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Multi-element (^{13}C , ^2H and ^{34}S) bulk and compound-specific stable isotope analysis for authentication of *Allium* species essential oils.

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Abstract

Pure and natural essential oils of *Allium* species are primarily used in the food industry, but due to their low yield, production quantities can be difficult to achieve and thus can be highly expensive. Due to these problems, adulterations are a reality in this field. The purpose of this research is to develop a multi element stable isotope analysis methodology for authentication of the essential oils of leek, onion, shallot, and chive. Naturalness of these essential oils was achieved by assessment of compound specific $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$: an addition of 5% of synthetic compound can be detected. Chemometric models were undertaken to assess purity by distinction of the *Allium* samples according to their species origin using bulk stable and compound specific $\delta^{13}\text{C}$, $\delta^2\text{H}$, $\delta^{34}\text{S}$, and compositions of their major metabolites dipropyl disulfide, dipropyl trisulfide, methyl propyl disulfide, and methyl propyl trisulfide.

Keywords

Allium essential oil; Authentication; Multicollector-inductively coupled plasma mass spectrometry; Isotope ratio mass spectrometry

1. Introduction

The *Allium* genus from the Alliaceae family includes many vegetal species principally cultivated as culinary ingredients, due to their strong aromas. *Allium* genus is widely distributed in North Africa, Europe, Asia and America (El-Wakil et al., 2015). Some of these species, such as bulbs of onion, garlic, and leek, are used to obtain essential oils by steam distillation and are incorporated as flavourings in the food industry. The essential oils extracted from these vegetal species have similar medicinal properties: diuretic properties (Satyal et al., 2017), antimicrobial activities (Casella et al., 2013), and antioxidant activities (Ye et al., 2013) which have been reported in previous studies. For instance, chinese chive and onion essentials oils (EOs) have a high antibacterial activity whereas shallot and leek EOs were the strongest antioxidants (Mnayer et al., 2014).

The composition of these oil extracts is relatively similar, as they all contain a high level of sulphur compounds. Dipropyl disulfide (DPDS) and dipropyl trisulfide (DPTS) are the main constituents of essential oils of the leek *Allium porrum*, the shallot *Allium ascalonicum*, the onion *Allium cepa*, and the chive *Allium schoenoprasum*. The relative compositions of DPDS are approximately 45% in leek, 30% in onion, 20% in chive and 15% in shallot, and the content of DPTS is close to 15% in leek, onion, and chive, and near 10% in shallot (Mnayer et al., 2014). Other major components of these essential oils are methyl propyl disulfide (MPDS) and methyl propyl trisulfide (MPTS). Essential oils of garlic *Allium sativum*, wild garlic *Allium ursinum* and garlic chive *Allium tuberosum* are principally composed of diallyle disulfides (DADS), diallyle trisulfides (DATS), methyl allyl disulfides (MADS) and methyl allyl trisulfides (MATS) (Dziri et al., 2014).

Similarly, with other natural products that present low extraction yields leading to expensive production costs, essential oils from *Allium* species are subject to fraud (e.g., the price of leek essential oil is near 1000 €/kg). To obtain a quantity of 100 g of leek essential oils, it is necessary to treat approximately 1 T of leek raw material. For the same quantity of essential oil, raw materials needed for chive, shallot, onion, and garlic are 2.5 T, 1.5 T, 0.8 T and 0.1 T respectively. It is then necessary to ensure the naturalness of these essential oils by highlighting any adulteration with synthetic compounds. Furthermore, their similar chemical composition makes the mixture detection of these essential oils more difficult.

Authentication of naturalness of *Allium* essential oils based on chiral properties cannot be achieved because these molecules of interest do not present any stereoselectivity. Identification of synthetic impurities is also hardly achievable as residual impurities such as halogens precursors can be eliminated with a purification process. Additionally, the complexity of the matrix, with many different metabolites (Mnayer et al., 2014) and the tendency of sulphur-containing molecules to rearrange during gas chromatography (GC) injections, lead to the formation of derived sulphur components (Cantrell et al., 2020). Among the various analytical techniques involved for the assessment of the authenticity of natural products, stable isotope analyses have been widely used to trace or to ensure the origins.

Multi-isotopic (^{13}C , ^2H , ^{18}O , ^{15}N , and ^{34}S) approaches sometimes associated with inorganic multi-elemental analyses have been used to track the geographical origin of allium products: Korean onions (Park et al., 2019), Korean garlic (Choi et al., 2020) or Italian red garlic (Pianezze et al., 2019). In these studies, the geographical origins are correlated to specific cultivation practices allowing the production of high quality of these food condiments. Regarding essential oils, bulk stable isotope analysis was recently used to authenticate naturalness of

wintergreen essential oils (Cuchet et al., 2019). Bulk stable isotope analyses are usually performed using an elemental analyser linked to an isotope ratio mass spectrometer in continuous flow mode for determination of $\delta^2\text{H}$ via pyrolysis (EA-P-IRMS) and $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ via combustion (EA-C-IRMS). Over a period of a few years, several systems have been developed and manufactured using a combustion approach to simultaneously analyse C, N and S either based on chromatographic separation of the gases with a flash Thermo system (Kracht, 2011) or with a purge-and-trap separation device for the Elementar system (Fourel et al., 2014).

However, these methods only produce bulk analyses, and the study of the targeted main secondary metabolites could supply new criteria to improve authentication on more complex essential oils matrices. Gas chromatography – combustion / pyrolysis – isotope ratio mass spectrometry (GC-C/P-IRMS) is an alternative technique allowing to assess compound-specific ^{13}C and ^2H compound-specific isotope ratios. Authentication of naturalness and purity of several essential oils have been achieved on this GC-IRMS system: oregano and thyme (Nhu-Trang et al., 2006a), lemongrass (Nhu-Trang et al., 2006b), or citrus (Schipilliti et al., 2012) essential oils for example.

However, due to the high oxidation degree of sulphur, a combustion interface with gas chromatography is difficult to set up for ^{34}S assessment, and therefore, it has not yet been achieved via GC-IRMS. An alternative is to use gas chromatography (GC) connected on line to a multi-collector-inductively coupled plasma mass spectrometry coupling system (GC/MC-ICP-MS) to assess the compound-specific isotopic ratio of this element. This technique has been developed during the past ten years (Amrani et al., 2009; Clough et al., 2006; Hanousek et al., 2016; Said-Ahmad and Amrani, 2013) essentially applied to petrochemical products. Several

recent papers also describe the use of GC/MC-ICP-MS for the analysis of organic sulphur compounds (Kümmel et al., 2020; Said-Ahmad et al., 2017) but not for food chemistry yet.

In this study, we propose a novel methodology to authenticate the naturalness and the purity of the extracted essential oils of *Allium* species based on $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\delta^{34}\text{S}$ performed with both bulk stable isotope analysis via EA-C/P-IRMS as well as compound-specific stable isotope analysis via GC-C/P-IRMS and GC/MC-ICP-MS. The aim of this work is to develop a method that supplies naturalness authentication of these essential oils and highlights the isotope differences between the different *Allium* species, using chemometric tools. Simultaneous determination of bulk and compound specific values for $\delta^{13}\text{C}$, $\delta^2\text{H}$ and $\delta^{34}\text{S}$ can lead to the discrimination of the origins of authentic essential oils and synthetic molecules. This method is tested on essential oils of leek, onion, shallot and chive that share similar metabolites.

2. Material and methods

2.1. Sample description

2.1.1. Essential oils

Six Egyptian leek (*Allium porrum* L.) essential oils (EOs), nine German Shallot (*Allium ascalonicum* L.) EOs, and eleven Egyptian onion (*Allium cepa* L.) EOs were provided by Albert Vieille SAS (Vallauris, Alpes Maritime, France). Two leek EOs, two shallot EOs, ten chives (*Allium ascalonicum* L.) EOs, and two onion EOs originating from France were supplied by Ferrand PHE (Rodelinghem, Pas de Calais, France). More information can be found in **Supplementary Table S1.**

2.1.2. Organosulfides standards

Commercial synthetic molecules of organosulfides (propyl sulfide, dipropyl disulfide, dipropyl trisulfide, methyl propyl disulfide, and methyl propyl trisulfide) were purchased from TCI (Tokyo, Japan) and Sigma Aldrich (Saint Louis, MO, USA). Chemosynthetic origin was confirmed by radiocarbon activity assessment. The undertaken method is fully described in a previous study (Cuchet et al., 2019).

2.2. Stable Isotope Ratio Analysis

Isotopic results are expressed in the delta unit (δ) according to the following formula (Brand et al., 2014):

$$\delta^i E = \frac{(^i R_{Sample} - ^i R_{Reference})}{^i R_{Reference}}$$

where i is the mass number of the heavier isotope of element E , R_{Sample} is the respective isotope ratio of a sample, $R_{Reference}$ is the relevant internationally recognized reference material. The delta values are multiplied by 1000 and are expressed in units "per mil" (‰). SI unit for isotopic ratio is the Urey (Ur), but for practical reasons, the unit used here is the per mil: 1 per mil = 1 mUr.

High-purity gases of carbon dioxide 99.998% (4.8 Messer), hydrogen 99.999% (5.0 Messer). and N45 high-purity SO_2 (Linde Gas France), previously calibrated versus international references (VPDB, VSMOW, and VCDT), were used as the working monitoring gas with each sample. Helium flow at 99.999% was used as a carrier gas.

International reference materials IAEA CH7 (polyethylene foil: $\delta^{13}C_{VPDB}$ (‰) -32.15, δ^2H_{VSMOW} (‰) -100), USGS40 (L-glutamic acid: $\delta^{13}C_{VPDB}$ (‰) -26.39), NBS127 (barium sulfate, $BaSO_4$: $\delta^{34}S_{VCDT}$ = +20.3‰), (Halas and Szaran, 2001) and S1 (silver sulfide, Ag_2S : $\delta^{34}S_{VCDT}$ = -0.3‰),

(Robinson, 1995), supplied by IAEA (International Agency of Atomic Energy, Vienna, Austria) were used to calibrate all of our studied samples, which allowed us to anchor our $\delta^{13}\text{C}$ results to the Vienna Pee Dee Belemnite (VPDB) scale, our $\delta^2\text{H}$ results to the Standard Mean Ocean Water (VSMOW) scale and our $\delta^{34}\text{S}$ results to the Vienna-Canyon Diablo Troilite (V-CDT) scale. Each sample was analysed versus a monitoring gas for which two pulses were admitted with each analyte. All sample values were analysed in duplicate and verified by bracketing the analytical sequence with international reference materials every six analyses. The $\delta^{13}\text{C}$ values are given within a maximum standard deviation of $\pm 0.28\text{‰}$, $\delta^2\text{H}$ within SD = $\pm 5\text{‰}$ and $\delta^{34}\text{S}$ within SD = $\pm 0.34\text{‰}$.

2.2.1. EA-C/P-IRMS

The $\delta^2\text{H}$ measurement was conducted using a TC-EA elemental analyser linked to a Delta V Plus via a Conflo IV interface (all Thermo Fisher Scientific, Bremen, Germany). Samples weighed in silver capsules (3.3 X 5 mm, Santis Analytical AG, Teufen, Switzerland) were dropped into the pyrolysis unit held at 1400°C. This high temperature reactor was constructed of an inner glassy carbon tube half filled with glassy carbon granules (IVA Analysentechnik, Meerbusch, Germany) integrated into an external ceramic tube. Hydrogen is converted into H_2 and separated on a gas chromatography column prior to the introduction in the IRMS via the same Conflo IV interface. The interface allows the introduction of monitoring gas pulses.

Carbon and sulphur isotope compositions were measured using a Vario PYRO cube elemental analyser (Elementar GmbH, Langenselbold, Germany) equipped with a “purge and trap” gas separation module in combustion mode. The elemental analyser was interfaced in continuous-flow mode with an Isoprime 100 isotope ratio mass spectrometer (Elementar UK Ltd, Cheadle, UK). The applied technique is fully described in a previous study (Fourel et al.,

2015). For each sample, 3 aliquots of 200 mg of essential oils were loaded in tin foil capsules.
Data are reported as $\delta^{34}\text{S}$ in ‰ vs. V-CDT.

2.2.2. GC-C/P-IRMS

GC-IRMS analyses were performed using a 6890N GC gas chromatograph system (Agilent) connected to an Isoprime GV isotope ratio mass spectrometer (Elementar UK Ltd, Cheadle, UK) via the GC5MK1 GV combustion or pyrolysis interface. The system can be configured for either ^{13}C or ^2H analyses. All samples were analysed in triplicate and verified by bracketing the analytical sequence with **organosulfides** standards every six analyses. Organosulfides standards were calibrated to the international references V-PDB and VSMOW using the EA-IRMS technique.

An amount of 1 μL of diluted liquid sample (10 μL in 1 mL of ethanol) was injected using a 7683 Agilent Technologies auto-sampler into the 6890N gas chromatograph equipped with an Agilent HP-5 column (60 m*0.320 mm*0.25 μm I.D film thickness) used in chromatographic separation. The injection port was held at 250°C, fitted with a split liner containing glass wool, and operated in split mode (10:1). The oven temperature programme started at 50°C during 2 min, increased to 120°C at a rate of 10 °C min⁻¹, increased to 210°C at a rate of 5°C min⁻¹, increased to 280°C at a rate of 20 °C min⁻¹, and held for 5 min. Compounds were separated at a flow rate of 1 mL.min⁻¹.

A heart split valve connected to the outlet of the GC column enabled the components to be obtained either in the combustion/pyrolysis tube or in the FID detector. This process allows elimination of the solvent peak as well as unwanted compounds.

The combustion of carbon in carbon dioxide was undertaken using a tube containing copper oxide maintained at 850°C in a furnace. Silver wool was placed in the second portion of the tube to trap halogens and sulphur. A Nafion water trap, placed between the combustion furnace and the IRMS, was used to remove water produced during the combustion.

Pyrolysis of the sample for ^2H measurement as H_2 was conducted at higher temperature (1050°C) using a tube containing chromium powder. Chromium was used as a pyrolysis catalyser to convert organic components into H_2 . Furthermore, chromium is not affected by any carryover effects and directly traps other elements such as halogens and sulphur (Kelly et al., 2001).

The targeted molecules present a high percentage of sulphur, which can generate disruptions to metabolite combustion and/or pyrolysis within the interface. To prevent such perturbations, it is necessary to retain the sulphur by addition of silver wool in the combustion tubes. The pyrolysis temperature is too high for silver wool use, but fortunately, the chromium pyrolysis catalyser has a sulphur-trapping property.

2.2.3. GC-MC-ICP-MS

A Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) was coupled to a Neptune Plus MC-ICP-MS (Thermo Fisher Scientific) using a GCI 300 interface (Thermo Fisher Scientific). GC setting parameters were as follows: 1 μL of each diluted sample (20 μL in 0.5 mL of ethanol) or standard (10 μL in 1 mL of ethanol) was injected in split mode (120:1) with the injector temperature maintained at 300 °C. The helium carrier gas was regulated at a constant flow rate of 2 mL/min. Separation of volatile compounds was realised on a TraceGOLD TG-5MS capillary column (30 m \times 0.25 mm \times 0.25 μm ; Thermo Fisher Scientific).

209 Initial oven temperature was held at 150 °C for 4 min, then ramped at 20 °C/min to 300 °C and
210 held for 2 min. The transfer line temperature was maintained at 280 °C.

211 The Neptune Plus MC-ICP-MS was used in high-resolution mode to avoid any interference for
212 ^{32}S (O_2) and ^{34}S ($^{33}\text{S}^1\text{H}$ and O_2). Due to the low abundance of ^{33}S (0.76%), ratio precision with
213 ^{33}S was poor and finally not recorded. After acquisition (parameters are described in
214 **Supplementary Table S2**), data were analysed using IsoDat Software Version 3.0.94.12
215 (Thermo Fisher Scientific) for background subtraction, peak integration, and ratio calculation.
216 MC-ICP-MS optimisation was performed prior to connection with the GCI 300 interface using
217 an Aridus II desolvating system (Teledyne CETAC Technologies, Omaha, NE, USA) with a 1 mg/L
218 sulphur solution. Argon (1 L/min) was added via T piece inside the Trace 1310 GC to enhance
219 the low helium flow rate for separation (2 mL/min). The capillary exit from the GC column
220 entered the GCI 300 interface for approximately 13 cm with this piece. Argon surrounded the
221 capillary tube for this length and was mixed with helium inside the GCI 300 interface before
222 entering the torch injector. **Table S2** describes the applied parameters.

223 All samples were analysed in triplicate and were verified by bracketing the analytical sequence
224 with organosulfide standards every six analyses. Organosulfide standards were calibrated to
225 the international standard V-CDT (Vienna Canyon Diablo Troilite) using the EA-IRMS
226 technique.

227 In order to obtain an accurate isotope ratio measurement (Yang et al., 2018), the MC-ICP-MS
228 mass bias needs to be corrected, according to the Russell law (Russell et al., 1978):

$$229 \quad R(^{34}\text{S}/^{32}\text{S})_{\text{True}} = R(^{34}\text{S}/^{32}\text{S})_{\text{Measured}} * \left(\frac{34}{32}\right)^f \Leftrightarrow f = \frac{\ln \left[\frac{R(^{34}\text{S}/^{32}\text{S})_{\text{True}}}{R(^{34}\text{S}/^{32}\text{S})_{\text{Measured}}} \right]}{\ln \left[\frac{34}{32} \right]}$$

Where $R (^{34}\text{S}/^{32}\text{S})_{\text{Measured}}$ is the integrated $^{34}\text{S}/^{32}\text{S}$ ion-current ratio of samples; $R (^{34}\text{S}/^{32}\text{S})_{\text{True}}$ is the true $R (^{34}\text{S}/^{32}\text{S})$ value after correction; and f is the correction factor.

2.3. Statistical Analysis

Univariate and multivariate statistical analyses were performed using Xlstat© (Addinsoft, Paris). The Pearson correlation test was carried out to determine pairwise correlations between isotope ratio values. Unidimensional test of equality was realized to determine significant differences between species, within a confidence level $p\text{-value} < 0.05$.

Principal component analysis (PCA) (Jolliffe, 2002) was preliminary used in this study to describe and to assess the quality and homogeneity of the 42 samples of interest. Machine learning algorithm linear discriminant analysis (LDA) (Haghighat et al., 2016), and partial least square method – discriminant analysis (PLS-DA) (Tenenhaus, 1998) were then used to classify the different *Allium* species on the basis of their isotopic signature and chemical composition. Due to a small number of data points, a 7-fold stratified cross validation was repeated 20 times to avoid overfitting the small number of data-points. Features were ranked according to the variable importance projection (VIP) from PLS-DA. The model's quality was evaluated based on the residuals ($R^2\text{X}$, $R^2\text{Y}$) and the model's predictive ability parameter (Q^2_{cum}) (Levet et al., 2013). More information can be found in **Supplementary data**. The overall quality of models was also assessed using confusion matrix.

3. Results and discussion

3.1. Variations of the isotopic composition

The Bulk Stable Isotope Analysis (BSIA) and Compound Specific Isotope Analysis (CSIA) $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\delta^{34}\text{S}$ values of leek, shallot, chive, and onion essential oils are reported in **Table 1** and **Supplementary Table S3**.

3.1.1. BSIA and CSIA $\delta^{13}\text{C}$ analyses

Bulk $\delta^{13}\text{C}$ stable isotope ratio analyses show differences amongst the studied species (p-value $p < 0.05$). Leek essential oils present $\delta^{13}\text{C}$ values in the range of -35.41‰ to -36.79‰, which are slightly depleted compared with the values measured for other species. Chive essential oils have $\delta^{13}\text{C}$ values measured between -33.36‰ and -34.17‰, and onion essential oils have $\delta^{13}\text{C}$ values between -31.67‰ and -33.34‰. Shallot essential oils have a wider range of $\delta^{13}\text{C}$ values, spreading between -31.73‰ and -35.19‰. These $\delta^{13}\text{C}$ isotopic values correspond to essential oils from C3 plant, which is in line with the C3 plant origin of *Allium* species (Choi et al., 2020; Park et al., 2019; Pianezze et al., 2019). $\delta^{13}\text{C}$ typical values of C3 plants generally range from -34‰ to -24‰ (Krueger and Reesman, 1982) but it has been reported in some cases more negative values for $\delta^{13}\text{C}$ in essential oils extracted from C3 plants (Cuchet et al., 2019). To our knowledge, stable isotopic analysis on *Allium* essential oils have never been referenced in the literature before. However, isotopic analyses were realized on *Allium* raw materials. Park et al. (2019) presented an average $\delta^{13}\text{C}$ value of Korean onions ranging from -26.5‰ to -25.4‰, which differs from the analyses undertaken on onion essential oils. This could be due to the fact that raw material isotope signature could be influenced by cellulose, proteins, carbohydrates, and other constituents which are not present in essential oils. Moreover, this can also be attributed to isotopic fractionation during distillation processes. These *Allium* essential oils are peculiar amongst essential oils as they are produced from degradation when exposed to air, and from heating processes. This generates organosulphur compounds from precursors, such as allicine (Ramirez et al., 2017), which could result to more negative $\delta^{13}\text{C}$ values.

The global isotopic values for $\delta^{13}\text{C}$ are similar to the measured CSIA values, especially for DPDS and DPTS. However, the $\delta^{13}\text{C}_{\text{MPDS}}$ and $\delta^{13}\text{C}_{\text{MPTS}}$ values are systematically depleted compared

with $\delta^{13}\text{C}_{\text{DPDS}}$ and $\delta^{13}\text{C}_{\text{DPTS}}$ in all samples analysed. The $\delta^{13}\text{C}$ CSIA values are different amongst several vegetal species of this study, especially for leek essential oils, for which it can be noticed that the $\delta^{13}\text{C}_{\text{MPTS}}$ values (-40.41‰ to -43.59‰) are significantly different from those of the other essential oils: -33.98‰ to -36.91 ‰ in shallot essential oils, -34.31‰ to -35.15‰ in chive essential oils, and -35.42‰ to -37.81‰ in onion essential oils. The $\delta^{13}\text{C}_{\text{DPDS}}$ (-33.50‰ to -35.22‰) and $\delta^{13}\text{C}_{\text{DPTS}}$ (-34.88‰ to -35.97‰) values for leek essential oils are also more depleted.

3.1.2. *BSIA and CSIA $\delta^2\text{H}$ analyses*

Bulk $\delta^2\text{H}$ analyses from all of the studied species are relatively similar (leek, shallot and chive essential oil $\delta^2\text{H}$ values are spread between -218‰ and -227‰, p-value > 0.05), including samples from various geographical origins. However, deuterium isotope fractionation is noticeably more important in the case of onion essential oils ($\delta^2\text{H}$ between -172‰ and -251‰). Onion samples with higher $\delta^2\text{H}$ isotopic ratios were resulting from a different extraction process (OE9, OE10, and OE11, see **Supplementary table S1**), where the whole onion bulb was crushed. This grinding process accelerates the organosulfur precursors oxidation (Tocmo et al., 2014; Zhang et al., 2021), leading to a possible isotopic enrichment. The CSIA $\delta^2\text{H}$ natural isotopic values are significantly lower than the $\delta^2\text{H}$ synthetic values. Amongst the essential oils, the CSIA $\delta^2\text{H}$ values are relatively similar, except for onion essential oil batches in which we noticed a larger isotopic fractionation.

3.1.3. *BSIA and CSIA $\delta^{34}\text{S}$ analyses*

The $\delta^{34}\text{S}$ isotopic fingerprint of sulphur-containing volatile metabolites which is the components of *Allium* essential oils is dependent on sulphur issued from rainwater, soil, and external supply. The sulphur $^{34}\text{S}/^{32}\text{S}$ isotope ratio in plants might be influenced by several

factors, including geological characteristics of a location (Sulfides abundance) (Choi et al., 2020), climatic conditions (Nehlich, 2015), sea spray effect in coastal regions (Norman et al., 2006), and soils bacterial processes (Pianezze et al., 2019). $\delta^{34}\text{S}$ values are also affected by anthropogenic effects such as the sulphur supplied by fertilizers (Mizota and Sasaki, 1996) under the SO_4^{2-} sulfate ion form. Depending on the origin of the fertilizers used, the global isotopic ratio of ^{34}S can theoretically differ, offering the potential to observe different agricultural practices.

Egyptian leek essential oils have $\delta^{34}\text{S}$ values ranging from 9.0‰ to 10.4‰, as compared with $\delta^{34}\text{S}$ values from French essential oils, which show significantly more depleted $\delta^{34}\text{S}$ values (from 1.0‰ to 2.6‰). Egyptian samples are located in the Nile Delta region, and are probably affected by sulphates from the Nile Delta, which could explain these higher values. These results are also observed amongst onion samples of Egyptian and French origins.

The CSIA $\delta^{34}\text{S}$ values of DPDS, DPTS, and MPTS were determined via GC/MC-ICP-MS. In order to validate this recent technique for our type of samples, comparison measurements between CSIA and BSIA analyses were first done. Similarities were observed between CSIA $\delta^{34}\text{S}$ and BSIA $\delta^{34}\text{S}$, as the isotopic values were on the same scale, between -2.3 and 15.2‰. Furthermore, similar isotopic trends were observed between species and geographical origin. For example, the $\delta^{34}\text{S}_{\text{Bulk}}$ values of French leek essential oils were depleted compared to their Egyptian equivalent, as well as the compound-specific $\delta^{34}\text{S}$ values for DPDS, DPTS, and MPTS. This demonstrates the accuracy of values measured with the ICP methodology. However, a systematic discrepancy was also observed between IRMS and MC-ICP-MS values. The MC-ICP-MS is a precise isotope ratio measurement method, which usually achieve precise ratio determination using 20 to 40 cycles (4 to 8 seconds per cycle). However, in our configuration,

with the fast-transient signal obtained from the GC system, the MC-ICP-MS technology was at the limit of the Faraday cup detector response time, and to correct for that drawback, we used a Tau correction for the amplifiers. It is well known that MC-ICP-MS suffers mass bias problems from the torch, the interface, the lens stack, or the mass spectrometer, and it should be corrected to obtain the true ratio (Yang et al., 2018). On Sr, Hf, or Nd isotope measurements, it is possible to use a stable ratio for mass bias correction. When this is not possible (Pb or Cu for example), a correction with another element (like Zn or Tl) can be used. However, for sulfur measurements, there is no stable ratio available, and the addition of another element for mass bias correction is not possible because the mass dispersion authorized by the spectrometer is not sufficient. The only way to do the measurement is the sample bracketing technique (Amrani et al., 2009; Clough et al., 2006, Said-Ahmad and Amrani, 2013). Unfortunately, the absolute value for $^{32}\text{S}/^{34}\text{S}$ cannot be obtained due to the impossibility to correct for mass bias, which explains the systematic discrepancy observed between IRMS and MC-ICP-MS results. The relative differences between the samples obtained with GC/MC-ICP-MS still supply significant $\delta^{34}\text{S}$ to characterise our samples.

Measured $\delta^{34}\text{S}$ isotopic values differed amongst species and geographical origins. Egyptian leek essential oils presented higher $\delta^{34}\text{S}_{\text{DPDS}}$ (11.7-15.2‰) and $\delta^{34}\text{S}_{\text{DPTS}}$ (10.4-12.8‰) values compared with French leek values ($\delta^{34}\text{S}_{\text{DPDS}}$, 6.9-7.7‰; $\delta^{34}\text{S}_{\text{DPTS}}$, 2.4-4.9‰). However, the ranges were similar for $\delta^{34}\text{S}_{\text{MPTS}}$ values, between 2.5 and 11.2‰. German shallot samples presented higher $\delta^{34}\text{S}_{\text{DPTS}}$ values (4.6-7.8‰) than French samples (2.7- 4.6‰). However, shallot essential oil values for $\delta^{34}\text{S}_{\text{DPDS}}$ (6.4- 8.8‰) and $\delta^{34}\text{S}_{\text{MPTS}}$ (0.1-5.4‰) were within the same range for both geographical origins. Sulphur isotope fractionation was noticeably important in the case of onion essential oils. Egyptian and French onion samples presented $\delta^{34}\text{S}_{\text{DPDS}}$ values between 5.1 and 10.7‰, $\delta^{34}\text{S}_{\text{DPTS}}$ values between 3.7 and 10.2‰, and $\delta^{34}\text{S}_{\text{MPTS}}$

values between -2.30 and 6.1‰. Moreover, chive essential oils presented $\delta^{34}\text{S}_{\text{DPDS}}$ values ranging between 3.92 and 8.8‰; $\delta^{34}\text{S}_{\text{DPTS}}$ values between 1.6 and 3.5‰; and $\delta^{34}\text{S}_{\text{MPTS}}$ values between -0.2 and 3.5‰.

3.2. Naturalness authentication

^{13}C , ^2H , and ^{34}S stable isotope signature can differ between natural and synthetic molecules. Synthetic-origin molecules are not subject to the same isotopic fractionation phenomenon than natural metabolites, as they are not produced from similar pathways. Whereas *Allium* natural organosulfides compounds are produced from biosynthesis pathways, synthetic components are produced from petrochemical processes. Alkyl and allyl sulphides, from *Allium* essential oils, are generated during heating process from allicin and other thiosulfinates degradation (Ramirez et al., 2017). These secondary metabolites represent the enzymatic hydrolysis product of alkyl cysteine sulfoxides (ACSO) (Jones et al., 2004) which are produced from several biosynthesis steps influenced notably by the assimilation of SO_4^{2-} , and the photosynthesis pathways of CO_2 . Syntheses of organic disulfides molecules has been usually prepared by nucleophile substitution reactions of alkyl halides with sulphur containing products (Abbasi et al., 2016; Bhaumik and Misra, 2017; Soleiman-Beigi and Mohammadi, 2012). All of these pathways generate symmetric alkyl disulfides with elevated yields. Other common synthetic pathways use a sulfonate (Filippi et al., 2007).

3.2.1. Analysis of synthetic organosulfides

In order to compare natural and synthetic compounds, synthetic $\delta^{13}\text{C}_{\text{DPDS}}$ were measured, generating values between -28.52‰ and -26.26‰, significantly different from natural values between -31.66‰ and -36.79‰. The same observations were noted for DPTS and MPTS. Nevertheless, the $\delta^{13}\text{C}$ value of one MPDS synthetic compound (-37.17‰) is comparable to

the $\delta^{13}\text{C}$ value from MPDS in essential oils, whereas, considering hydrogen data, its $\delta^2\text{H}$ value is enriched, which outlines the power of multi-isotope approaches. Most synthetic commercial standards have shown enriched $\delta^{13}\text{C}$ values (-26.18‰ to -28.68‰) compared with the natural essential oils reported in the study. Those results are in line with the literature. Synthetic compounds are petrochemicals by-products, which have generally a $\delta^{13}\text{C}$ value ranging between -25 and -30‰ (Krueger and Reesman, 1982). Based on this significant $\delta^{13}\text{C}$ isotopic gap between natural and synthetic origins for those compounds, adulteration of essential oils with addition of synthetic molecules to natural products could be clearly established with $\delta^{13}\text{C}$ bulk analyses. Therefore, we have established an efficient indicator of authentication for *Allium* essential oils.

As it was the case for $\delta^{13}\text{C}$ values, synthetic standard molecules show more positive $\delta^2\text{H}$ values (ranging from -105‰ to -170‰), and thus adulteration of natural *Allium* extract with synthetic compounds is expected to increase the $\delta^2\text{H}$ bulk value and could also be detected. A majority of synthetic samples present $\delta^{34}\text{S}$ values close to 0‰ (ranging from 2.0‰ to -3.1‰), except for one MPDS sample that has a more positive value (7.3‰). Differentiation was realised between synthetic DPDS and DPTS molecules and natural essential oils of *Allium* species. Synthetic DPDS values of $\delta^{34}\text{S}$ (-0.8‰ and -3.0‰) and $\delta^{13}\text{C}$ (-28.52‰ and -26.26‰) and the synthetic DPTS $\delta^{13}\text{C}$ value (-26.18‰) differed significantly from natural isotopic values. However, synthetic (2.0‰) and natural $\delta^{34}\text{S}_{\text{DPTS}}$ values were within the same range. Comparisons between $\delta^{34}\text{S}$ values from natural and synthetic compounds are also promising.

3.2.2. Correlation study

A univariate Pearson's correlation is realized on the data set to determine correlation coefficients and pairwise correlations, described in **Supplementary Table S4**. It appears that

several data are linearly correlated with each other: a correlation of $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ values determined on *Allium* metabolites DPDS, DPTS, MPDS, and MPTS, was established, especially $\delta^{13}\text{C}_{\text{DPDS}}$ and $\delta^{13}\text{C}_{\text{DPTS}}$ ($r=0.94$), $\delta^{13}\text{C}_{\text{DPDS}}$ and $\delta^{13}\text{C}_{\text{MPTS}}$ ($r=0.84$), and $\delta^{34}\text{S}_{\text{DPDS}}$ and $\delta^{34}\text{S}_{\text{DPTS}}$ ($r=0.84$). These $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ isotopic filiations between organosulphur metabolites were expected as these metabolites are sulphur-enriched from the same sulphite SO_3^{2-} source. Furthermore, the biosynthesis pathways at the origin of their formation during plant photosynthesis are composed of similar precursors. All metabolites DPDS, DPTS, MPDS, and MPTS were affected by the same isotopic fractionation effects. However, no correlation was observed with $\delta^2\text{H}$ analysis, which is probably because $\delta^2\text{H}$ values are mainly affected by climatic effects / geographical origins. This $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ linear correlation is useful in authentication as the potential addition of the same metabolite from a different source (synthetic or natural) will not show the same correlation. For instance, this means that an addition of DPDS or DPTS from another source can be highlighted. This is an important result for the use of the CSIA method in an authentication approach.

3.2.3. Adulteration of samples by synthetic samples

To evaluate the detection limits for the addition of synthetic molecules in essential oil, a known sample of leek essential oil was adulterated with 5%, 10%, and 20% of DPDS and DPTS from synthetic origins. Results are presented in **Fig. 1**. It is observed that due to a different isotopic ratio between DPDS/DPTS from synthetic and natural origins, the adulterated samples no longer fall on the previously obtained correlation line.

The CSIA approach allows the comparison between metabolites contained in the various essential oil that were investigated. The result of those isotopic measurements is that the combination of $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\delta^{34}\text{S}$ values supply useful information for deciphering the

authentication of *Allium* natural products. Moreover, the combination of $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ values can be used to discriminate synthetic molecules and natural essential oils and thereby indicate potential adulterations.

3.3. Multivariate analyses – Chemometric study

In order to understand the capability of our variables to predict the species origin of the *Allium* essential oil samples, a multivariate analysis was carried out on the entire sample data set (42 samples), with all the 17 following parameters as variables: BSIA ($\delta^{13}\text{C}_{\text{Bulk}}$, $\delta^2\text{H}_{\text{Bulk}}$, $\delta^{34}\text{S}_{\text{Bulk}}$) and CSIA ($\delta^{13}\text{C}_{\text{DPDS}}$, $\delta^{13}\text{C}_{\text{DPTS}}$, $\delta^{13}\text{C}_{\text{MPDS}}$, $\delta^{13}\text{C}_{\text{MPTS}}$, $\delta^2\text{H}_{\text{DPDS}}$, $\delta^2\text{H}_{\text{DPTS}}$, $\delta^2\text{H}_{\text{MPDS}}$, $\delta^{34}\text{S}_{\text{DPDS}}$, $\delta^{34}\text{S}_{\text{DPTS}}$, $\delta^{34}\text{S}_{\text{MPTS}}$) stable isotope data, as well as relative concentration of major organosulfides (DPDS, DPTS, MPDS, and MPTS). This statistical analysis consisted in a preliminary PCA to describe the dataset, followed by a LDA and a PLS DA analyses to classify the samples and exhibit clusters. The main objective of this statistical multivariate analysis is to authenticate the species origin of the samples. Although many studies describe the possibility to assess the geographical origin of samples using isotopic data and chemometric (Drivelos and Georgiou, 2012; Diomande et al., 2015), it has not been realised here as the dataset does not have enough representative samples of each origin.

3.3.1. Principal Component Analysis

We depict scores and loadings plots of the first two principal components (F1 and F2) of the PCA in **Fig. 2**, which constituted 63.5% of the total variance. PCA supported our earlier observations. Species differentiation was observed, especially for leek essential oils. Onion essential oils were highly scattered, because of their large isotopic fractionation. Individual clusters were identified for shallot and chive essential oils. Shallot and chive essential oils were

closely grouped however, even if a slight separation was exhibited on F2. These two essential oils have closely related isotopic range values and share similar compositions.

Essential oils from leek were also grouped according to the geographical origin of the raw material, being the two samples from France allocated in more negative values of F2. The variables involved in this separation are hydrogen isotopic ratios ($\delta^2\text{H}_{\text{Bulk}}$, $\delta^2\text{H}_{\text{DPDS}}$, $\delta^2\text{H}_{\text{DPTS}}$, and $\delta^2\text{H}_{\text{MPDS}}$), which are known to be highly dependent on geographical origins (Pianezze et al., 2019; Hoefs, 2018). Shallot essential oils were also geographically separated in component F2, making the use of $\delta^2\text{H}$ ratios more robust for geographic assignments.

3.3.2. Discriminant models (LDA and PLS-DA)

Classification of the oil extracts according to allium species was first addressed by means of linear discriminant analysis (LDA) using all the stable isotopes and the chemical composition. This statistical model is one of the most popular multivariate analytical tool to highlight data clusters in authentication. Unlike PCA, LDA considers differences between data classes. The analytical target was **set** to increase the separation between species. LDA scores and loadings plots can be observed in **Fig. 3**; grouped by species. The confusion matrix for estimated samples is displayed on **Table 2**. Only BSIA isotope values were first included in the LDA study, but only 85.7% of samples were well classified, with several shallot and onion essential oils samples wrongly attributed. This study showed that the use of CSIA isotope values and relative concentration of principal metabolites lead to 100% of classification of species origin. With regard to species, score plots of the linear discriminants F1 (80.0%) and F2 (14.5%) constituted 94.5% of the total variance. Several parameters highly contribute to the clustering. F1 correlated positively notably with $\delta^{34}\text{S}_{\text{Bulk}}$ (0.602), $\delta^{34}\text{S}_{\text{DPDS}}$ (0.622), $\delta^{34}\text{S}_{\text{DPTS}}$ (0.534), $\delta^{34}\text{S}_{\text{MPDS}}$ (0.542), %DPDS (0.909), and %MPDS (0.721), and negatively with $\delta^{13}\text{C}_{\text{Bulk}}$ (-0.819), $\delta^{13}\text{C}_{\text{DPDS}}$ (-

0.701), $\delta^{13}\text{C}_{\text{DPTS}}$ (-0.726), $\delta^{13}\text{C}_{\text{MPDS}}$ (-0.552), $\delta^{13}\text{C}_{\text{MPTS}}$ (-0.937), and $\%_{\text{MPTS}}$ (-0.653); whereas F2 correlated negatively with $\delta^{34}\text{S}_{\text{DPTS}}$ (-0.531), and positively with $\%_{\text{MPTS}}$ (0.568).

In order to conclude the study and establish a predictive statistical model, a partial least square – discriminant analysis (PLS-DA) was also conducted. PLS-DA scores and loadings plots are represented in **Fig. 4**. The component F1 is mostly correlated positively with $\delta^{13}\text{C}$ isotopic values ($\delta^{13}\text{C}_{\text{Bulk}}$ (0.814), $\delta^{13}\text{C}_{\text{DPDS}}$ (0.839), $\delta^{13}\text{C}_{\text{DPTS}}$ (0.886), and $\delta^{13}\text{C}_{\text{MPDS}}$ (0.934)), and negatively with $\delta^{34}\text{S}$ isotopic values and organosulfides composition ($\delta^{34}\text{S}_{\text{Bulk}}$ (-0.738), $\delta^{34}\text{S}_{\text{DPDS}}$ (-0.828), $\delta^{34}\text{S}_{\text{DPTS}}$ (-0.691), $\delta^{34}\text{S}_{\text{MPTS}}$ (-0.710), $\%_{\text{DPDS}}$ (-0.811), and $\%_{\text{MPDS}}$ (-0.797)). The component F2 is mostly correlated negatively with $\delta^2\text{H}$ isotopic values ($\delta^2\text{H}_{\text{Bulk}}$ (-0.520), $\delta^2\text{H}_{\text{DPDS}}$ (-0.710), $\delta^2\text{H}_{\text{DPTS}}$ (-0.535), and $\delta^2\text{H}_{\text{MDPS}}$ (-0.484)). Essential oils from leek presented more negative values on F1 than other *Allium* species, whereas shallot, onion, and chive essential oils were separated in component F2. According to the **Table 2**, varieties were significantly clustered with 100% of sample classification. The performances of this model in this study are comparable to the LDA. Both models successively allow to distinguish the different classes of *Allium* species.

Conclusion

Authentication of essential oils of *Allium* varieties was performed using bulk stable and compound specific isotope analysis of carbon, hydrogen, and sulphur elements determined in EA-IRMS, and GC-IRMS, as well as GC/MC-ICP-MS. This last technique was used for the first time to measure organic compounds in essential oils. The isotopic filiation demonstrated in $\delta^{13}\text{C}$ and $\delta^{34}\text{S}$ produces better precision in authenticating naturalness of these types of essential oils. We demonstrated that an addition of 5% of synthetic-origin DPDS or DPTS can be detected. The results also showed that this methodology is applicable to assess **EOs** purity. Combination of BSIA, CSIA, and metabolite composition leads to varieties discrimination: LDA

and PLS-DA models performances achieved 100% of sample classification. Furthermore, the methodology could be employed to improve the authentication of other sulphur component-based essential oils, such as garlic *Allium sativum* and other *Allium* essential oils composed of allyl components, such as *A. ursinum* and *A. tuberosum* essential oils.

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Declaration of Competing Interests

The authors declare that there are no conflicts of interest.

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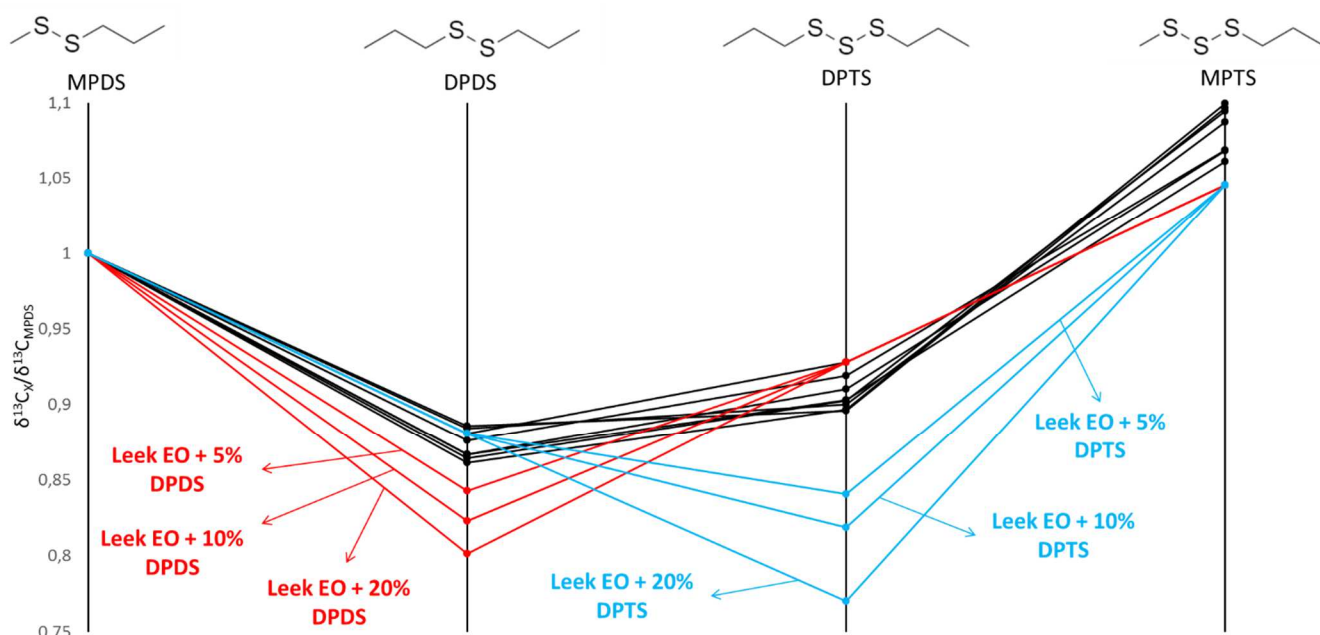
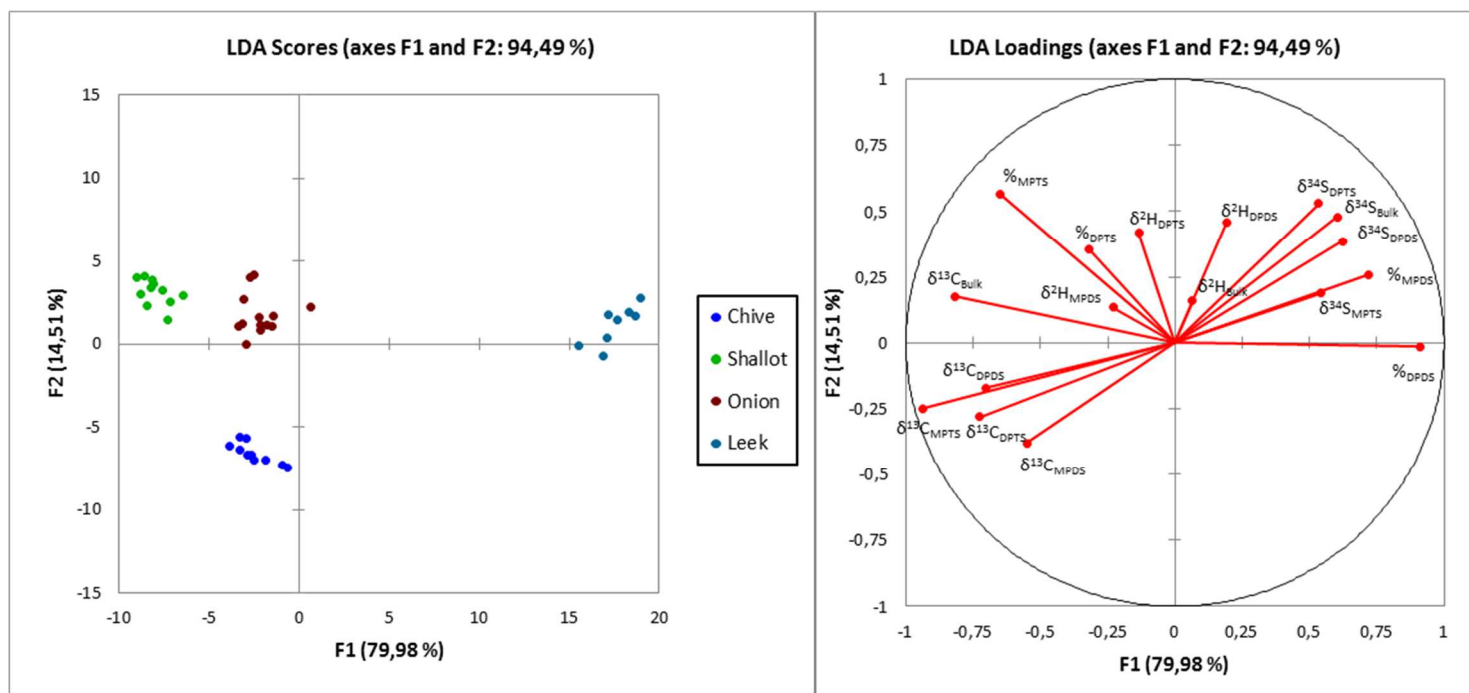


Fig. 1. Parallel plots of $\delta^{13}\text{C}_{\text{MPDS}}$, $\delta^{13}\text{C}_{\text{DPDS}}$, $\delta^{13}\text{C}_{\text{DPTS}}$, and $\delta^{13}\text{C}_{\text{MPTS}}$ values compared with $\delta^{13}\text{C}_{\text{MPDS}}$ and measured on 8 natural leek essential oils (in black), one essential oil adulterated with 5, 10, and 20% of synthetic DPDS (in red), and one essential oil adulterated with 5, 10, and 20% of synthetic DPTS.



667

668 **Fig. 3.** Linear Discriminant Analysis (LDA) scores and loadings plots of all isotopic values (BSIA

669 and CSIA) and DPDS, DPTS, MPDS, and MPTD chemical composition, determined on *Allium*

670 essential oils samples grouped by species

671

Table 1. Mean, max, min, and standard deviation (SD) of bulk stable and compound-specific $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\delta^{34}\text{S}$ isotope values, and DPDS, DPTS, MPDS, and MPTD chemical composition, of leek, shallot, chive, and onion essential oils (n=number of samples).

Isotopic Values	Leek essential oils (n=8)				Shallot essential oils (n=11)				Chive Essential oils (n=10)				Onion essential oils (n=13)			
	Mean	Max	Min	SD	Mean	Max	Min	SD	Mean	Max	Min	SD	Mean	Max	Min	SD
$\delta^{13}\text{C}_{\text{bulk}} (\text{‰})$	-36,02	-34,63	-36,79	0,73	-32,41	-31,73	-35,19	1,35	-33,61	-33,31	-34,17	0,29	-32,63	-31,67	-34,32	0,65
$\delta^2\text{H}_{\text{bulk}} (\text{‰})$	-218	-211	-227	6	-223	-218	-226	2	-223	-217	-227	3	-209	-172	-251	25
$\delta^{34}\text{S}_{\text{bulk}} (\text{‰})$	7,5	10,4	1,0	3,7	3,0	4,1	-0,2	1,6	0,7	1,4	0,0	0,5	3,9	7,2	1,2	1,7
$\delta^{13}\text{C}_{\text{DPDS}} (\text{‰})$	-34,27	-33,50	-35,22	0,64	-32,14	-31,52	-34,11	0,96	-32,08	-31,66	-32,44	0,25	-32,43	-30,00	-34,13	1,00
$\delta^2\text{H}_{\text{DPDS}} (\text{‰})$	-189	-145	-251	42	-198	-184	-216	9	-227	-216	-252	12	-199	-164	-253	30
$\delta^{34}\text{S}_{\text{DPDS}} (\text{‰})$	12,1	15,2	6,9	3,2	8,0	8,8	6,4	0,8	6,1	8,8	3,9	1,4	7,9	10,7	5,1	1,9
$\delta^{13}\text{C}_{\text{DPTS}} (\text{‰})$	-35,59	-34,88	-35,97	0,41	-33,16	-32,36	-35,43	1,12	-32,68	-32,15	-33,14	0,32	-33,27	-31,12	-34,95	1,02
$\delta^2\text{H}_{\text{DPTS}} (\text{‰})$	-217	-204	-251	15	-196	-154	-228	25	-236	-164	-266	36	-208	-152	-273	37
$\delta^{34}\text{S}_{\text{DPTS}} (\text{‰})$	9,7	12,9	2,4	3,9	5,8	7,8	2,7	1,4	2,9	3,5	1,6	0,6	6,8	10,2	3,7	1,8
$\delta^{13}\text{C}_{\text{MPDS}} (\text{‰})$	-39,24	-38,66	-39,81	0,45	-37,55	-36,97	-38,87	0,66	-37,19	-36,10	-38,17	0,63	-38,56	-35,69	-40,13	1,02
$\delta^2\text{H}_{\text{MPDS}} (\text{‰})$	-229	-211	-253	13	-220	-184	-237	16	-221	-171	-249	20	-201	-162	-231	21
$\delta^{13}\text{C}_{\text{MPTS}} (\text{‰})$	-42,29	-40,41	-43,59	1,13	-34,78	-33,98	-36,91	1,00	-34,67	-34,31	-35,15	0,24	-36,37	-34,65	-37,81	0,73
$\delta^{34}\text{S}_{\text{MPTS}} (\text{‰})$	6,5	11,2	2,5	3,0	2,9	5,4	0,1	1,8	1,5	3,5	-0,2	1,3	1,0	6,1	-2,3	2,5
%DPDS	38,4	46,2	34,7	3,5	18,5	20,7	14,0	2,3	22,5	26,6	17,0	3,0	21,5	28,3	14,4	4,1
%DPTS	12,7	14,2	10,4	1,5	16,0	18,5	8,5	3,5	12,7	15,7	8,6	2,1	14,9	17,9	9,5	2,9
%MPDS	5,5	7,9	2,1	2,1	2,4	3,9	1,5	0,7	1,6	2,3	0,7	0,5	1,9	4,6	1,2	0,9
%MPTS	4,9	5,7	4,4	0,6	8,7	9,2	7,9	0,4	5,4	6,3	4,9	0,4	6,9	10,7	5,5	1,3

683 **Table 2.** LDA and PLS-DA confusion matrices using BSIA, CSIA, and chemical composition as
684 variables (17 variables)

Model	from \ to	Chive	Shallot	Onion	Leek	%correct
LDA	Chive	10	0	0	0	100%
	Shallot	0	11	0	0	100%
	Onion	0	0	13	0	100%
	Leek	0	0	0	8	100%
PLS-DA	Chive	10	0	0	0	100%
	Shallot	0	11	0	0	100%
	Onion	0	0	13	0	100%
	Leek	0	0	0	8	100%

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