

δ 180 compound-specific stable isotope assessment: An advanced analytical strategy for sophisticated adulterations detection in essential oils - Application to spearmint, cinnamon, and bitter almond essential oils authentication

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 strategy for sophisticated adulterations detection in essential oils Application to spearmint, cinnamon, and bitter almond essential oils
 authentication.

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20 Abstract

Natural plant extracts are primarily used as raw materials in the cosmetic and perfumery 21 industry. However, adulterations with petrochemical products are occurring in the market, 22 leading to non-100% natural products. Several analytical techniques such as impurity 23 detection or enantioselective ratio assessments have been previously described as good 24 indicators to detect any addition of synthetic products, but these techniques are ineffective 25 with novel type of synthetic pathways such as semisynthesis. 26 In order to improve authentication, development of advanced analytical strategies such as δ^{18} O stable isotopic 27 ratios assessment was tested on spearmint, cinnamon and bitter almond essential oils major 28 metabolites (carvone, (E)-cinnamaldehyde, and benzaldehyde). Natural δ^{18} O mean values 29 $(\delta^{18}$ OCarvone = 18.4‰; δ^{18} OCinnamaldehyde = 13.9‰; δ^{18} OBenzaldehyde = 16.5‰) were 30 found to be 29 higher than semisynthetic origin for the 3 studied molecules (δ^{18} OCarvone = 31 9.2%; 30 δ^{18} OCinnamaldehyde = 8.8%; δ^{18} OBenzaldehyde = 10.9%). These measurements 32 proved to be efficient to discriminate natural and semisynthetic origins of these components 33 34 and therefore potentially lead to a novel way to authenticate natural products.

35 Keywords

Semisynthesis; Essential oil; Gas chromatography; Isotope ratio mass spectrometry;
 Authentication

39 1. Introduction

Natural aromatic raw materials market is growing exponentially nowadays to supply food 40 industry, perfumery and cosmetics. To satisfy this increasing demand, researches have been 41 42 focused on compounds issued from biotechnology processes used to produce aromas and fragrances from other plant origins. Unfortunately, these new technologies can lead to new 43 44 sophisticated frauds. The adulterations found on the natural product market are constantly 45 evolving and it is nowadays possible to observe additions of biotechnological origin molecules. These new processes have the capability to create seemingly natural molecules 46 47 such as the vanillin, which has been regularly obtained from the ferulic acid contained in rice [1], or the frambinone obtained from 4-hydroxybenzaldehyde contained in *Escherichia coli* 48 [2,3]. 49

In the regulation field of essential oils, molecules must come from the raw material of the 50 51 plant reference to be considered and labelled 100% pure and natural. Molecules produced 52 from natural reagents, also called semisynthetic compounds, are suspected of being used to adulterate specific essential oils. Several examples are cited in the literature. 53 Cinnamaldehyde molecules can be made from bitter almond benzaldehyde and used to 54 55 adulterate an essential oil of cinnamon [4]. Greule et al. reported the use of carvacrol, produced from the carvone of caraway essential oil, to adulterate an essential oil of oregano 56 57 [5]. Semisyntheses enable fraudsters to bypass many analytical controls.

An essential oil that has undergone an addition of a semisynthetic molecule will be more difficult to detect than traditional synthetic molecule additions with usual analytical methods of authentication. One of the most common analysis is the determination of enantiomeric ratios to control chiral molecules such as linalool, because synthetic chiral

62 molecules are produced in racemic proportion, whereas natural molecules are generally stereospecific [6]. However, enantiomeric analyses will not be effective in the case of 63 semisyntheses because the stereochemistry will be preserved. Another common analytical 64 technique is the determination of radiocarbon activity, which determine the presence of 65 fossil carbon in the sample [7]. However, a semisynthesis will eventually conduct to a 100% 66 67 bio based carbon molecule because no petrochemicals would be used. Finally, stable isotope analyses of carbon and hydrogen will generally be inefficient to demonstrate this addition, 68 69 because the transformations carried out will not generate significant isotopic variations of δ^{13} C and δ^{2} H ratios. Typical examples are the conversion of limonene into carvone or 70 benzaldehyde into cinnamaldehyde. 71

72 However, several analytical strategies are possible. A first possibility is the detection of 73 residual impurities from semisyntheses. This approach has numerous flaws, as many 74 synthetic pathways do not produce any impurities, and these residual molecules can be removed with a purification process [7]. A second analytical approach is the Site-Specific 75 76 Natural Isotope Fractionation-Nuclear Magnetic Resonance (SNIF-NMR) technique. This 77 system allows the measurement of isotopic fractionations of specific sites of a molecule, 78 which can lead to the origin of the product [8]. Several authentication studies have been conducted using SNIF-NMR [4,9,10]. However, this analytical tool requires that the analysed 79 molecules are almost entirely pure, which is difficult to achieve on complex matrices such as 80 essential oils. In addition, there is a risk of isotopic fractionation during the purification step. 81 Since SNIF-NMR is a relatively insensitive technique, it also requires using a large amount of 82 83 sample for analysis.

Analysis of oxygen stable isotopes can be an interesting approach. In many cases of semisynthesis of oxygenated terpenes or phenylpropanoids, the synthesis is characterized by the addition of an external oxygen atom from the original molecule. The δ^{18} O ratios can therefore be different, and it seems relevant to measure them. To determine this isotope ratio, the use of a GC-P-IRMS instrument is necessary to be used on most of the essential oils. This analytical system to operate is rarely used in authentication on natural products due to difficulties in the analytical process.

This article describes the development of the δ^{18} O analysis by GC-P-IRMS to highlight the additions of semisynthetic-origin compounds (carvone, (E)-cinnamaldehyde, and benzaldehyde) in essential oils of spearmint, cinnamon and bitter almond.

94

2. Material and methods

97 **2.1.** *Materials*

98 2.1.1. Essential oils

99 Forty-eight samples of spearmint *Mentha spicata var crispa*, from India, USA, China, and 100 France were analysed for this study. From these samples, 11 certified 100% pure and natural 101 samples were provided by Albert Vieille SAS (Vallauris, France) and Young Living (Lehi, Utah, 102 USA), and one sample was obtained from hydrodistillation process from fresh spearmint 103 leaves at the Institute of Analytical Sciences (ISA Lyon, UMR5280 - CNRS, Villeurbanne, 104 France) laboratory. Other essential oils (n=36) were commercial samples, obtained from 105 various sources.

106 Fifty-five samples of cinnamon essential oils were analysed for this study. These samples were obtained from various botanical and geographical origins: Cinnamomum verum 107 108 essential oils were sourced from Sri Lanka and Madagascar, Cinnamomum cassia essential 109 oils were sourced from China and Cinnamomum burmanii (Nees & T.Nees) Blume (Indonesia) came from Indonesia. Authentic samples (n=11) were provided by Albert Vieille SAS, Astérale 110 111 (Madagascar), and Young Living. Moreover, homemade distillations of cinnamon bark were 112 realized in the ISA Lyon laboratory to obtain cinnamon essential oils from several botanical origins. Other samples were commercial essential oils (n=44). 113

In addition, 12 pure and natural samples of bitter almond essential oils, obtained from steam distillation apricots almonds *Prunus armeniaca* L. have also been analysed. These essential oils were provided by Albert Vieille SAS and are originated from Morocco. For information, these samples were subjected to a cyanhydric acid disposal process prior to analysis.

2.1.2. Commercial molecules

120 Commercial molecules of carvone (R) (n=3), (E)-cinnamaldehyde (n=1), and benzaldehyde 121 (n=3) were purchased from TCI (Tokyo, Japan), Alfa Aesar (Ward Hill, MA, USA) and Sigma 122 Aldrich (Saint Louis, MO, USA).

123

2.1.3. Syntheses

124

2.1.3.1. Synthesis of carvone (R) from limonene (S)

Several carvone molecules were obtained from limonene conversion. The synthesis of the carvone has been carried out in 3 steps based on Rothenberger et al. protocol [11] (Figure 1). The first step leads to a first intermediate molecule: the limonene nitrosochloride (1). The second step leads to the synthesis of carvoxime (2). This compound is then used to produce the carvone (3).

In order to perform this synthesis, the limonene is firstly diluted in isopropanol at a 130 131 temperature comprised between 0°C and 10°C. A solution of 37% chloridric acid and 132 isopropanol is then gradually added to the mixture. The obtained solution is then mixed with NaNO₂ in deionised water. Limonene nitrosochloride product is then purified using Buchner 133 vacuum filtration and traces of water were removed using a vacuum desiccator. This product 134 135 is then dissolve in dimethylformamide and isopropanol. The mixture is heated to reflux. Carvoxime product is then extract using diethyl ether and the organic phase is washed with 136 137 ultra-pure water until neutral pH. The solution is then dried using anhydrous sodium sulfate, 138 then filtered. Diethyl ether is then evaporated and the carvoxime is dissolved in an aqueous solution of oxalic acid (5%), then heated at reflux for 30 minutes. The carvone final product 139 is then obtained by steam distillation. 140

2.1.3.2. Aldol reaction of benzaldehyde

143 Ten samples of (E)-cinnamaldehyde were produced in laboratory using aldolisation of 144 benzaldehyde from synthesis origin and natural origin from essential oils of bitter almond 145 (Figure 2). The synthesis protocol is described below.

A volume of 600 μL of benzaldehyde was diluted in 3 mL of ethanol 96%. The mixture was
maintained at 0°C. 1 mL of NaOH 15% was ten added and the solution was stirred for 5
minutes. A volume of 200 μL of acetaldehyde (TCI and Sigma Aldrich) was then added at 0°C.
This step is renewed four times every 15 minutes to make sure that the acetaldehyde is
maintained in excess during the reaction entire time. The mixture is then heated at 40°C
during 10 minutes. Highly concentrated hydrochloric acid is then added to acid pH. Diethyl
ether extraction was then carried out to collect the (E)-cinnamaldehyde final product.

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2.1.3.3. Retro aldol reaction of cinnamaldehyde

Five samples of benzaldehyde have been produced in laboratory by retro aldol reaction of synthetic origin (E)-cinnamaldehyde (Fluka) and natural origin (E)-cinnamaldehyde from essential oils of *Cinnamomum verum, Cinnamomum cassia,* and *Cinnamomum burmanii*.

157 Ten grams of (E)-cinnamaldehyde are firstly diluted in an aqueous solution of sodium 158 carbonate Na_2CO_3 (3%). This mixture was heated at reflux during 7 hours. A steam 159 distillation was then carried out to extract the benzaldehyde.

160

161

2.2. Isotopic Analyses

164 **2.2.1.** Data Expression

165 Isotopic results are expressed in delta (δ) units according to the following formula [30]:

$$\delta^{i} \mathbf{E} = \frac{({}^{i} \mathbf{R}_{sample} - {}^{i} \mathbf{R}_{Reference})}{{}^{i} \mathbf{R}_{Reference}}$$

166 where i is the mass number of the heavy isotope of element E, R_{Sample} is the isotope ratio of 167 the sample, and $R_{Reference}$ is the isotope ratio of a relevant internationally recognized 168 reference material. The δ values were multiplied by 1000 and are expressed in units "per 169 mil" (‰).

High-purity gases of carbon dioxide (99.998%, 4.8 Messer), hydrogen (99.999%, 5.0 Messer),
and carbon monoxide (99.997%, 4.7 Messer) were used as the working reference gases
injected with each sample. Helium (99.999% purity) was used as a carrier gas.

All studied metabolites in the essential oils of interest were calibrated with commercial molecules (carvone, (E)-cinnamaldehyde, and benzaldehyde) previously calibrated against international standards in elemental analysis – IRMS (EA-IRMS).

International reference materials IAEA CH7 (polyethylene foil: $\delta^{13}C$ = -32.15‰, $\delta^{2}H$ = -176 100‰), IAEA 601 (benzoic acid: δ^{18} O = 23.3‰), and USGS40 (L-glutamic acid: δ^{13} C = -177 26.39‰), supplied by the International Agency of Atomic Energy (IAEA, Vienna, Austria), 178 were used to calibrate each molecule in the mixture, which allowed us to express our δ^{13} C 179 results relative to the Vienna Pee Dee Belemnite (VPDB) scale and our δ^2 H and δ^{18} O results 180 relative to the Standard Mean Ocean Water (VSMOW) scale. Each sample was analyzed 181 versus a monitoring gas for which two pulses were admitted with each analyte. All sample 182 values were analyzed in duplicate and verified by bracketing the analytical sequence with 183

international reference materials every six analyses. δ^{13} C isotopic analyses of the standards molecules were conducted using a Flash HT elemental analyzer connected to a Delta V Plus via a Conflo V interface. The δ^2 H and δ^{18} O measurements were carried out using a TC-EA elemental analyzer linked to a Delta V Plus via a Conflo V interface (all Thermo Fisher Scientific). The method is fully explained in a previous study [7].

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Isotope Ratio Mass Spectrometry (GC-MS-C/P-IRMS)

2.2.2. Gas Chromatography - Mass Spectrometry - Combustion/Pyrolysis -

191 The δ^{13} C, δ^{2} H, and δ^{18} O values of targeted spearmint, bitter almond, and cinnamon essential 192 oils metabolites were determined by GC-IRMS using a GC Trace 1310 simultaneously 193 connected to an ISQ MS quadrupole mass spectrometer and to a Delta V Plus isotope ratio 194 mass spectrometer via a GC Isolink II interface. (Thermo Fisher, Bremen, Germany). This 195 equipment enabled the identification of compounds via the ISQ MS instrument and specific 196 compound isotopic ratio measurements of δ^{13} C, δ^{2} H, and δ^{18} O via IRMS.

197 One microliter of diluted essential oil (1 µL into 100 µL of ethanol) was injected using a Tri Plus RSH autosampler in a Trace 1310 gas chromatograph equipped with an Agilent HP-1 198 capillary column (50 m * 0.320 mm * 0.52 µm internal diameter film thickness) used for 199 chromatographic separation. The injection port was held at 260°C, fitted with a split liner 200 201 containing glass wool and operated in split mode (10:1). The oven temperature program 202 started at 80°C for 2 min, increased to 150°C at a rate of 10°C/min, increased to 250°C at a 203 rate of 20°C/min, increased to 325°C at a rate of 20°C/min. Components were separated at a flow rate of 1.1 mL/min and divided in 2 ways using a Sil Flow connector. 204

205 δ^{13} C analyses requires to perform the combustion of carbon in carbon dioxide. It was 206 achieved using an alumina tube containing nickel oxide maintained at 1000°C in a furnace.

Pyrolysis of the sample for $\delta^2 H$ measurement in H₂ was carried out at a high temperature 207 (1400°C) using an alumina conversion tube (Thermo Fischer Scientific). Undecane, C₁₁H₂₄ 208 (Sigma Aldrich), was regularly added to regenerate the alumina pyrolysis tube. For δ^{18} O 209 measurements, it was necessary to ensure the conversion of oxygen atoms into carbon 210 monoxide. The pyrolysis unit was designed by an inner platinum tub partly filled with nickel 211 212 wires incorporated in an outer ceramic tube (Thermo Fisher) hold at 1280°C and work under a column flow 1.1 ml/min Helium added by 1.1 ml/min of a mix (98% Helium +2% H_2) 213 214 necessary in this configuration. Pyrolysis tube was conditioned by injecting 1 μ l of high purity undecane paraffin (sigma Aldrich) in duplicate, allowing to create an inner homogenous 215 coating into the platinum tube. This layer of carbon created would be necessary bring 216 217 enough carbon reactant in the pyrolysis reaction and the production of carbon monoxide. 218 This coating process has been monitored every week enabling to reconstitute the carbon layer. 219

220

2.3. Enantiomeric study and impurity detection

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2.3.1. Carvone enantioselective analysis using GC-FID

222 Samples of spearmint essential oils have been analysed in Enantio GC-FID to control the 223 enantioselective ratio of carvone. Enantioselective analyses were performed on a gas chromatograph 6890N GC System (Agilent Technologies) equipped with a flame ionization 224 detector (FID) with the detector temperature set at 250°C. Separation of the carvone 225 enantiomers were performed on a β-DEX 225 column (30 m * 0,250 mm * 0,25 μm, Supelco 226 227 Inc.) coated with cyclodextrin stationary phase. The GC conditions were programmed as 228 follows: 0.2 µL of diluted essential oil (1:100 in ethanol) was injected into the GC in split mode (100:1) with the injector temperature set at 250°C. The carrier gas was H₂ at a 229 constant flow rate of 1.2 mL/min. The initial oven temperature was held at 80°C for 5 min, 230

ramped to 130°C at a rate of 2°C/min, then ramped to 180°C at a rate of 5°C/min and held
for 2min.

233

234

2.3.2. Impurity detection using GC-MS analysis

235 GC-MS analyses were performed on a gas chromatograph 6890 N GC System (Agilent 236 Technologies) interfaced with a 5975 inert XL MSD mass spectrometer with electron impact ionization (70 eV). 1 µL of pure cinnamon essential oil was injected into the GC in the split 237 mode (100:1), with the injector temperature set at 250 °C. Helium was used as the carrier 238 gas with a constant flow rate of 1.0 mL/min. Separation of the volatile compounds in 239 cinnamon essential oils was achieved using a HP-5 capillary column (30 m*0.250 mm*0.25 240 241 μm, Agilent Technologies). The initial oven temperature was held at 60 °C for 3 min and then 242 ramped at a rate of 5°C/min to reach 160°C, then ramped to 300°C at a rate of 10°C/min and held for 4 min. The SIM parameters were as follows: the targeted mass ions (m/z) were 128, 243 244 129 and 158 (molecular ion) to detect 5-phenylpenta-2,4-dienal. NIST library and lab-made library, combined with retention time, retention indices, literature references, and standards 245 injections were used to identify the impurity produced during benzaldehyde aldolisation. 246

247

3. <u>Results and discussion</u>

250 **3.1.** Spearmint Essential Oil

251 **3.1.1.** Context

Essential oils of spearmint *Mentha spicata* L. are mainly composed of carvone molecule. These extracts are generally processed with a fractional distillation to obtain more than 80% of carvone in the composition of the essential oil [12,13]. These products are usually originated from China, USA, and India.

256 These essential oils are amongst the most important product in the essential oil market. Therefore, these extracts are highly subjects to be adulterated. Addition of synthetic carvone 257 is a known adulteration and is easily highlighted with the control of the enantiomeric ratio 258 259 [14,15]. An olfactory control is also very efficient, because enantiomers of carvone present 260 different odours. Carvone (R) has a minty smell and is abundant in spearmint essential oils, 261 whereas carvone (S) has a spicy aroma and is found in caraway essential oils. However, 262 carvone (R) isomer can also be synthesized from natural origin limonene (S) from sweet orange essential oils [16]. This extract is currently the best-selling essential oil in the world 263 264 and the cheapest in the market. Limonene represents over than 90% of the composition of 265 sweet orange essential oil.

The semisynthesis of carvone from limonene has the particularity to introduce an oxygen [11] (Figure 1). An isotopic monitoring of δ^{18} O may potentially be of interest to highlight the semisynthetic origin of carvone, because this newly introduced oxygen is from a different source and has not been impacted by the same isotopic fractionations as the original oxygen from spearmint carvone.

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272 **3.1.2.** Analysis of results

273 Enantiomeric and isotopic data are depicted in Table 1 and Figure 3.

The determination of the enantiomeric ratio has been carried out in order to control that carvone essential oils had not been adulterated with petrochemical origin carvone. The enantiomeric content of carvone (R) is measured between 97.3% and 100.0%. All the analysed samples present the carvone enantiomer (R) in excess, which is in line with the literature [14,15]. No significant differences were observed between authentic and commercial samples.

 δ^{13} C isotopic composition of carvone molecules derived from essential oils of genuine 280 spearmint (-29.35 ± 1.54‰) is similar to the δ^{13} C of carvone molecules from commercial 281 samples (-29.63 ± 0.87‰). These values are in line with the C3 origin [17] of the spearmint 282 plant. According to the literature, C3 plants presents a δ^{13} C comprised between -20‰ and -283 30‰ [18]. The δ^{13} C of the carvone is not efficient to discriminate natural and synthetic 284 origins of the carvone molecule according to the analyses. Indeed carvone molecules from 285 petrochemical origins have δ^{13} C values comprised between -30.24‰ and -26.40‰, which 286 coincide with δ^{13} C range value of authentic spearmint essential oils. Same result is observed 287 with the carvone synthesized from limonene ($\delta^{13}C = -29.81\%$). 288

As regards with δ^2 H isotopic values, significant isotopic fractionations are noticed between samples: authentic samples -262 ± 31‰; commercial samples -242 ± 29‰). These differences are probably explained by the different geographical origins, because δ^2 H stable isotope ratios are linked to the geographical location of the harvest [19]. USA spearmint essential oils have a mean value of -282 ± 28‰; China samples mean isotopic value is more enriched in deuterium (-235 ± 18‰); as well as India samples (-226 ± 27‰). These natural values are significantly depleted by comparison with δ^2 H stable isotopic values of carvone molecules synthesized from petrochemical products (-190 ± 24‰). The study of stable isotope ratios of hydrogen potentially highlights an origin of synthesis. However, this is unfortunately not possible in the case of a carvone obtained from limonene conversion, because the measured δ^2 H value (-204‰) is within the range of authentic sample values.

Finally, the study of δ^{18} O isotopic values highlights significant isotopic differences between 300 authentic and commercial samples. The δ^{18} O value of 100% pure and natural carvone 301 molecules are comprised between 14.1‰ and 22.1‰. These values are more positives than 302 the δ^{18} O value measured on the carvone (R) obtained from limonene (9.2‰). From the 303 panel of commercial samples, several essential oils presents $\delta^{18}\text{O}$ values similar to the 304 measured range determined to authentic samples, but other samples present a lower $\delta^{18}O$ 305 306 value, which potentially indicate that these samples have been adulterated with 307 semisynthetic carvones.

308

3.2. Cinnamon Essential Oil & Bitter Almond Essential Oil

309 **3.2.1.** *Context*

Essential oils of cinnamon and bitter almond are amongst the most sold essential oils of the market. Bitter essential oils are obtained from the extraction of almond (*Prunus amygdalus* var. amara (DC.)) and apricot (*Prunus armeniaca* L.) kernels. This essential oil presents a composition with more than 95% of benzaldehyde. Cinnamon essential oil comes in two forms: oil cinnamon essential *Cinnamomum cassia* (L.) J. Presl, mainly from China and from distillation of wood and / or leaves, and cinnamon essential oil *Cinnamomun verum* J. Presl (syn. *Cinnamomum zeylanicum* Blume), mainly from Sri Lanka, India, and Madagascar, and

317 made from the distillation of cinnamon bark. These two essential oils are rich in (E) -318 cinnamaldehyde.

319 There are many frauds associated with the essential oils of bitter almond and cinnamon. Since the cassia origin of cinnamon is much cheaper than the Ceylon origin, the mixtures 320 between these two essential oils are common. However, it is possible to highlight this type 321 322 of addition by studying the differences in composition between the two essential oils [20], or 323 by NIR and FTIR spectroscopic analysis [21,22]. Additions of (E) -cinnamaldehyde and benzaldehyde are also identifiable by radiocarbon analysis [23]. However, as shown in 324 literature with the example of methyl salicylate or benzaldehyde [7,24], a labelling of 325 synthetic molecule makes the measurement of ¹⁴C activity inefficient. Isotopic methods by 326 δ^2 H GC-P-IRMS are applicable to highlight the synthetic additions to benzaldehyde (Ruff et 327 328 al., 2000) and (E) -cinnamaldehyde [25], as well as SNIF-NMR [4]. The synthesis of (E) -329 cinnamaldehyde from benzaldehyde produces a residual impurity, 5-phenylpenta-2,4-dienal [26], which is identifiable by GC, if the product has not been sufficiently purified. 330

331 In the same way as for mint, (E) -cinnamaldehyde can also be produced by semisynthesis, 332 from benzaldehyde obtained from the essential oil of bitter almond. The scheme of reaction, presented in Figure 2, shows that (E)-cinnamaldehyde is obtained by aldolization of 333 334 benzaldehyde (1) [4]. During the reaction, the oxygen in the benzaldehyde is removed and 335 oxygen from acetaldehyde is introduced to form (E) -cinnamaldehyde. A stable isotopic 336 assessment is therefore again of real interest. As prices fluctuate, essential oil bitter almond is sometimes cheaper than cinnamon bark essential oil (Cinnamomum verum). The fraud can 337 be reversed. Benzaldehyde can also be hemi-synthesized from (E)-cinnamaldehyde [4], 338 339 according to the retro-aldolization reaction (2). This time, the oxygen in the hemi-

340 synthesized benzaldehyde is obtained from water. However, the use of carbonates in the341 reaction suggests isotopic exchanges [27].

Therefore, adulteration can be realized in either ways, depending on market prices. The essential oil of cassia is ten times cheaper than the other two; it remains the main source of fraud.

345

3.2.2. Cinnamon Essential Oils

Some reference isotopic values of (E) -cinnamaldehyde have been published. The value $\delta^{13}C$ 346 of (E) -cinnamaldehyde from natural cinnamon essential oil is between -28.20 and -28.60 ‰ 347 according to Culp [23,24] and between -24.20‰ and -27.10‰ according to Sewenig [25], 348 while the values $\delta^{13}C$ of synthetic (E) -cinnamaldehyde obtained from the oxidation of 349 toluene, are between -24.61‰ and -25.70‰. According to the literature, the values $\delta^2 H$ of 350 natural (E) -cinnamaldehyde are given between -142‰ and -101‰ [23-25]. (E) -351 352 cinnamaldehyde of petrochemical origin are much more enriched in deuterium, which results in values δ^2 H much higher, between 464‰ and 569‰ after Culp [23,24]. Finally, the 353 naturally occurring (E)-cinnamaldehyde, a product of the shikimic pathway of biosynthesis 354 [28], is expected to exhibit value δ^{18} O around 14.9‰ [29]. 355

The isotopic values of (E) -cinnamaldehyde of the various essential oils of cinnamon, determined by GC-C / P-IRMS, are described in Table 2 and Figure 4.

The (E) -cinnamaldehydes of essential oils of cinnamon, which did not present impurities, have isotope ratios δ^{13} C of -28.37 ± 1.14 ‰ and δ^{2} H of -142 ± 10 ‰. These results are in agreement with the natural isotopic values presented by Culp and Sewenig[23–25]. δ^{2} H stable isotopic values are sometimes more negative however. The mean δ^{18} O value is

measured around $13.9 \pm 2.2\%$, which is close to the value proposed by Schmidt [29]. All of these values are consistent with a natural origin.

Cinnamon essential oils that had impurities present various isotopic values. We can 364 distinguish those which have $\delta^2 H$ indicators of one synthetic origin and others. The first ones 365 have isotope ratios δ^{13} C -24.70 ± 1.66‰ and δ^{2} H between -42 and 844‰. These stable 366 isotopic values demonstrate enrichment in ²H and ¹³C compared to natural samples. The 367 highest $\delta^2 H$ values are indicative of an origin of synthesis from toluene, while the lower 368 values seem to correspond to benzyl chloride origin [25]. The measured δ^{18} O values are very 369 variable, and fluctuate from 1.2 to 21.8‰. Isotope analyses confirm the detection of 370 371 synthetic residues: these samples have undergone a partial or total addition of (E) cinnamaldehyde of synthetic or semisynthetic origin. 372

However, other samples, which nevertheless had traces of 5-phenylpenta-2,4-dienal, exhibit naturally occurring (E) -cinnamaldehyde isotopic values (δ^{13} C -27.54 ± 0.65‰ and δ^{2} H -147 ± 12‰). However, we can see that the values in δ^{18} O are lower (4.0 ± 3.3‰). The oxygen values do not correspond to a natural origin from of the phenylpropanoid biosynthetic pathway from shikimic acid. The oxygen of (E) -cinnamaldehyde was probably altered, from a naturally occurring reagent: acetaldehyde from ethanol oxidation.

To verify this hypothesis, several semisyntheses of (E) -cinnamaldehyde were carried out, by aldolization of benzaldehydes, from different samples of almond essential oils bitter. Isotopic values δ^{13} C and δ^{2} H obtained correspond to a natural origin of (E) -cinnamaldehyde, but this is not the case with δ^{18} O, which is measured between 7.9‰ and 9.5‰. These values are lower than the values measured previously on essential oils of cinnamon natural, nor does it correspond to the value given by Schmidt [29]. The oxygen introduced during the aldolization of benzaldehyde comes from acetaldehyde used as reagent. It is the latter that will influence the final value of δ^{18} O of (E) cinnamaldehyde produced by this semisynthesis. To verify this claim, the two commercial acetaldehyde samples used to produce the samples by aldolization, are analyzed by EA-P-IRMS, by direct introduction. The measure of their value δ^{18} O is 8.7 ± 0.1 ‰. By comparison, the mean value δ^{18} O measured on the 7 samples of (E) -cinnamaldehyde obtained by aldolization of benzaldehyde natural is 8.8 ± 0.6‰.

These results confirm the origin of the oxygen molecule introduced during the synthesis from benzaldehyde. If the δ^{18} O value of acetaldehyde is more negative, the (E) cinnamaldehydes produced during aldolization will also have more negative δ^{18} O values, which may explain the values very low measured on some suspect samples. Indeed, acetaldehyde is produced industrially from agricultural ethanol, whose values δ^{18} O are low (between 3.0‰ and 8.1‰ after Schmidt [29]). It is therefore possible that syntheses can be carried out with acetaldehydes weakly enriched in ¹⁸O.

399

3.2.3. Bitter Almond Essential Oil

For benzaldehyde, the isotopic values of natural origin indicated in the literature are 400 between -27.06‰ and -29.58 ‰ for δ^{13} C, and between -145‰ and -84‰ for δ^{2} H from the 401 study by Culp and Noakes [23,24], and between -113‰ and -148‰ for δ^2 H from Ruff et al 402 [30], for a bitter almond origin. Schmidt et al. [29] present a value δ^{18} O for one naturally 403 occurring benzaldehyde from the shikimic route, around 19.3‰. Ruff's work and al. [30] also 404 focused on samples of natural benzaldehyde ex-*Cinnamomum cassia*, the values $\delta^2 H$ data is 405 between -83 and -144 ‰, which merges with the range of values natural benzaldehyde from 406 bitter almond essential oil. The isotopic values of Synthetic benzaldehyde are different 407

depending on the method of synthesis. A benzaldehyde from the acid or basic hydrolysis of (dichloromethyl) benzene has values between -28.52‰ and -29.99‰ for δ^{13} C [23,24], between -47 and -85‰ for δ^{2} H [23,24,30], and around 6.8‰ for δ^{18} O. While a benzaldehyde obtained by oxidation of toluene will have a value δ^{13} C between -24.70 and -26.50‰ according to Culp and Noakes [23,24], a strongly positive δ^{2} H isotopic value, between 420 ‰ and 720‰ approximately, and a value δ^{18} O around 14.1‰ according to the publication by Schmidt et al. [29].

The isotopic values of benzaldehyde in various bitter almond essential oils analyzed,
determined by GC-C / P-IRMS, are described in Table 3.

Bitter almond essential oils have isotopic values corresponding to an origin natural benzaldehyde, isotopic values varying from -31.81‰ to -29.80‰ for δ^{13} C, from -131‰ to -97‰ for δ^{2} H, and from 13.4‰ to 19.4‰ for δ^{18} O.

These values differ from those measured on benzaldehydes of petrochemical origin. Indeed, it is possible to observe more positive isotopic values on the three elements analyzed, which puts in evidence a greater enrichment in ¹³C, ²H, and ¹⁸O in the synthetic benzaldehydes. These values are characteristic of a synthesis from toluene.

Finally, various samples of benzaldehydes, obtained by retro-aldolization of (E) – cinnamaldehydes from different origins were analyzed. Semisyntheses, carried out from (E) –cinnamaldehydes natural oils derived from cassia and verum cinnamon essential oils, have isotopic ratios stable δ^{13} C (-29.92 ± 0.54‰) and δ^{2} H (-108 ± 11‰) similar to the values obtained on oils bitter almond essentials. However, the isotopic values δ^{18} O are lower (10.9 ± 0.4‰) than those of natural essential oils.

The oxygen introduced during the retroaldolization reaction of (E) -cinnamaldehyde comes 430 from water. However, isotopic exchanges occur between water and the carbonate used in 431 the synthesis (Na₂CO₃), at 100°C [27]. The δ^{18} O value of benzaldehyde obtained by 432 retroaldolization is therefore dependent on the isotopic value of the carbonate used during 433 434 the synthesis, which explains why we find similar isotopic values on the different benzaldehydes produced by retroaldolization, despite the use as a starting reagent from 435 different origins of (E) -cinnamaldehydes (synthetic or natural from essential oils of bitter 436 almond). 437

The δ^{18} O of tap water, used for this synthesis, cannot be measured in EA-P-IRMS. To be able to carry out the measurement, we used an OA-ICOS system (Off Axis - Integrated cavity output spectroscopy) [31–33]. The δ^{18} O of water is measured at -11 ‰, while carbonates are generally highly enriched in ¹⁸O (between 25‰ and 30‰, [27]). The isotopic exchange of oxygen between the carbonate ion CO₃²⁻ and H₂O lead to an intermediate value, which corresponds to the δ^{18} O values that we measure on benzaldehydes produced by retroaldolization.

445

447 <u>Conclusion</u>

The results presented in this study are promising, as there are currently no analytical solutions to highlight evidence of semisynthetic product additions with δ^{13} C and δ^{2} H measurements, radiocarbon assessment, and enantiomeric ratio determination. In addition, biotechnologies will continue to grow with production of molecules from various origins using novel processes. δ^{18} O isotopic measurements will be of greater interest depending of technological advances.

Other leads of investigation can be considered, such as the authentication of Oregano 454 Origanum vulgare essential oils, primarily constituted of carvacrol. This molecule is easily 455 synthesized from limonene (predominantly compound of sweet orange essential oil) and / or 456 carvone (primarily found in spearmint and caraway essential oils)[34]. Another future 457 458 prospect could be the study of the semisynthesis of (E)-anethol (Star anise Illicium verum, Anise Pimpinella anisum, and fennel Foeniculum vulgare essential oils) from methyl chavicol 459 (basil Ocimum basilicum, tarragon Artemisia dracunculus, and chervil Anthriscus cerefolium 460 461 essential oils).

Furthermore, δ^{18} O isotopic study is not restricted to semisynthetic frauds. Many applications are potentially applicable, depending of the matrix of interest. δ^{18} O compound specific analyses using GC-P-IRMS can potentially be a new complementary tool for authentication, especially since many oxygenated constituents are present in the composition of essential oils, and that most of these metabolites have not yet been studied in δ^{18} O compound specific GC-P-IRMS analysis. As reported in previous studies [7], δ^{18} O metabolite specific values are efficient to highlight a petrochemical origin. In addition, this measure can provide

geographical origin authentication, as the isotopic fractionations of oxygen are linked to the
atmospheric cycles of water, similar to those of hydrogen.

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477 Declaration of Competing Interests

The authors declare that there are no conflicts of interest.

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Table 1: Carvone compound-specific δ^{13} C, δ^{2} H, and δ^{18} O isotopic values and enantiomeric ratios measured in spearmint authentic and

590	commercial essential oils, and carvone from	petrochemical and from limonene conve	rsion origins (n=number of samples).
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Samples		Enantiomeric values of carvone				Compound-specific isotopic values of carvone (GC-C/P-IRMS)										
		% Carvone (R)			δ ¹³ C				δ ² H				δ ¹⁸ Ο			
	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD
Spearmint essential oils – Authentic samples (n=12)	99.7	98.7	100.0	0.4	-29.35	-33.34	-27.24	1.54	-262	-294	-211	31	18.4	14.1	22.1	2.6
Commercial spearmint essential oils (n=36)	99.1	97.3	100.0	0.7	-29.63	-30.96	-28.15	0.87	-242	-321	-201	29	9.0	-1.5	18.4	5.6
Carvone (R) – Petrochemical origin (n=3)	100.0	100.0	100.0	0.0	-27.94	-30.24	-26.40	2.03	-190	-217	-176	24	13.2	10.9	14.5	2.0
Carvone – Limonene conversion origin (n=1)	99.4	NA	NA	NA	-29.81	NA	NA	NA	-204	NA	NA	NA	9.2	NA	NA	NA

Table 2: (E)-Cinnamaldehyde compound-specific δ^{13} C, δ^{2} H, and δ^{18} O isotopic values in cinnamon authentic and commercial essential oils, and

596 (E)-Cinnamaldehyde from petrochemical and from benzaldehyde aldol origins (n=number of samples). Indication of the eventual detection of

597 the 5-phenylpenta-2,4-dienal aldol reaction impurity using GC-MS and compliance of δ^2 H value (maximal value of -101‰ based on [23–25]).

	ג ² ⊔	Impurity	Compound-specific isotopic values of (E)-cinnamaldehyde (GC-C/P-IRMS)											
Samples		dotoctod?	δ ¹³ C		δ ² H				δ ¹⁸ Ο					
	complieu:	uelecleu!	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD
Cinnamon essential oil – Authentic samples (n=11)	Yes	No	-28.37	-27.60	-26.13	1.14	-142	-156	-123	10	13.9	10.9	16.9	2.2
Cinnamon essential oil - Suspected hemi synthetic (E)-	Voc	Yes	-27.54	-28.68	26.00	0.65	-147	-168	175	12	4.0	-2.8	10 E	2.2
Cinnamaldehyde adulteration	res				-20.00	0.05			-125				10.5	5.5
Cinnamon essential oil - Synthetic (E)-Cinnamaldehyde	No	Voc	24 70	27 57	22.20	1 66	146	10	011	201	11 7	1 7	21 0	76
adulteration	NO	res	-24.70	-27.57	-25.29	1.00	140	-42	044	391	11.7	1.2	21.0	, 7.0
(E)-Cinnamaldehyde – Petrochemical origin (n=1)	No	Yes	-25.96	NA	NA	NA	540	NA	NA	NA	16.1	NA	NA	NA
(E)-Cinnamaldehyde – natural benzaldehyde (bitter almond oil)	l) Yes	Voc	26.22	20 E 1	24.02	1 60	125	120	100	0	0 0	7.9	0 5	0.6
aldol reaction origin (n=7)		Tes	-20.25	-20.51	-24.05	1.00	-125	-138	-109	Э	0.0		9.5	
(E)-Cinnamaldehyde – synthetic benzaldehyde aldol reaction		Voc	26.10	26 60	25 70	0 5 1	200	270	407	15	0.4	0.0	0.0	0.5
origin (n=3)	162	Yes	-20.10	-20.09	-25.79	0.51	209	519	407	13	9.4	0.0	9.8	0.5

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599

- **Table 3:** Benzaldehyde compound-specific δ^{13} C, δ^{2} H, and δ^{18} O isotopic values in bitter almond authentic essential oils, and benzaldehyde from
- 602 petrochemical and from (E)-cinnamaldehyde retro aldol origins (n=number of samples).

	Compound-specific isotopic values of benzaldehyde (GC-C/P-IRMS)											
Samples	δ ¹³ C	δ ² H					δ ¹⁸ Ο					
	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD
Bitter almond essential oil – Authentic samples (n=12)	-30.72	-31.81	-29.80	1.00	-117	-131	-97	12	16.5	13.4	19.4	2.1
Benzaldehyde – Petrochemical origin (n=3)	-26.17	-26.91	-25.26	0.84	494	389	546	91	20.9	19.2	23.7	2.4
Benzaldehyde – Natural (E)-cinnamaldehyde (cinnamon oil) retro-aldol reaction origin (n=4)	-29.92	-30.42	-29.18	0.54	-108	-120	-99	11	10.9	10.3	11.2	0.4
Benzaldehyde – Synthetic (E)-cinnamaldehyde retro-aldol reaction origin (n=1)	-26.57	NA	NA	NA	705	NA	NA	NA	11.2	NA	NA	NA



Figure 1: Synthesis of carvone from limonene in 3 steps: (1) Synthesis of limonene nitrosochloride from limonene. (2) Production of carvoxime.

607 (3) Synthesis of carvone.



- 612 Figure 2: Synthesis of (E)-cinnamaldehyde from aldolisation of benzaldehyde. This synthesis leads to the production of an impurity : 5-
- 613 phenylpenta-2,4-dienal
- 614
- 615



δ¹⁸O_{GC-P-IRMS} (‰)

Figure 3: Distribution of $\delta^{18}O_{GC-P-IRMS}$ carvone isotopic values measured on 48 spearmint essential oils and 4 semisynthetic origin carvones from

618 sweet orange limonene.



Figure 4: Distribution of $\delta^{18}O_{GC-P-IRMS}$ (E)-cinnamaldehyde isotopic values measured on 50 *Cassia* and *Verum* cinnamon essential oils and 10 (E)-

624 cinnamaldehyde obtained from benzaldehyde aldolisation (Bitter almond and petrochemical origins).