51}V, ^{56}Fe, ^{55}Mn, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{138}Ba, and ^{208}Pb.

2.3.2. Soil samples

As a pre-treatment step prior to subsequent analyses, soil samples were dried in a thermostatic oven at 40 °C for 24 h to remove moisture. Major and trace elements in soil samples were measured at the facilities of the Geological Survey of Japan (GSJ) of the National Institute of Advanced Industrial Science and Technology (AIST) in Japan, according to a method developed by the GSJ. The method consisted of preparing high-dilution-ratio fused glass beads for X-ray fluorescence (XRF) determination of major element oxides (Ejima, Kon, Kawano & Araoka, 2018) and using the same beads for trace element measurements by femtosecond ultraviolet Laser Ablation ICP-MS (LA-ICP-MS) (Kon & Hirata, 2015).

2.3.2.1. XRF analysis

The glass beads were prepared by mixing 0.5 g of powdered soil sample with 5.0 g of lithium tetraborate flux. The mixture was heated to 1200 °C for 10 minutes in a 95% Pt-5% Au crucible using a semi-automatic fusion device (HAG-M-HF, Herzog, Germany). Four major elements in soil samples (Fe, Mn, Mg, and Na) were quantified by an XRF instrument (ZSX Primus III+, Rigaku Corp., Japan) with an Rh tube. The calibration curves of each element were prepared using 14 GSJ geochemical reference samples (JA-1, JA-2, JA-3, JA-1a, JB-3, JB-1b, JG-2, JG-3, JG-1a, JGb-1, JGb-2, JR-1, JR-2, and JR-3) with their chemical compositions varying from mafic to felsic (Imai et al., 1995). The accuracy was verified each day using two reference samples (JB-1b and JG-3). The analytical uncertainties for each element were better than 1.5%, as estimated from the long-term reproducibility of measurements of JB-1b.

2.3.2.2. LA-ICP-MS analysis

To obtain reliable abundance data for the seven trace elements in the soil samples, we used a quadrupole ICP-MS system (Agilent 7500cx, Agilent Technologies Japan Ltd., Japan) coupled with a 260 nm (UV) titanium-sapphire femtosecond laser ablation system (IFRIT, Cyber Laser Inc., Japan). The laser ablation was operated using a crater size of 20 µm, a pulse energy of 10 J cm^{-2}, an emission repetition rate of 1000 Hz, and a pulse duration of 150 s. A galvanometric optical scanner was employed to minimize elemental fractionation and for effective ablation of the glass beads, with a rastering speed of 10 000 µm s^{-1} in a 400 x 400 µm rastered area. The plasma parameters of the ICP-MS instrument were optimized to minimize the production of $^{232}Th^{16}O^{+}$ ($^{232}Th^{16}O/^{232}Th$) and the doubly charged ion interferences ($^{103}Rh/^{206}Pb$) and to maximize the intensity of ^{7}Li, ^{29}Si, ^{89}Y, ^{139}La, and ^{208}Pb by using the NIST SRM 610 glass standard. To quantify the concentration of each element, GSJ geochemical reference samples (JP-1, JB-1b, JB-2, JB-3, JA-1, JA-2, JA-3, JR-1, JR-2, JR-3, JGb-1, JGb-2, JG-1a, JG-2, and JG-3) were used to build the calibration curves. Calibration curves were established by plotting Li-normalized signal intensities against the reference values of the standards. The ICP-MS operating conditions and parameters were as follows: Rf power 1600 W; cool gas flow rate 15 L min^{-1}; auxiliary gas flow rate 1 L min^{-1}; carrier gas flow rate 0.8 L min^{-1}; peak jump scanning mode; time-resolved analysis mode; integration time 240 s/sample; dwell time 10 ms for Li, 20 ms for V, Cr, Rb, Sr, Ba, and Pb and 40 ms for other elements; sweep time 1.6 s; detector mode, analog for Li and P/A mode for other elements. The isotopes measured were: ^{6}Li, ^{51}V, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{137}Ba, and ^{208}Pb. The repeatability was better than 5% for V, Rb, Sr, Pb, and Ba, and 9% for Zn and As.

2.4.Data analysis

The approach used in this study to reveal related features and samples distributions in the olive oil and soil datasets according to geographical provenance included uni- and multivariate statistical algorithms. The analysis of variance (ANOVA) is a parametric test that can be used to determine the statistical effect of geographical origin on the multielemental profile of the different groups based on the variability in their means. After checking for normality in the distributions and homogeneity of the variance assumptions in the oil dataset, the non-parametric Kruskal-Wallis test was applied to detect location-dependent changes in the distribution. The unsupervised dimension-reducing principal component analysis (PCA) procedure creates a new feature space defined by non-correlated principal components that are linear combinations of the original variables, in order to reduce redundancy. PCA with Varimax rotation was applied to assess multielement associations in the oil dataset and reveal the underlying processes responsible for such associations. Linear discriminant analysis (LDA), a supervised class modeling approach, was applied to evaluate the capability of multielements to classify the olive oils and soils according to their origin, thereby providing a method for validating the prediction and generalization abilities of the model. LDA maximizes the ratio of between-class variance to within-class variance based on a defined classification. The sensitivities of the established LDA models were evaluated using the leave one out cross-validation (LOOCV) technique, in consideration of the relatively small number of cases. Spearman's rank correlation coefficient was calculated to evaluate the relationship between the chemical compositions of the soil and that of the olive oil.

Kruskal-Wallis, PCA, LDA, and Spearman's correlation were performed using IBM SPSS Statistics software for Windows, version 24.0 (IBM Corp., Armonk, N.Y., U.S.A.).

3. Results and discussion

3.1. Quality control

As can be seen from Table 2, accuracy results were in the range 84–102% for almost all elements measured in the oil standard, excluding Mg for which the accuracy was 66%. All of the reported elements had concentrations 100% greater than the LOD, except for Zn and Pb whose concentrations were 95% and 32% greater than the LOD, respectively. In terms of the LOQ, the results were less satisfactory: only Na, Rb, and Sr were quantifiable in olive oil samples. The rest of the elements were present in concentrations between 0% (Pb) and 76% (As) higher than the LOQ. The linearity was satisfactory, with $R^2 \geq 0.999$ for all the elements

measured. For the statistical analyses, we used the original concentrations of all 11 elements (Table 1), even those with values below the LOD and/or LOQ.

Table 2. Quality control of ICP-MS analysis of olive oil samples (21 samples three times replicated).

Element	Unit	Isotope	Operation mode	Linearity R²	Accuracy (%)	LOD	Samples > LOD (%)	LOQ	Samples > LOQ (%)
Na	mg kg^{-1}	23	Standard	0.9998	84	0.35	100	0.12	100
Mg	mg kg^{-1}	24	Standard	0.9991	66	0.47	100	0.16	6
Fe	mg kg^{-1}	56	DRC	0.9999	88	0.12	100	0.39	52
Zn	mg kg^{-1}	66	DRC	0.9997	97	0.11	95	0.36	19
V	µg kg^{-1}	51	Standard	1.0000	97	1.7	100	5.6	25
Mn	µg kg^{-1}	55	Standard	1.0000	103	6.0	100	20	24
As	µg kg^{-1}	75	Standard	0.9990	-	0.73	100	2.4	76
Rb	µg kg^{-1}	85	Standard	0.9996	-	0.30	100	1.0	100
Sr	µg kg^{-1}	88	Standard	0.9999	-	5.1	100	17	100
Ba	µg kg^{-1}	138	Standard	1.0000	102	4.6	100	15	11
Pb	µg kg^{-1}	208	Standard	1.0000	97	6.9	32	23	0

R^2: least square regression coefficient; - corresponds to elements not contained in the oil standard.

3.2. Elemental profile of olive oils

Table 2 shows descriptive statistics of the elements detected in olive oil samples from the four geographical origins, with all cultivars taken together. As expected, Na and Mg were the most abundant elements, followed by Fe, Zn, Sr, As, Mn, Ba, Pb, V and Rb. As most of Tunisia's bedrock is of sedimentary nature, Na, Mg, and Fe are found in high concentrations in various types of soils (Table 4). Therefore, finding them as the principal elements in olive oils as well testifies to their absorption from soil by olive trees in higher proportions compared to the absorption of micronutrients and trace elements.

Table 3. Element contents in Tunisian olive oil samples from the four geographical origins independently of the cultivar.

Element	Unit	Origin Stat.	Gafsa n=5	Medenine n=2	Sfax n=2	Monastir n=5	*P* Value
Na	mg kg^{-1}	Median	4.3 a	4.0 a	1.6 b	2.3 ab	***
		SD	1.2	0.62	0.33	1.8	
Mg	mg kg^{-1}	Median	1.2 a	1.0 ab	0.85 b	0.93 ab	*
		SD	0.080	0.030	0.080	0.36	
Fe	mg kg^{-1}	Median	0.57	0.43	0.39	0.28	Ns
		SD	0.49	0.18	0.15	0.17	
Zn	mg kg^{-1}	Median	0.29 a	0.27 ab	0.18 bc	0.17 c	***
		SD	0.050	0.027	0.050	0.040	
V	µg kg^{-1}	Median	5.2	5.2	5.1	4.8	Ns
		SD	0.73	0.50	1.0	0.81	
Mn	µg kg^{-1}	Median	18 a	13 b	12 b	13 b	***
		SD	4.3	0.74	0.020	4.0	
As	µg kg^{-1}	Median	25 ab	29 a	21 ab	10 b	***
		SD	15	22	1.9	3.3	
Rb	µg kg^{-1}	Median	3.4 ab	3.5 ab	2.5 a	3.5 b	*
		SD	0.76	0.040	0	3.9	
Sr	µg kg^{-1}	Median	37 a	35 ab	33 b	33 ab	*
		SD	2.4	1.3	1.3	8	
Ba	µg kg^{-1}	Median	8.7 a	9.6 ab	12 b	11 b	***

Table 2. (Continued)

		SD	0.79	0.10	0.42	6.0	
Pb	µg kg⁻¹	Median	6.0 ab	6.2 ab	7.4 a	5 b	*
		SD	1.0	0.90	0.13	0.96	

SD: standard deviation. Nonparametric Kruskal-Wallis test was applied: ns. not significant at $p > 0.05$; *. $p < 0.05$; **. $p < 0.01$; ***. $p < 0.001$. Dunn's multiple comparison adjusted by Bonferroni correction was used to make pairwise comparisons; different letters a. b. c in the same row indicate significant differences ($p < 0.05$).

The elemental profiling of olive oils can provide valuable information about their quality, expressed in terms of the total content of specific elements. A quality criteria known as the maximum residue limit (MRL) was established by the International Olive Council (IOC, 2016), which sets maximum acceptable levels of metal ions known to increase the rate of oil oxidation, for example 100 µg kg⁻¹ for As and Pb or 3000 µg kg⁻¹ for Fe. All of the olive oil samples had concentrations of Fe, As, and Pb far below their respective MRLs, which demonstrates the inherent quality of Tunisian olive oils.

Since data on Tunisian olive oil is practically non-existent, concentrations measured in the present work were compared to concentrations measured in olive oils from several other Mediterranean countries (Table 3). A literature review indicated a wide variability in the concentrations of many elements, even within the same country. Our results agreed the most with those from studies on Tunisian olive oils from the Sfax region (Benincasa et al., 2012), southern and central Italian olive oils (Benincasa et al., 2007), and Croatian olive oils (Zeiner, Steffan & Juranovic-Cindric, 2005; Juranovic-Cindric, Zeiner & Steffan, 2008; Zeiner et al., 2010) but differed significantly from other results concerning the majority of the elements investigated. Concentrations of Na found in this study were in the range of 1.6-4.3 mg kg⁻¹, which is similar to that found in Sfax olive oils (4.6 mg kg⁻¹) as reported by Benincasa et al. (2012) and in Croatian olive oils (3.4 mg kg⁻¹) as reported by Zeiner et al. (2010), but 50 times lower than the levels reported in Portuguese olive oils (Gouvinhas et al., 2016) and 1000 times higher than the levels reported in Spanish olive oils (3.8-7.8 mg kg⁻¹) (Beltran et al., 2015). The concentrations of Mn measured in the present study ranged between 12-18 µg kg⁻¹, which is 10 times lower than that previously reported in Sfax oils (Benincasa et al.,

2012), in a similar range as those of olive oils from five different geographic regions of central and southern Italy (Rossano, Andria, Lamezia, Spoleto and Pescara) (n.d.-25 µg kg^{-1}) (Benincasa et al., 2007), and about 100 times higher than those of Italian olive oils from Sicily and Tuscany (< 0.20 µg kg^{-1}) (Camin et al., 2010a). Mn concentrations varied from 1.8 times lower to 100 times higher than the level of Mn quantified in Spanish olive oils. If this variability had been solely due to the geochemistry of the provenance soil, then this evidence would further support the usefulness of elemental fingerprinting. However, we presumed that such variability could have been at least partially affected by the use of different analytical methods for elemental quantification in olive oils. As previously proved in a comparative study, significant differences were found between element concentrations in olive oils using different extraction methods (Bakircioglu et al., 2013; Kara, Fisher & Hill, 2015). This situation highlights the need for a standardized method of multielement quantification in olive oils for the purpose of geographical traceability in order to allow rigorous comparisons.

The non-parametric Kruskal-Wallis test was applied to evaluate differences between the four origins, since data failed the normality of distribution and homogeneity of variance assumptions as assessed by Shapiro-Wilk and Levene's tests, respectively. Mn and Zn concentrations were significantly different among three pairs of origins ($p < 0.01$) (Table 2). Nine out of eleven elements exhibited significant differences between the mean ranks of at least one pair of origins ($p < 0.05$), demonstrating that oils from different regions have a characteristic elemental profile. There was no evidence of variation in the concentrations of V or Fe ($p > 0.05$) between any pair of origins. Among all origins, the highest concentrations of all elements were found in oils from Gafsa and Medenine, except for Rb, Ba, and Pb, which had higher concentrations in the oils from Monastir and Sfax. As pointed out in a previous study on wine traceability, soil and dust are probable sources of Ba and Sr in food, and thus variations in the Ba, Rb, and Pb concentrations in the olive oils from different origins can likely be explained by differences in regional soil geochemistry (Fiket, Mikac & Kniewald, 2011).

Table 4. Element contents in olive oils from different Mediterranean countries quantified by different analytical methods (µg/kg).

	Element	Na	Mg	Fe	Zn	V	Mn	As	Rb	Sr	Ba	Pb	Reference
Tunisia	Min	1600	850	280	170	4.8	12	10	2.5	33	8.7	5	This
	Max	4300	1200	570	290	5.2	18	29	3.5	37	12	7.4	work
	Max	4654	50290	3882	122980		102			13	178		a
Italy	Min		56	89.3			n.d.	1.72		n.d.			b
	Max		1032	550.9			25.2	26.65		48.9			
	Min	49	15				0.134		0.04	1.12	0.309	0.18	c
	Max	280	87			<0.016	3.42		4.19	3.85	2.49	8.46	
	Min				7				0.043				d
	Max	36	12		170	0.016	<0.2		0.133	<0.3	0.13		
Spain	Min												d
	Max		14		7	<0.007	1.1		0.157	<0.3	0.18		
	Min												e
	Max			n.d.		100		50				50	
	Min			67.52									f
	Max			115.80			35.87						

Table 3. (Continued)

Country													
	Min												g
	Max	299.00	78.00			16.73					4.56	2.16	
	Min	3.80	0.60	0.01			5.30	0.80			9.90	48.30	h
	Max	7.80	0.90	0.01		0.20	7.70	2.60	n.d.		22.40	74.20	
	Min												i
	Max					5.40						74.60	
Portugal	Min	150000	10000	190	250	30	250					30	j
	Max	200000	20000	1090	2800	310	670					250	
Croatia	Min	28770	2910	13100	2820	n.d.							k
	Max	38030	3620	18460	4030	130						n.d.	
	Min												l
	Max		3500	15400	3400	n.d.						n.d.	
	Min												m
	Max	3350	80	1810	n.d.	n.d.	n.d.					n.d.	
Greece	Min		13		11				0.031		<0.12		d
	Max	<20	28		12	<0.007	0.6		0.077	<0.3	0.37		

Table 3. (Continued)

Turkey	Min	12.5	0.7	n.d.	n
	Max	29.5	82	7.4	
Morocco	Min		14.7	94.2	i
	Max		25.8	103.7	

a, Benincasa et al. (2012) ; b,Benincasa et al. (2007); c, Camin et al. (2010a); d, Camin et al. (2010b); e, Llorent-Martínez et al. (2014); f, Cabrera-Vique et al. (2012); g, Jiménez et al. (2004); h, Beltrán et al. (2015); i, Bakkali et al. (2009); j, Gouvinhas et al. (2016); k, Zeiner et al. (2005); l, Juranovic-Cindric et al. (2008); m,Zeiner et al. (2010) n, Pehlivan et a. (2008).

3.3. Multivariate analyses

3.3.1. PCA

Univariate methods alone are insufficient to obtain a global overview of the discriminating potential of elements, considering the complex interactions between different elements. Multivariate approaches such as PCA can assist the understanding of the relationship between various predictors. PCA can be useful in depicting associations between elements and evaluating whether such associations are conditioned upon natural or anthropogenic processes. In our study, PCA was applied to the correlation matrix with Varimax rotation, and four components were extracted which together explained 75% of the original variance in the dataset. Examination of the component loadings (Table 4) shows that Rb, Mg, Sr, and Ba were highly loaded in the first principal component (PC), which explained 27% of the overall variance. Bearing in mind that strong associations between the elements in olive oil would likely reflect similar behavior or bioavailability in the soil, the first component represents elements grouped according to Goldshmidt's geochemical classification (Greenough et al., 2010).

Table 5. Loadings of PCA for element concentrations in Tunisian olive oils.

Component	1	2	3	4
C.V.%	27	21	15	11
Rb	0.899	0.059	-0.041	-0.212
Mg	0.789	0.378	0.144	-0.116
Sr	0.782	0.196	0.041	-0.089
Ba	0.716	-0.150	-0.298	0.244
Mn	0.371	0.835	0.073	0.001
Fe	0.074	0.806	-0.326	-0.342
Zn	-0.183	0.629	0.162	0.280
Na	0.335	0.606	0.334	0.125
V	0.195	-0.009	0.841	-0.144
As	-0.336	0.135	0.783	0.042
Pb	-0.099	0.061	-0.104	0.934

C.V.; Cumulative variance.

Rb, Sr, Mg, and Ba are classified as lithophile elements and occupy positions in the first two groups of the periodic table, which consist of elements with similar chemical characteristics such as solubility. Considering the Kruskal-Wallis test results, it is interesting to note that the elements of the first PC were found in higher concentrations in the Monastir and Sfax olive oils, except for Mg and Sr. Thus, it appears that this component groups the elements which exhibit significant variation between Monastir and Sfax oils and those of the other origins. This occurrence can be related to the composition of the soils in which the olive trees were grown. As shown on the geological map (Fig. 1), samples originating from Monastir and Sfax were grown in soils situated on the same mixture of clay, conglomerates, and sand. Clays can accumulate rare elements such as Rb and Ba as reported by Krauskopf (1995). The second component was dominated by Mn, Fe, Zn, and Na. Jimenez et al. (2004) mentioned that the relationship between Mn and alkali elements in soil has been proven and used in geographical traceability issues. On the other hand, Mn and Fe exhibit very similar geochemical behavior and are common in Fe-Mg silicates and carbonates, given the similar ionic radii of Mn, Fe, Mg, and Ca, which facilitates substitution in minerals (Pohl, 2011). Similarly to what was observed for the first PC, the elements grouped in the second PC, except for Fe, were more concentrated in Gafsa olive oils derived from olives grown in sandy soils rich in silicates, showing significant differences from the oils from the rest of the origins. The distribution of elements in the third and fourth components were less clear, and did not permit determination of the predominant cause of the associations. The grouping of elements in the PCA suggests that the elemental profile of olive oils is substantially shaped by geochemical processes. This agrees with the findings of Ariyama et al. (2006) concerning Japanese onions, and of Greenough et al. (2010) regarding Canadian maple syrups and wines, wherein the tendency for elements of the same group in the periodic table or from similar geochemical groups to form clusters was linked to similar behaviors of the elements in the foodstuff in question. This information supports the utility of using multielements as tracers of olive oil provenance.

3.3.2. Classification and prediction

PCA provides evidence that could only indicate how the elements behave. In our study, PCA was inappropriate for assessing patterns in the data points of a bidimensional plot, since the variance explained by the first two components was deemed to be too small to successfully substitute the original dimensions without information loss. In order to gain information about the classification potential of multielements, LDA was chosen among a wide range of multivariate classifiers based on its capability and relevance to the application area of this

study. In particular, two main outputs are of a predominant importance: The first one is the graphical output which is a bi-plot showing the reciprocal location of the samples in the canonical space and allowing to visually assessing the discriminatory power (Granato et al., 2018). The second relevant output is the standardized coefficients table that indicates the relative importance of each variable in each function; thereby, it shows the variables with the greatest discriminating ability. The recognition of the most discriminating variables for olive oils and soils from the four origins simultaneously will help to check for a possible correspondence of elements. Such correspondence, if identified, will indicate a transfer of elements from the soil to the olive oil and that this soil's transferred geochemical signature is responsible for the geographical traceability (Beltran et al., 2015).

LDA was applied to 63 cases (21 samples in triplicate, to allow the smaller groups to have a reasonable ratio of cases to variables) organized in four groups corresponding to the four origins and 11 independent variables. The LDA calculated three canonical linear discriminant functions, of which the first two accounted for 98% of the total variance. A total of 92.1% of the original samples were correctly classified by their origin. The bidimensional plot (Fig. 2) shows that olive oils from Gafsa, Medenine, and Monastir could be differentiated on the basis
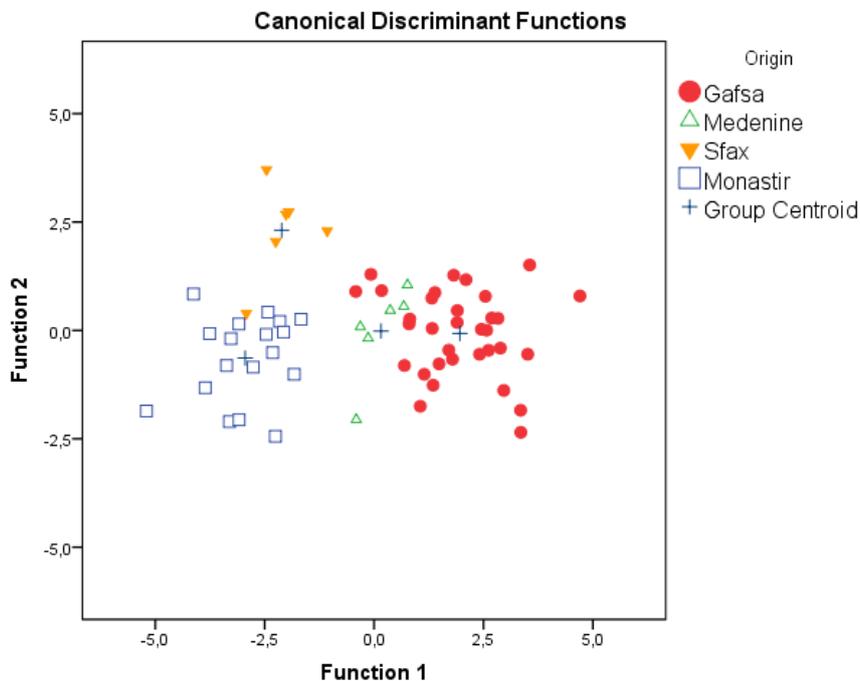
Figure 2. LDA analysis of element concentrations. Scatterplot of the first two canonical functions in olive oils using 4 groups corresponding to each origin with all cultivars taken together as inputs.

of the first function alone. The samples from Sfax and Monastir could not be clearly separated on the basis of the first function, but were easily differentiated by the second function. Analysis of the standardized coefficients indicated that Ba (-1.1), Rb (0.70), Mg (0.53), and Zn (0.45) had the largest absolute values, i.e., the greatest discriminating ability, in the first function, while Fe (0.84), Pb (0.74), As (0.52), and V (0.47) exhibited the largest absolute values in the second function. Indeed, the first set of variables (Ba, Mg, and Zn especially) exhibited significant differences between olive oils from Gafsa and those from the rest of the regions, while Pb, which was featured in the second function, exhibited a significant difference only between the olive oils from Sfax and Monastir. To verify the prediction and generalization powers of the model, LOOCV was applied, and 87.3% of the sample origins were correctly predicted. The lower prediction rate was probably due to the small number of samples used to train the model, but the sensitivities achieved are still considered satisfactory. The fact that olive oils from the same origin were plotted together, meaning that they are chemically similar, demonstrates the important influence of provenance on olive oil composition. This composition can be attributed to a significant extent to the geochemistry of the soils, as we showed in the PCA analysis. The homogeneity of the primarily sandy soils in Gafsa could have played a positive role in achieving the high classification rate, despite the fact that various cultivars came from that region. This again proves the importance of soil composition. However, olive oils from Sfax, Monastir, and Medenine were mostly produced from the same cultivar within each origin, which could also contribute to the classification success achieved in the case that the elemental profile also depends on the cultivar. At this point it is important to note that Beltran et al. (2015) reported that the matching of south-western Spanish olive oils to their municipality of origin was not influenced by the cultivar used. In another study on olive oils from central and southern Italy, Benincasa et al. (2007) showed that the influence of origin was more significant than that of the cultivar used.

3.3.3. Influence of cultivar

In order to test this hypothesis, we applied LDA again, this time using 9 groups corresponding to each single cultivar and origin. The hypothesis was verifiable primarily by the data on Koroneiki olive oils coming from Gafsa and Monastir, since other cultivars were also sampled from these origins. The LDA calculated 8 canonical linear discriminant functions, of which the first two accounted for 77% of the total variance. A total of 97% of the original samples were correctly classified by their origin. Analysis of the standardized coefficients indicated
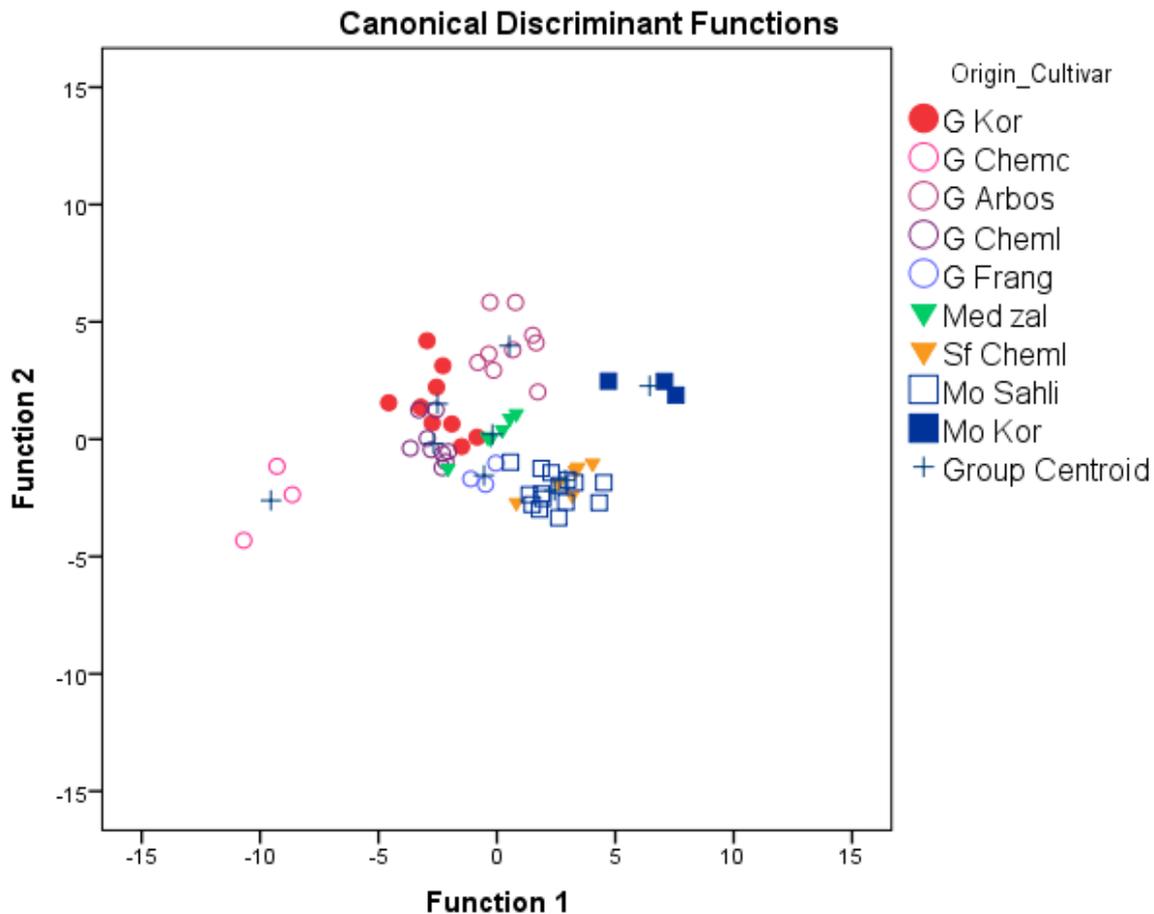
Figure 3. LDA analysis of element concentrations. Scatterplot of the first two canonical functions in olive oils using 9 groups corresponding to each single origin and cultivar as inputs (G., Gafsa; Med., Medenine; Sf., Sfax; Mo., Monastir; Kor., Koroneiki; Chemc., Chemchali; Arbos., Arbosana; Cheml., Chemlali; Frang., Frangivento; Zal., Zalmati).

that Na (-1.4), Sr (-1.33), Ba (-1.05), Mn (-1.04), and Fe (0.98) had the largest absolute values in the first function, while Zn (0.87) and Mg (0.83) had the largest absolute values in the second function. The bidimensional plot (Fig. 3) shows that olive oils from the same origin tended to occupy the same region, even when the differences in cultivar were taken into account. This explains the short distance on the plot between Koroneiki from Gafsa (100% correct classification) and other cultivars from Gafsa compared to the distance between Koroneiki from Gafsa and Koroneiki from Monastir (100% correct classification). This observation was further supported by LOOCV results, wherein 100% of the Koroneiki olive oils samples from Monastir were ascribed to the correct origin, and Koroneiki samples from Gafsa were attributed correctly to Gafsa, albeit with some misclassifications (56% Koroneiki Gafsa, 22% Chemlali Gafsa, 11% Frangivento Gafsa, and 11% Zalmati Medenine); nonetheless, 89% of the samples were still classified as originating from Gafsa. These results

agree with the findings of Benincasa et al. (2007) and Beltran et al. (2015). In future work, sufficient data on coexisting cultivars in different regions should be collected such that this trend can be confirmed.

3.4. The link between soil and oil

The key point in using multielements in geographical traceability matters lies in the hypothesis that soil chemistry is the main factor that influences the elemental composition of olive oil. Accordingly, it is relevant to check the strength of the link between the elemental composition of olive oil and that of the soil. Table 5 shows the concentration of elements in soil samples from both depths at the four origins. Welch's test, an equivalent to ANOVA used when group variances are not equal, was employed to test the differences between the soils from the two depths and the four origins. The element concentrations did not exhibit any significant differences between the two depths, but all elements showed significant differences between at least two origins. Spearman's rank correlation coefficients were calculated for the elements in olive oils and soils taken from the two layers. Out of the 11 determined elements, only Zn exhibited a statistically significant correlation between its content in olive oil and that in soil, regardless of the soil depth, but the correlation signs were negative (c.c. = -0.62, p = 0.019 for 0-30 cm and c.c. = -0.74, p = 0.0030 for 30-60 cm). In the 0-30 cm layer, Pb (c.c. = -0.58, p = 0.031) showed statistically significant correlations between its contents in olive oils and soils. The comparison of elemental concentrations in soil and olive oil showed that Fe, Mn, Zn, Na, and Pb had the highest concentrations in the soils of Monastir and lower concentrations in the soils of Gafsa. Contradictorily, these elements were found in higher concentrations in the olive oils from Gafsa than in those from Monastir. Thus, the significant negative correlation coefficients obtained for Zn and Pb likely signifies that their content in olive oils depends not only on their contents in the soil, but also on other determinants, with the closest related factor being climate (Greenough et al., 2010). In fact, Gafsa is located in a middle arid bioclimatic zone characterized by higher temperatures and evapotranspiration rates which favor increased water and element uptake, as opposed to Monastir which is situated in a lower semi-arid climate marked by lower temperatures and higher precipitation (Kefi, Dat Pham, Kashiwagi & Yoshino, 2016). The other elements did not exhibit any significant correlations in their concentrations between soil and olive oil, which could be explained by many reasons: the use of total element concentrations rather than the bioavailable fraction of elements in soil; elemental fractionation

Table 6. Mean ± standard deviation in ppm of the element contents in soil samples from the four geographical origins at 0-30 and 30-60 cm depths.

Element	Gafsa		Medenine		Sfax		Monastir	
	0-30 cm	30-60 cm	0-30 cm	30-60 cm	0-30 cm	30-60 cm	0-30 cm	30-60 cm
Na	1500 ± 870	880 ± 220	2000 ± 110	1900 ± 270	1100 ± 280	860 ± 150	2000 ± 860	1900 ± 720
Mg	3500 ± 1000	3600 ± 680	4600 ± 17	4400 ± 210	2300 ± 680	1700 ± 260	4200 ± 1400	4500 ± 1400
Fe	6400 ± 1700	6700 ± 1300	8600 ± 600	8200 ± 15	6000 ± 1500	4100 ± 300	14000 ± 4400	15000 ± 4700
Zn	18 ± 2.8	18 ± 3.0	20 ± 1.0	19 ± 1.6	23 ± 9.9	15 ± 0.48	41 ± 12	38 ± 7.2
V	29 ± 7.9	25 ± 3.6	34 ± 4.0	32 ± 7.2	23 ± 6.0	17 ± 1.3	45 ± 11	47 ± 17
Mn	84 ± 28	84 ± 18	95 ± 11	87 ± 0	79 ± 13	53 ± 16	170 ± 81	170 ± 76
As	2.0 ± 0.53	2.2 ± 0.45	3.2 ± 0.33	3.1 ± 0.13	2.3 ± 0.83	1.4 ± 0.14	4.2 ± 1.6	3.9 ± 1.2
Rb	23 ± 2.8	22 ± 3.3	34 ± 2.7	33 ± 0.35	21 ± 5.3	15 ± 1.7	40 ± 13	41 ± 12
Sr	110 ± 27	130 ± 30	170 ± 14	180 ± 2.0	130 ± 39	100 ± 12	140 ± 88	140 ± 100
Ba	190 ± 9.5	190 ± 16	290 ± 17	290 ± 11	210 ± 46	160 ± 17	270 ± 92	270 ± 84
Pb	n.d.	n.d.	0.17 ± 0.25	0.18 ± 0.25	0.31 ± 0.41	n.d.	4.4 ± 3.6	4.7 ± 3.7

n.d.; not detected.

during the olive oil extraction process; physiological aspects of the cultivars that impose a specific elemental uptake pattern and partitioning within the different olive tree tissues; and differences in climate conditions which affect water and element uptake by olive trees.

Although most of the elements did not exhibit significant correlations between the soil compositions and the oil profiles, it was necessary to check whether olive oil profile differences could be explained by the variations in the soil composition. When stepwise LDA analysis was applied to the elemental composition of the soils, soil samples were correctly classified in 100% of cases, and 97% were validated in terms of their origin, including the soil depth; the results for the soils showed less scattering than the results for the olive oil samples (Fig. 4). Analysis of the standardized coefficients of the first two of three total calculated functions indicated that Fe (6.4), Rb (-5.06), Mg (-4.37), Sr (2.52), and Pb (1.85) had the largest absolute values in the first function, while Mn (4.61) had the largest absolute value in the second function. Thus, Fe, Mg, Sr, Rb, Mn, and Pb were identified by the stepwise LDA as the elements with the most discriminatory power. This result agrees to some extent with the elements with the most discriminatory power for olive oil, thereby revealing Fe, Rb, Mg, and Pb as elements with common discriminatory power for both olive oils and their provenance soils.

4. Conclusions

In conclusion, this study provided evidence of the validity of multielements as markers for the geographic authentication of olive oils.

This work showed that the concentrations of Fe, As, and Pb in Tunisian olive oils were far below the MRLs, which certifies the high quality of Tunisian olive oils.
The classification of olive oils according to their origin via a multielement fingerprinting approach proved to be successful. LDA yielded 92.1% and 87.3% classification and prediction rates, respectively, demonstrating that multielements are promising discriminating markers of Tunisian olive oils from different regions. The attribution of olive oils to their origin was not hindered by the effect of the cultivar, as the origin proved to have a more significant effect than cultivar on the variation of multielement concentrations in olive oils.
The correspondence identified between the primary discriminatory elements in both olive oils and soils, Fe, Rb, Mg, and Pb, undoubtedly indicates that the geochemical signature of the soil is transferred to the oil. Nevertheless, some discrepancies in that signature were found

since no clear correlation between the elemental composition of olive oils and soils could be established.

This work can serve as a reference for further research in which olive oils representative of various Tunisian producing origins are characterized by their elemental composition. This will be a crucial starting point for building comprehensive datasets for each producing region in order to support authentication of geographic origin. In future work, correlation of the elemental profile of the olive oil with other soil parameters, such as the bioavailable fraction of the total metal content and climatic factors, need to be investigated to further substantiate the validity of multielements as markers of provenance.
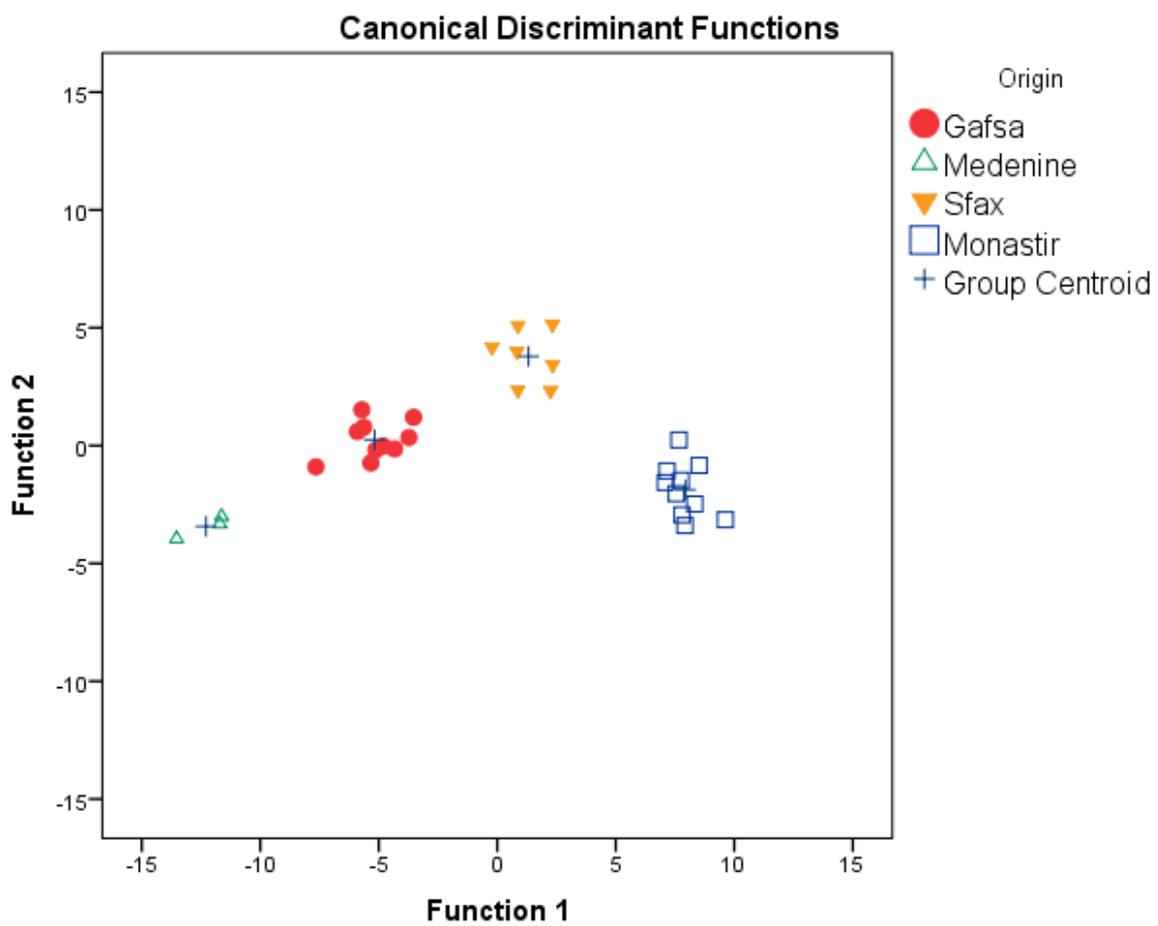
Figure 4. LDA analysis of element concentrations. Scatterplot of the first two canonical functions in soils using 4 groups corresponding to each origin as inputs.

Chapter 2

Method development and comparison of the performance of three analytical methods: Microwave digestion, evaporation of pre-digested solutions and ultrasonic extraction

1. Introduction

The determination of the concentration of metallic light and heavy elements in all sorts of edible oils has gained an important place in the scientific community due to their implication in safety and quality issues of these oils. In fact, one of the most important quality parameters of olive oil quality is its heavy metal content. Specifically, the concentration of heavy elements in vegetable oils is known to have an adverse effect on the rate of oil oxidation (Frazin & Esmail-Moassesi, 2014) therefore deteriorating their freshness and storability if exceeding the allowable limits fixed by national and international regulations and constituting a big risk to the human nutrition and health. For instance, catalytic oxidation of oils with oxygen through copper leads to the rancidification of oils, a phenomenon characterized by the deterioration of their smell and taste. Copper and iron significantly reduce the oxidative stability of oils. In addition, transition metals catalyze the decomposition of some of the oil's organic compounds increasing the risk of pathological effects development on the digestive tract and developing carcinogenic effect by reacting with proteins and pigments (Frazin & Esmail-Moassesi, 2014).

On the other hand, the determination of elements in edible oils is also of paramount importance because of the potentialities for adulteration detection and origin authentication. Mario Giaccio & Assia Vicentini (2008) stated that numerous works have shown that the mineral composition of plants is affected by the presence and concentration of elements in the soil and underlying bedrock, by fertilization practice, by the physiological and metabolic pathways of the species in question and by the technological effects resulting from transformation and/ or conditioning of the derived foodstuff.

Therefore it is crucial to determine the presence and concentration of elements in olive oils in pursuance of preserving its quality and value and most importantly in our case make use of their fingerprinting power.

Determination of metals in oils is a difficult analytical problem due to the high viscosity of the oil matrix leading to problems in leaching and dissolving (Szyczewski et al., 2016). It is therefore necessary to choose or develop specific analytical procedure that ensures the accurate and reproducible generation of results.

During the beginning of the pilot study, we had compiled all the literature on elements determinations in edible oils to screen the analytical methods used to extract elements from the oil matrix. Microwave digestion of the olive oil samples in HNO_3 and sometimes with addition of H_2O_2 turned out to be the most widely proposed, validated and used method (Ni, Chen, Yu, Sun & Tang, 2019; Benincasa, Lewis, Perri, Sindona & Tagarelli, 2007; Zeinera, Steffana & Juranovic-Cindric, 2005; Llorent-Martínez, Ortega-Barrales, Fernández-de Córdova, Domínguez-Vidal & Ruiz-Medina, 2011; Mendil, Dogan-Uluözlüa, Tüzena & Soylak, 2009; Juranovic-Cindric, Zeiner & Steffan, 2007 and Gonzálvez, Ghanjaoui, Rhazi & De la Guardia, 2010).

The use of microwave energy as a heat source for wet digestion was first proposed by Abu-Samra et al. in 1975. Today, the use of microwave heating for sample decomposition prior to elemental analysis is an established technique. Closed-vessel systems capable of sustaining high pressures are advantageous because they can achieve higher temperatures as well as withstand increases in pressure resulting from sample decomposition. In addition, samples are isolated from the laboratory environment during closed-vessel digestion, reducing the risk of contamination (Rhoades, Levine, Salido & Jones , 1998).

Therefore, we choose the microwave digestion as an extraction method of the elements in the olive oil samples used in the pilot study. However, after examining the results and comparing the elements concentrations to those reported in previous studies, whether for Tunisian or extra-Tunisian olive oils, a wide variability in the concentrations of many elements, even within the same country has been identified. We presumed that such variability could have been at least partially affected by the use of different analytical methods for elemental quantification in olive oils. This situation highlights the need for a standardized method of multielement quantification in olive oils for the purpose of geographical traceability in order to allow rigorous comparisons. Moreover, we noticed that the microwave digestion method presented some limitations. In fact, of the initially analyzed thirty-two elements in standard and CH_4 DRC modes (excluding multi-isotopes) (^{11}B, ^{23}Na, ^{24}Mg, ^{27}Al, ^{28}Si, ^{39}K, ^{44}Ca, ^{46}Ti, ^{51}V, ^{52}Cr, ^{53}Cr, ^{54}Fe, ^{56}Fe, ^{57}Fe, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{64}Zn, ^{66}Zn, ^{69}Ga, ^{72}Ge, ^{75}As, ^{77}Se, ^{82}Se, ^{85}Rb, ^{88}Sr, ^{90}Zr, ^{98}Mo, ^{107}Ag, ^{114}Cd, ^{120}Sn, ^{121}Sb, ^{133}Cs, ^{138}Ba, ^{184}W, ^{208}Pb) only eleven

elements could be reported in the article after passing quality control screening. High detection limits and low precision were amongst the most problematic factors leading to eliminate almost two-thirds of the elements of interest. It is well-known that edible oils analysis by ICP-MS is one of the most challenging analytical procedures. This is due to many reasons pertaining to the edible oils complex matrix characterized by a high organic load which increases the possibility of polyatomic molecular interferences, a high viscosity which may raise matrix effects and the very low concentration of elements which makes it extremely prone to contamination during preparation and may require clean room for ultra-trace metal analysis. As previously reported by Ilyes Dammak et al (2015), minerals constitute only 1.5% of the total mass balance of the olive fruit and even lower level in olive oil. To overcome these difficulties, different sample preparation procedures (such as digestion, emulsification, extraction, and dilution) have been proposed and applied and have been reviewed by Lepri et al. (2011).

From our own experience, it was necessary to test and adopt an alternative method of multielements quantification in olive oils to achieve these two main goals:

1/ Improve the method's performance especially the detection and quantification limits so more elements can be detected and then used with higher confidence in the subsequent statistical analyses.

2/ Apply a more time-saving method considering the high number of samples gathered and the necessity of a rapid, accurate and simple method for food traceability subject matter.

Because metals constitute only an extremely small fraction of the total olive oil mass, the development of an accurate and precise method for quantification of multielements is usually very challenging. The most widely used procedure is based on the principal of total/ complete decomposition of the olive oil samples using concentrated HNO_3 and H_2O_2 in a microwave at high temperature and pressure conditions as we previously stated. Sometimes other acids are employed like HCl. Concentrated HNO_3 is a strong oxidizing agent whose oxidizing capability is enhanced at high temperatures. The addition of H_2O_2 to the digestion mixture ensures the complete oxidation of any organic matter present in the sample as well as the decrease in the production of nitrogen monoxide (NO) fumes and reduction of pressure in the closed microwave vessel (Aldabe, Santamarıa, Elustondo, Lasheras & Santamar, 2013). Usually, the volumes of HNO3 and H2O2 used in each digestion vessel vary from 5 to 9 ml and 1 to 2 ml respectively. The direct implications of the use of this considerable volume of

concentrated nitric acid are: (i) the corrosive nature of the digests to the ICP-MS components that make contact with the solutions such as the nebulizers and nebulisation chambers (Bressy, Brito, Barbosa, Teixeira & Graças, 2013) and (ii) the high viscosity of the analytical solutions which may result in matrix effects. In fact, the matrix (refers to the components of a sample other that its analytes) can have significant effects on the way the analysis is carried out and consequently on the quality of the results. The approach usually used to counter these effects is to make further dilutions to ensure a maximal residual acidity of 1 to 5% which is the usual acid medium of choice for most ICP-MS analyses and to approximate the matrix of the samples as much as possible (Chudzinska, Debska & Baralkiewicz, 2012). The final solution volume is usually brought around 25 ml which means that for 0.5 g of maximal allowable mass of oil inside the digestion vessel, the oil's analytes are left diluted up to fifty-folds. Additional dilution is usually required with a dilution factor of 5 so the final dilution factor is usually around 250. While the actual concentration of major elements (e.g. Na, K, Mg, Ca, Fe, Zn) can reach the ppm level, that of trace and rare earth elements can be as low as the ppt level (Camin et al., 2010). Diluting these extremely low elements to additional 250 times would result in going below the ICP-MS detection capabilities which is comprised between 0.1 and 1 ppt for rare earth elements for example according to the manufacturer's descriptions.

The first solution one can think about is to try to decrease the acid concentration so that we reduce the volume of deionised or ultrapure water added to dilute the samples. This is possible by: (i) dispelling the excess of the residual acid in the digest by evaporation to near dryness or (ii) decreasing the initially used concentration of nitric acid. The latter possibility implies that the complete decomposition of the organic matrix may not be possible, so another type of method should be used: it is the liquid-liquid ultrasonic-assisted extraction. This strategy has the benefit of speed and simplicity of application. It can also reduce the amount of reagents through the use of dilute acid solutions. Amelioration in the analytes recoveries in liquid–liquid extraction can be obtained through the use of ultrasonic energy (Trindade, Dantas, Daniel, Lima, Ferreira & Teixeira, 2015).

The main objective of this study is to develop a robust preparation method which allows the simultaneous accurate and precise quantification by ICP-MS of major and trace elements in olive oil samples. The method and programs for: microwave-assisted digestion, evaporation of pre-digested samples and ultrasonic-assisted extraction of major and trace elements are

described and their results compared to aid choosing the method that reaches lower detection limits, higher sensitivity and higher precision.

2. Materials and methods

2.1. Equipment

A microwave oven equipped with a 10 positions rotor and capable of delivering 1600W of power (ETHOS 1600, Advanced Microwave Labstation, Milestone Inc., Italy) was used for closed-vessel digestion of samples, method blanks and spiked samples. Capped Teflon tubes were used to decompose samples and also evaporate them when necessary.

An ultrasonic bath capable of delivering 300W of power and 55°C of maximal temperature was used to carry out the liquid-liquid extraction of elements.

A commercial hotplate (80°C) placed inside a fume hood was used to evaporate the residual acid to near dryness after digestion. The microwave vessels were used to evaporate the samples after their digestion and they were covered with a laboratory clean tissue to form a vertical cone that protects the vessels from air-borne contamination by isolating the vessels from the environment to a certain extent.

DigiTUBES that have an ultra-low leachable metal content, of class A tolerance at the 25 ml graduation (SCP Science, Canada) were used to collect samples after microwave digestion and dilute them to volume with ultrapure water.

Milli-Q Integral 3 (Nihon Millipore, Tokyo, Japan) was used to prepare ultrapure water that was used to prepare all solutions, make dilutions and rinse material at all times during the experiment.

The quantification of the elements was carried out with an Elan DRC-e ICP-MS instrument (Perkin-Elmer SCIEX, Canada). Samples were introduced by means of a borosilicate glass nebulizer. The ICP torch was a standard torch (Fassel type torch) with ceramic injector. ICP-MS is known to suffer from unwanted polyatomic isobaric interferences. Therefore, the elements had been monitored in standard, kinetic energy discrimination (KED: He collision) and CH_4 dynamic reaction cell (DRC) modes to check for and reduce polyatomic interferences and the appropriate isotopes were used. Instrument performance was checked by a midrange continuing calibration verification (5 μg L^{-1}) every ten samples. Indium was used as an internal standard in all the three methods and added to all samples, calibration solutions, method blanks and solutions prepared for quality control to yield a concentration of 1 μg L^{-1}. The operating conditions and parameters of ICP-MS are shown in table 7 for each method.

Monitored isotopes: ^{23}Na, ^{24}Mg, ^{51}V, ^{56}Fe, ^{55}Mn, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{138}Ba, ^{208}Pb.

2.2. Samples

A commercially available Tunisian olive oil sample was used in all experimental methods to: (i) optimize the combined microwave digestion and evaporation analytical method parameters, (ii) test the repeatability (precision) expressed as RSD% in all of the three

Table 7. ICP-MS operating conditions.

Parameter	Method		
	Microwave digestion	Digestion / evaporation	Ultrasonic extraction
TQ or Q	Q	Q	Q
ICP Rf power (W)	1100	1600	1600
Plasma Ar flow rate (L min-1)	15	18	18
Auxiliary Ar flow rate (L min-1)	1.30	1.20	1.20
Nebulizer (carrier gas) flow rate (L min-1)	0.77	0.98	0.98
Sampler and skimmer cones	nickel	Nickel	Nickel
lens voltage(Deflector voltage) (V)	7.5	-11.50	-11.50
Analog stage voltage (V)	-1700	-1800	-1800
Pulse stage voltage (V)	950	1100	1100
Discriminator threshold (V)	70	13	13
Quadrupole rod offset (V)	-1.50	0.50	0.50
Detector	pulse	Pulse	Pulse
Speed of peristaltic pump (rpm)	20	20	20
Sweeps/reading	20	20	20
Replicates	3	1	1
Dwell time (ms)	50	50	50
Scan mode	peak hopping	peak hopping	peak hopping
STD: rejection parameter a and Rejection parameter q	0, 0.25	0, 0.25	0, 0.25

Table 7. (Continued)

DRC mode: CH4 reaction gas flow (L min-1)	0.60	0.60	0.60
DRC mode: rejection parameter a and Rejection parameter q	0, 0.65	0, 0.65	0, 0.65
KED mode He reaction gas flow (L min-1)	-	3.5	3.5
KED mode: rejection parameter a and Rejection parameter q	-	0, 0.25	0, 0.25

TQ, semi-quantitative; Q, quantitative; STD, standard mode; DRC, dynamic reaction cell mode; KED, kinetic energy discrimination mode.

methods proposed and (iii) serve as a base for spiking with multielemental standard solutions (aqueous or oily) in the accuracy check for the ultrasonic extraction experiment.

2.3. Chemicals

Evaluating reliability of trace elements measurement results in olive oil is hindered by the absence of a matrix matching certified reference material. The accuracy of the method was therefore evaluated using a multielement oil standard of 100 ppm concentration (S23-100Y, SPEXCertiPrep) to spike olive oil samples. Spiking was also done using aqueous multielement standard solutions that were used for calibration of ICP-MS (10 mg L^{-1} XSTC-622B and 10 mg L^{-1} XSTC-1, SPEXCertiPrep, USA) because some of the elements are not contained in the oil standard. 61% electronic-grade (EL) nitric acid HNO$_3$ (Cica-Merck, Kanto Chemicals, Tokyo, Japan), 30% atomic absorption spectrometry-grade hydrogen peroxide H$_2$O$_2$ (Wako Pure Chemical Industries, Osaka, Japan) for ultratrace analysis and 35% HCl were used to prepare samples. Influence of instrumental drift was corrected by using Indium (In) as internal standard prepared from 10 mg L^{-1} CLISS-1, SPEX CertiPrep, USA to yield a concentration of 1 ppb in the samples, method blanks and calibration solutions.

2.4. Description of the methods

2.4.1. Microwave digestion

Mineralization of olive oil samples was carried out according to the method described by Llorent-Martinez, Fernandez-de Cordova, Ortega-Barrales, and Ruiz-Medina (2014), with minor modifications. The method consists of weighing 0.5 g of vigorously shaken sample and placing it directly into the digestion vessel, and adding 7 mL of HNO$_3$ and 1 mL of H$_2$O$_2$. The vessels were placed in the microwave digestion system. The program of the microwave

consisted of a ramp of 15 minutes to reach 200 °C and 1000 W, where the system was maintained for an additional 15 minutes. After being cooled to room temperature, samples were transferred into DigiTUBES and diluted to volume with ultrapure water. Samples were filtered using a 0.20 μm pore size syringe filter (Captiva econofilter, Agilent Technology, USA). Vessels were cleaned using the same microwave operating program after each digestion batch and successively rinsed with Milli-Q water.
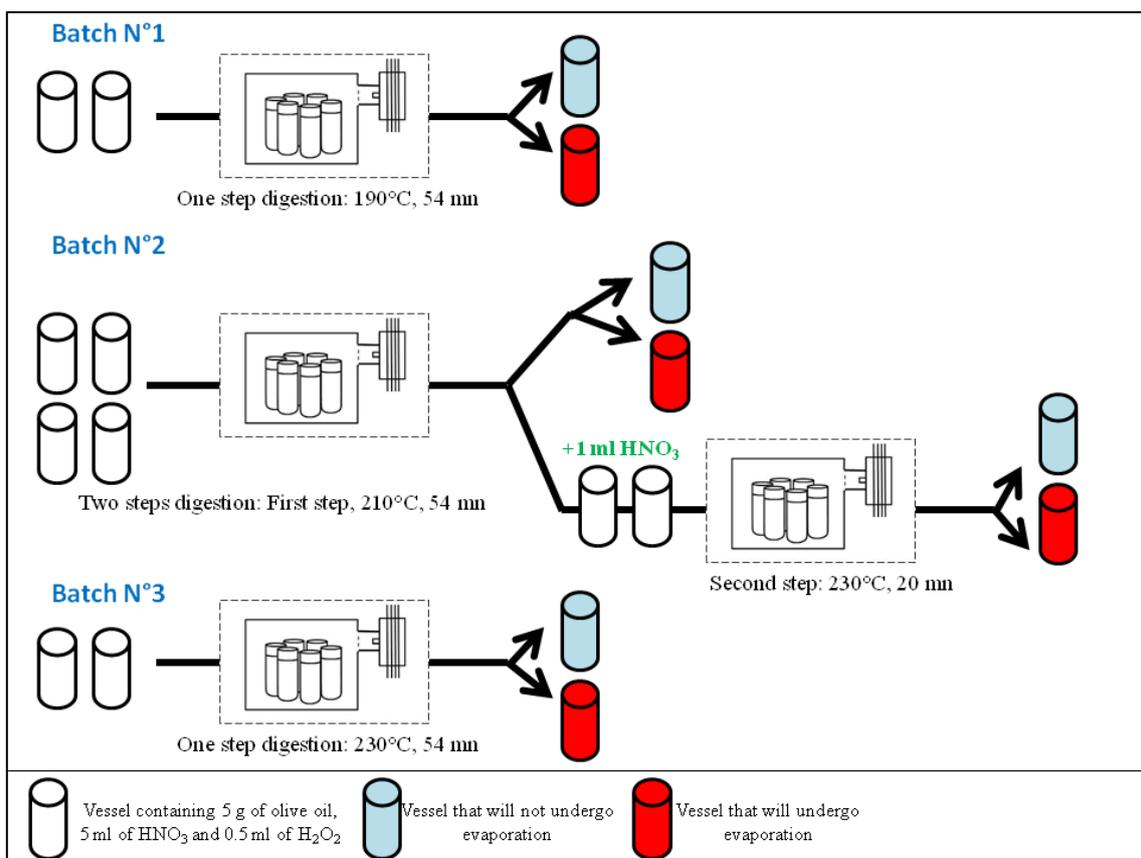
Figure 5. Scheme of optimization procedure for sample pre-treatment prior to multielements determination by ICP-MS

2.4.2. Combined evaporation after digestion: Optimization

2.4.2.1. Description of the protocol

Three batches have been designed with precise samples and reagents amounts specified and microwave digestion parameters defined (Fig. 5.). The first step of this method development was focused on optimizing the microwave working parameters (i.e. temperature (°C), time, number of digestion steps) and digestion vessels were prepared in duplicate in each batch so that one can be used to test the evaporation effect later on.

Microwave program was chosen according to the manufacturer's application note. The matrix that was the closest to that of olive oil was that of egg oil, so we have chosen the microwave program for egg oil digestion to digest samples of the first batch and then we increased the temperature by 20°C two times in each step of the programs of the following two batches. Table 8, table 9 and table 10 show the microwave program used for digesting the oil samples in each of the three batches.

Table 8. Operating program for the microwave system used for batch N°1.

Step	Time (mn)	Power (W)	Temp 1 (°C)	Temp 2 (°C)	Pressure (bar)
1	2	1000	50	0	0
2	1	0	30	0	0
3	31	1000	190	0	0
4	1	0	160	0	0
5	6	1000	190	0	0
6	13	1000	190	0	0
7	5	Ventilation			

Table 9. Operating program for the microwave system used for batch N°2.

Step	Time (mn)	Power (W)	Temp 1 (°C)	Temp 2 (°C)	Pressure (bar)
1	2	1000	70	0	0
2	1	0	50	0	0
3	31	1000	210	0	0
4	1	0	180	0	0

Table 9. (Continued)

5	6	1000	210	0	0
6	13	1000	210	0	0
7	5	Ventilation			

Table 10. Operating program for the microwave system used for batch N°3.

Step	Time (mn)	Power (W)	Temp 1 (°C)	Temp 2 (°C)	Pressure (bar)
1	2	1000	90	0	0
2	1	0	70	0	0
3	31	1000	230	0	0
4	1	0	200	0	0
5	6	1000	230	0	0
6	13	1000	230	0	0
7	5	Ventilation			

Following the digestion, the vessels that will not undergo evaporation were opened and their contents collected. The collected samples were then transferred to a DigiTube and their volume was adjusted to 25 ml. Samples were then centrifuged and dilutes two times prior to ICP-MS analysis.

The other vessels, those that will undergo evaporation, were opened and the cup removed from the bomb body and placed on a hotplate at 80°C until the residual digest volume becomes approximately equal to 1 ml (It takes around 30 minutes to reach that state). After that, 20 ml of 1% HNO_3 were gradually added to each vessel (10 ml at the first step that were left to dissolve the residual digest for about 5 minutes and then the total volume transferred to DigiTube and additional 10 ml added to wash the vessel). The volume was finally brought to 25 ml.

2.4.2.2. Selection criteria

The quality of organic matrix decomposition was evaluated by determining the residual carbon content of the digested solutions. For the determination of carbon content in digest (CCD), digested solutions were analyzed by ICP-MS using the semi-quantitative mode.

2.4.3. Ultrasonic-assisted extraction

The samples were prepared according to the method of Camin et al. (2010) with minor modifications. Briefly, 10 g of olive oil sample – previously centrifuged at 3500 g and the clear olive oil supernatant carefully collected - was weighed into a 50 ml conical bottom polypropylene centrifuge tube and 10 ml of the extracting aqueous solution was added. The extracting water solution was prepared with: 1% HNO_3, 0.2% HCl and 6.7% H_2O_2. The mixture was then vortex-shaken for 30 s and placed in a an ultrasonic cleaning bath (300W, 27°C) with a capacity of 30 liters for 20 minutes to extract the inorganic elements from the oil to the aqueous solution. The mixture was then centrifuged (3500 g x 5 min) to separate the two phases. The upper oil layer was carefully aspired and discarded and the lower aqueous phase collected and 5 ml of it were poured into a 15 ml conical bottom polypropylene centrifuge tube and subjected to ICP-MS analysis.

2.5. Quality control for method validation

According to Piotr Konieczka (2007), validation of an analytical procedure refers to the precise characterization of the procedure so the most valid, well founded, reliable and precise measurement results can be acquired with it. It is one of the most critical steps in the process of introducing a new method into practice. The method validation must be conducted using the evaluation of at least the basic performance criteria which include: the estimation of the accuracy, the limits of detection and quantification, and the precision of the analytical method under repeatability conditions.

2.5.1. Accuracy

The accuracy means the nearness of test results to the true value. The most used and common procedure of accuracy determination is based on the independent measurements of 10 replicates of a certified reference material (CRM) and it is reported as the percent recovery of the known certified value. It can also be determined by a comparison of its results to those of another, well-characterized method. Sometimes, CRMs are not easily available and this may

complicate the validation of analytical methods. An alternative to CRMs would be to analyze samples spiked with known amounts of the analytes of interest

2.5.2. Limit of detection (LOD) and limit of quantification (LOQ)

The values of LOD and LOQ are strongly related to measurement noise. LOD is the lowest concentration that can be measured (detected) by using a specified analytical procedure with statistical significance. LOQ is the lowest concentration that can be determined or quantified by using a specified analytical procedure with the established accuracy, precision and uncertainty.

LOD and LOQ of each element are calculated as three and ten times, respectively, the standard deviation of the measurement of the specific element in ten independent method blank samples. Each method blank solution must be prepared with the reagents used to prepare the samples and must undergo the same analytical conditions as the samples (Piotr Konieczka (2007).

2.5.3. Precision (RSD)

It refers to the degree of variability among the independent measurements obtained by analysis of a specific sample by using a specific analytical method. Precision is usually expressed as a repeatability measurement of ten repeated determinations under the same conditions of a given sample on the same day (Khan et al., 2013) or it can be expressed as reproducibility measurement of ten different sample preparations on different days. In that case, it is expressed as a relative standard deviation.

2.6. ICP-MS calibration

Table 11 shows the calibration range of elements in olive oil samples in the three methods. External calibrations curves were built using a range of different mass concentrations prepared by a mixture of the following single-element and multi-element standard stock solutions: 10000 mg L^{-1} Ca (SPEXCertiPrep, USA), 1000 mg L^{-1} Na (SPEXCertiPrep, USA), 1000 mg L^{-1} Fe (SPEXCertiPrep, USA), 1000 mg L^{-1} Mg (SPEXCertiPrep, USA), 10 mg L-1 XSTC-622B (SPEXCertiPrep, USA) and 10 mg L^{-1} XSTC-1 (SPEXCertiPrep, USA) (all in 5% HNO$_3$).

Remarks:

(i) Semi quantitative mode (without calibration of ICPMS) has been employed in the carbon content in digest (CCD) analysis for the optimization of digestion/ evaporation method and also in the KED Helium mode during RSD check for the combined digestion at 190°C and evaporation method.

(ii) XSTC-622B contains the following elements: Li, B, Na, Mg, Al, Si, K, Ca, Ti, V, Cr, Fe, Mn, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Zr, Mo, Ag, Cd, Sn, Sb, Cs, Ba, W and Pb.

(iii) XSTC-1 contains the following rare earth elements: Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sm, Sc, Tb, Tm, Yb and Y.

Table 11. Calibration range of elements in the three analytical methods analysis by ICP-MS.

	Solution name (concentrations in µg L^{-1})						
Method	Na	Ca	Mg	Fe	622B*	XSTC-1	N**
Digestion[a]	-	-	-	-	0-50	-	8
Combined[b]	0.05-100	0.025-50	0.01-20	0.01-20	0.005-10	-	11
Ultrasonic extraction[c]	5-10000	5-10000	0.5-1000	0.5-1000	0.05-100	0.005-10	12

*, XSTC-622B; **, number of calibration points evenly distributed across the corresponding range; a, assessment of LOD, LOQ, RSD and accuracy of oil standard's elements recovery; b, assessment of LOD, LOQ and RSD only; c, assessment of LOD, LOQ, RSD and accuracy based on spiked natural olive oil sample with an oil and aqueous standards: levels of spiking indicated in 2.5.1.

3. Results and discussion

3.1. Microwave-assisted digestion

The table 12 shows the results of performance criteria determinations for microwave-assited digestion.

Table 12. Results of determination of analytical procedure parameters (Microwave digestion at 200°C, modified according to Llorent-Martinez et al., 2014).

Element	Unit	Isotope	Operation mode	Linearity R²	Accuracy (%)	LOD	LOQ
Na	mg kg⁻¹	23	Standard	0.9998	84	0.12	0.35
Mg	mg kg⁻¹	24	Standard	0.9991	66	0.16	0.47
Fe	mg kg⁻¹	56	DRC	0.9999	88	0.12	0.39
Zn	mg kg⁻¹	66	DRC	0.9997	97	0.11	0.36
V	µg kg⁻¹	51	Standard	1.0000	97	1.7	5.6
Mn	µg kg⁻¹	55	Standard	1.0000	103	6.0	20
As	µg kg⁻¹	75	Standard	0.9990	-	0.73	2.4
Rb	µg kg⁻¹	85	Standard	0.9996	-	0.30	1.0
Sr	µg kg⁻¹	88	Standard	0.9999	-	5.1	17
Ba	µg kg⁻¹	138	Standard	1.0000	102	4.6	15
Pb	µg kg⁻¹	208	Standard	1.0000	97	6.9	23

The results shown above are the ones collected during our pilot study. Precision assessment was not conducted back then, but by examining the concentrations of the three replicates analyzed for each sample, their RSD was considered very high. The linearity was very satisfactory with a linear regression coefficient greater than 0.999 for most of the elements. The accuracy results obtained in our study are very similar to those obtained in the original research study of Llorent-Martinez et al., 2014 using the oily multi-elements standard. As for the LOD and LOQ, our results were significantly better than those reported in the original research for the elements Fe, V and As and relatively similar for the element Pb (Table 13).

However, these detection limits remain high especially for the elements that were not reported (11 out of 32 initially analyzed elements) because their concentrations in olive oil samples were lower than the determined corresponding LOD value and this method appears not

suitable for rare earth elements determination as well. Therefore, another method capable of reaching lower detection limits and higher precision and suitable for rare earth elements determination is highly required.

Table 13. Comparison of the limits of detection of the elements extraction method between our pilot study and the original research.

Element	LOD (μg Kg-1)	
	Our study	Original study
Fe	120	600
V	1.7	15
As	0.73	15
Pb	6.9	4

3.2. Development of the combined microwave digestion and evaporation method

3.2.1. Carbon content in digest

Sample digestion or decomposition is an important step in analytical methods for the routine determination of chemical elements in foodstuff. The particular degree to which decomposition is complete was assessed by measuring the residual carbon content in digests (RCCD). The study with a one step digestion at 190°C followed by evaporation on a hotplate at 80°C showed the lowest RCCD values compared to those obtained by higher temperatures or a two-steps digestion process (Table 14). Overall, all the vessels that underwent evaporation after the microwave-digestion step showed lower residual carbon content. This result was expected as the prolonged exposure of the sample to heat over time in its acidic environment increases its decomposition rate and the release of the volatile carbon to the atmosphere. As for vessels that didn't underwent evaporation, we noticed that the residual carbon content increases as the temperature increase and it exceeded the range when a two-steps digestion process was employed. We presume that the high temperatures and pressure inside the closed vessels would have caused recalcitrant organic compounds formation.

According Castro et al. (2009) efficient digestions should allow a complete decomposition of organic material leading to low residual carbon contents. The results presented in this study support the fact that digestion at 190°C followed by evaporation confirms the observations made by (Castro et al., 2009). Consequently, we will determine the performance criteria of this method namely: LOD, LOQ and precision as a first step of method development procedure before continuing with the complementary criterion (accuracy).

Table 14. Counts at mass number 12 corresponding to carbon: a comparative way of residual carbon content estimation in different decomposition conditions.

	One step						Two steps	
	190°C		210°C		230°C		210°c then 230°c	
Mass numbe r	NE	E	NE	E	NE	E	NE	E
12	172,855,74 3	13,216,17 9	217,036,88 1	77,300,75 2	219,254,25 0	191,976,92 7	over range	76,931,31 4

NE, no evaporation; E, evaporation

3.2.2. Validation of the proposed combined method: first step of validation based on LOD, LOQ and precision

The table below (table 15) shows the results of the performance criteria determination for the proposed method.

3.2.2.1. Range of linearity and linearity

The ICP-MS analysis was calibrated using eleven external standards including the blank. Although ICP-MS is well known for a wide linear dynamic range, we checked the linear regression coefficient of the calibration curves. Linearity is considered satisfactory if the coefficient exceeds 0.999 (Chudzinska et al., 2012). R^2 was greater than 0.999 for Mg, Ca, V, Cr, Ni, Zn, As, Rb, Sr, Mo and Pb. The rest of the elements had lower coefficients especially Fe which had the lowest one.

Table 15. Results of determination of analytical procedure parameters (Microwave digestion at 190°C followed by evaporation).

Element	Isotope	Mode	LOD (µg L^{-1})	LOQ (µg L^{-1})	Repeatability (RSD %)	Range of linearity (µg L^{-1}) and R^2
Na	23	He	13.5	45.00	5.4	0-10; 0.9985
Mg	24	He	3.46	11.50	26.6	0-2; 0.9991
Ca	44	CH_4	192.0	641.00	6.61	0-50; 0.9990
V	51	He	0.0124	0.041	52.9	0-1; 0.9996
Cr	52	CH_4	0.466	1.550	24.54	0-10; 0.9996
Mn	55	He	0.487	1.620	51.7	0-1; 0.9989
Fe	56	He	5.770	19.20	45	0-2; 0.9957
Ni	60	CH_4	2.470	8.21	25.6	0-10; 0.9998
Cu	63	He	2.302	7.68	16.2	0-1; 0.9989
Zn	64	CH_4	12.70	42.30	11.9	0 -10; 0.9997
As	75	He	0.093	0.312	98.6	0-1; 0.9993
Rb	85	STD	0.036	0.121	38.7	0-10; 1.000
Sr	88	STD	0.098	0.327	15.3	0-10; 1.000
Mo	98	CH_4	0.153	0.510	46.3	0-10, 0.9999
Pb	208	STD	2.44	8.14	22.3	0-10; 0.9999

He, Helium collision mode; CH_4, CH_4 dynamic reaction cell mode; STD, no gas standard mode; R^2, linear regression coefficient of calibration for LOD and LOQ determinations.

3.2.2.2. LOD and LOQ

When compared to the microwave digestion method, the combined digestion followed by evaporation method proposed here offers significantly better detection limits and consequently better quantification limits (Table 16).

3.2.2.3. Precision (Repeatability)

The results of repeatability obtained for the microwave followed by evaporation method were

Table 16. Comparison of the limits of detection of the elements between microwave only and microwave followed by evaporation methods.

Element	Isotope	Microwave method	Microwave and evaporation method
		LOD (μg L^{-1})	LOD (μg L^{-1})
Na	23	120	13.5
Mg	24	160	3.46
Ca	44	-	192.0
V	51	1.7	0.0124
Cr	52	-	0.466
Mn	55	6	0.487
Fe	56	120	5.770
Ni	60	-	2.470
Cu	63	-	2.302
Zn	64	110	12.70
As	75	0.73	0.093
Rb	85	0.3	0.036
Sr	88	5.1	0.098
Mo	98	-	0.153
Pb	208	6.9	2.44

not satisfactory and showed high spread around the mean. Elements such as V, Fe, Rb and Mo had RSDs higher than 30%. V, Mn and As had RSDs higher than 50% (98.6% for As). Since RSD estimation was based on counts and not on real concentrations, the RSD is sometimes not representative of the real spread, for example in the case of As, counts varied between 0 and 9 which means the As was almost absent from the method blank solutions so an RSD of 98.6% is not representative of the real situation.

3.3. Ultrasound-assisted extraction

Table 17 shows the results of the performance criteria determination for the ultrasound-assisted extraction method.

Table 17. Results of determination of analytical procedure parameters for ultrasonic extraction of elements.

Element	Isotope	Mode	LOD ppb	LOQ ppb	Repeatability (RSD %)	Range of linearity ($\mu g\ L^{-1}$) and R^2
Na	23	He	0,418	1,39	10.6	0-50; 0.9985
Mg	24	He	0,105	0,350	10.7	0-10; 0.9986
Ca	44	He	1,470	4,920	13.4	0-25; 0.9811
Ti	47	He	0,135	0,450	10.9	0-5; 0.9977
V	51	He	0,069	0,230	1.85	0-5; 0.9975
Cr	52	He	0,0350	0,117	12.4	0-5; 0.9987
Mn	55	He	0,006	0,0205	10.34	0-5; 0.9979
Fe	56	He	0,140	0,467	5.05	0-10; 0.9984
Ni	60	CH_4	0,179	0,596	40.3	0-5; 0.9974
Cu	63	He	0,198	0,660	29.6	0-5; 0.9931
Zn	66	He	0,0766	0,255	18.4	0-5; 0.9988
As	75	CH_4	0,0360	0,120	6.19	0-5; 0.9987
Rb	85	STD	0,00061	0,0021	8.91	0-5; 0.9993
Sr	88	STD	0,0025	0,0085	9.10	0-5; 0.9993
Mo	98	STD	0,0047	0,0160	8.62	0-5; 0.9970
Ba	138	STD	0,0014	0,0049	12.6	0-5; 0.9990
Pb	208	STD	0,0035	0,0116	13.4	0-5; 0.9989

He, Helium collision mode; CH_4, CH_4 dynamic reaction cell mode; STD, no gas standard mode; R^2, linear regression coefficient of calibration for LOD and LOQ determinations.

3.3.1. Range of linearity and linearity

The ICP-MS analysis was calibrated using thirtheen external standards including the blank. Although ICP-MS is well known for a wide linear dynamic range, we checked the linear regression coefficient of the calibration curves.

R^2 obtained for most of the elements was not very satisfactory as it was under 0.999 which is considered the optimal coefficient value. Cu had the lowest coefficient (0.9931) and only the alkaline Rb, Sr and Ba had coefficients equal or higher than 0.999.

3.3.2. LOD and LOQ

When compared to the microwave digestion method, the ultrasound-assisted extraction method offers significantly better detection limits and consequently better quantification limits (Table 18). The sensitivity improvement varied from eight-fold for Rb to as high as 20,000 fold for Fe.

Table 18. Comparison of the limits of detection of the elements between microwave digestion and sonication methods.

		Microwave method	Sonication method
Element	Isotope	LOD ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)
Na	23	120	0,418
Mg	24	160	0,105
Ca	44	-	1,470
V	51	1.7	0,135
Cr	52	-	0,069
Mn	55	6	0,0350
Fe	56	120	0,006
Ni	60	-	0,140
Cu	63	-	0,179
Zn	64	110	0,198
As	75	0.73	0,0766
Rb	85	0.3	0,0360
Sr	88	5.1	0,00061
Mo	98	-	0,0025
Pb	208	6.9	0,0047

3.3.3. Precision (Repeatability)

The results of repeatability obtained for the sonication method were very satisfactory compared to the two previous methods. Fourteen elements had RSDs lower than 13.4% whereas Ni, Cu and Zn had the least satisfactory RSDs (40.3%, 29.6% and 18.4% respectively).

3.4. Comparison between the three methods

Based on the results above-presented, the combined microwave digestion-evaporation and the ultrasound assisted extraction performed better than the microwave digestion as judged by the detection limits. So it is advantageous to replace the microwave digestion only by one of these two proposed methods to improve the detection limits and widen the range of the mass numbers that can be detected by the ICP-MS in the olive oil samples. Our results agree with those recently reported by Filip Poscic et al. (2019) stating that the microwave acid digestion does not allow measurement of elements present at very low concentrations even if ICP-MS technique is used for detection. This is because of the limited amount of sample that can be processed. The high pressure that develops during digestion owing to the high organic matter and high fatty acid content of the olive oil restrain the amount of sample allowed into the digestion vessel. Moreover, the residual acidity in the digests makes it mandatory to dilute samples prior to ICP-MS analysis which lowers the concentration of the elements and may make it at the same range of the blank for some elements that are initially present at trace level and therefore make it undistinguishable from the uncertainty of the blank (i.e. very close or below the detection limit). Moreover, the same authors reported similar findings to our pilot study regarding the high detection limits obtained by the microwave digestion only as they could only detect seven (Cu, K, Mg, Mn, P, Rb and S) out of the twenty-nine measured elements (Filip Poscic et al., 2019). In another study comparing the performance of microwave digestion and simple dilution of wine samples prior to ICP-MS measurement, the authors reported that the detection limits for most elements in the digested samples were between 2 and 10 times higher than those for the diluted samples and attributed these findings to the higher background in the method blanks of the digested samples. This higher background is due to the impurities in the reagents used to mineralize the samples and to possible contamination from the digestion vessels (Coetzee et al., 2005).

When compared together, the ultrasound-assisted extraction performed better than the combined microwave digestion-evaporation method as judged by the detection limit and the precision expressed as repeatability. These lower detection limits and higher precision can be explained by the fact that the sonication method uses fewer steps to extract the elements from the samples or to prepare the method blanks. On top of that, sonication exposes the samples to the laboratory environment for a shorter amount of time meaning it reduces the contamination risk. In fact, the dependable determination of elements at very low levels in organic materials is significantly determined by the ability to control and reduce contamination in the method

blanks and samples solutions during sample preparation because detection limits estimation is based on the variability of the blanks. This confirms the statements of Khan et al. (2013) that an effective sample digestion or decomposition method is the one that involves a minimal preparation steps since the risks of loss or contamination would be limited (Khan et al., 2013).

4. Conclusions

The accurate determination of trace metals in organic materials is often limited by contamination encountered during sample preparation rather than sensitivity of the analytical technique.

It was concluded that the ultrasound-assisted extraction of major, trace and ultra-trace elements from olive oil samples was to be preferred to the closed microwave-assisted digestion procedure because of the following advantages: lower detection limits, higher precision, less risk of contamination due to exposure to the laboratory surrounding environment, less time-consuming, and simple sample preparation. The ultrasound-assisted extraction will accordingly be the method of choice for multielements extraction from the olive oil samples in the rest of this thesis.

Methods for reducing and eliminating sources of contamination must regularly be controlled and improved to move at the same speed with advances in equipments used for the end analysis. The analyst, reagents, materials, and laboratory environment are all potential sources of contamination that must be assessed during the sample preparation step.

Chapter 3

Establishing a database of Tunisian olive oils based on the elemental composition: Implication of the geochemical and edaphological characteristics of the provenance soil

1. Introduction

The pilot study – carried out using the 2015/2016 sampling campaign's olive oil and soil samples – has shown the potential of multielements as successful geochemical markers. These markers are capable of discriminating olive oils coming from different regional climatic and geological contexts characterized by their soil geochemical signature. These preliminary results encouraged us to extend the measurement to a greater number of olive oil samples coming from different Tunisian regions and also from Italy to verify their real capability for the geographical origin identification. Therefore, a second extensive and almost-exhaustive sampling campaign that aimed to cover the whole olive-growing regions in Tunisia was conducted.

The first goal was to extend the database of the multielemental composition of Tunisian monovarietal olive oils to the major olive-growing regions. The exact location of the olive orchards from which these olive oils are produced (in the laboratory in the same industrial procedure) is recorded by a GPS to be confident about their true origin. This database is crucial because it will constitute the reference list of the composition of olive oils from all these sampled regions. The reference list can serve in two ways: (i) screen – by means of visual interpretation of graphical representations of the analytical results and sophisticated multivariate statistical analyses also called chemometrics - the elemental profiles of olive oils from all the major olive-growing regions in Tunisia in view of identifying regions with unique elemental profile and consequently qualifying as potential protected geographical indication (PGI) area based on the uniqueness of its chemical signature, (ii) used to verify - in the future when Tunisia establishes a PGI label for some olive oil producing area – the authenticity of a suspicious product claiming to originate from this area by analyzing its composition and comparing it to that of the authentic samples constituting the database. This approach requires a representative sampling from all the relevant regions.

The second goal was to enhance and deepen the scientific understanding of the relationship between the elemental composition of olive oil and its environment. Multielemental-based

geographical traceability technique is deeply rooted in the hypothesis that the mineral content of soils from two or more regions subject to discrimination is the driving factor that determines the elemental composition of olive oils. Additionally, and taking this hypothesis into account, the differences in the elemental composition of soils between these regions should be large enough – which presumably allow for a larger difference in the elemental profile of olive oils - to permit a successful discrimination. Therefore it is necessary to identify the elements that can characterize the geographical origin of soils and act as geographical indicator. However, the mineral content of soil may be influenced by some agricultural practices such as irrigation, chemical application (fertilizers, amendments, pesticides, etc…) which could influence the elemental profile of olive oils. This can deteriorate the credibility and effectiveness of the model built based on the generated analytical datasets if these practices change (Zhao et al, 2013). Therefore, it is essential to evaluate the closeness of the soils composition to that of their parent material and the geochemical background levels and show their predominant geochemical origin whether in soils or olive oils. However, little attention has been paid to this aspect in previous researches (Damak et al., 2019). Our previous results showed that the total concentration of the inorganic elements contained in the soils on which the olive trees are grown is not capable of explaining the elemental profiles of their paired olive oils (Damak et al., 2019). For this reason, other environmental factors need to be investigated. These include edaphological, climatic and geographical variables. The former set of variables involves the soil's inorganic elements bioavailable fraction, the soil's chemical characteristics such as its pH, its electrical conductivity (EC), its organic matter content and its inorganic carbon (in the form of calcium carbonate $CaCO_3$) content. The latter variables are the temperature, precipitations, humidity and elevation.

The aims of this study were:

(1) to extend the multielemental measurements to a greater number of Tunisian monovarietal olive oil samples coming from areas with different geologic (outcrops) and climatic conditions (different mean annual temperature, mean annual precipitation, relative humidity, elevation);

(2) to verify the real possibility of identifying the geographical origin of oils in a restricted area by comparing and studying the correlation of the olive oil's trace elements with the geochemical, edaphological and climatic factors previously mentioned.

2. Materials and methods

2.1. Sampling

2.1.1. Area extent, local geology, climat and samples collection (soil and olive)

Within the framework of SATREPS project, 85 olive oils and 137 soil samples have been collected in December 2017. Figure 6 shows the location of the sampling points. The sampling covered a large area spanning the country from north to south and from east to west and focusing on the main olive producing regions. The sampling regions can roughly be administratively divided into eleven governorates namely Beja (3 sites), Zaghouan (7 sites), Nabeul (8 sites), Sousse and Mahdia (a.k.a. the Sahel) (6 sites), Sfax (10 sites) including Kerkennah island (5 sites), Sidi Bouzid (8 sites), Gasserine (5 sites), Gafsa (6 sites), Medenine and Tataouine (the south) (9 sites) and Kairouan (2 sites) accounting for a total of 68 sites. The sampling in Zaghouan, Nefza, Nabeul and Kerkennah was less dispersed than in the large Sahel and Sfax area, Gasserine, Sidi Bouzid and the south where the sampling tried to include points from the "four corners" of the regions but remained sparse. It remains important to say that the sampling points in each region tried to cover all the possible variability in the bedrock and include the maximal number of producing farms but there are still lots of unsampled areas considering the enormous and outsize surface area and the practical limitations of the time and resources. On the other hand, our main objective was to make sort of a first rough screening of the olives from each producing-region.

The table 19 shows the name of the sampling points, number and the assigned codes to olive fruit and soil samples. The table 20 as well as figures 7, 8 and 9 show the geological (age and lithology of the underlying outcrop) characteristics deduced from the intersection of the point feature corresponding to the sampling point with the shapefiles layers of the different outcrops on ArcMAP (ArcGIS, 10.4.1).
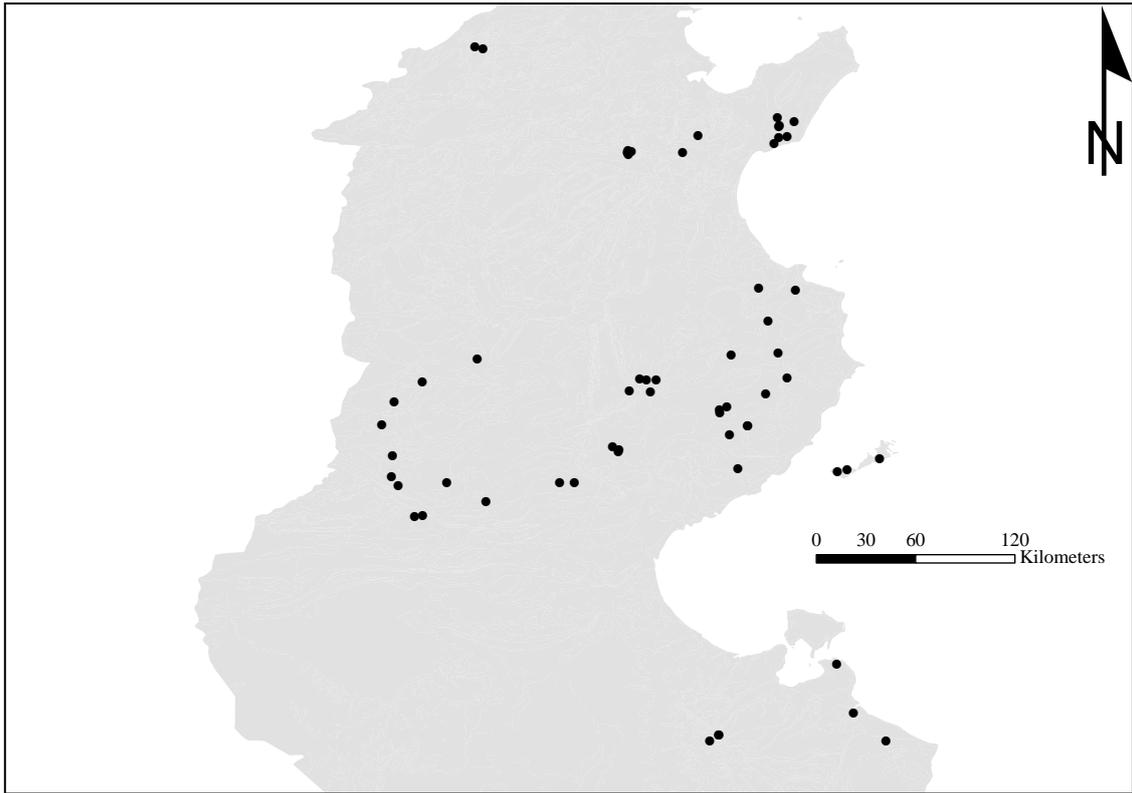
Figure 6. Map showing the location of the totality of the sampling points of 2017/2018 olive harvesting campaign.

Table 19. The origin and name of the sampling sites, the number of olive and soil samples and the codes assigned to each sample and site.

Origin			Soil and olive samples				
Site code	Governorate	Site	Olive code	Olive cultivar	N° O.O.S*	Soil code	N° S.S**
F1	Gafsa	Sidi Yaiich	1,2,3,4,5	Arbosana, Chetoui, Chemchali, Chemlali, Koroneiki	5	15,16, 17,18,19	5
F2		Oasis de Gafsa	6	Chemchali	1	20	1
F3		Gafsa Sud	7	Chemchali	1	21	1
F4		Zannouch	8, 9	Zarrazi, Gafsi	1	22,23,24	3
F5		Essakouia	15	Sahli	1	74-75	2
F6		Sidi Boubaker	16	Sahli	1	76-77	2
				Total	10	Total	14
F7	Gasserine	Mejel Bel Abbes	17, 18	Sahli, Gafsi	2	78-79	2
F8		Feryena	19	Sahli	1	82-83	2
F9		Thelepte	20	Sahli	1	80-81	2
F10		Belhijet	21	Sahli	1	84	1
F11		Sbeitla	22	Sahli	1	85-86	2
				Total	6	Total	9
F12	Zaghouane	El Fahs	44	Chetoui	1	87, 88-89,90-91,92-93	7
F13		El Fahs	42, 45	Sayali, Chetoui	2	94, 95-96	3

Table 19. (Continued)

F14		El Fahs	43, 46	Chetoui, Oleaster	2	97-98	2
F15		El Fahs	47	Oleaster	1	No soil	0
F16		Jemla	40	Chetoui	1	99-100	2
F17		Echahda	37,38,39	Beldi, Sahli, Tkoubri	3	101-102	2
F18		Zaghouan Centre	36	Chetoui	1	103-104	2
				Total	11	Total	18
F19	Nabeul	El Halfa	48	Sahli	1	72-73	2
F20		Dar Châabane centre	49	Chaiibi	1	66-67	2
F21		Mrezga 1	50	Sahli	1	62-63	2
F22		Somâa	51	Sahli	1	166-167	2
F23		Bir Challouf	52	Sahli	1	64-65	2
F24		El Amroun-Dar Châabane 1	53	Sahli	1	68-69	2
F25		El Amroun-Dar Châabane 2	65	Chaiibi	1	70-71	2
F26		Mrezga 2	66	Sahli	1	60-61	2
				Total	8	Total	16
F27	Tataouine/Medenine	Krerachfa	60	Chemlali	1	7-8	2
F28		Ksar Ouerjijen	71	Chemlali	1	9-10	2
F29		Ksar Ouerjijen	72	Zarrazi and Dhokar blend	1	No soil	0
F30			74	Zalmati	1	1-2	2

Table 19. (Continued)

F31		Chammakh	64	Zalmati	1	3-4	2	
F32		Chammakh CZ	67, 73	Chemlali, Zalmati	2	5-6	2	
F33		Alandaya	68, 70	Zalmati, Chemlali	2	11-12	2	
F34		Ben Gardane	69	Zalmati	1	13-14	2	
				Total	12	Total	16	
F35	Beja (Nefza)	Fatnassa-Nefza	33	Oleaster	1	No soil	0	
F36		Nefza centre	34	Chetoui	1	105-106	2	
F37		Errmila	35, 41	Chetoui, Jerbouii	2	107-108	2	
				Total	4	Total	4	
F38	SidiBouzid	Menzel Bouzaiane-Ennasr	10	Chemlali	1	121	1	
F39		Meknassi	11	Chemlali	1	122	1	
F40		Kaf Arrayet- Cherarda	57	Chemlali	1	117-118	2	
F41		Ouled Haffouz	59	Chemlali	1	119-120	2	
F42		Kaf Arrayet- SidiBouzid	62	Chemlali	1	115-116	2	
F43		Regueb	54, 55	Koroneiki, Arbosana	2	109-110	2	
F44		Ouled Ayouni 1	61	Chemlali	1	111-112	2	
F45		Ouled Ayouni 2	56	Arbosana	1	113-114	2	
				Total	9	Total	14	

Table 19. (Continued)

F46	Sfax	Menzel Chaker-Triaga 1	12,13	Sahli, Chemlali	2	55-56,57-58	4
F47		Menzel Chaker-Triaga 2	14	Chemlali	1	59	1
F48		Menzel Chaker-Bir El Mallouli	28	Chemlali	1	30,31	2
F49		Agareb-Torba	29, 30, 31	Chemlali, Chetoui, Arbequina	3	27-28	2
F50		Menzel Chaker-Bir El Mallouli	32	Chemlali	1	29-36	2
F51		Agareb	75	Chemlali	1	25-26	2
F52		El Hencha-Baltech	82	Chemlali	1	47-48	2
F53		Menzel Chaker-Boujarbouea 1	80	Chemlali	1	49-50	2
F54		Menzel Chaker-Boujarbouea 2	76	Chemlali	1	51-52	2
F55		Menzel Chaker	85	Chemlali	1	53-54	2
				Total	13	Total	21
F56	Kairouan	Cherarda-Chraitia	58	Chemlali	1	123-124	2
F57		Cherarda	63	Chemlali	1	125-126	2
				Total	2	Total	4
F58	Sahel	Mahdia-Kerker	77	Chemlali	1	43-44	2
F59		Sousse-Bourjine	78	Chemlali	1	39-40	2
F60		El Djem 1	81	Chemlali	1	32-33	2
F61		El Djem 2	79	Chemlali	1	34-35	2
F62		Ouled Chemekh-Ouled Hannachi	83	Chemlali	1	41-42	2

Table 19. (Continued)

F63		Jammel	84	Chemlali	1	37-38	2
				Total	6	Total	12
F64	Kerkennah	Mellita 1	23	Chemlali	1	127-128	2
F65		Mellita 2	24	Chemlali	1	129-130	2
F66		Abbasiya	25	Chemlali	1	131-132	2
F67		Erramla	26	Chemlali	1	133	1
F68		Sidi Fredj	27	Chemlali	1	134-135	2
				Total	5	Total	9

NA, Not available; *, Number of olive oil samples; **, Number of soil samples.
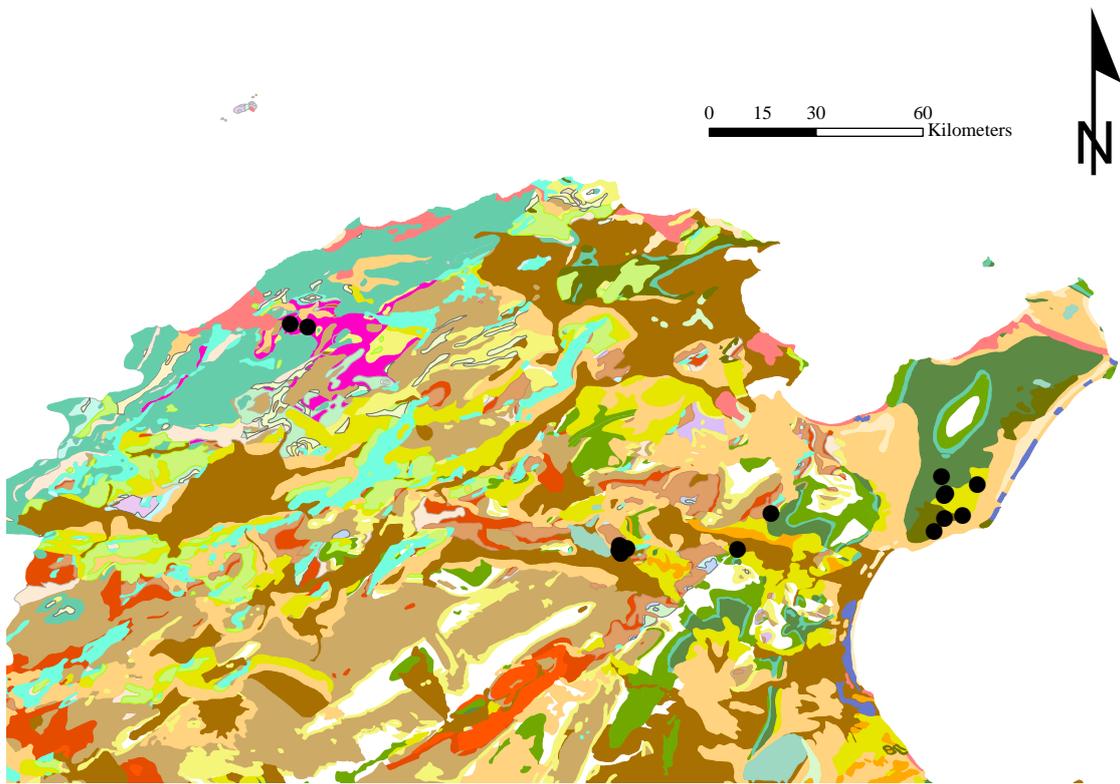
Figure 7. Geological map showing the sampling points from the northern regions of Tunisia.
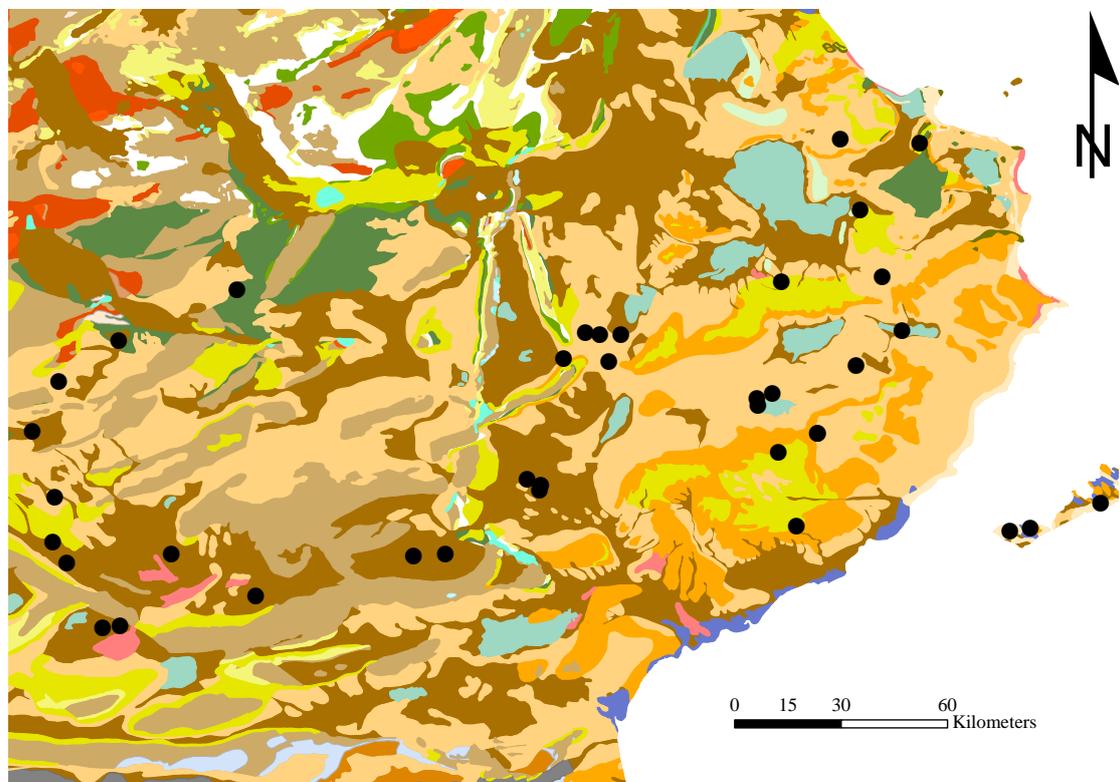
Figure 8. Geological map showing the sampling points from the central regions of Tunisia.
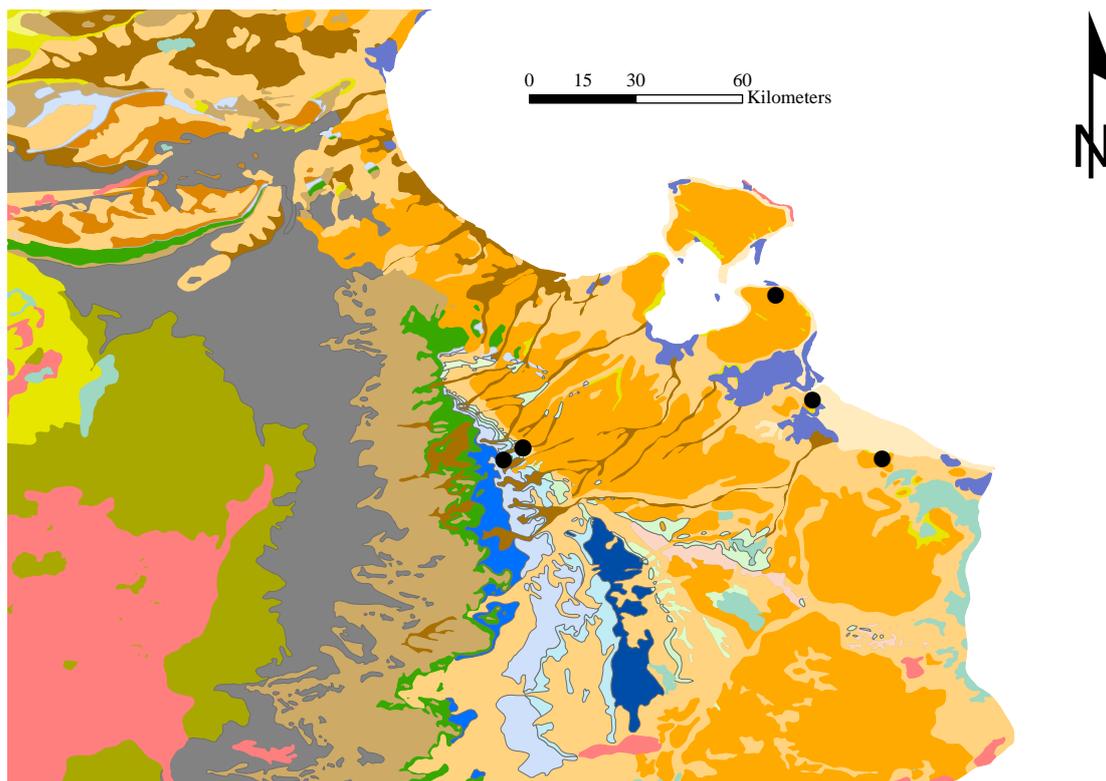
Figure 9. Geological map showing the sampling points from the southern regions of Tunisia.

2.2. Samples preparation and analysis

2.2.1. Olive oil samples

2.2.1.1 Olive oil extraction

Each batch of olive oil was extracted in the Sfax Olive Institute from 1.5 kg of whole washed olive fruits by mechanical means alone. A laboratory-scale 2-phase oil mill was used following the standard methods employed in industrial olive oil mills. This method consists of crushing, malaxation for 30 min at ambient temperature, centrifugation without addition of water, and finally natural decantation. This process yields an oily phase and a very wet pomace phase. Olive oil samples were stored in amber glass bottles at 4 °C until their inorganic elements analyses, which were performed at the Advanced Analysis Center, National Agriculture and Food Research Organization (NARO) in Japan.

2.2.1.2. Ultrasound-assisted extraction of multielements

Table 20. Age and lithology of the outcrops at the sampling sites.

Governorate	Site's code	Age	Lithology (Symbol)**
Gafsa	F1	Holocene	aQ
	F2	Holocene	aQ
	F3	Holocene	aQ
	F4	Holocene	aQ
	F5	Holocene	aQ
	F6	Holocene	aQ
Gasserine	F7	Continental middle and upper Pleistocene	cQ
	F8	Continental middle and upper Pleistocene	cQ
	F9	Continental middle and upper Pleistocene	cQ
	F10	Holocene	aQ
	F11	Upper Miocene	aM3
Zaghouane	F12	Jurassic-lower Cretaceous	J
	F13	Lower Cretaceous	sC1
	F14	Holocene	aQ
	F15	Holocene	aQ
	F16	NA	
	F17	Lower Cretaceous	sC1
	F18	Continetal Mio-Pliocene	M-PL
Nabeul	F19	Late Miocene	aM3
	F20	Pléistocène moyen et supérieur continental	cQ
	F21	NA	
	F22	Continetal Mio-Pliocene	M-PL

Table 20. (Continued)

	F23	Marine Pliocene	Pl
	F24	Upper Miocene	aM3
	F25	Upper Miocene	aM3
	F26	Marine Pliocene	Pl
Tataouine/Medenine	F27	Holocene	aQ
	F28	Holocene	aQ
	F29	Holocene	aQ
	F30	NA	NA
	F31	Villafranchian-Pleistocene	Qv
	F32	NA	NA
	F33	Pléistocène moyen et supérieur continental	cQ
	F34	Villafranchian-Pleistocene	Qv
Beja (Nefza)	F35	NA	NA
	F36	Oligocène-Aquitanien	O-M1
	F37	Maestrichtian-Paleocene	Cm-P
SidiBouzid	F38	Holocene	aQ
	F39	Holocene	aQ
	F40	Continental middle and upper Pleistocene	cQ
	F41	Continental middle and upper Pleistocene	cQ
	F42	Continental middle and upper Pleistocene	cQ
	F43	Holocene	aQ
	F44	Holocene	aQ
	F45	Holocene	aQ
Sfax	F46	Continental middle and upper Pleistocene	cQ

Table 20. (Continued)

	F47	Continental middle and upper Pleistocene	cQ
	F48	Continental middle and upper Pleistocene	cQ
	F49	Continetal Mio-Pliocene	M-PL
	F50	Continental middle and upper Pleistocene	cQ
	F51	Holocene	aQ
	F52	Holocene	aQ
	F53	Continental middle and upper Pleistocene	cQ
	F54	NA	NA
	F55	Continental middle and upper Pleistocene	cQ
Kairouan	F56	Holocene	aQ
	F57	Continental middle and upper Pleistocene	cQ
Sahel	F58	Holocene	aQ
	F59	Continental middle and upper Pleistocene	cQ
	F60	NA	NA
	F61	Continental middle and upper Pleistocene	cQ
	F62	Continetal Mio-Pliocene	M-PL
	F63	Continetal Mio-Pliocene	M-PL
Kerkennah	F64	Middle and upper marine Pleistocene (Mainly Tyrrhenian)	mQ
	F65	Villafranchian-Pleistocene	Qv
	F66	Holocene (Flandrian)	spQ
	F67	NA	NA
	F68	NA	NA

NA: Not available; **: aQ, Recent and Actual sandy alluviums; cQ, Old Alluviums, limestone and gypsum crusts; aM3, Alternation of sandstones and marls often with lignite (Oum Douil formation); J, Non-subdivided calcareous Jurassic; sC1, Marls, altrenating marl-limestone and sandstone; M-PL, Conglomerates, sand and clay; Pl, Marls and sandstone; Qv, Conglomerates, limestone crusts and red beds; O-M1, Argillaceous sandstone (Numidian flysch); Cm-P, Marls and clay with yellow balls; mQ, Beaches and consolidated costal dunes; spQ, Paralic Sebkha (Old lagunes).

The samples were prepared according to the method firstly proposed and partially validated by Camin et al. (2010) and approved and totally validated by Pošćić et al. 2019.

10 g of olive oil sample previously centrifuged at 3000 g for 10 minutes were transferred into a 50 ml polypropylene (PP) centrifuge tube. 10 ml of extracting aqueous solution prepared with 6.7% H_2O_2 (30%, Atomic absorption spectrometry (AAS) grade, Kanto chemical Co., INC., Tokyo, Japan), 1% HNO_3 (61%, Electronic grade (EL), Hayashi pure chemical IND.,LTD. (HPC), Japan) and 0.2% HCl (35%, AAS grade, Kanto chemical Co., INC., Tokyo, Japan) was added to the oil. The mixture was thoroughly shaken by a vortex for 30 seconds and then placed in an ultrasonic bath (300W, 27°C) for 10 minutes. After the ultrasonic extraction, the mixture is centrifuged at 3000 g for 5 minutes and the supernatant depleted oily phase is meticulously removed by aspiration. The enriched aqueous phase is then transferred into clean PP tube to which Indium ([115]In) was added as an internal standard yielding a concentration of 1 µg L^{-1}. Sample preparation and analysis were carried out in triplicate to assess the repeatability.

2.2.1.3. Quality control

The limit of detection (LOD) and limit of quantification (LOQ) of each element were calculated as three and ten times the standard deviation of the average concentrations determined in ~~10~~ independent method blank samples, respectively. The method blank sample was prepared using ultrapure water (18.2 MΩ.cm resistivity, ADVANTEC, RFD280NC, Japan) as a substitute for the olive oil in the extraction step. Each olive oil sample was extracted and measured three times to ensure precision (repeatability). Each extraction batch contained one method blank to monitor contamination. The influence of instrumental drift was corrected by using a 1 µg L^{-1} internal standard solution of [115]In prepared from 1000 mg L^{-1} single standard solution (SPEX CertiPrep, USA). The precision (RSD%) of the analytical method was evaluated by extracting and analyzing a commercial oil sample 10 times.

2.2.1.4. Calibration

External calibration curves were built using twelve different mass concentrations including the blank. Standard solutions were prepared by diluting a calibration mixture prepared by mixing:

1) A multielement solution (XSTC-622B) containing 34 elements: Ag, Al, As, B, Ba, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, Ge, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, Sb, Se, Si, Sn, Sr, Ti, V, W, Zn, Zr at 10 mg L^{-1} concentration (SPEXCertiPrep, USA) in 5.0% w/w nitric acid matrix and,

2) A multielements solution (XSTC-1) containing 16 rare earth elements: Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sm, Sc, Tb, Tm, Yb, Y at 10 mg L^{-1} concentration (SPEXCertiPrep, USA) in 5.0% w/w nitric acid matrix and,

3) Four single element solutions of Fe, Mg, Ca and Na at 1000, 1000, 10,000 and 1000 mg L^{-1} concentration respectively (SPEXCertiPrep, USA) in 5.0% w/w nitric acid matrix.

The resultant concentrations for the elements in the calibration mixture was 5500 µg L^{-1} for Ca and Na, 1500 µg L^{-1} for Fe and Mg, 500 µg L^{-1} for the elements coming from the XSTC-622B and 2 µg L^{-1} for the elements coming from XSTC-1. This calibration mixture was further diluted in the aqueous solution used to extract the oil samples to matrix-match the calibration solutions and the sample solutions. Thirteen solutions were consequently prepared to calibrate the ICP-MS. The range of concentrations is presented in table 21.

2.2.1.5. ICP-MS measurements

The quantification of the elements was carried out by inductively coupled plasma-mass spectrometry (ICP-MS; Elan DRC-e, Perkin-Elmer SCIEX, Canada). Oil samples were introduced by means of a borosilicate glass nebulizer. The ICP torch was a standard Fassel-type torch with a ceramic injector. ICP-MS is known to suffer from unwanted polyatomic isobaric interference. Therefore, the elements were monitored in Kinetic Energy Discrimination (KED) He collision mode to reduce polyatomic interference, and the appropriate isotopes were used. Instrument performance was checked by a midrange continuous calibration verification every ten samples. The operating conditions and

Table 21. Concentration range of the calibration solutions for quantification of the elements in the olive oil samples (ultrasound-assisted extraction of elements).

Calibration solution number	Name of the stock solution (and concentration in µg L^{-1})					
	Ca	Na	Fe	Mg	XSTC-622B	XSTC-1
1	0	0	0	0	0	0
2	1,375	1,375	0,375	0,375	0,125	0,0005
3	2,75	2,75	0,75	0,75	0,25	0,001
4	6,875	6,875	1,875	1,875	0,625	0,0025
5	13,75	13,75	3,75	3,75	1,25	0,005
6	27,5	27,5	7,5	7,5	2,5	0,01
7	68,5	68,5	18,75	18,75	6,25	0,025
8	137,5	137,5	37,5	37,5	12,5	0,05
9	275	275	75	75	25	0,1
10	687,5	687,5	187,5	187,5	62,5	0,25
11	1375	1375	375	375	125	0,5
12	2750	2750	750	750	250	1

parameters of ICP-MS were as follows: Rf power 1100 W; plasma Ar flow rate 15 L min−1; auxiliary Ar flow rate 1.3 L min−1; nebulizer (carrier gas) flow rate 0.77 L min−1; sampler and skimmer cones of nickel; lens voltage 7.5 V; analog stage voltage −1700 V; pulse stage voltage 950 V; discriminator threshold 70 V; quadrupole rod offset −1.5 V; dual detector; speed of peristaltic pump 20 rpm; 20 sweeps/reading; 3 replicates; dwell time 50 ms; peak hopping scan mode; rejection parameter a 0, and rejection parameter q 0.65.

2.2.2. Soil samples

Soil samples have been taken from two layers: the surface layer from 0 to 30 cm and the subsurface layer from 30 to 60 cm, except for soil samples from Gafsa (Sidi Yaiich samples corresponding to sample codes 15, 16, 17, 18 and 19; Oasis de Gafsa: 20; Gafsa sud: 21), from Sidi Bouzid (Menzel Bouzaiane-Ennasr: 121; Meknassi: 122), from Sfax (Menzel Chaker-Triaga 2: 59) and from Kerkennah (Erramla: 133) whose only the top layer was sampled. Two layers have been taken because we want to investigate the influence of soil depth on the elemental concentration and because we experienced a higher olive root density in the 30-60 layer than in the surface layer while taking samples by using the hand auger.

2.2.2.1. Total concentration measurements

Soil samples have been dried at 40°C to remove moisture. Major and trace elements in soil samples were measured at the facilities of the Geological Survey of Japan (GSJ) of the National Institute of Advanced Industrial Science and Technology (AIST) in Japan, according to a method developed by the GSJ. The method consisted of preparing high-dilution-ratio fused glass beads for X-ray fluorescence (XRF) determination of major element oxides (Ejima, Kon, Kawano, & Araoka, 2018) and using the same beads for trace element measurements by femtosecond ultraviolet Laser Ablation ICP-MS (LA-ICP-MS) (Kon & Hirata, 2015). The method is well described in Damak et al. 2019.

XRF analysis

The glass beads were prepared by mixing 0.5 g of powdered soil sample with 5.0 g of lithium tetraborate flux. The mixture was heated to 1200 °C for 10 min using a semi-automatic fusion device (HAG-M-HF, Herzog, Germany). Ten major elements in soil samples (Si, Ti, Al, Fe, Mn, Mg, Ca, Na, K and P) were quantified by an XRF instrument (ZSX Primus III+, Rigaku Corp., Japan) with an Rh tube. The accuracy was verified each day using two reference samples (JB-1b and JG-3). The analytical uncertainties for each element were better than 1.5%, as estimated from the long-term reproducibility of measurements of the geochemical CRM JB-1b.

LA-ICP-MS analysis

To obtain reliable abundance data for the trace elements in the soil samples, we used a quadrupole ICP-MS system (Agilent 7500cx, Agilent Technologies Japan Ltd., Japan)

coupled with a 260 nm (UV) titanium-sapphire femtosecond laser ablation system (IFRIT, Cyber Laser Inc., Japan). The laser ablation was operated using a crater size of 20 μm, a pulse energy of 10 J cm−2, an emission repetition rate of 1000 Hz, and a pulse duration of 150 s. A galvanometric optical scanner was employed to minimize elemental fractionation and for effective ablation of the glass beads, with a rastering speed of 10,000 μm s−1 in a 400×400 μm rastered area. To quantify the concentration of each element, GSJ geochemical reference samples (JP-1, JB-1b, JB-2, JB-3, JA-1, JA-2, JA- 3, JR-1, JR-2, JR-3, JGb-1, JGb-2, JG-1a, JG-2, and JG-3) were used to build the calibration curves. The ICP-MS operating conditions and parameters were as follows: Rf power 1600 W; cool gas flow rate 15 L min−1; auxiliary gas flow rate 1 L min−1; carrier gas flow rate 0.8 L min−1; peak jump scanning mode; time-resolved analysis mode; integration time 240 s/ sample; dwell time 10 ms for Li, 20 ms for V, Cr, Rb, Sr, Ba, and Pb and 40 ms for other elements; sweep time 1.6 s; detector mode, analog for Li and P/A mode for other elements. The isotopes measured were: 6Li, 51V, 52Cr, 59Co, 61Ni, 63Cu, 66Zn, 75As, 85Rb, 88Sr, 89Y, 90Zr, 93Nb, 95Mo, 111Cd, 118Sn, 121Sb, 133Cs, 137Ba, 139La, 140Ce, 141Pr, 146Nd, 147Sm, 151Eu, 153Eu, 157Gd, 159Tb, 163Dy, 165Ho, 166Er, 169Tm, 172Yb, 175Lu, 178Hf, 181Ta, 182W, 204Pb, 205Tl, 206Pb, 207Pb, 208Pb, 209Bi, 232Th, 238U .

2.2.2.2. Bioavailable fraction extraction and measurement

The bioavailable fraction was important to determine because the olive trees cannot extract all of the elements within the soil of growth since some elements are strongly bound to the soil structure (Marisa, Almeida & Vasconcelos, 2003).

Soil samples have been air-dried as in the total concentration analyses and passed through a 2 mm nylon-screen sieve before extraction. The 0.01 M $CaCl_2$ single extraction procedure has been chosen to analyze the bioavailable fraction of the elements in soil samples (Houba, Temminghoff, Gaikhorst & Vark, 2000; Bakircioglu, Bakircioglu-Kurtulus & Ibar, 2011) In fact, the 0.01M CaCl2 extracting solution has an ionic strength that is comparable to that of the soil solution especially that Ca^{2+} is its main cation. In addition, 0.01M CaCl2 is unbuffered and therefore, the necesssary reactions and interactions occur at the pH of the soil. The calcium cation from the $CaCl_2$, once in the soil suspension, provides the advantage of preventing the coagulation of the soil's colloidal material by enhancing the aggregates stability. Finally, the 0.01M CaCl2 procedure is used in the Dutch legislation for the

assessment of nutrients and heavy metals in soils (Amoakwah, Van Slycken, Tack & Essumang, 2013).

As what concerns the experimental protocol, briefly, a 1:10 soil: 0.01M $CaCl_2$ mixture was shaken for 2 hours using a mechanical shaker at room temperature, after that the mixture was centrifuged at 1800 g to separate the solid and liquid phases. Consequently, 15 ml of the supernatant was carefully collected, transferred to a PP tube and acidified with 0.71 ml of 20% HNO_3 (yielding 1% HNO_3 in the final sample solution volume which matrix-match the calibration solutions) to preserve the extract and prevent adsorption of the ions on the tubes surface or bacterial growth until the metals determination by ICP-MS analysis.

2.2.2.3. Chemical characterization of the soil samples

pH and electrical conductivity (EC)

The pH and EC were measured in the same suspension of 1:5 soil/distilled water ratio as described by Rayment & Higginson (2002). The mixture was shaken for 1 hour in a mechanical shaker at room temperature. The suspension was allowed to rest for 1 hour before measuring its pH. The EC was measured after 24 hours in the same suspension (Wali et al., 2015).

Organic matter content (OM) and inorganic carbon content ($CaCO_3$)

The OM and CaCO3 were determined based on the loss on ignition (LOI) procedure. 1 g of soil was added to ceramic crucibles, placed in muffle furnace (Yamato FP410, Japan) at 500°C for 4 hours, cooled to room temperature in desiccators and weighed. LOI at 500°C was measured as the difference between dry soil at 105°C mass and the soil mass after calcinations divided by the mass at 105°C. LOI at 1050°C (2 hours hold) was measured as the difference between combusted soil mass at 500°C and the soil mass at 1050°C divided by the soil mass at 500°C (Heiri, Lotter & Lemcke, 2001; Wright, Wang & Reddy, 2008).

2.3. Data analysis

All statistical analyses were performed using IBM SPSS Statistics software for Windows, version 24.0 (IBM Corp., Armonk, N.Y., U.S.A.)

3. Results and discussion

3.1. Soils charecteristics: A geochemical characterization of the olive terroirs.

3.1.1. Influence of soil depth on the elemental concentration

The first investigation of the soil result concerned the influence of the sampling depth on the elemental content. The normality of the data was tested using the Shapiro-Wilk test and we concluded that the data comes from a non-normal distribution. Therefore, we analyzed the variance based on the non-parametric test. The non-parametric Kruskall-Wallis test revealed that all the elements did not display significant differences ($p < 0.05$) in their concentrations at the two soil depths (Table 3). Taking into account that most of the sampled soils are not tilled and that tillage contributes to mixing of the different soil layers, this result indicates the absence of contamination in surface soil layer usually enriched with heavy elements resulting from chemicals application to the agricultural soil surfaces. Indeed, many national governmental reports and authors have reported that Tunisia has the largest organic agricultural surface area in the world (Willer et al., 2011).Therefore, only the concentration values of each element in 0-30 cm layer was used for the remaining statistical analyses because in some areas we were only able to sample the 0-30 cm layer. The absence of contamination can further be confirmed by comparisons with literature data on the background levels of elements in soils.

3.1.2. The influence of origin on the elemental concentration

The origin herein denotes the administrative subdivision of the Tunisian territory. The non-parametric Kruskall-Wallis test indicated that all elements displayed significant differences between at least two pairs of origin (Table 21) except for Cd, K and Tl ($p<0.05$) and Cd, K, Tl and Ti ($p<0.01$).
To simplify the interpretation of the data, the most famous data reduction method: the principal component analysis (PCA) with Varimax rotation was applied to the dataset composed of 51 variables X 76 cases (case corresponds to sample and variable corresponds to inorganic element in our case). The first PCA conducted used the eigenvalue > 1-cut-off criteria for the selection of the number of generated components. This PCA generated 7

principal components (PC) as a substitute of the original 51 variables and all the 7 PCs explained 88.8% of the original variance. Examination of the associated scree plot and the

Figure 10. Loading plot of the original 51 variable on PC1 and PC2.

table of the total variance explained suggested that only the first 2 PCs are sufficient to explain the largest part of the original variance (52.2% and 12.4% for PC1 and PC2 respectively). Therefore, the analysis was conducted a second time with a forced selection of 2 PCs. 73.7% was the cumulative variance explained by the 2 extracted PCs which means that nearly three quarters of the information in the dataset (initially 51 variables) was encapsulated by just two PCs. Examination of the variables loading plots on these two PCs (Fig. 10) reveals that:

- PC1 was dominated by rare earth elements (RRE), some heavy elements like Th, U, Nb, Ta, W, Rb, Ti, Pb, Sn, V, Cr, Al, Fe, Mn, Zn, and Co and also LOI at 500°C which represents the organic matter content of the soils.

- Ca, LOI at 1050°C which represents the inorganic carbon content of the soils (mostly as $CaCO_3$) and Sr loaded positively high on PC2 and Si loaded negatively high.

- On the plot, it is clear that Zr and Hf formed a separate group together.

- On the other hand, P plotted next to Ba, Mg, Cu, Sb, As, Bi, Mo and Ni.

- Cd and Tl formed another group together.

These associations indicate some geochemical processes, some of them are clear like the associations displayed on PC2 which show the phenomenon of the Sr enrichment by substitutions with major cations (Ca and Mg) in the carbonate minerals. Enrichment of Sr up to concentrations of ca. 1000 mg kg^{-1} is common in limestone and evaporates, although the Sr:Ca ratio in most types of limestone is less than 1:1000. (Kulp et al. 1952). Strontium is strongly associated with calcium and is indicative of calcareous rocks, especially in association with Ca, Mg and Ba http://weppi.gtk.fi/publ/foregsatlas/text/Sr.pdf.

In fact, Sr is alkaline lithophile element just as Ca which are characterized by similar hydrated ionic radius (0.272 nm and 0.274 nm for Ca and Sr respectively) and ionic charge. In addition to that Ca/Sr ratio is used as a tracer in geochemistry, hydrogeochemistry, and bioavailability studies to identify the sources of Ca in plants alongside 87Sr/86Sr ratio which is an excellent source tracer because of the absence of fractionation of these isotopes during biogeochemical processes (Pett-Ridge, Derry & Barrows, 2009; Maria V. Baroni et al., 2015).
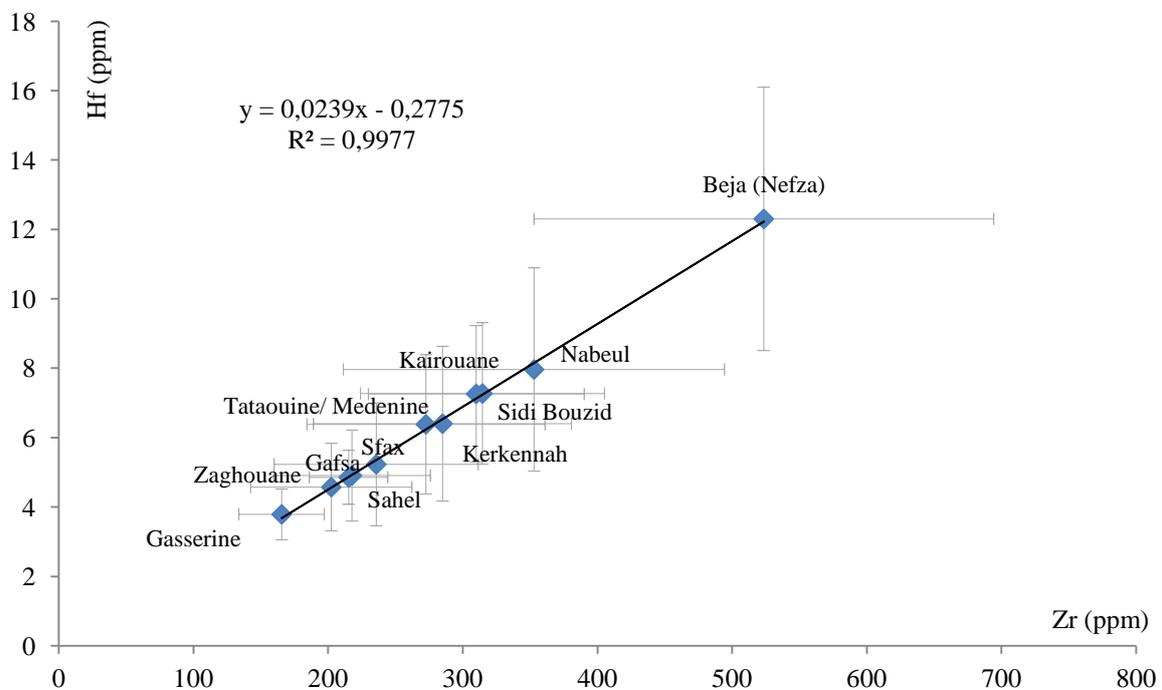

Figure 11. Bivariate plot of the abundances of Hf vs Zr in soil samples from all the 11 Tunisian regions. Values are in ppm and the median values as well as the related standard deviations are shown herein.

The PC1 also reveals the enrichment of rare earth elements in organic matter and clay rich soils: argilo-humic complexes (Al, Fe and LOI at 500°C) that may indicate the high adsorption capacity of cations on the negatively charged sites on the surface of the soil particles.

Zr and Hf are from the same group on the periodic table and thus present similar properties (valence, of 4, atomic radii size around 0.72) as evidenced by their linear relationship (R^2= 0.9977) (Fig. 11) confirmed through a pearson bivariate correlation (cc= 0.993, p<0.001). As previously mentioned by Chandrajith, Dissanayake & Tobschall (2005), Hf occurs mainly in zircons minerals where it substitutes Zr and they display strong linear relationship.

Next, the position of each sample in relation to PC1 and PC2 was graphically represented by a score plot (Fig. 12). The positions of the samples originating from Zaghouane (F12 to F18) can be superposed to those of Ca, LOI1050 and Sr on PC2. Especially, the soil samples coming from site F12 had the highest scores on PC2. Examination of the concentrations of Ca and Sr and also CaCO3 content in these samples shows that they had the highest concentrations of these parameters. However, F14 and F18 also coming from Zaghouane (Table 1) had low scores on PC2 probably because they had lower concentrations of Ca. Although some of the samples from each group are spread out but in general, the samples from each group show a tendency to populate a particular quadrant of the plot. As for PC1, samples from sampling sites F37, F14 and F36 had the highest positive scores on it. F36 and F37 originate from Beja (Nefza) and F14 originates from Zaghouane. As we previously mentioned, PC1 is dominated by REEs and heavy elements such as Th, U, Nb, Ta, W, Rb, Ti, Pb, Sn, V, Cr, Al, Fe, Mn, Zn, and Co and also LOI at 500°C. Examination of the medians of these elements across all the 11 origins, shows that Beja (Nefza) and Zaghouane displayed the highest concentrations of all the RRE, Th, U, Al and Fe etc... The differences were especially more pronounced for the light rare earth elements (LREE) (Fig. 12) compared to the heavy RRE (HREE); more specifically Ce, La and Nd were the most abundant RREs in all the 11 regions.

As can be seen in figures 14 and 15, La, Ce and Nd also show strong linear relationships (R^2=0.9855 and 0.9973 for La vs Ce and La vs Nd respectively) attesting to their similar properties across all ranges of soils of the 11 origins (Fig. 14; Fig. 15). These figures also confirm the highest abundances of these RRE in Beja followed by Zaghouane compared to lowest abundances in soil especially those coming from Tataouine/ Medenine and Sfax.

Examination of the bar graph (Fig. 11) of the main major elements (Si, Ca, Fe and Al) in all the 11 regions shows 2 main features:
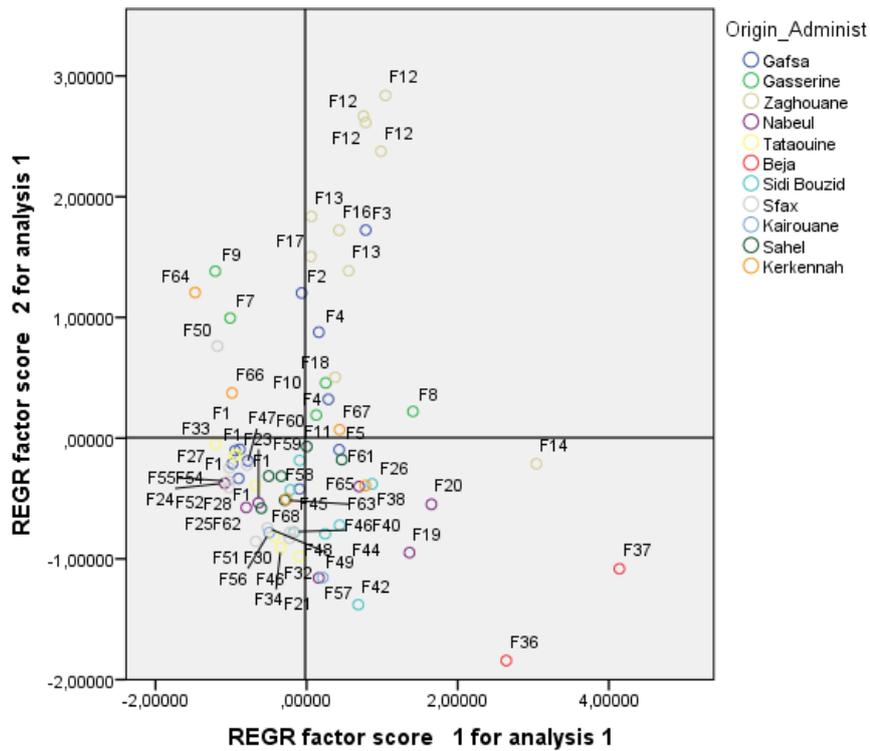

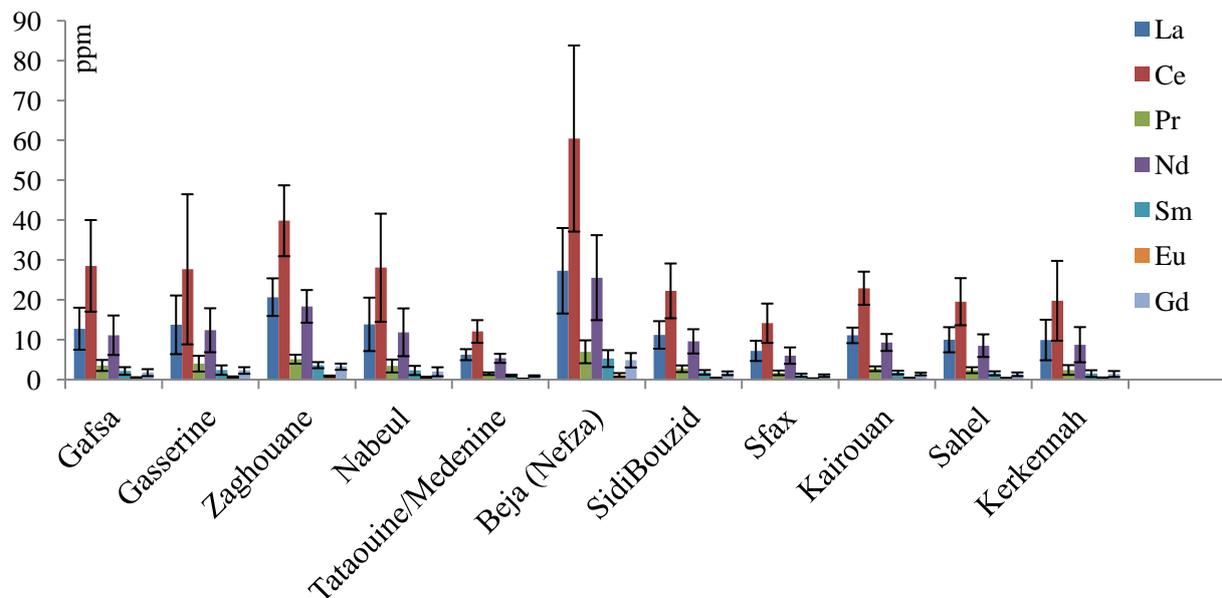**Figure 12.** Score plot of the samples on PC1 and PC2.

Figure 13. The distribution of light rare earth elements (LRRE) concentrations (in ppm) in the 11 sampled Tunisian origins. Values are in ppm and the median values as well as the related standard deviations are shown herein.

(i)   Zaghouane had the lowest concentrations of Si and the highest concentrations of Ca.

(ii)   Al and Fe had the highest concentrations in soils of Beja (Nefza).

(iii)   The highest Si concentrations are found in Kairouan, Sfax, Sidi Bouzid and Nabeul respectively.

As previously stated by Jiyan & Ruidong (2010), the enrichment of RREs in soils of Beja (Nefza) can be explained by the following reasons: the higher the degree of soil development the higher the leaching loss of Ca, Mg, K and Na except for Al, Fe and REEs because of their lower mobility. Indeed, Nefza soils show the lowest concentrations of Ca and the highest concentrations of Al, Fe and RREs which also points out the degree of soil development in that region that can be due to the humid climate of that region that has one of the highest precipitation volumes in Tunisia .
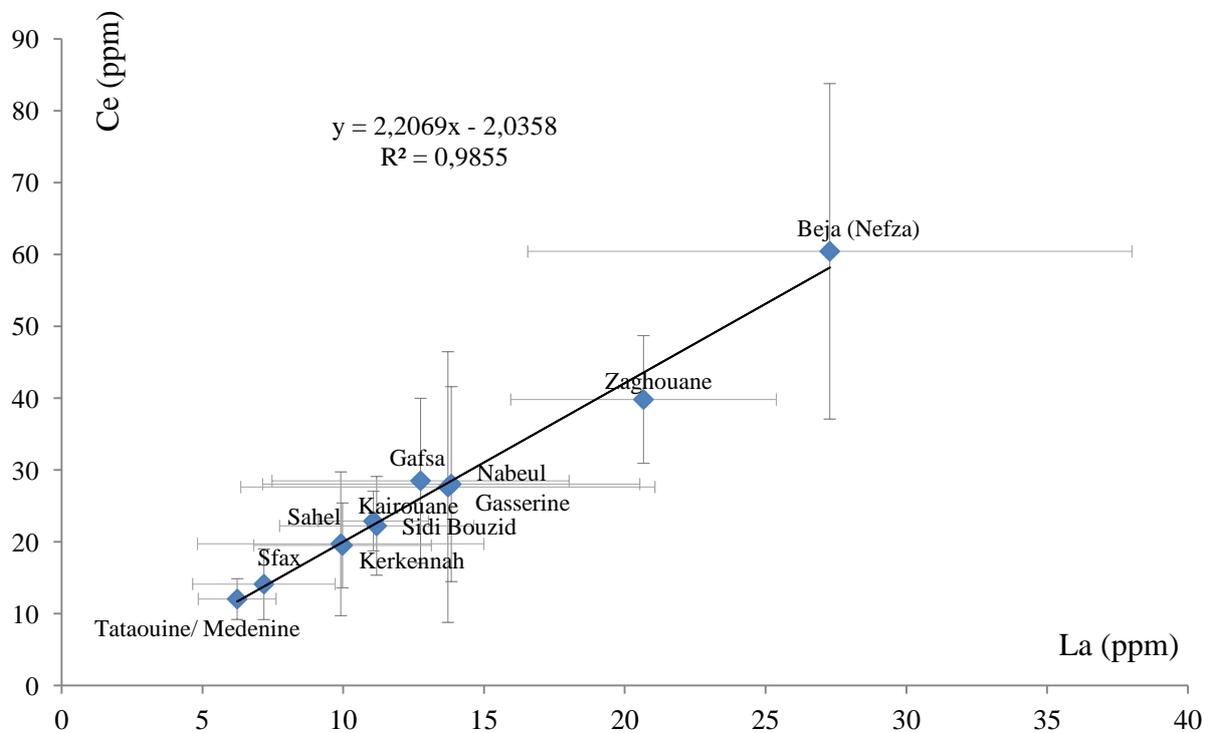

Figure 14. Bivariate plot of abundances of La vs Ce in soil samples from the 11 origins. Values are in ppm and the median values as well as the related standard deviations are shown herein.
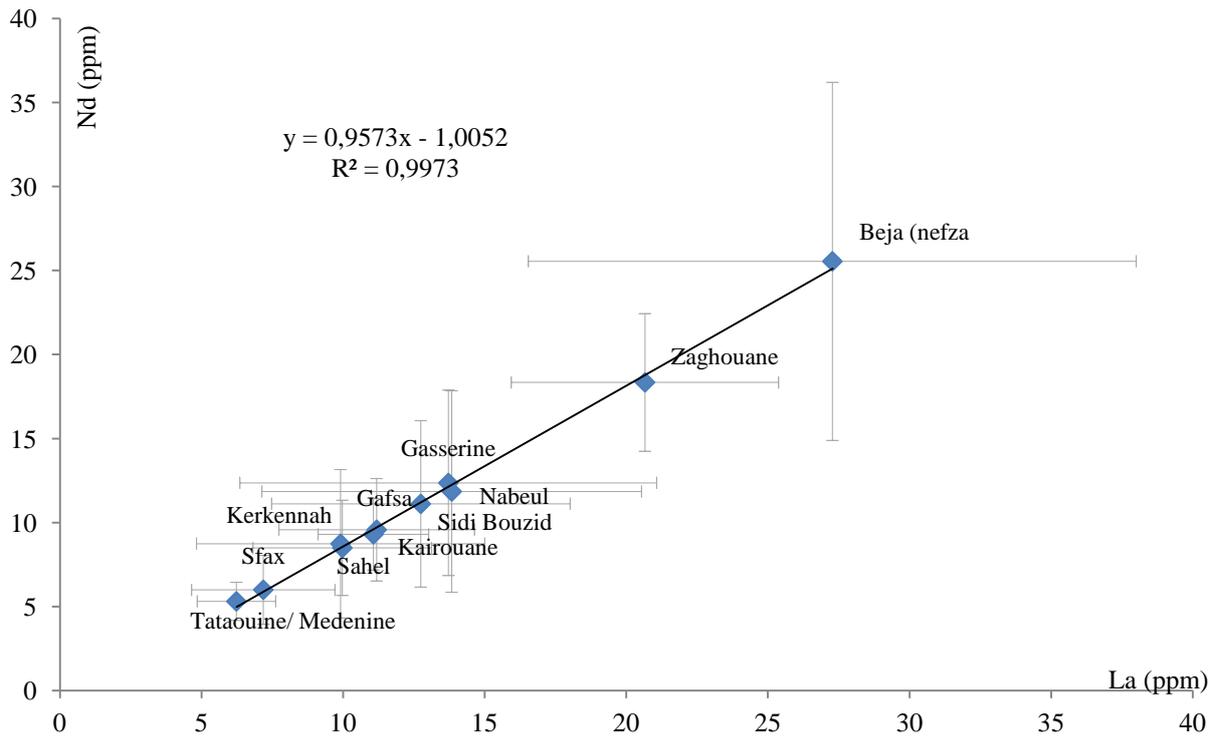
Figure 15. Bivariate plot of abundances of La vs Nd in soil samples from the 11 origins. Values are in ppm and the median values as well as the related standard deviations are shown herein.
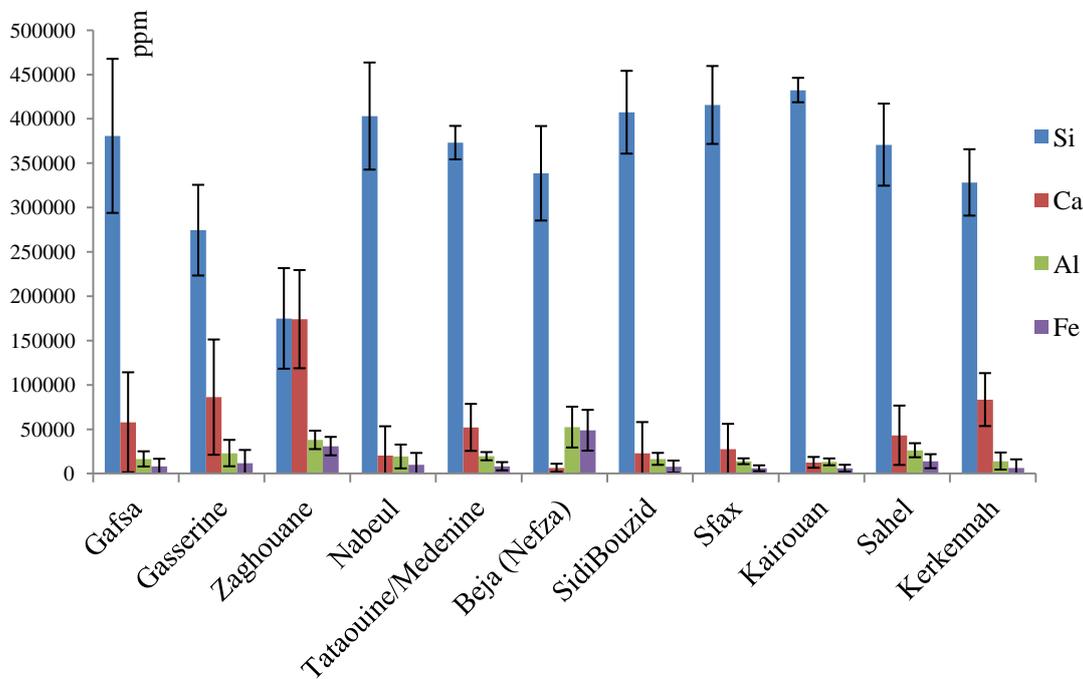

Figure 16. The distribution of main major elements concentrations (in ppm) in the 11 sampled Tunisian origins. Values are in ppm and the median values as well as the related standard deviations are shown herein.

On the other hand, this particularly high abundance of RREs in Nefza can also be related to their highest OM content (Fig. 14). In fact, Pearson correlation analysis conducted between the LOI at 500°C which represents the OM content of the soils and their RREs concentrations revealed significant positive correlations (cc from 0.771 to 0.879, p< 0.001). This finding agrees with that reported by De Sá Paye, De Mello, De Magalhães MascarenhasT& Gasparon (2016) who found significant positive correlations of REEs in Brazilian soils with soil's OM.

As for Zaghouane, the enrichment of REEs encountered particularly in F14 is probably due to the alkaline barrier located at the soil horizon near the bedrock that causes REE cations to precipitate and enrich. Indeed, this alkaline barrier can be proved by the fact that the position of the F14 sampling sites from Zaghouane on the geological map (Table 21) revealed that it is located above lower Cretaceous Marl/ alternating marl-limestone and sandstone formation. Moreover, the position of F14 on the pedological map revealed that it is located above brown carbonated calcimorphic soil. The $CaCO_3$ determined in the laboratory by loss on ignition (LOI) method at 1050°C shows that the highest $CaCO_3$ contents are found in soils of Zaghouane region (Fig. 18).

On the other hand, a soil profile conducted at F18 of Zaghouane (Fig. 17) showed that three horizons can be distinguished: A horizon, Bk1 horizon and Bk2 horizon. The soil carbonate in-situ reaction with 10% HCl showed that the upper two horizons (till 30 cm depth) are strongly calcareous horizons (10%>$CaCO_3$>25%) with a soft to hard consistency and sub-angular blocky to massive structure. The lower horizon (from 30 to 65cm) is extremely calcareous ($CaCO_3$>25%) with a very hard consistency and massive structure. The soil texture revealed by the soil profile in all three horizons belongs to the heavy clay (H.C.) textural class. In addition, carbonates in soil are either residues of the parent material or secondary carbonates (the result of neo-formation).
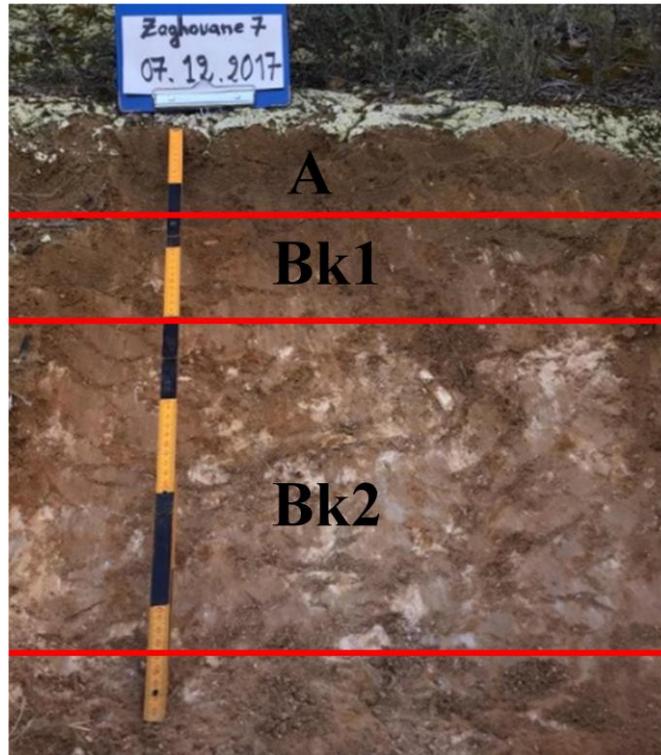
Figure 17. Soil profile conducted at F18 in zaghouane showing the three horizons: A, from 0 to 15 cm; Bk1 from 15 to 30 cm and Bk2 from 30 to 65 cm.
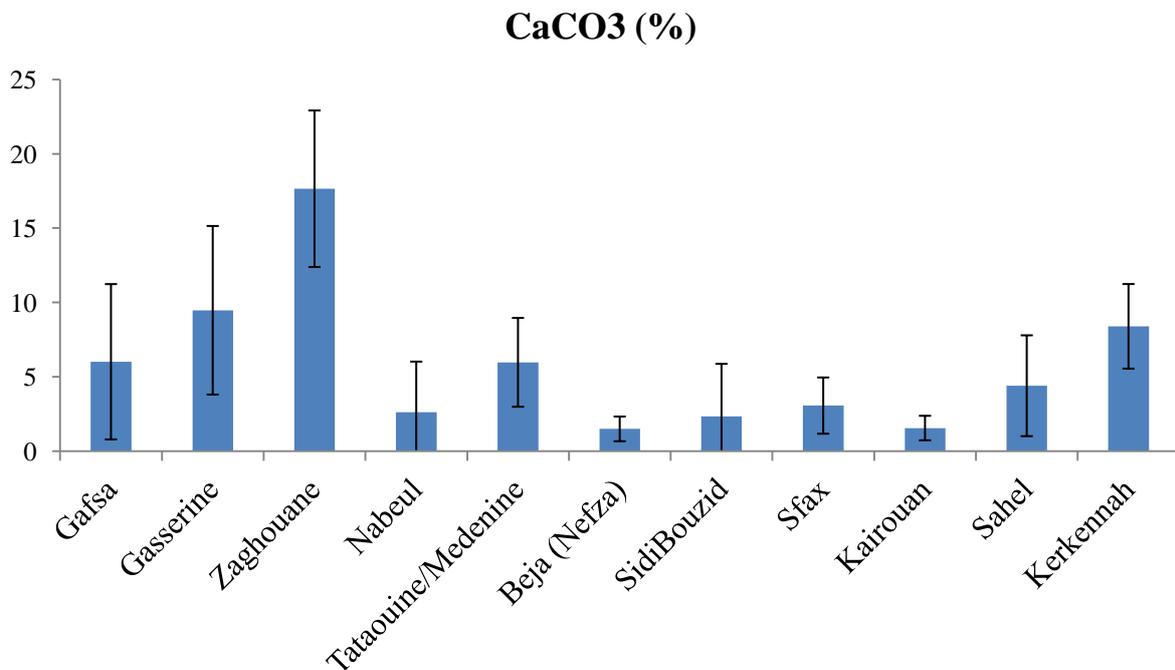

Figure 18. The inorganic carbon content (CaCO3) in soil samples of the 11 regions. Values are in % and the median values as well as the related standard deviations are shown herein.
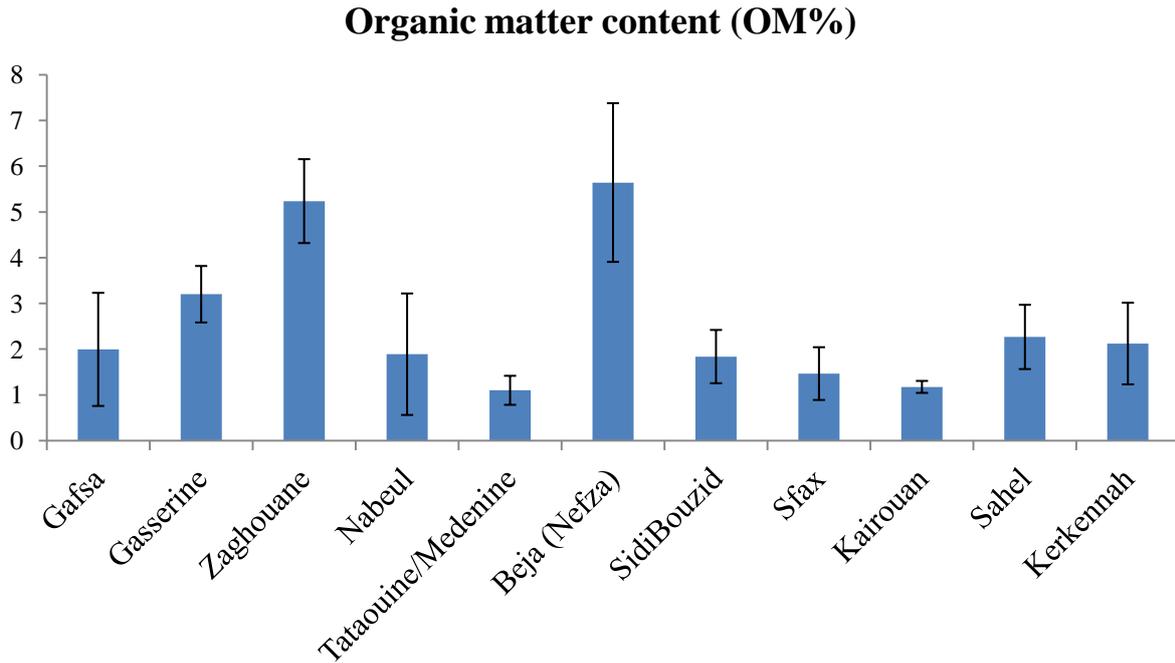
Organic matter content (OM%)

Figure 19. The organic matter content(OM%) in soil samples of the 11 regions. Values are in % and the median values as well as the related standard deviations are shown herein.

Coming back to the rest of the origins soil samples scores on PC1 and PC2, we noticed that the samples that dominate both positive quadrants of PC2 are those from Zaghouane, Kerkennah, Gasserine, Gafsa and only F50 from Sfax. Looking back at their geological setting, Ca, Sr or $CaCO_3$ content, these sites present high values of at least one of the previously mentioned variables or a limestone lithology. The negative quadrants of PC2 are mainly dominated by samples from Nabeul, Sfax, Kairouane, Beja (Nefza) and Tataouine/ Medenine. These findings further confirm the role of PC2 in separating samples based on their carbonate content and Sr enrichment phenomenon. As for the positive quadrants of PC1, as we previously mentioned they are dominated by samples from Beja (neza) and zaghouane presenting high levels of REEs and OM content. As for the negative quadrants of PC1, the samples are much spread and interpretation of their distribution is a complex task at this level. However, we can draw some interpretations as what concerns some samples. For example, in Gasserine F8, F10 and F11; in Nabeul F19 to F22 and F26 showed the highest concentrations of some RREs and this also confirms that PC1 is responsible for elucidating patterns of REEs levels.

3.2. Soils classification

3.2.1 Classification based on the administrative subdivision

Stepwise LDA (SLDA) was applied to 51 variables and 76 cases (soil samples from 0-30 cm) grouped into 11 classes corresponding to the 11 governorates. SLDA extracted 9 variables from which the first two canonical functions explained 88,4% of the total variance contained in the original dataset. Out of the 51 variables, the SLDA selected 21 variables: Co, Mg, Si, Sr, K, Ti, Rb, U, Nd, Pb, Mn, Ca, Ta, W, Hf, Er, Ni, Tb, Al, Zn and La as most discriminatory elements. The distribution of soil samples on the scatterplot of discriminant function 1 (DF1) vs discriminant function 2 (DF2) is shown on Fig. 20. SLDA resulted in 92.8% of original samples correctly classified and 71% correctly validated to their governorate of origin. The classification results table can be seen in table 22 (Table 22). As can be seen in the table, most of misclassifications come from Gafsa samples being wrongly assigned to Sfax, Gasserine and Sahel. Sidi Bouzid samples are misclassified as coming from Sfax and kerkennah, Sfax samples have been misclassified as coming from Gafsa, Sidi Bouzid and Kerkennah, Sahel samples are misclassified as coming from Sfax and finally Kerkennah samples are misclassified as coming from Sidi Bouzid.

These misclassifications are certainly due to similar elemental composition found in samples from different governorates. We assume that the parent material plays a significant role in determining the soils chemical composition, so we believe that a subdivision of samples into geological classes instead of administrative classes (governorates) would result in a better classifications and predictions. However, since the sampling did not include representative cases from each geological class, and because soil characteristics (pH, EC, OM, CEC etc…), morphology, climate, etc… can also play a significant role in determining the elemental profile of soils, soils coming from adjacent geographical locations will be merged into a single class to reduce misclassifications.

Different scenarios of grouping have been tested merging each time more samples into a
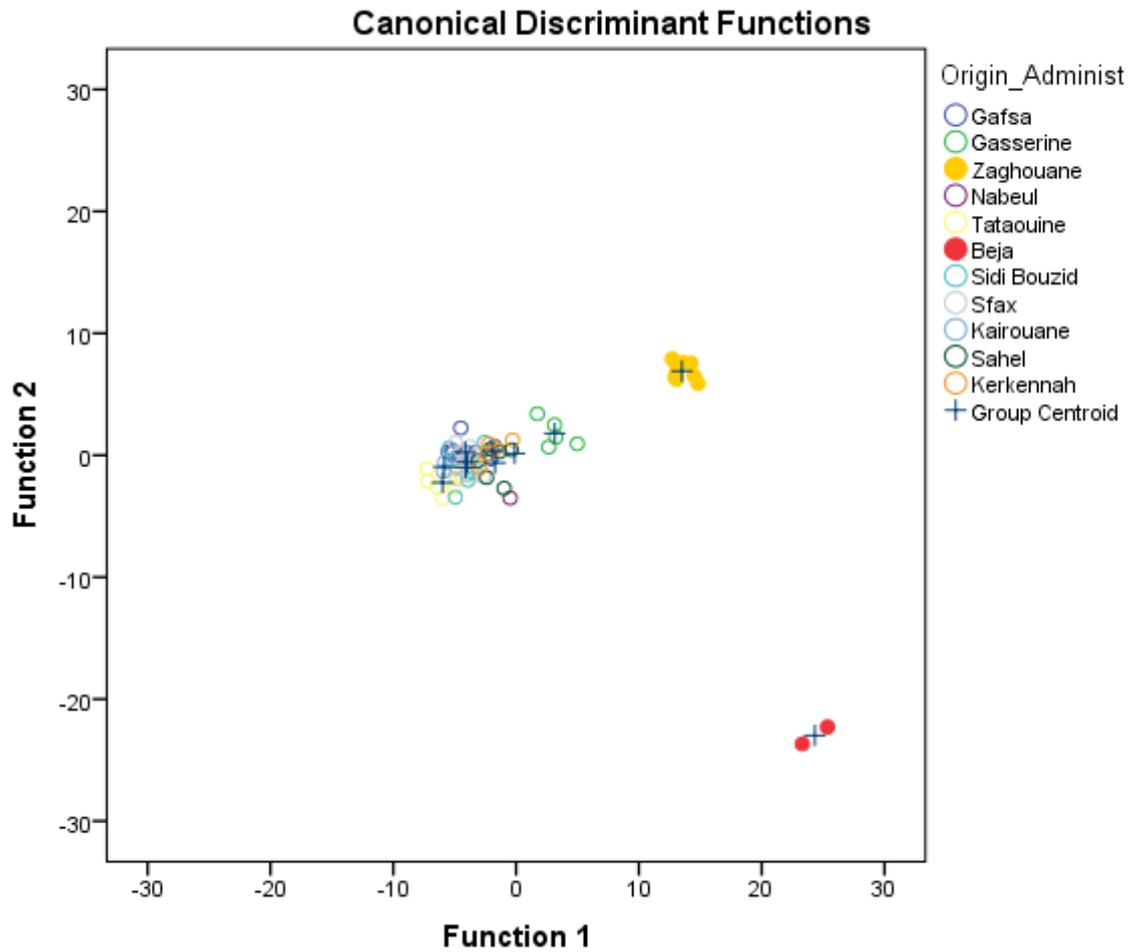
Figure 20. SLDA analysis of elements concentrations in soil samples (0-30 cm): Scatterplot of the first two canonical functions using 11 groups corresponding to administrative subdivisions of sampling sites as input.

Table 22. Classification results of soils samples (0-30 cm) from 11 Tunisian governorates based on cross validation method.

Origin_Administ		Gafsa	Gasserine	Zaghouane	Tataouine	Beja	Sidi Bouzid	Sfax	Kairouane	Sahel	Kerkennah	Total
Count	Gafsa	5	1					4		1		11
	Gasserine	1	4									5
	Zaghouane			10								10
	Tataouine				6			1				7
	Beja					2						2
	Sidi Bouzid						6	1			1	8
	Sfax	2					2	7			1	12
	Kairouane								2			2
	Sahel							2		4		6
	Kerkennah						2				3	5
%	Gafsa	45,5	9,1					36,4		9,1		100
	Gasserine	20,0	80									100
	Zaghouane			100								100
	Tataouine				85,7			14,3				100
	Beja					100						100
	Sidi Bouzid						75	12,5			12,5	100
	Sfax	16,7					16,7	58,3			8,3	100
	Kairouane								100			100
	Sahel							33,3		66,7		100
	Kerkennah						40				60	100

Single group in view of obtaining a model with the highest possible prediction accuracy (Table 23).

Table 23. Scenarios of soil samples grouping into new classes.

Governorate	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Scenario 5	Scenario 6
Gafsa	1	1	1	1	1	1
Gasserine	2	2	2	1	1	1
Zaghouane	3	3	3	2	2	2
Nabeul	4	4	4	3	3	3
Tataouine/Medenine	5	5	5	4	4	4
Beja (Nefza)	6	6	6	5	5	5
Sidi Bouzid	7	7	7	6	6	1
Kairouane	8	7	7	6	6	1
Sfax	9	8	7	6	6	1
Sahel	10	9	8	7	6	1
Kerkennah	11	10	9	8	6	1
Classification (%)	92.8%	93.4%	93.4%	97.4%	98.7%	98.7%
Prediction (%)	71%	80.3%	76.3%	84.2%	85.5%	93.4%

Governorates with the same number in the same column indicate they were assigned to the same group in the SLDA.

This table shows that the highest prediction percentage (93.4%) and thus the lowest error rate were obtained when all the central Tunisian regions (Gafsa, Gasserine, Sidi Bouzid, Kairouane, Sfax, Sahel and Kerkennah) were merged into a single class. Table 24 details the classification results obtained with the 6th scenario.

Table 24 shows that 2 out of 49 of central Tunisian regions samples were misclassified as coming from Tataouine. 2 out of 7 samples from Tataouine samples still get misclassified as

coming from central Tunisia. 1 out of 8 samples from Nabeul was mis-assigned to Tataouine. Zaghouane and Beja samples were predicted with 100% accuracy as in the first scenario. The SLDA selected Co, Sb, Zn, Ca, K, Ti, Cs, Pb, U, La, Sm, Cr, Sn, Sr, W, Y, Ho, Er, Ta, Rb, Fe, Si, Lu, Tl and Zr as most discriminating elements between these 5 classes.
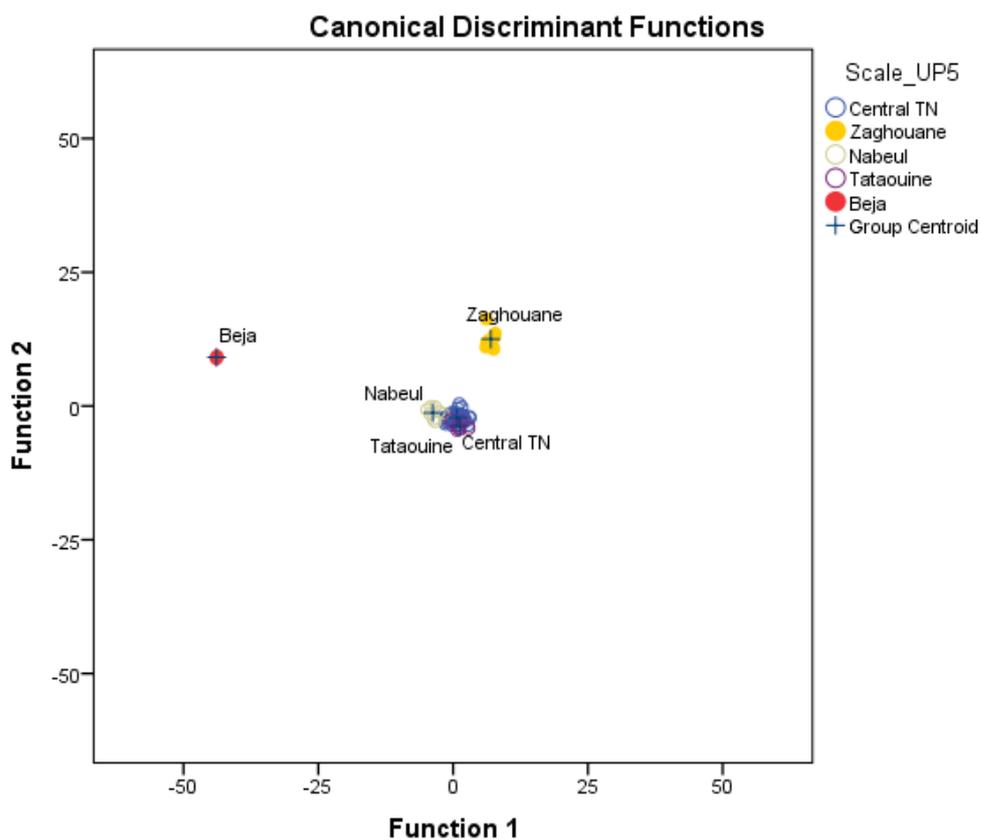

Figure 21. SLDA analysis of elements concentrations in soil samples (0-30 cm): Scatterplot of the first two canonical functions using 5 groups as input.

Table 24. Classification results of soils samples (0-30 cm) from 5 Tunisian geographical classes based on cross validation method.

		Central TN	Zaghouane	Nabeul	Tataouine	Beja	Total
Count	Central TN*	47			2		49
	Zaghouane		10				10
	Nabeul			7	1		8
	Tataouine	2			5		7
	Beja					2	2
%	Central TN	95,9			4,1		100
	Zaghouane		100				100
	Nabeul			87,5	12,5		100
	Tataouine	28,6			71,4		100
	Beja					100	100

TN*, Tunisia; Central TN includes: Gafsa, Gasserine, Sidi Bouzid, Kairouane, Sfax, Sahel and Kerkennah

3.3. Olive oil elemental characterization

Figures 22 and 23 show the median values and standard deviations of elements data for olive oil samples from the 11 studied governorates. In all the analyzed 105 samples (including Italian oils that will be used later for an inter-country discrimination study) V, Cr, Co, Ga ,Ag and Sb could not be detected in all of the analyzed samples. Li, Al and Zr were detected in 1 sample each only. Ge and Cs were detected in 2 samples each only. As, Se, Y, Cd, Sn and Sm were detected in less than 11 samples. Finally, Gd, Tb, Er, Tm, Yb and Pb were detected in less than 17 samples. Therefore these elements will be excluded from the statistical analyses. The elemental data of 105 olive oil samples was characterized by a non-normal distribution for all the elements based on Shapiro-Wilk test ($p<0.05$). Therefore, Kruskall-Wallis test was run to analyze the distribution of the remaining 22 elements across the different 11 origins. We observed some differences between the geographical origins. Out of 22 elements (B, Na, Mg, P, Ca, Ti, Mn, Fe, Ni, Cu, Zn, Rb, Sr, Mo, Ba, La, Ce, Pr, Eu, Dy, Ho, Lu), only 13 (Na, Mg, Ca, Ti, Fe, Cu, Zn, Rb, Sr, Mo, Ba, La and Ce) had a significant difference ($p< 0.05$)

between at least two pairs of the 11 origins. For the major elements, Mg, Na, P, Ca, Fe, Al, Ti and Mn had decreasing concentrations respectively. For trace elements, Li, Y, B, Ba, Rb, Sr, Zn, Mo, Cu and Ni had decreasing concentrations respectively. As for RREs, Eu, Lu, Pr, La, Ce, Er, Ho and Dy had decreasing concentrations respectively. The Sahel region had the highest concentrations of Mg and Fe. Na presented highest concentrations in Nabeul followed by Kerkennah and Tatouine. Ca had the highest concentrations in Tataouine followed by Sahel and the lowest values in Beja and Zaghouane.

Apart from the geographical origin assessment, the elemental composition of olive oils can give insights into environmental contamination or contamination during processing. The contents of selected heavy elements (Fe<3 mg L^{-1} Cu <0.1 mg L^{-1}, etc…) shows that the analyzed olive oils from representative 11 Tunisian olive producing governorates are far below the maximum allowable limit established by the international olive oil council (IOC). This finding agrees with our previous result on olive oils collected in December 2015 from four governorates (Sfax, Monastir, Medenine and Gafsa). This demonstrates the good quality of Tunisian olive oils regarding the level of potentially toxic heavy metals.

3.3.1. Principal component analysis

PCA was conducted to study the elements associations in olive oil. Such associations are expected to reveal geochemical processes.
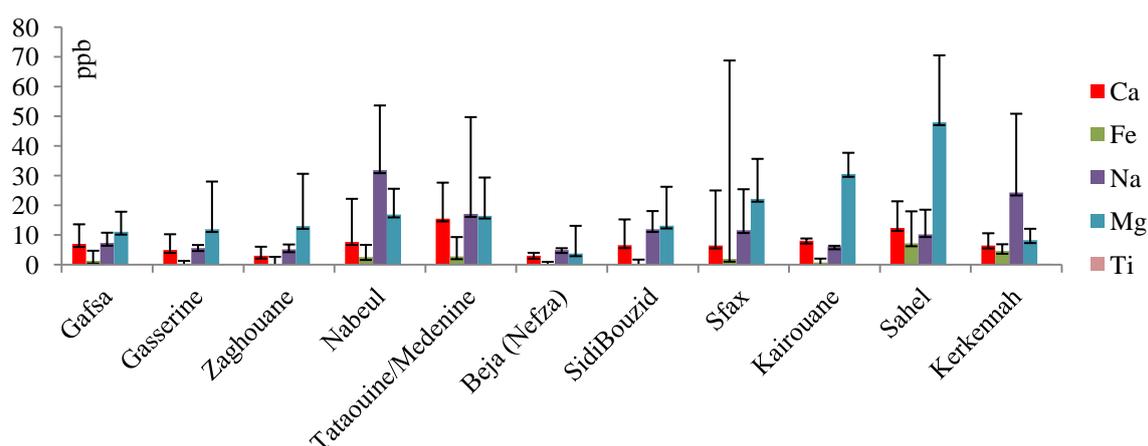

Figure 22. The distribution of main major elements concentrations (in ppb) in the 11 sampled Tunisian origins. Values are in ppb and the median values as well as the related standard deviations are shown herein.
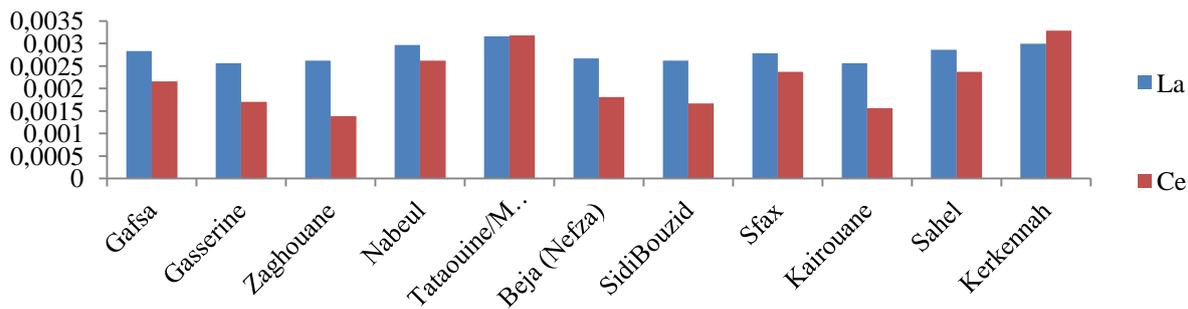
Figure 23. The distribution of some RREs concentrations (median in ppb) in the 11 sampled Tunisian origins.

Similarly to what was observed in the soil PCA analysis, PC1 was dominated by RREs whereas on PC2, lithophile elements such as Na, Rb, Mg, Ca, Sr, Ba had high loadings along other elements (P, Mn, Fe and Zn). These elements are characterized by similar properties and thus these associations reveal common prevailing geochemical origin of the elements in olive oils.
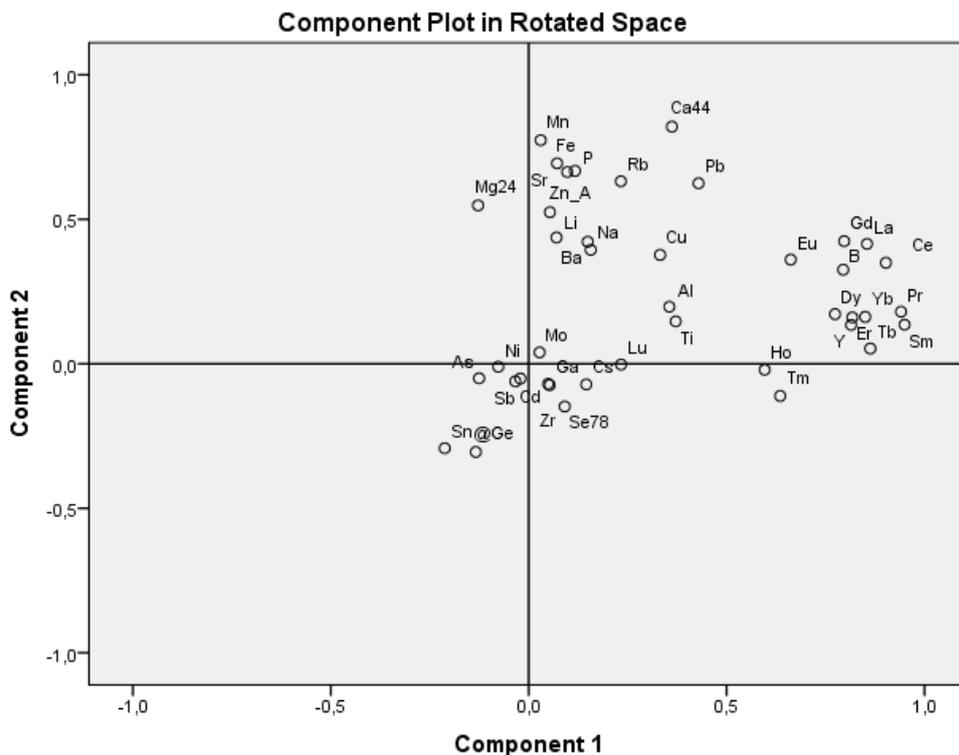

Figure 24. PCA of the elements contained in olive oil samples coming from 11 regions.

3.4. Classification of the olive oil samples according to their origin

The proposed study of simultaneously classifying olive oil samples from 11 different regions based on their elemental composition is expected to be a very difficult task taking into consideration the previous results obtained with soil samples. If 48 out of 51 variables showed a significant difference in soil samples across the 11 regions with very clear differences in elements concentrations especially in Zaghouane and Beja (Nefza), only 13 elements showed a significant difference between at least two pairs of origin in olive oil samples and differences in the elemental concentrations were less clear. Tunisian olive producing regions form a contiguous area especially in the central part of the country where most of the productions take place.

 A first attempt to classify all olive oil samples into the 11 governorates of origin was conducted using a SLDA with the least strict conditions (Wilk's Lambda with an F to enter = 1). The SLDA showed that 10 functions are fit automatically, but the Wilk's Lambda test of functions revealed that only the four first functions contributed significantly to the model and had an eigenvalue greater than 1. The first 4 functions account for 73.3% of the variance explained by the SLDA model, but the first two functions accounted for only 45.8% of the total variance. This is why the bivariate plot of F1 vs F2 (Fig. 25) might not fairly display the classification performance achieved by the model. The SLDA achieved 80.2% and only 40% of correct classifications for the original grouped samples and cross-validated samples, respectively.

In a similar approach to that done for soil classification, to increase classification success and avoid errors, samples from adjacent regions have been merged into a single group using the sixth scenario (Table 23). Therefore, 5 Groups have been used as input for the next SLDA analysis which correspond to Beja (Nefza), Zaghouane, Nabeul, Tataouine and all the rest of the regions (Sfax, Kerkennah, Sidi Bouzid, Kairouane, Gafsa and Gasserine) have been merged into a single class representing central Tunisia. This SLDA extracted 4 functions of which, only the first three had significant Wilk's Lambda value. Only the first two functions had eigenvalues greater than 1 and together they accounted for 69.3% of the total variance
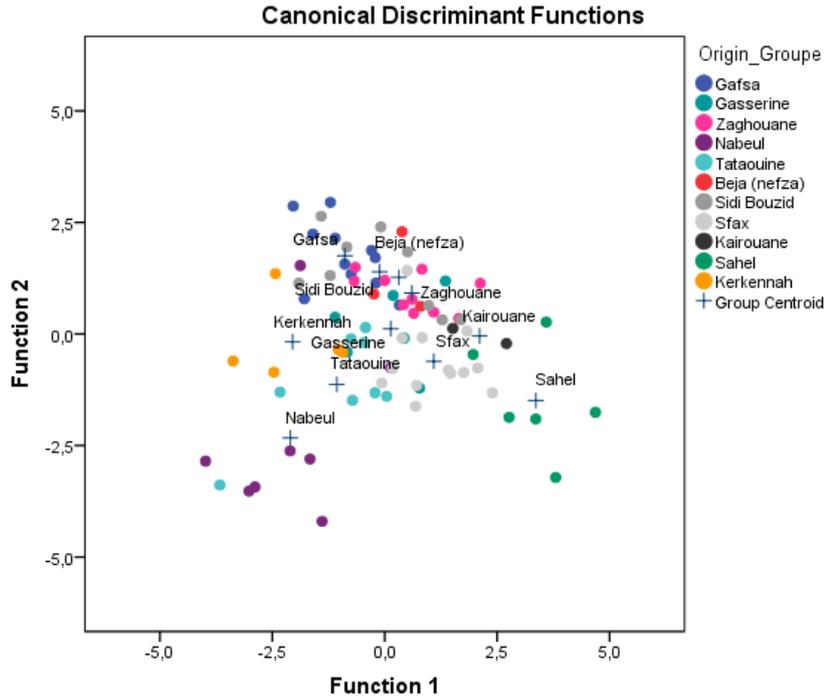
Figure 25. Scatterplot of the first two canonical discriminant functions of SLDA of olive oil samples using 11 groups as input.

explained by the model. The classification of original and predicted samples improved by decreasing the number of input groups into the model; they were 93.8% and 64.2% respectively. The improvement can also be seen in the scatterplot of the first two discriminant functions (Fig. 26). Table 25 shows that the model performed well in the case of predicting the true origin of samples from central Tunisia (78.8%) and Beja (66.7%); mediocrely for Nabeul (50%) and badly for Zaghouane (30%) and Tataouine (25%). Most of the misclassifications are due to samples being wrongly attributed to central Tunisia group. The most discriminating variables selected by the SLDA are Na, Dy, Sr, Rb, Mg, Ti, Cu, B, Ni, Ca, Zn, Lu, Ba, Ho and Eu.

Thus, 6 elements: Zn, Ca, Sr, Ho, Rb, Lu can establish a valid correspondence between the geochemical composition of the provenance soils and that of the paired olive oils of these 5 origins.

Table 25. Classification results of olive oil samples from 5 Tunisian origins based on cross validation method.

			Predicted Group Membership					
		Origin	Central TN	Zaghouane	Nabeul	Tataouine	Beja	Total
Cross-validated	Count	Central TN	41	5	1	2	3	52
		Zaghouane	7	3	0	0	0	10
		Nabeul	2	1	4	1	0	8
		Tataouine	3	2	1	2	0	8
		Beja	1	0	0	0	2	3
	%	Central TN	**78,8**	9,6	1,9	3,8	5,8	100
		Zaghouane	70	**30**	0	0	0	100
		Nabeul	25	12,5	**50**	12,5	0	100
		Tataouine	37,5	25	12,5	**25**	0	100
		Beja	33,3	0	0	0	**66,7**	100

3.5. Correlation study between olive oil and soil elemental composition and influence of climatic factors on olive oils' elemental profile

Multielemental-based geographical traceability technique is deeply rooted in the hypothesis that the mineral content of soils from two or more regions subject to discrimination is the driving factor that determines the elemental composition of olive oils. Therefore, we were interested in studying the correlation between the elemental profile of olive oil and that of soil as well as other environmental factors such as climatic parameters.

3.5.1. Correlation with the soil's total elemental concentrations

Since data was not characterized by a normal distribution, Spearman correlation coefficients have been calculated to quantitatively estimate the relationships between the elemental content of olive oils and the total elements concentrations of soils. The results show that no single element in olive oil samples was significantly correlated to that in soil. This finding

confirms our previous results of the pilot study where we concluded that the total content of the elements in soil samples cannot explain that of olive oils.
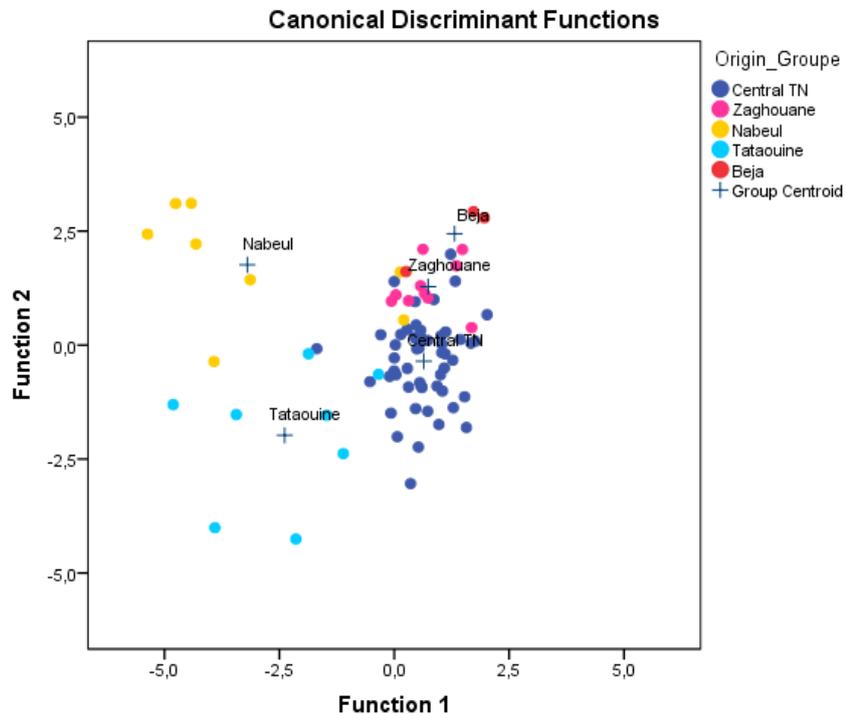

Figure 26. Scatterplot of the first two canonical discriminant functions of SLDA of olive oil samples using 11 groups as input.

3.5.2. Correlation with the soil's bioavailable fraction of the total content.

Spearman's correlation coefficients (table 25) (deciphering monotonic relations) calculated for elements in soil bioavailable fraction and olive oil elements revealed that Ti, Fe, Ni and Ba had significant positive correlations between both datasets (cc=0.221, p=0.04; cc=0.284, p=0.01; cc=0.261, p=0.02; and cc=0.240, p=0.031, respectively). The significant positive Spearman correlation was not found for the rest of the elements (B, Na, Mg, P, Ca, Mn, Cu, Zn, Rb, Sr, Mo, La, Ce, Pr, Eu, Dy, Ho and Lu). Since the relationship of Ti, Fe, Ni and Ba between olive oil and soil bioavailable elements is not linear, in addition to the fact that the elements are not normally distributed, we could not get significant Pearson (linear) correlation coefficients for the same element in oil and soil datasets. Therefore, higher contents in soil

samples do not imply higher contents in olive oils within the same region. The following figures demonstrate this statement.
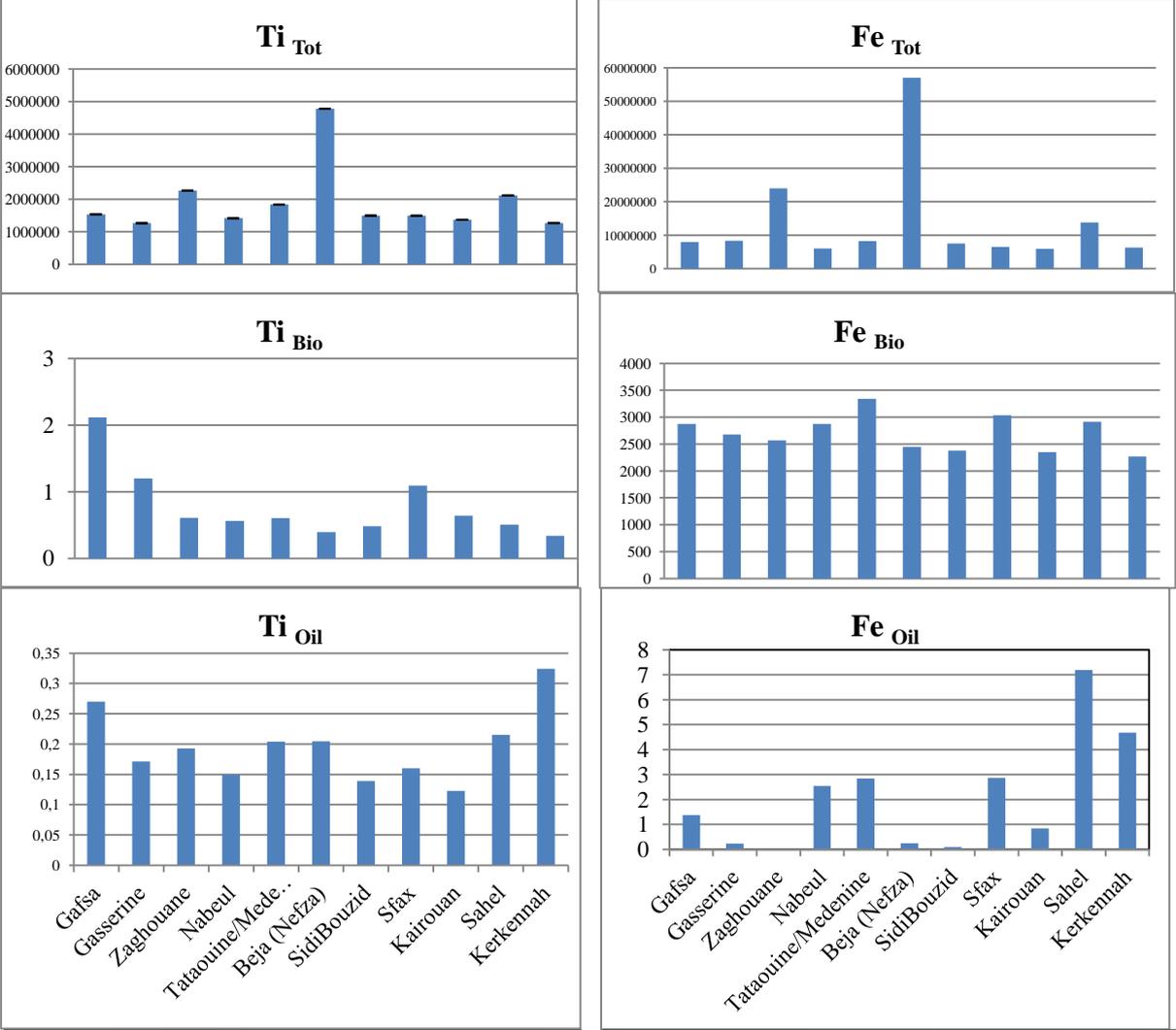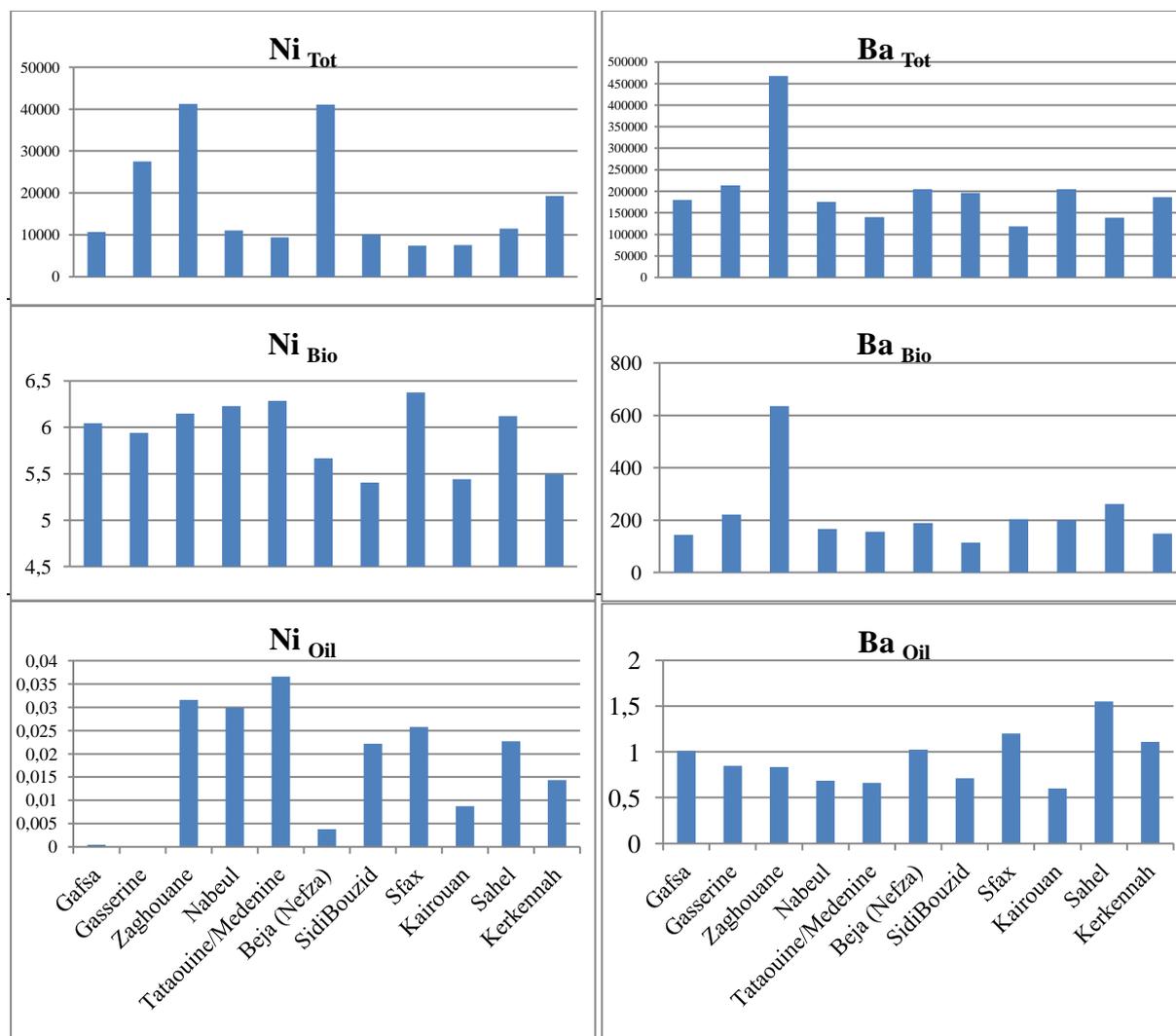
Fig. 27 (Continued)

Figure 27. Bar graphs of the medians of the four elements: Ti, Fe, Ni and Ba that displayed a significant positive Spearman correlation between their content in olive oil and soil bioavaiolable fraction across the 11 regions. Values are in $\mu g\ L^{-1}$. Tot, total contentration; Bio, bioavailable concentration; Oil, concentration in oil samples.

These figures show that the bioavailable concentrations are clearly better reflected in the olive oil profile than the total concentrations. The highest and most significant Spearman correlation coefficient was obtained for Fe. Fig. 27 shows that the trends in the concentrations of Fe across the 11 regions are similar between the bioavailable form and olive oil. The lowest concentrations in both datasets are encountered in Gasserine, Zaghouane, Beja, Sidi

Bouzid and Kairouane while the highest were observed in Tataouine (Medenine), Sahel, Sfax, Nabeul and Gafsa. Kerkennah is an exception because it had low Fe bioavilable concentrations in soil but high Fe concentrations in oil. The same applies to Ti for Kerkennah where low values in soil bioavailable fraction implied high concentration in olive oils. But overall, the monotony of the relationship is quiet obvious and clear. Considering the complexity of interactions between elements, Spearman correlation coefficients were further calculated for the matrix defined by the elements in olive oil (B, Na, Mg, P, Ca, Ti, Mn,Fe, Ni, Cu, Zn, Rb, Sr, Mo, Ba, La, Ce, Pr, Eu, Dy, Ho and Lu) and the bioavailable elements in soil samples (Na, Mg, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Mo, Ba, La,Ce, Pr, Nd, Eu, Dy, Lu, Ho and W). The results (table 25) have shown that monotonic (Spearman) relationships existed between olive oil and bioavaiable soil elements. All the elements in olive oil sample displayed significant Spearman correlations with at least one bioavailable soil element except for Ho. For example, contents of Mo in olive oils were significantly and positively influenced by the contents of available Ti, La, Ce, Pr, Nd, Dy, Ho and Lu. Contents of Zn in olive oil were significantly positively influenced by the contents of bioavailable Cr and Co and negatively influenced by the contents of bioavailable Na and W.

The results of Kruskall Wallis, LDA and Spearman correlation analyses point out some elements that can establish a valid correspondence between olive oil and their provenance soils and that are significantly correlated between olive oils and the bioavailable form in their paired soil. These elements are Zn, Rb, Sr and Lu. Therefore these elements have a proven usefulness in geographical traceability of Tunisian olive oils to their provenance soils.

Table 25. Spearman correlation coefficients of the elements in soil (bioavailable form) and olive oils.

Oil												Bioavailable elements in soil samples												
	Na	Mg	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Sr	Mo	Ba	La	Ce	Pr	Nd	Eu	Eu	Dy	Ho	Lu	W
B	-0,054	0,145	0,023	0,160	,221*	,221*	0,002	0,118	-0,055	0,146	0,155	0,120	0,036	0,158	-,251*	0,033	0,056	0,003	0,000	-,293**	-,295**	-0,067	-0,046	-0,072	0,007
Na	-0,043	-0,003	-0,074	0,120	0,028	,237*	0,140	,219*	0,089	0,088	-0,116	-0,162	-0,017	-0,170	-,353**	0,033	0,050	-0,003	-0,012	-,333**	-,361**	-0,071	-0,054	-0,098	0,143
Mg	0,029	0,196	,225*	0,148	0,068	0,031	0,104	-0,002	0,044	0,071	0,107	-0,027	0,096	0,070	0,163	0,115	0,115	0,090	0,094	0,157	0,164	0,136	0,123	0,093	-0,024
P	-0,146	-0,011	-0,048	0,154	0,177	,246*	0,140	0,185	0,006	-0,030	-0,168	-0,167	-0,161	-0,141	-,227*	0,104	0,119	0,075	0,063	-0,215	-,235*	-0,009	-0,001	-0,012	0,050
Ca	-0,128	-0,004	0,094	,257*	0,166	0,213	0,156	0,161	0,036	0,100	-0,112	-0,085	-0,102	-0,151	-0,139	0,135	0,161	0,111	0,108	-0,112	-0,149	0,101	0,092	0,075	0,032
Ti	0,000	0,068	0,054	,221*	0,101	0,008	0,181	-0,014	0,133	-0,015	0,081	0,166	0,141	0,092	0,069	0,158	0,174	0,140	0,159	0,075	0,051	0,139	0,146	0,177	-0,072
Mn	-,249*	0,014	0,110	0,045	,294**	,267*	0,004	,265*	0,111	0,170	-0,108	-0,099	-0,050	-0,181	0,109	0,008	-0,003	-0,021	-0,031	0,027	0,052	-0,051	-0,060	-0,048	-0,158
Fe	-,299**	-0,163	-0,026	0,026	,240*	,299**	0,043	,284*	0,059	0,070	-,301**	-0,095	-0,096	-,270*	0,148	-0,042	-0,024	-0,051	-0,059	-0,206	-0,189	-0,125	-0,118	-0,102	-0,128
Ni	0,061	0,155	,244*	0,106	-0,012	0,136	,312**	0,199	,344**	,261*	0,198	0,140	0,112	0,157	-0,087	-0,012	-0,025	-0,009	-0,008	-0,070	-0,078	0,047	0,039	0,053	-0,080
Cu	-,337**	-0,147	0,032	-0,127	-0,066	0,101	-0,024	0,118	-0,087	0,091	-0,154	-,332**	-0,165	-0,123	0,048	-0,177	-0,192	-0,173	-0,170	-0,023	0,029	-0,113	-0,139	-0,165	-0,134
Zn	-,225*	-0,082	0,145	-0,044	0,063	,249*	0,101	,281*	0,081	0,206	-0,152	-0,184	-0,150	-0,193	0,047	-0,114	-0,123	-0,111	-0,112	-0,017	0,015	-0,076	-0,088	-0,110	-,229*

Table 25. Continued

Rb	-0,100	0,118	0,018	0,097	0,092	,222*	0,128	0,187	-0,008	0,082	-0,149	-0,190	-0,171	-0,189	-,228*	0,133	0,126	0,119	0,099	-,224*	-,233*	0,036	0,043	-0,017	0,017
Sr	-0,065	0,023	-0,073	0,193	0,202	,219*	0,161	0,163	0,040	-0,032	-0,171	-0,117	0,029	-0,163	-,357**	0,064	0,104	0,024	0,008	-,337**	-,373**	-0,065	-0,051	-0,081	0,142
Mo	0,017	0,005	0,141	,278*	0,191	0,089	0,090	0,040	0,075	-0,124	-0,159	0,001	-0,002	-0,137	-0,139	,260*	,281*	,257*	,258*	-0,057	-0,103	,241*	,228*	,219*	0,015
Ba	-0,121	-0,025	0,052	-0,019	0,162	0,092	-0,121	0,044	-0,077	-0,039	-0,127	0,000	0,082	-0,103	,240*	0,027	0,010	0,023	0,043	0,188	0,210	0,031	0,036	0,051	-0,139
La	-,235*	-0,108	-0,012	0,103	0,035	,290**	0,120	,250*	0,154	0,168	-0,101	0,041	-0,122	-0,159	-0,159	0,037	0,041	0,020	0,042	-0,134	-0,165	0,023	0,022	0,029	-0,060
Ce	-,318**	-0,200	-0,050	0,015	0,203	,320**	-0,023	,245*	-0,075	0,020	-,291**	-,254*	0,218	,238*	-0,121	-0,069	-0,055	-0,081	-0,088	-0,200	-0,176	-0,129	-0,126	-0,102	-0,063
Pr	-0,060	0,026	-0,152	0,010	-0,013	0,196	0,146	0,132	0,118	0,067	-0,010	-0,039	-0,087	-0,022	,231*	-0,029	0,002	-0,041	-0,041	-0,217	-,246*	-0,083	-0,054	-0,041	0,012
Eu	-0,027	0,124	-0,046	-0,068	0,041	0,031	,249*	0,054	0,033	0,092	0,078	0,060	0,086	0,014	0,112	-0,011	-0,022	0,018	0,033	0,113	0,121	0,044	0,050	0,016	-0,087
Dy	-0,109	0,066	0,201	0,008	0,029	0,166	0,077	0,190	0,175	,277*	0,079	0,042	-0,059	0,063	0,104	0,008	-0,004	0,023	0,038	0,129	0,119	0,082	0,065	0,006	-0,062
Ho	0,092	0,127	0,149	0,142	-0,130	-0,103	0,089	-0,081	0,003	0,003	0,108	0,122	0,187	0,194	0,069	-0,067	-0,040	-0,052	-0,050	0,052	0,066	0,005	-0,014	-0,039	0,106
Lu	-,241*	-,353**	-,222*	-0,121	-0,107	-0,102	0,018	-0,002	-0,099	-0,080	-,244*	-,238*	-,276*	-0,122	-0,077	-0,095	-0,071	-0,082	-0,100	-0,055	-0,069	-0,103	-0,098	-0,079	0,026

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

3.6. Discrimination power between Tunisian and European olive oils based on the multielemental profile
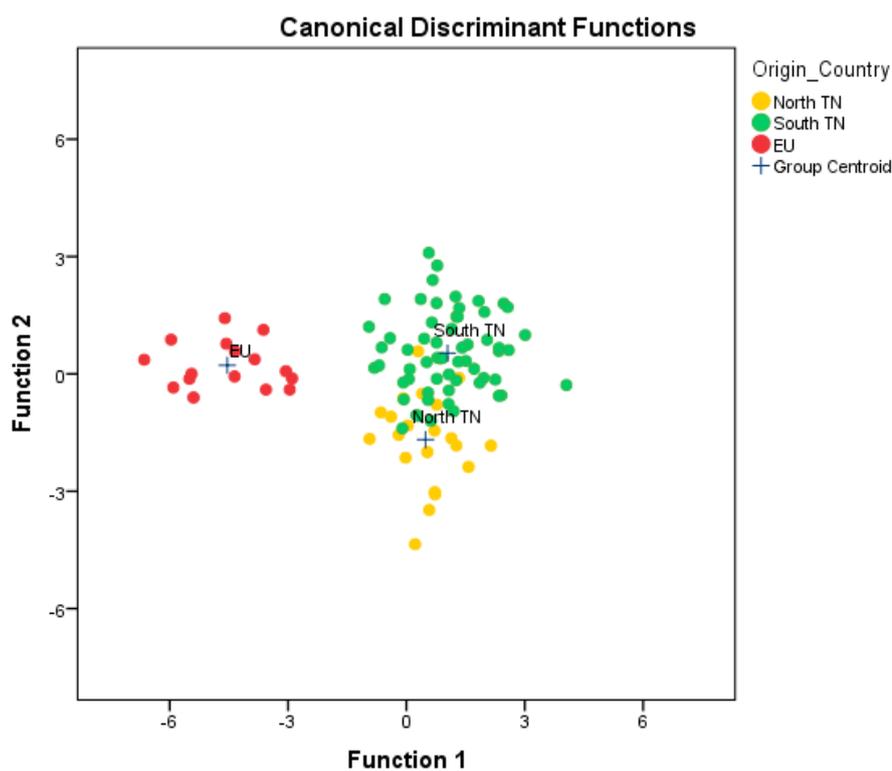

Figure 28. Scatterplot of the first two canonical discriminant functions of SLDA of olive oil samples from Tunisia (north and south regions) and Europe (15 from Italy and 1 from Greece).

SLDA was conducted to differentiate Tunisian from European olive oil most of them coming from Italy (15 samples) and Greece (1 sample). 2 functions have been automatically calculated by the SLDA accounting for 100% of the total variance. 100% of correct training

Table 26. Classification results of olive oil samples from Tunisian northern and southern origins and European olive oils based on cross validation method.

			Predicted Group Membership			
		Origin_Country	North TN	South TN	EU	Total
Cross-validated	Count	North TN	12	9	0	21
		South TN	8	52	0	60
		EU	0	0	16	16
	%	North TN	57,1	42,9	0	100
		South TN	13,3	86,7	0	100
		EU	0		100	100

and validation rates have been achieved by this SLDA (Table 26). Li, Sr, Na, Dy, Ga, Ni, Mg, Cu, Rb, Al, B, Y, As, Yb, Lu, Sm, Mo, Eu, Ti and Zn have been selected by SLDA as the most discriminatory elements between North and South Tunisian and Italian oils.

Chapter 4

Carbon and oxygen stable isotope ratios analysis as a potential tool for the geographical traceability of Tunisian olive oils: implication of climatic conditions and comparison with Italian oils

1. Introduction

In this research, we are exploring the efficiency of chemical markers of olive oils origin. Previous research efforts aiming at verifying the geographical origin of Tunisian olive oils have focused on indirect markers, those that characterize the biochemical composition of olive oils such as its polyphenols content, fatty acid profile, etc… But these parameters do not allow verification of the geographical origin of olive oils (Bontempo et al., 2019).

In addition, direct markers that have a direct link with the environment were proved to be more useful in geographical traceability subject matter. Those markers are the inorganic composition and stable isotope ratios of light or bio- (C, O, H, etc…) and heavy or geo- (Sr, Pb, etc…) elements. In the previous chapters we have demonstrated that the multielemental fingerprinting approach is promising but presents some limitations in the case where the geological and soil characteristics of the regions to be discriminated are similar. In fact, when a specific driving factor (geology, soil, climate, variety, etc…) affects different geographical regions in the same manner, the geographical traceability problem may be difficult to solve if the olive oil marker analyzed to solve the traceability issue is largely and predominantly determined by that driving factor. To overcome this challenge, exploring different marker that reflects a different driving factor constitutes an attractive and promising alternative. Portarena et al. (2017) stated that an improved discrimination of foodstuff can be achieved by the multivariate analysis of the data generated from different analytical techniques (Portarena et al., 2017).

Carbon and Oxygen stable isotope ratio analyses have shown a tremendous usefulness in verifying the geographical origin of olive oil in the last decades. But they have neither been used to characterize and distinguish Tunisian olive oils at the intra-country scale (different Tunisian regions) nor to distinguish Tunisian and Italian olive oils. Indeed, there is an increasing demand for the development of an analytical method capable of characterising and efficiently identifying the geographical provenance of Tunisian olive oil to contribute to the

creation of GIs by proving their uniqueness, and to distinguish Tunisian and extra-Tunisian olive oils to protect our national productions from fraudulent practices.

Carbon stable isotope ratio analysis has gained success in traceability studies owing to the fact that $^{13}C/^{12}C$ reflects local atmospheric CO_2 and the local climate conditions where the olive trees were grown. These climatic conditions are mainly the annual temperature and climatic conditions such as humidity (Angerosa et al., 1999). Accroding to Portarena et al. (2015), the transition from hot and dry summer conditions to cold and humid autumn conditions favors the uptake by olive trees of the light carbon isotope during the photosynthesis due to diffusive and biochemical effects. Accordingly, under mild conditions, the photoassimilates are characterized by a relatively depleted carbon isotope ratio (Portarena et al., 2015). The oxygen stable isotope composition of olive oils reflects that of the water source (soil water) that depends on the isotopic composition of meteoric precipitation water which in turns is related to geo-climatic conditions such as latitude, altitude, distance from the coast, annual and/or seasonal temperatures and precipitations. In addition to the isotopic composition of water source, the olive oil d18O may be affected by additional fractionations (enrichment and depletion) that result from physiological phenomena such as transpiration (Portarena et al., 2015).

In this study $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios were analysed in the bulk of different monovarietal olive oils using Isotope Ratio Mass Spectrometry (IRMS). These olive oil samples were derived from olive trees grown in 11 different Tunisian governorates. As for the Italian oils, these were commercial oils acquired from the internet.

The aim of this study was to: i) characterize for the first time in the literature the carbon and oxygen stable isotopes of Tunisian olive oils from different regions spanning the "four corners" of the country, ii) study the effect of the geographic and climatic conditions on carbon and oxygen isotopic fingerprints of monovarietal Tunisian olive oils and iii) investigate the possible inter-regional discrimination of Tunisian olive oils on one hand and the Tunisian and Italian oils discrimination on the other hand based on d13C, d18O.

2. Materials and methods

2.1. Olive oil samples

The Tunisian olive oil samples used in this study were the same used in the third chapter (Please refer to pages 62-66). These olive oil samples were all collected during the same season, in December 2017, to limit the seasonal effect and from 11 Tunisian governorates to

investigate the influence of the geographical variability on carbon and oxygen isotope ratios composition.

2.2. Climatic and geographic data collection

To study the effect of the geographical and climatic conditions on the Tunisian olive oil C and O stable isotope composition, the latitude and longitude were recorded at the time of samples collection by a handheld GPS receiver. The elevation and the distance from the sea were determined by Google Earth after inputting the latitude and longitude previously measured by the GPS. As for the average annual climatic conditions in 2017 (precipitation, relative humidity and the maximum, minimum and mean temperatures), these data were obtained from the NASA Langley Research Center (LaRC) POWER Project funded through the NASA Earth Science/Applied Science Program and that can be freely accessed through: https://power.larc.nasa.gov/data-access-viewer/?fbclid=IwAR394V0I-XLDJ 8ywx5Km_y1U6gRvdN3GYpqkd4K-HiUj3tc_dljjThFc0o

2.3. Carbon and oxygen stable isotopes analyses

The $\delta^{13}C$ of olive oils ($\delta^{13}C$ oil) was determined using the combustion mode of an elemental analyzer/isotope ratio mass spectrometer (Elementar GmbH, Vario PYRO Cube elemntar analyzer linked to an Iso-Prime 100 IRMS, Hanau, Germany). Subsamples of olive oil (1.0 mg) were placed into tin capsules (5 X 9 mm) and loaded into the auto-sampler of the EA/IRMS. The obtained CO_2 was separated on a PorapakQS column (3 m long) at 40° C. The δ notation defined in Eq. (1) was used to describe the isotopic composition.

$$\delta\ (‰) = (R_{sample} / R_{standard} − 1)\ x\ 1000 \qquad (1)$$

where R_{sample} is the isotope ratio of the sample, and $R_{standard}$ is the isotope ratio of the international standard, Vienna Pee Dee Belemnite. Each sample was determined in duplicate, and analytical errors in $\delta^{13}C$ (standard deviation (SD)) were < 0.1 ‰

The $\delta^{18}O$ of olive oils ($\delta^{18}O$ oil) was determined by EA/IRMS in the pyrolysis mode. Subsamples of olive oil (0.5 mg) were placed into silver capsules (5 X 3 mm). Pyrolysis was performed in a ceramic tube of glassy carbon at 1,450° C under a continuous flow of He at 100 ml min^{-1}. The gas obtained was separated from residual gases by molecular sieving through a 5-Å column (length, 3 m) at 50° C. $\delta^{18}O$ of olive oil was calculated according to Eq. (1) and expressed relative to that of Vienna Standard Mean Ocean Water (V-SMOW).

The measured $\delta^{18}O$ values were obtained using known isotope benzoic acid standards (+71.4, +23.2 ‰) purchased from Indiana University (Indianapolis, IN). The accuracy of the obtained $\delta^{18}O$ values was checked independently against V-SMOW and Vienna Standard Light Antarctic Precipitation standards distributed by the International Atomic Energy Agency. Four working standards—dibenzo-24-crown-8 (15.7 ‰), dibenzo-18-crown-6 (+1.7 ‰), β-D-galactose pentaacetate (+12.7 ‰), and D-(+)-sucrose octaacetate (+26.8 ‰) were determined every six samples to confirm the reproducibility of the measurements. The analytical error of the isotope measurements had a SD < 0.3‰ with a minimum sample amount of 10 μmol oxygen (Suzuki et al. 2008; Akamatsu et al., 2014).

2.4. Data analysis

Median and standard deviation values for olive oil $\delta^{13}C$ and $\delta^{18}O$ values and geo-climatic parameters (latitude, longitude, altitude, distance from the sea, average annual: maximum, mean and minimum air temperatures, precipitation and relative humidity) values were calculated for each governorate. Correlations between olive oil ($\delta^{13}C$ and $\delta^{18}O$) and geo-climatic data were tested using Pearson's product-moment correlation coefficient with the individual values for each sampling point. The data was statistically evaluated using IBM SPSS Statistics software for Windows, version 24.0 (IBM Corp., Armonk, N.Y., U.S.A.).

3. Results and discussion

3.1. Isotope ratios results

Stable isotope data (median and standard deviation) for all the olive oil samples as well as their corresponding climatic data are shown in table 27. Additionally, the $\delta^{13}C$ values have been plotted against $\delta^{18}O$ values and the results can be seen in Fig.29.
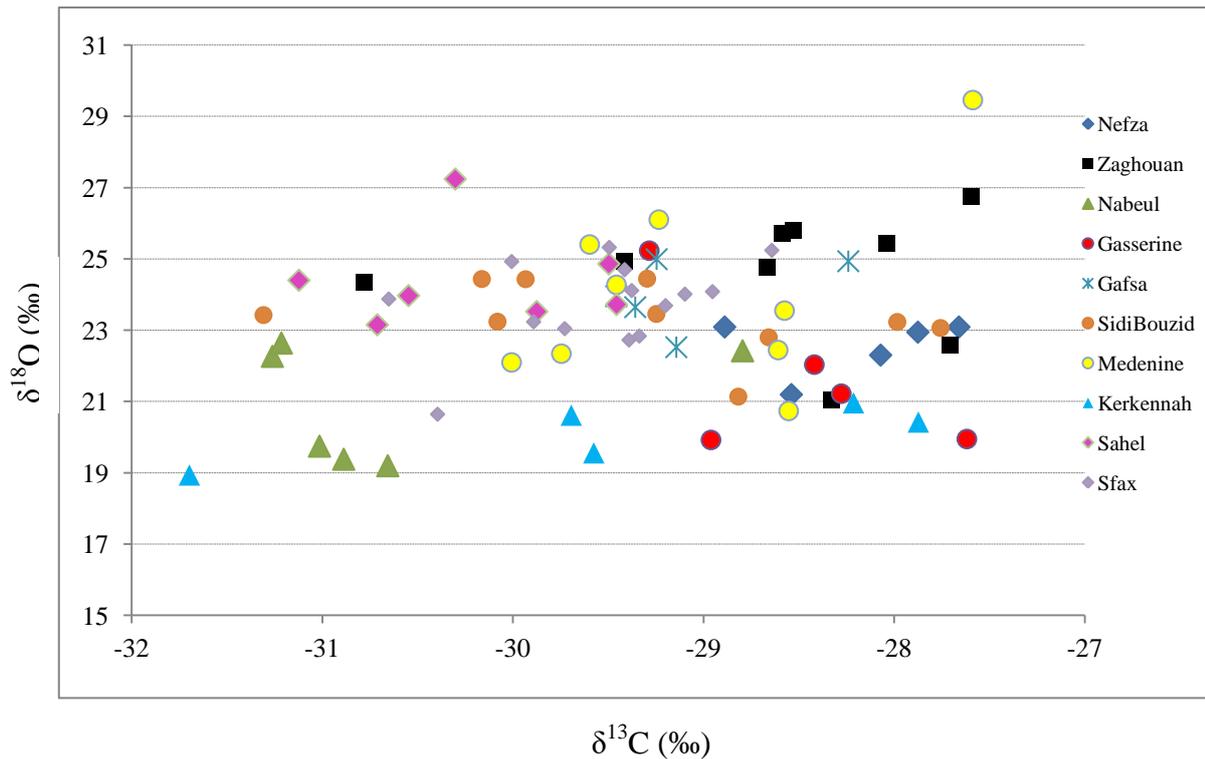
Figure 29. δ18O vs δ13C values of 77 olive oil samples from ten different Tunisian governorates. Samples from the same geographical region are represented by the same symbol, according to the legend.

The range of variation of δ^{13}C was about 4.11‰ (from -31.69‰ to -27.58‰) while a wider range of 10.53‰ was found for δ^{18}O (from 18.93‰ to 29.46‰). Our results revealed that most of the Tunisian olive oils presented δ^{13}C and δ^{18}O values that were in agreement with some other isotopic studies about Mediterranean olive oils.

The isotopic composition was further mapped by using Surfer software. The spatial interpolation method used was the Kriging method. The results of the spatial interpolation can be seen in Fig.30.

Table 27. Stable isotope composition and climatic conditions in the 11 Tunisian governorates.

Governorate		δ13C	δ18O	Distance from the sea (km)	Elevation (m)	Precipitations (mm)	Relative humidity (%)	Max Temperature	Min Temperature	Mean Temperature
Gafsa	MEDIAN	-29,1428339	24,0829776	112,69	396	199,5	50,6761644	24,5686849	11,4166849	17,4016712
	STDEV	1,940292	0,91655571	15,3285174	80,2385081	42,1992638	1,56453524	1,00422313	0,95858697	1,02283182
Gasserine	MEDIAN	-28,3482178	21,4256388	149,185	654	249,815	52,4651096	24,5416575	11,3148219	17,2970685
	STDEV	0,96232292	1,95228784	7,43944263	100,815508	57,3103442	2,28988056	0,7854548	0,81056183	0,80293063
Zaghouane	MEDIAN	-28,5287548	25,5691953	55,135	271	429,79	59,2956438	25,0057808	12,0828219	17,8524384
	STDEV	0,51479321	0,80071686	11,1858	43,76	42,9786	1,13031781	0,13177644	0,76367507	0,36699945
Nabeul	MEDIAN	-30,6568216	19,7495032	6,45	102	307,58	69,0284932	22,4147123	16,4711507	19,1543288
	STDEV	0,93491717	1,6578793	5,10736286	67,9536344	23,8610837	0,52487179	0,17006602	0,08045662	0,04311327
Tataouine/Medenine	MEDIAN	-28,9216554	22,9994446	3,53	29	355	62,3885205	24,2766301	16,7118356	20,1793425
	STDEV	0,78880198	2,77301624	23,3315108	122,450962	28,0181508	7,32031075	1,87453656	2,33530937	0,51234772
Beja (Nefza)	MEDIAN	-27,8755646	22,9489039	NA	NA	571,93	62,3314247	24,1736712	12,9690685	18,0187123
	STDEV	0,20534871	0,42024093	NA	NA	0	0	0	0	0
SidiBouzid	MEDIAN	-29,2961892	23,4437668	59,3	155	190,47	53,9626575	25,8252055	13,1898904	18,8999452
	STDEV	1,23947282	1,10034535	13,3113561	52,4152756	42,63	0,12432877	0,03641096	0,40412329	0,2590411
Sfax	MEDIAN	-29,3912893	23,6934396	31,83	126	198,755	58,4107671	25,2615068	14,648	19,4544658
	STDEV	0,68338269	1,4621028	8,92133176	36,0935394	12,2355183	2,8749718	0,74429512	0,85693071	0,15895312
Kairouan	MEDIAN	-29,3696564	23,0239122	78,395	124	235,88	56,5049589	26,021863	13,5083288	19,1337534
	STDEV	1,00598837	0,31094704	2,82135606	26,8700577	0	0	0	0	0
Sahel	MEDIAN	-30,089017	23,8499961	31,27	75,5	219,72	62,0031233	24,7468493	15,1210411	19,4506027
	STDEV	0,53597293	1,50040916	14,3235396	22,4469746	11,0295127	3,11359707	0,8542485	0,84495628	0,13596999
Kerkennah	MEDIAN	-29,5760381	20,4254496	1,34	1	217,23	71,308274	21,3976164	18,6412603	19,9988493
	STDEV	1,51121816	0,83015043	0,98693127	0	0	0	0	0	0

NA: Not available

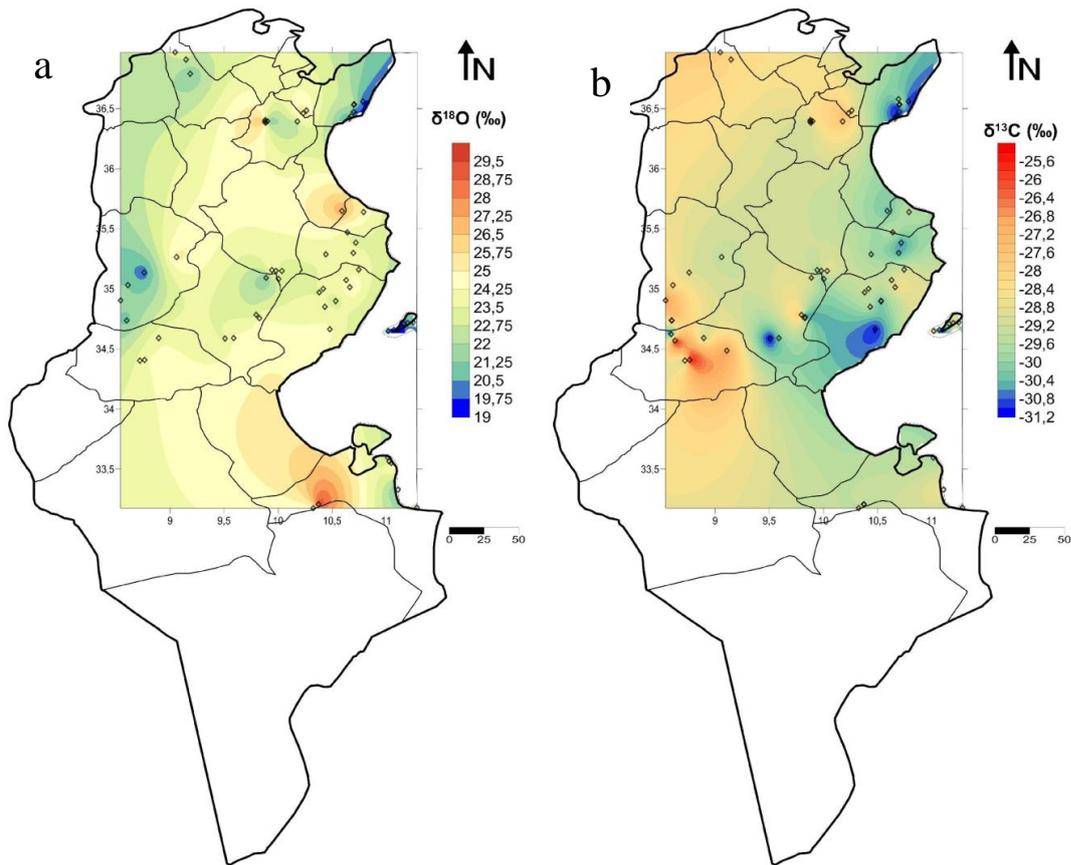**Figure 30.** δ18O (a) and δ13C (b) spatial interpolation maps using Kriging method.

These maps clearly show the tendencies of δ^{18}O and δ^{13}C variations across the country.

For δ^{18}O, Medenine, the Sahel and Zaghouane regions have shown the highest values whereas Gasserine, Nabeul, Nefza and Sidi Bouzid have shown the lowest values. The results of oxygen isotope indicate a tendency of enrichment in the coastal eastern and southern Tunisian regions and depletion in the northern and western regions. As for δ^{13}C, the lowest values were registered for Sfax, Nabeul and one site in Gafsa. This highlights a tendency of enrichment in the western and northern regions and depletion in the coastal and southern regions.

To further understand these results, correlation analyses with the climatic data have been carried out.

The results of the correlation analysis revealed that δ^{18}O presented significant positive correlations with precipitations and maximum temperatures and significant negative correlations with humidity and minimum temperature.

As for δ^{13}C, significant positive correlations have been registered for distance from the sea, altitude, humidity and maximum temperature while significant negative correlations were found with longitude, minimum and mean temperature.

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Our results have shown that with just two variables (δ^{18}O and δ^{13}C) the inter-regional discrimination between the 10 regions may be difficult to achieve. In fact, since the carbon and oxygen isotope composition in organic matter strongly depends on the water cycle (evaporation, condensation, and precipitation), which is mediated by the evaporative demand of the region, stomatal conductance, and photosynthesis, stable isotope analysis may not succeed in distinguishing olive oils coming from regions where the meteorological cycle of water is impacted by similar climate parameters (Portarena et al., 2017).

However, these two analytical parameters were clearly demonstrated to depend on the climatic conditions and they are capable of discriminating Tunisian olive oils according to two macro-areas: the north-western regions characterized by depleted δ^{18}O and enriched δ^{13}C values and the coastal/east-southern regions characterized by enriched δ18O and depleted δ^{13}C values. These two macro-areas are delimited by the line passing by the south of Nabeul to east of Gafsa.

These results allowed us to conclude that carbon and oxygen stable isotope analysis could be used as a fingerprint of the geographical origin of Tunisian olive oils.

General discussion

The authentication of the geographical origin of olive oils has become a crucial factor in terms of valorizing this key foodstuff originating from certain geographic origins that have longly been appreciated for the organoleptic or health-associated qualities of their olive oils. The authentication of the geographical origin brings on other advantages especially the fight against fraudulent practices and the strengthening of the consumers' confidence and trust in the product they buy. In the case of valorizing olive oils from specific geographical areas, whether at the country or municipality scale, the specificity, typicality and uniqueness of these oils in terms of quality attributes and chemical composition need to proven. Additionally, the specifications regulating the registration of foodstuff as protected GI, requires the justification of the causal link between the characteristics of the provenance environment (climate, soil, etc…) and those of the derived foodstuff such as olive oil. In that case, it becomes mandatory to develop scientific techniques to ensure an efficient traceability of the olive oils from producer to consumer. Such traceability systems have been used in the EU and other countries for many years and they have proven their efficacy in adding value to olive oils and lots of other foodstuff including wine, cheese, meat, vinegar, etc… Other advantages include the preservation of biodiversity and the local know-how. Moreover, GIs can help increasing price premium which have in part contributed in the economic development of rural agricultural areas in some EU countries (in fact, on average the price of a GI product is 2.23 times the price of a comparable non-GI product: e.g., the case of Baena olive oil in which the GI label "PDO" resulted in 2.75 Euro increase in 1998) (Berenguer Reguant, 2016).

Taking these facts into account, Tunisia has been trying to follow the path of the EU since the last two decades by establishing the regulatory and legal framework for GI. This has encouraged researchers to develop scientific methodologies aiming at proving the typicality of olive oils from selected regions. These methodologies were based on the characterization of the organoleptic and sensory profile, volatile composition, fatty acid composition, triglycerides and even the molecular markers of olive oils. These compounds, that have been used as tracers or markers, when used in a uni- or multivariate statistical approach can distinguish two or more regions. However, these markers are highly dependent on the varietal origin, can vary with cultivation practices, ripeness of the olive fruits and the conservation conditions and cannot also truly prove the causal link with the provenance. Consequently, the use of these analytical parameters as geographical markers could be unreliable.

Numerous studies have shown that multielemental and isotopic (for instance carbon and oxygen) fingerprinting of various foodstuffs including olive oil are among the most powerful tools for elucidating their geographical origin. In the case of multielements, a study on Japanese Welch onions has demonstrated that the various cultivation practices and the use of fertilizers don't disrupt the discrimination model efficacy and reliability. We built our work on that piece of evidence and we considered a negligible effect of the variability of cultivation practices and anthropogenic inputs on the multielemental profile. Moreover, it is well know that Tunisia was the top first country of organic olives area in the world in 2017 with 254.4 thousand hectares of organic olive area (Willer & Kilcher, 2011).

Additionally, Tunisian olive oil is mostly produced without any pesticides and can be easily certified as organic production (Laroussi-Mezghani et al., 2015). This means insignificant anthropogenic inputs and influence on the olives composition. Therefore, the previously mentioned hypothesis in addition to the recent statistics lead to believe that multielemental fingerprinting of olive oil can be one of the most useful tools to identify its geographical origin. However, contrary to the information available for stable isotope ratios of carbon and oxygen, whose variability in olive oils according to geographical origin was correlated with the climatic and geographical parameters of the involved regions and the oxygen stable isotope ratio of the source water, this information is not available for multielements. This information is crucial in order to prove the causal link with the environment to qualify as a GI on one hand and to prove that multielements are indeed valid tracers of the provenance environment on the other hand. In this research, we studied the origin of the elements contained in olive oil to identify the potential influence of anthropogenic activities on the oils' multielemental profile as a first step towards proving the validity of inorganic elements. For this reason, the analysis of the correlation between the olive oils' major, minor and trace elements has been carried out to reveal the underlying processes responsible for such relations and associations. This study has been conducted twice with samples collected in December 2015 (21 samples from 4 regions) and validated a second time with samples collected in December 2017 (85 samples from 11 regions). In each time, the correlations between elements in the oil compartment revealed that geochemical processes are the main modulators of elements cycling in the soil-oil system (i.e. uptake by olive trees, transport until olive fruits and transfert to olive oils). In fact, elements from the same geochemical group classified according to Goldschmidt rule showed a tendency to cluster together. Our findings agree with previous studies on Canadian wines and maple syrups that showed that if such correlations between elements with similar chemical properties would be demonstrated, the multielements

could be considered as valid and useful markers for the geographical traceability of the foodstuff in question. This agreement proves for the first time the validity of multielement as markers of the geographical origins of olive oils.

The next step in the approach of proving the usefulness of multielements as valid provenance markers consists in studying and quantifying the strength of the relationship of the multielemental profile of olive oils and their environment characteristics. In the pilot study, the geochemical composition (i.e. the total concentration of elements) of the soils in which the olive trees - from which the olive oils were derived - were planted was considered as the most significant precursor and proxy of the elements in olive oils. Consequently, a correlation analysis was conducted with the aim of quantifying the relationship between the total concentration of olive oils and their paired soils. This analysis revealed that among 11 measured elements in soils and olive oils, only Zn exhibited a statistically significant correlation between these two compartments. Moreover, the correlation coefficient sign was negative which pointed out other possible modulating factors, with the closest related one being climate confirming as so the works of Greenough et al. (2010).

Therefore, in the second extensive study on the geochemical characterization of the 11 Tunisian olive producing regions, we considered the climatic factors (temperatures, precipitations and relative humidity) and the bioavailable fraction of the total soil elemental composition in the correlation with olive oil elemental contents study. For the first time in the scientific literature, selected elements: Ti, Fe, Ni and Ba demonstrated a significant positive Spearman correlation between olive oil and soil bioavailable elements. The absence of significant linear relationships (Pearson correlation coefficients) can be explained by many reasons: i) the non-normal distribution of the variables in the datasets of olive oil and soil bioavailable concentrations, ii) the influence of other determinants on the olive oil elemental profile like we have showed earlier regarding climatic factors potential intervention and iii) the complexity of the of interactions between elements in both geological and biological compartments. Considering this last reason, the bivariate correlations between all the elements in olive oil and soil samples have been studied and the results have shown that all elements in olive oils (22 elements), except Ho, displayed significant Spearman correlations with at least one bioavailable soil element. Specifically, Mo, Ce, Lu, Fe, Na, Mn, Ni and Zn had significant correlations with up to 8 bioavailable elements in soils. These findings constitute important pieces of evidence of the direct and tight relationship between the elemental composition of olive oil and its environment. Therefore this study provided an additional and consequential proof on the validity and usefulness of multielements in the geographical

traceability of Tunisian olive oils. However, in addition to the proven link with the environment, a marker could be deemed efficient only if it can show a significant discriminatory power.

The approach used to study the discriminatory power of multielements in the Tunisian geographical and geological context was based on the following hypothesis: Since the link between the olive oil and its provenance soil was proven, if the elemental composition of soils of origin is large enough that it can discriminate them, then the paired olive oils will be also successfully discriminated and a correspondence between the elements used to discriminate the soils an olive oils will be detected. Accordingly, the pilot study based on 21 olive oil samples and 28 soil samples from four geographic regions (Gafsa, Sfax, Medenine and Monastir) have shown the promising discriminatory power of multielements for both soils and olive oils as demonstrated by the high prediction rates achieved and have also demonstrated the existence of correspondence between the most discriminatory elements in the two compartments represented by Fe, Rb, Mg and Pb. This study has also shown that the origin effect is more dominant than that of cultivar on the elemental profile of olive oils which agrees with previous works of Benincasa et al. (2007) and Beltran et al. (2015) and which proves again the power of multielements as tracers of the origin despite the existence of any potential induced interference. The results of this preliminary work encouraged us to explore the discriminatory power of multielements in more Tunisian olive producing regions. Therefore, the same approach was applied to the 85 and 136 olive oil and soil samples, respectively, sampled in December 2017. But in an effort to increase the classification performance, the inclusion of additional trace and even RREs was highly desirable. For this reason and taking into account that the microwave digestion of olive oils to extract its inorganic elements is characterized by high detection limits, we performed a comparative study. Three methods were compared among which one was originally proposed in this research. The three methods are the microwave-assisted extraction, the ultrasound-assisted extraction and the combined microwave-assisted extraction and evaporation of the elements. Results showed that despite reaching lower detection limits as compared to the microwave only, the combined microwave-assisted extraction and evaporation was characterized by very high relative standard deviations caused by the prolonged preparation time and contact with the environment resulting in increased contamination. The ultrasound-assisted extraction resulted in even lower detection limits and higher precision and was therefore selected as the preparation method of choice for the next part.

The main objective of the second extensive sampling campaign was to include more representative samples from the main Tunisian producing regions and identify if some one or more regions will have a very distinct and unique elemental profile to help it/them qualify for a potential GI labelling. The first step dealt with the study of the discrimination of soils based on their geochemical composition. Our results showed that out of the 11 Tunisian regions (Sfax, Sahel, Medenine, Nabeul, Zaghouane, Nefza, Gafsa, Gasserine, Sidi Bouzid, Kairouane and Kerkennah) only Nefza and Zaghouane had an outstanding unique profile (50+ elements) that allowed them to be successfully discriminated against the rest of the regions This was attributed to their unique soil geochemical composition that agreed with their edaphic characteristics. Zaghouane is characterized in fact by high carbonates content that was reflected in their high soil Ca concentrations while Nefza is characterized by high clay content and developed soils favoring the retention and enrichment of the less mobile Fe, Al and REEs. The rest of the regions had very similar soil characteristics and therefore could not be discriminated with a success similar to that reached with Nefza and Zaghouane. We report similar finding for the soil bioavaialble fraction where Nefza and Zaghouane had the most unique elemental composition. As for the olive oil multielements' discriminatory power, that is the core of this study, the results of classification and prediction based on LDA revealed overlapping between most of the regions even for Nefza and Zaghouane that had very distinct soil composition. However the discrimination results improved when all the central Tunisian regions were merged into a single class. In fact, Tunisian olive producing regions form a contiguous area especially in the central part of the country where most of the productions take place. These regions have very similar geological setting characterized by the predominance of sedimentary deposits of alluvial origin that cover large parts of the country especially the central Tunisia. These findings reveal limitations of the traceability based on analytical parameters when similar driving factors affecting the presence and concentration of these parameters in the foodstuff in question prevail in the regions to be discriminated. As suggested by other authors, to overcome this challenge, combining two markers each of them reflecting a different driving factor can effectively solve this problem.

The last part of this study deals with the carbon and oxygen stable isotope ratio analysis of olive oil samples. This marker was introduced to improve separation between olive oil samples based on multielements when similar geological settings exist in the regions to be discriminated. Tunisia is characterized by a large variation of its bioclimatic zones varying from Saharan in the south to humid in the north. The isotopic fingerprinting of Tunisian olive oil samples was capable of distinguishing olive oils originating from two macro-areas: : the

north-western regions characterized by depleted $\delta^{18}O$ and enriched $\delta^{13}C$ values and the coastal/east-southern regions characterized by enriched $\delta^{18}O$ and depleted $\delta^{13}C$ values. These two macro-areas can roughly be delimited by the line passing by the south of Nabeul to the east of Gafsa.

The study on the efficiency of these two kinds of markers to discriminate Tunisian and Italian oils has demonstrated that multielements performed better that carbon and oxygen stable isotopes in the identifying of the geographical origin of oils coming from these two countries. This was attributable to the fact that the carbon and oxygen isotope composition in organic matter strongly depends on the water cycle (evaporation, condensation, and precipitation), which is mediated by the evaporative demand of the region, stomatal conductance, and photosynthesis. Therefore, stable isotope analysis may not succeed in distinguishing olive oils coming from regions where the meteorological cycle of water is impacted by similar climate parameters which is the case of Tunisian and south-Italian regions.

Conclusion

This study is the first in the scientific literature reporting the multielemntal and stable isotopic fingerprinting of Tunisian olive oils from various Tunisian olive oil-producing regions. Multielemental fingerprinting turned out to be an important parameter regarding the verification of the safety and the authentication of the origin of the oils. Our results have shown that all of the 106 analyzed oils in this study collected during two sampling campaigns in December 2015 and December 2017 have very low concentrations of potential toxic heavy metals far below the maximum allowable limits which shows the good quality of Tunisian olive oils based on the maximum residue limit criteria established by the IOC.

Multielements presence and concentrations in olive oils were also proven to significantly depend on their provenance environment characteristics. They displayed associations defined by Glodschmidt geochemical rule and they all showed a significant correlation with the soil bioavailable fraction. Moreover, a correspondence between the most discriminatory elements of olive oils and soils is manifested every time inorganic composition of these two compartments is submitted to the classification model. This undoubtedly attests to the fact that the geochemical signature of the soils is transferred to the oils. Therefore, our study demonstrated for the first time the validity of multielements as markers of the olive oil's geographical provenance.

The identification of the geographical origin of olive oils based on the combination of multielements and multivariate statistical analyses was shown to be very a powerful tool. However, the task may become challenging when the geological setting and soil characteristics on the regions to be discriminated are similar. This is particularly true for the central Tunisian regions where the predominant outcrops are mainly of a sedimentary alluvial origin which results in very similar soil chemical composition and consequently olive oil multielmental profile.

The combination of multielemnts with stable isotope ratios of carbon and oxygen can further separate the olive oil regions coming from two macro-areas: the north-western regions and the coastal/east-southern regions that can roughly be delimited by the line passing by the south of Nabeul to the east of Gafsa. This combination can therefore improve the discrimination of central Tunisian regions that are situated on the opposite sides of this virtual line.

Finally, to identify the Tunisian or Italian origin of olive oils, multielements have shown a very good performance by achieving 100% correct validation of the true origin.

This work can serve as a base and reference for future research intending to prove the uniqueness of potential GIs of olive oils as it constitutes a country-wide comprehensive dataset that can be used to check and compare the composition of olive oils from the main Tunisian producing regions and support the goal of geographical authentication of Tunisian olive oils.

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