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Determination of Enantiomeric and Stable Isotope Ratio Fingerprints of Active Secondary Metabolites in Neroli (*Citrus aurantium* L.) Essential Oils for Authentication by Multidimensional Gas Chromatography and GC-C/P-IRMS

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Aurélien Cuchet, Anthony Anchisi, Frédéric Schiets, Yohann Clément, Pierre Lantéri, et al.. Determination of Enantiomeric and Stable Isotope Ratio Fingerprints of Active Secondary Metabolites in Neroli (*Citrus aurantium* L.) Essential Oils for Authentication by Multidimensional Gas Chromatography and GC-C/P-IRMS. *Journal of Chromatography B - Analytical Technologies in the Biomedical and Life Sciences*, 2021, pp.123003. 10.1016/j.jchromb.2021.123003 . hal-03403765

HAL Id: hal-03403765

<https://hal.science/hal-03403765v1>

Submitted on 5 Jan 2024

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1 **Determination of Enantiomeric and Stable Isotope Ratio Fingerprints of Active**
2 **Secondary Metabolites in Neroli (*Citrus aurantium* L.) Essential Oils for**
3 **Authentication by Multidimensional Gas Chromatography and GC-C/P-IRMS**

4 **Authors**

5 Aurélien Cuchet^{a,b,*}; Anthony Anchisi^b; Frédéric Schiets^b; Yohann Clément^b; Pierre Lantéri^b;
6 Christelle Bonnefoy^b, Patrick Jame^b; Elise Carénini^a; Hervé Casabianca^b

7 **Affiliations**

8 ^aAlbert Vieille SAS, 629 Route de Grasse, 06220 Vallauris, France

9 ^bUniversité de Lyon, CNRS, Université Claude Bernard Lyon 1, Institut des Sciences
10 Analytiques, UMR 5280, 5 rue de la Doua, F-69100 Villeurbanne, France

11 ***Corresponding author**

12 Aurélien Cuchet, aurelien.cuchet@isa-lyon.fr; aurelien.cuchet@gmail.com

13

14

15 **Abstract**

16 Neroli essential oil (EO), extracted from bitter orange blossoms, is one of the most expensive
17 natural products on the market due to its poor yield and its use in fragrance compositions,
18 such as cologne. Multiple adulterations of neroli EO are found on the market, and several
19 authentication strategies, such as enantioselective gas chromatography (GC) and isotope ratio
20 mass spectrometry (IRMS), have been developed in the last few years. However, neroli EO
21 adulteration is becoming increasingly sophisticated, and analytical improvements are needed
22 to increase precision. Enantiomeric and compound-specific isotopic profiling of numerous
23 metabolites using multidimensional GC and GC-C/P-IRMS was carried out. These analyses
24 proved to be efficient for geographical tracing, especially to distinguish neroli EO of Egyptian
25 origin. In addition, $\delta^2\text{H}$ values and enantioselective ratios can identify an addition of 10% of
26 petitgrain EO. These results demonstrate that enantioselective and stable isotopic metabolite
27 fingerprint determination is currently a necessity to control EOs.

28 **Keywords**

29 **Isotope ratio mass spectrometry, Multidimensional gas chromatography, Enantioselective**
30 **analysis, Stable isotope analysis, Neroli essential oil, Authentication**

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33 1. Introduction

34 The essential oil (EO) of neroli is produced via hydrodistillation or steam distillation of bitter
35 orange (*Citrus aurantium* L.) blossoms. This EO is highly expensive due to its very low
36 production yield (0.1%) [1]: at least one metric ton of fresh orange blossoms is required to
37 produce only one kilogram of pure EO. The price of EO can fluctuate between 2,500 and 6,000
38 €/kg depending on the country and the year. The quantity of EO produced worldwide is
39 estimated between 4.5 to 6.5 MT per year. Neroli EO is nowadays an iconic product in the
40 fragrance industry, due to its olfactive profile (Floral, orange blossom, green, methyl-like, and
41 honeyed) in demand in the perfume industry [2]. Previous pharmacological studies have
42 reported neurotonic properties [3] and antimicrobial, antioxidant [4,5], anticonvulsant [6],
43 analgesic and anti-inflammatory activities [7] for this EO. Currently, this EO (*C. aurantium ssp.*
44 *amara var. pumilla*) is mainly produced in Morocco, Tunisia, and Egypt but also in Spain, and
45 Lebanon. The EO of another variety (*C. aurantium* L *ssp amara var. daidai*) is produced in
46 China.

47 Adulteration is a common problem in the EO industry, especially with expensive EOs such as
48 neroli. These adulterations are generally carried out to meet standard requirements, increase
49 artificial EO quantities, or develop economic strategies. Orange blossom harvesting requires
50 caution, and the production quality is a function of the weather and temperature conditions
51 throughout the year. In the case of neroli EO, the main adulterations include blending with
52 several known adulterations have been reported in the literature: blending with inexpensive
53 EOs such as sweet orange EO (*Citrus sinensis*) [8], bitter orange EO [8], and petitgrain EO [9];
54 addition of synthetic components such as linalool and linalyl acetate; and dilution with
55 vegetable oils or solvents, such as sunflower oil [10] or ethanol [11].

56 Typical identification and characterization techniques, such as gas chromatography (GC)
57 quality control or olfactive panel tests, cannot always differentiate a natural molecule from
58 various origins (species or geographical) or a natural molecule from its synthetic equivalent.
59 EO authentication requires the combination of several advanced analytical techniques to
60 accurately identify adulteration with high precision. Currently, several methods are commonly
61 used, such as the determination of synthetic residual impurities and natural markers [12],
62 enantioselective analyses [13,14], radiocarbon activity assessment [12,15], and stable isotope
63 analyses [12,13,16–19].

64 Enzymatic synthesis of asymmetric carbon molecules provides stereoselectivity. In most cases,
65 one enantiomer is significantly in excess compared to the other enantiomer. However, organic
66 synthesis will lead to a racemic mixture of both enantiomers. Adulteration of an EO can also
67 be determined with a chiral study if some enantiomer proportions are reversed between the
68 two EOs. Analysis of chiral molecules with enantioselective GC, monitored on cyclodextrin-
69 based capillary columns, enables efficient determination of the enantiomeric ratios of
70 compounds of interest. Chiral analysis is an effective tool to authenticate neroli EO purity.
71 Several studies have been performed to determine the enantiomeric ratios of major
72 metabolites in neroli EO, including linalool and linalyl acetate [14], α -pinene, β -pinene,
73 limonene [8,19], and other components [20].

74 Isotope ratio mass spectrometry (IRMS) is another powerful tool used to highlight the addition
75 of synthetic molecules to an EO, blending with another natural extract, or assessment of its
76 geographical origin. GC – combustion/pyrolysis – IRMS (GC-C/P-IRMS) allows for the
77 determination of compound-specific $\delta^{13}\text{C}$ and $\delta^2\text{H}$ isotope ratios. IRMS has been recently used
78 to identify adulterations of food products such as honey [21,22] or wine [23]. This analytical

79 technique is especially useful in the EO field, which presents complex matrices with several
80 metabolites in nonnegligible quantities. GC-C/P-IRMS has been successfully used in the past
81 for the authentication of several essential oils, including rose [17], oregano and thyme [24],
82 *Allium* species [16], lemongrass and citronella [13], and lemon [18]. Several studies have also
83 determined stable isotopic ratios on several secondary metabolites in neroli EOs, such as
84 linalool and linalyl acetate [25], and other components (β -pinene, myrcene, limonene,
85 terpinen-4-ol, α -terpineol, nerol, neryl acetate, geranyl acetate, β -caryophyllene, (E)-
86 nerolidol, and (E)-(E)-farnesol) [20,26].

87 In the present study, chiral and isotopic data of some compounds of neroli EOs have been
88 used in combination with chemometrics to trace their geographical origin and to assess their
89 authenticity. The first part of this study illustrates the use of these data to geographically
90 discriminate neroli EO samples. Geographical traceability is relevant because an EO is defined
91 by its extraction process, its species, and its location. Furthermore, prices vary with the
92 geographical origin of harvest. The second part of this study is a focus on the purity of neroli
93 EO samples. Although EO naturalness can be easily authenticated by ^{14}C activity assessment
94 or chiral studies, purity is much harder to detect, especially the addition of petitgrain EO,
95 which is also manufactured from bitter orange. Petitgrain EO is obtained from the distillation
96 process of bitter orange leaves and twigs. Neroli and petitgrain EOs are mainly composed of
97 monoterpenic hydrocarbons and oxygenated monoterpenoids. The principal components of
98 neroli and petitgrain EOs are linalool, limonene, linalyl acetate, β -pinene, (E) β -ocimene, (E)-
99 nerolidol, (E) (E) farnesol, and α -terpineol [27]. Neroli and petitgrain EOs share similar
100 compounds, except for nitrogen-containing trace compounds (indole and methyl
101 anthranilate), which have only been reported in neroli EO [28,29].

102 2. MATERIALS AND METHODS

103 2.1. Essential Oil Information

104 All studied samples (*C. aurantium* L ssp *amara* var. *pumilla*) were provided by Albert Vieille
105 SAS (Vallauris, France). The traceability and provenance of the EOs were certified.

106 Neroli EOs came from Morocco (26 samples), Lebanon (4 samples), Tunisia (16 samples), and
107 Egypt (22 samples) via several official producers.

108 In addition, Spanish samples (9 samples) were directly manufactured by Albert Vieille SAS from
109 their aromatic raw material production facility localized in Sevilla region (Spain). Flowers of
110 *Citrus aurantium* were mechanically harvested at the beginning of spring. Fresh flowers were
111 manually sorted to remove leaves and twigs and quickly conveyed to production. Two
112 different processes of distillation were undertaken. Steam distillation conditions applied were
113 as follows: 1 ton of fresh flowers were loaded in a 4000 L still apparatus, with a water steam
114 flow set between 100 and 150 L/h. After 3 hours of distillation, obtained yields comprised
115 between 0.03 and 0.06%. Hydrodistillation conditions applied were as follows: 800 kg of fresh
116 flowers were loaded in a 4000 L still apparatus, with a water flow set between 120 and 135
117 L/h. After 3 hours of distillation, obtained yields comprised between 0.01 and 0.04%.

118 Petitgrain EOs were from Madagascar (3 samples) and Paraguay (6 samples). More
119 information can be found in **Supplementary Table S1** including the year of production, harvest
120 location details, the vegetative stage at time of harvest, and the extraction process.

121 2.2. Enantioselective Analyses

122 2.2.1. Sample Preparation

123 Two hundred microliters of each EO sample were settled on silica gel 60A (35-70 μm particle
124 size, Carlo Erba Reagents, Val-de-Reuil, France). A primary fraction was collected by elution
125 with 200 mL of pentane. This nonpolar fraction, largely composed of monoterpene and
126 sesquiterpene molecules, was called Fraction 1. A second fraction was obtained by elution
127 with 200 mL of diethyl ether. This polar fraction, mainly composed of alcohols and esters, was
128 called Fraction 2. Fractions 1 and 2 were then evaporated with a rotary evaporator (Rotavapor
129 R200, Büchi, Villebon-sur-Yvette, France) and diluted in 1 mL of hexane. This sample
130 preparation was applied to dispose of 1-8 cineole from Fraction 1, which can interfere with
131 limonene and β -phellandrene detection in Enantio-GC.

132 **2.2.2. Multidimensional Gas Chromatography (MDGC) Analyses**

133 MDGC analyses were performed on a 7890A GC gas chromatograph system (Agilent
134 Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) operating
135 at a detector temperature of 250°C.

136 One microliter of each Fraction 1 was injected into the GC in split mode (120:1). Helium was
137 used as the carrier gas with a constant inlet pressure of 26.4 psi. The flow rate was set to 0.7
138 mL/min in the first column and 2 mL/min in the second column. Separation of volatile
139 compounds was realized on an HP-1 primary capillary column (15 m x 0.200 mm x 0.5 μm film
140 thickness, Agilent Technologies) combined with a Dean Switch system (heart cutting program)
141 with a second Cyclosil β column (30 m x 0.250 mm x 0.25 μm film thickness, Agilent
142 Technologies) coated with cyclodextrin stationary phase. The initial oven temperature was
143 55°C held for 15 min, followed by an initial increase at 1°C/min to reach a temperature of 85°C.
144 An additional increase in temperature at 10°C/min was then applied to reach a final
145 temperature of 200°C and held for 10 min. Enantiomeric ratios were determined for α -

146 thujene, α -pinene, camphene, sabinene, β -pinene, δ -3-carene, limonene, and β -phellandrene,
147 as reported in **Figure 1a**.

148 An aliquot 0.2 μ L in volume of each Fraction 2 was injected into the GC in split mode (60:1).
149 Helium was used as the carrier gas (18.8 psi applied inlet pressure). The flow rate was set to
150 0.7 mL/min in the primary column and 1.7 mL/min in the secondary column. Separation of
151 volatile compounds was realized on an HP-1 column (15 m x 0.200 mm x 0.5 μ m film thickness,
152 Agilent Technologies) combined with a DEtTBuSilBetaCDX PS086 cyclodextrin stationary phase
153 column (25 m*0.25 mm*0.25 μ m, Mega, Legano, Italy) and a Dean Switch system (heart
154 cutting program). The oven temperature program was as follows: the initial temperature was
155 held at 60°C for 5 min, increased at a rate of 1°C/min to reach a temperature of 95°C, and held
156 for 9 min. An additional increase at 1°C/min was applied to reach 150°C. Then, a final increase
157 at 10°C/min was carried out to reach 200°C, which was held for 10 min. Enantiomeric ratios
158 were determined for linalool, terpinen-4-ol, α -terpineol, linalyl acetate, and (E)-nerolidol, as
159 reported in **Figure 1b**.

160 **2.3. Isotopic Analyses**

161 **2.3.1. Data Expression**

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

$$163 \quad \delta^i E = \frac{(^i R_{Sample} - ^i R_{Reference})}{^i R_{Reference}}$$

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

167 High-purity gases of carbon dioxide (99.998%, 4.8 Messer) and hydrogen (99.999%, 5.0
168 Messer) were used as the working reference gases injected with each sample. Helium
169 (99.999% purity) was used as a carrier gas.

170 All studied metabolites in the EOs of interest were calibrated with a mixture of molecules (β -
171 pinene, limonene, (E)- β -ocimene, linalool, linalyl acetate, α -terpineol, neryl acetate, geranyl
172 acetate, nerol, geraniol, and (E)-nerolidol) purchased from Sigma Aldrich (Saint Louis, MO,
173 USA) and TCI (Tokyo, Japan). These molecules were previously calibrated against international
174 standards in elemental analysis – IRMS (EA-IRMS).

175 International reference materials IAEA CH7 (polyethylene foil: $\delta^{13}\text{C}$ (‰) -32.15, $\delta^2\text{H}$ (‰) -100)
176 and USGS40 (L-glutamic acid: $\delta^{13}\text{C}$ (‰) -26.39), supplied by the International Agency of Atomic
177 Energy (IAEA, Vienna, Austria), were used to calibrate each molecule in the mixture, which
178 allowed us to express our $\delta^{13}\text{C}$ results relative to the Vienna Pee Dee Belemnite (VPDB) scale
179 and our $\delta^2\text{H}$ results relative to the Standard Mean Ocean Water (VSMOW) scale. Each sample
180 was analyzed versus a monitoring gas for which two pulses were admitted with each analyte.
181 All sample values were analyzed in duplicate and verified by bracketing the analytical
182 sequence with international reference materials every six analyses. The $\delta^{13}\text{C}$ values are
183 expressed within a maximum standard deviation of $\pm 0.28\text{‰}$ and those of $\delta^2\text{H}$ within $\text{SD} = \pm$
184 5‰ . $\delta^{13}\text{C}$ isotopic analyses of the standards molecules were conducted using a Flash HT
185 elemental analyzer connected to a Delta V Plus via a Conflo V interface. The $\delta^2\text{H}$
186 measurements were carried out using a TC-EA elemental analyzer linked to a Delta V Plus via
187 a Conflo V interface (all Thermo Fisher Scientific). The method is fully explained in a previous
188 study [12]

189 **2.3.2. Gas Chromatography - Mass Spectrometry - Combustion/Pyrolysis - Isotope**
190 **Ratio Mass Spectrometry (GC-MS-C/P-IRMS)**

191 The $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of targeted neroli and petitgrain EO metabolites were determined by
192 GC-IRMS using a GC Trace 1310 simultaneously connected to an ISQ MS quadrupole mass
193 spectrometer and to a Delta V Plus isotope ratio mass spectrometer via a GC Isolink II
194 interface. (Thermo Fisher, Bremen, Germany). This equipment enabled the identification of
195 compounds via the ISQ MS instrument and specific compound isotopic ratio measurements of
196 $\delta^{13}\text{C}$ and $\delta^2\text{H}$ via IRMS. Isotopic ratios were determined for β -pinene, limonene, (E)- β -ocimene,
197 linalool, linalyl acetate, α -terpineol, neryl acetate, geranyl acetate, nerol, geraniol and (E)-
198 nerolidol.

199 One microliter of diluted EO (1 μL into 100 μL of ethanol) was injected using a Tri Plus RSH
200 autosampler in a Trace 1310 gas chromatograph equipped with an Agilent HP-Innowax column
201 (60 m * 0.250 mm * 0.50 μm internal diameter film thickness) used for chromatographic
202 separation. The injection port was held at 250°C, fitted with a split liner containing glass wool
203 and operated in split mode (120:1 for linalool and linalyl acetate assessment, 10:1 for the
204 other compounds). The oven temperature program started at 60°C for 2 min, increased to
205 100°C at a rate of 2°C/min, increased to 150°C at a rate of 5°C/min, increased to 200°C at a
206 rate of 2°C/min, and increased to 260°C at a rate of 1°C/min (which was for 5 min).
207 Components were separated at a flow rate of 1.2 mL/min and divided in 2 ways using a Sil
208 Flow connector.

209 The combustion of carbon in carbon dioxide was achieved using an alumina tube containing
210 nickel oxide maintained at 1000°C in a furnace. Pyrolysis of the sample for ^2H measurement in
211 H_2 was carried out at a high temperature (1400°C) using an alumina conversion tube (Thermo

212 Fischer Scientific). Undecane, $C_{11}H_{24}$ (Sigma Aldrich), was regularly added to regenerate the
213 alumina pyrolysis tube.

214 **2.4. Statistical analysis**

215 Multivariate statistical analyses were performed using R version 4.0.3 (R Core Team, Vienna,
216 Austria) [31] with corrplot [32], ropls [33], Factoextra [34], and FactoMiner [35] packages. The
217 Pearson correlation test was carried out to determine pairwise correlations between isotope
218 ratio values. Univariate analysis was realized to determine significant differences between
219 species, within a confidence level p-value < 0.05.

220 For multivariate data analyses, data were first analysed with unsupervised method such as
221 principal component analysis (PCA) [36] and clustering. PCA was used in this study to describe
222 and to assess the quality and homogeneity of the 90 samples of interest (81 neroli EO and 9
223 petitgrain EO). An autoscaling was realized prior to PCA and Ward's minimum variance
224 clustering method was applied to perform the hierarchical clustering [34].

225 Supervised methods were then carried out, including orthogonal partial least square–
226 discriminant analysis (OPLS-DA), to link models and variables responsible for the separation
227 observed on the unsupervised method [37].

228 A 7-fold stratified cross validation was repeated 50 times to avoid overfitting the small number
229 of data-points. Features were ranked according to the variable importance projection (VIP)
230 from OPLS-DA. S-plot verifies the selectivity and the specificity of the selected features. The
231 model's quality was evaluated based on the residuals (R^2X , R^2Y) and the models predictive
232 ability parameter (Q^2_{cum}) [38]. The statistical significances of models were also assessed using
233 confusion matrix.

234 3. Results and Discussion

235 3.1. Enantiomeric Ratio Fingerprint

236 3.1.1. Neroli Essential Oils

237 Enantiomeric ratio determination was conducted in this study to minor chiral compounds in
238 neroli EOs to obtain a complete enantiomeric signature of the studied EOs to improve
239 authentication. This study was achieved using a MDGC approach. This technique was
240 previously applied to authenticate other EOs, such as rosemary EO [39]. Thirteen chiral
241 components were identified in the EO: α -thujene, α -pinene, camphene, β -pinene, δ -3-carene,
242 sabinene, limonene, β -phellandrene, linalool, linalyl acetate, terpinen-4-ol, α -terpineol, and
243 (E)-nerolidol.

244 The results are depicted in **Table 1**. The neroli EOs from Morocco, Tunisia and Lebanon showed
245 the same chiral profile with the dominance of levorotary (-) form: α -thujene, α -pinene,
246 camphene, sabinene, β -pinene, δ -3-carene, β -phellandrene, linalool, linalyl acetate, and
247 terpinen-4-ol. Limonene, α -terpineol, and (E)-nerolidol were predominantly dextrorotary (+).
248 The α -pinene, β -pinene, limonene, linalool, linalyl acetate, terpinene-4-ol, α -terpineol and (E)-
249 nerolidol chiral results were in good agreement with those of previous enantioselective
250 studies of neroli EOs [8,14,19].

251 Several samples from Egypt featured different chiral signatures: racemization of α -pinene and
252 β -phellandrene was observed, and reversions of sabinene and terpinen-4-ol enantiomer
253 amounts were also detected. These results were in good agreement with those found in the
254 literature [20], except for a contradiction with the α -terpineol enantiomeric ratio previously
255 indicated in the literature, as we reported the R enantiomer as the major enantiomer. These
256 results suggest differences in varieties between neroli EO samples from Egypt and other

257 geographical origins. It is also hypothesized that Egyptian samples were produced from
258 flowers of a citrus hybrid *aurantium x sinensis*, based on our harvested area data. Sweet
259 orange *Citrus sinensis* cultivars are the most widely grown among *Citrus* species in Egypt
260 (accounting for 69% of the total cultivated area) [40] and bitter orange *Citrus aurantium* tree
261 is the main *Citrus* rootstock used in Egypt.

262 Several enantiomeric discrepancies were detected for minor compounds (α -pinene, sabinene,
263 terpinen-4-ol, and β -phellandrene), which were all present at less than 2% of the total
264 composition of neroli EOs. Differences between neroli EOs of Egyptian and other origins would
265 have been undetected if only chiral analyses of major compounds were conducted. This
266 highlights the interest in carrying out a complete enantiomeric profiling approach to
267 authenticate EOs. These results are compelling, as neroli EOs from Egypt also present
268 perceptible olfactive profiles. Indeed, EO is more fresh, lemony, and less methylated than
269 Morocco origin and more green and less honeyed than Tunisian.

270 **3.1.2. Petitgrain Essential Oils**

271 Petitgrain EO is a common adulterant in neroli EO. This adulteration can be unintentional if
272 producers are not meticulous enough during harvest, as they must harvest only flowers of *C.*
273 *aurantium* and not the leaves. It can be tricky to detect such adulterations, as major
274 compounds, including linalool and linalyl acetate, are similar between the two types of
275 hydrodistilled *C. aurantium* EOs. Enantiomeric fingerprints of chiral metabolites can also
276 highlight EO blending. Several studies have established chiral databases on petitgrain, sweet,
277 and bitter orange EOs [8,19,41]. By comparison with chiral data for sweet orange and bitter
278 orange EOs, adulteration of neroli EO can be detected. However, literature databases suggest
279 that the petitgrain chiral profile is similar to that of neroli.

280 The petitgrain EO chiral signature is detailed in Table 1. Enantiomeric ratios were determined
281 in 9 samples from Madagascar and Paraguay. α -pinene, β -pinene, limonene, linalool, linalyl
282 acetate, terpinen-4-ol, and α -terpineol chirality were consistent with previous investigations
283 [8,19,42].

284 No significant differences were observed between petitgrain and neroli profiling, except for
285 limonene and nerolidol (+) forms, which were less predominant in the petitgrain profile than
286 in the neroli profile. Enantiomeric profiles of petitgrain and neroli EOs are not sufficiently
287 distinct to highlight an addition of petitgrain EO in neroli EO. A complementary analysis is
288 required to detect such adulteration.

289 3.2. $\delta^{13}\text{C}$ and $\delta^2\text{H}$ Isotopic Composition

290 Multiple isotope (^{13}C and ^2H) analysis of multiple components results in a large amount of
291 information about the samples: compound-specific isotope analysis with GC-C/P-IRMS
292 enables the assessment of the complete isotope fingerprint of neroli EO samples via
293 determination of the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ ratios of major metabolites.

294 Carbon isotope ratios are dependent on the metabolic pathways for carbon fixation in
295 photosynthesis. *C. aurantium* is a C3 plant (Calvin cycle [43]). Its $\delta^{13}\text{C}$ is between -20 and -33
296 ‰ [44]. By comparison, a C4 plant (Hatch and Slack pathway [45]), such as lemongrass [13],
297 has a $\delta^{13}\text{C}$ value slightly more positive, between -9 and -16 ‰ [46]. The $\delta^{13}\text{C}$ stable isotopic
298 ratios of several metabolites of the samples are detailed in **Table 2**. These values are in line
299 with the C3 plant range stated in the literature. The results were also consistent with those of
300 previous $\delta^{13}\text{C}$ analyses conducted with neroli EOs [20]. Furthermore, the results were not
301 significantly different among geographical origins. It was expected that all the samples were
302 neroli EOs and came from the same *C. aurantium* natural plant variety.

303 Hydrogen isotopic ratios are predominantly influenced by the geographical origin of a plant
304 due to the hydrological cycle [47], resulting in $\delta^2\text{H}$ being dependent on the geographical area.
305 Neroli EO $\delta^2\text{H}$ values are detailed in **Table 3**. Several differences were observed among the
306 different geographical origins. It is important to note that the samples from Egypt were
307 different than other samples, with mean $\delta^2\text{H}$ values higher than those of samples with other
308 origins. According to Hör et al. (2001) [25], neroli EOs have $\delta^2\text{H}$ values (measured in 4 samples)
309 ranging between -234‰ and -253‰ for linalool and -270‰ and -272‰ for linalyl acetate. In
310 this study, $\delta^2\text{H}$ values of the 86 samples were higher than the previously reported values, with
311 mean values between -200‰ (Egypt) and -214‰ (Tunisia) for linalool and between -218‰
312 (Egypt) and -240‰ (Tunisia) for linalyl acetate.

313 In conclusion, compound-specific stable isotopic data are relevant because they provide a
314 stable isotopic fingerprint that provides geographical information, as the samples from Egypt
315 were clearly different than those from Morocco, Lebanon, and Tunisia.

316 **3.3. Chemometric Analyses**

317 **3.3.1. Geographical Origin Authentication**

318 Principal component analysis (PCA) was carried out on the entire sample data set (83 samples
319 of neroli), with 33 parameters as variables (enantiomeric ratios and $\delta^{13}\text{C}$ and $\delta^2\text{H}$ isotopic
320 ratios) to obtain a visual representation of the collected data (**Figure 2a**). Two different
321 clusters could be observed: one comprising neroli EOs from Morocco, Lebanon, and Tunisia,
322 and another including neroli EOs from Egypt. Most Spanish samples were in the first cluster,
323 but two commercial samples were found in the second cluster. The Moroccan and Tunisian
324 samples were also slightly separated from each other.

325 Hierarchical clustering was then realized to distinguish the clusters with more accuracy. This
326 clustering technique calculates the difference in distance between each sample. The cluster
327 plot (**Figure 2b**) helps to distinguish three different clusters: one comprising samples from
328 Spain, Morocco, Tunisia, and Lebanon (Cluster 1) and two clusters including samples from
329 Egypt (Clusters 2 and 3). Both Egyptian clusters corresponded to different providers.
330 Geographical location differences might explain these differences: Cluster 2 includes only
331 samples from the specific Egyptian region of Gharbia Governorate, whereas Cluster 3 regroups
332 other samples from Egypt.

333 As described in **Supplementary Table S1**, neroli essential oils were extracted from two
334 different several processes: steam distillation and hydrodistillation. Based on the clustering,
335 the extraction process does not affect the enantiomeric and isotopic compositions, as no
336 differences were observed between steam distilled and hydrodistilled samples. The vegetative
337 stage at time of harvest, the type of resources (wild or cultivated flowers), and the year of
338 production do not impact chirality and isotopy of neroli EO metabolites too.

339 This study highlights the differences in quality between Egyptian and other neroli EOs.
340 Authentication criteria need to be different among the two groups of EO samples to avoid
341 misinterpreting analytical results from Egypt samples.

342 **3.3.2. Purity Authentication**

343 An additional PCA process was employed, this time including petitgrain samples. **Figure 3a**
344 depicts the score plot of the principal components F1 and F2, which accounted for 49% of the
345 total variance in the data. Differences between neroli and petitgrain EOs were successfully
346 visualized.

347 Orthogonal partial least squares – discriminant analysis (OPLS-DA) was conducted to identify
348 discriminant variables. This model was built from all samples using all parameters. The
349 projection of the individual samples is represented in **Figure 3b**: neroli and petitgrain are
350 significantly separated on the OPLS-DA score plot. The S-Plot visualization represented in
351 **Figure 3c** highlights discriminant variables. The S-plot represents the variable confidence
352 (correlation) against the variable contribution (covariance). Variables with the highest
353 contributions and reliability are $\delta^2\text{H}$ stable isotope values, including nerol, geranyl acetate,
354 geraniol, and α -terpineol. The geographical origins of petitgrain samples (Madagascar,
355 Paraguay) probably affecting this discrimination, but this large isotopic fractionation is also
356 explained by the differences between leaves and flowers. (E)-Nerolidol and limonene
357 enantiomeric distributions also present high contributions. The $\delta^{13}\text{C}$ parameter did not seem
358 to contribute to clustering based on this S-Plot representation.

359 Finally, petitgrain was added to one Moroccan neroli EO sample. Three levels of adulteration
360 were applied (10%, 20%, and 50% adulteration). PCA (**Figure 4**) was then performed to
361 observe the effects of this addition on the data. Since neroli EOs presented geographical
362 variations, as seen earlier, only Moroccan samples were represented. As observed in the
363 figure, adulterated samples were no longer in the neroli cluster. Even 10% adulteration can
364 be detected with the determination of enantiomeric and stable isotope ratio fingerprints of
365 the major metabolites of neroli EO.

366 This study shows the importance of determining the most complete enantiomeric and isotopic
367 signature to investigate adulterations, especially if the matrices are similar to those of neroli
368 and petitgrain EOs.

369 **Conclusion**

370 Measurements of enantiomeric and isotopic ratios of major metabolites of neroli essential oils
371 were performed using MDGC and GC-C/P-IRMS. The data banks were treated with
372 chemometric models, leading to geographical and authentication of varieties.

373 Geographical discrimination was achieved using PCA and hierarchical clustering models.

374 We demonstrated that an adulteration of 10% of petitgrain could be highlighted in neroli
375 essential oil.

376 The work carried out in this study highlighted the interest to use simultaneously enantiomeric
377 and isotopic compound-specific analyses to authenticate geographical and botanical origins
378 of an essential oil sample. This type of multianalytical approach can be reproduced to other
379 type of complex essential oils in the future to improve authentication and detect fraudulent
380 products.

381 **Acknowledgments**

382 This work was conducted in partial fulfilment of the PhD requirements for Aurélien Cuchet
383 who received a grant from the Association Nationale de la Recherche et de la Technologie
384 (ANRT, Paris, France). The research was supported by a contract between Albert Vieille SAS
385 and ISA (Institut des Sciences Analytiques, Villeurbanne, France).

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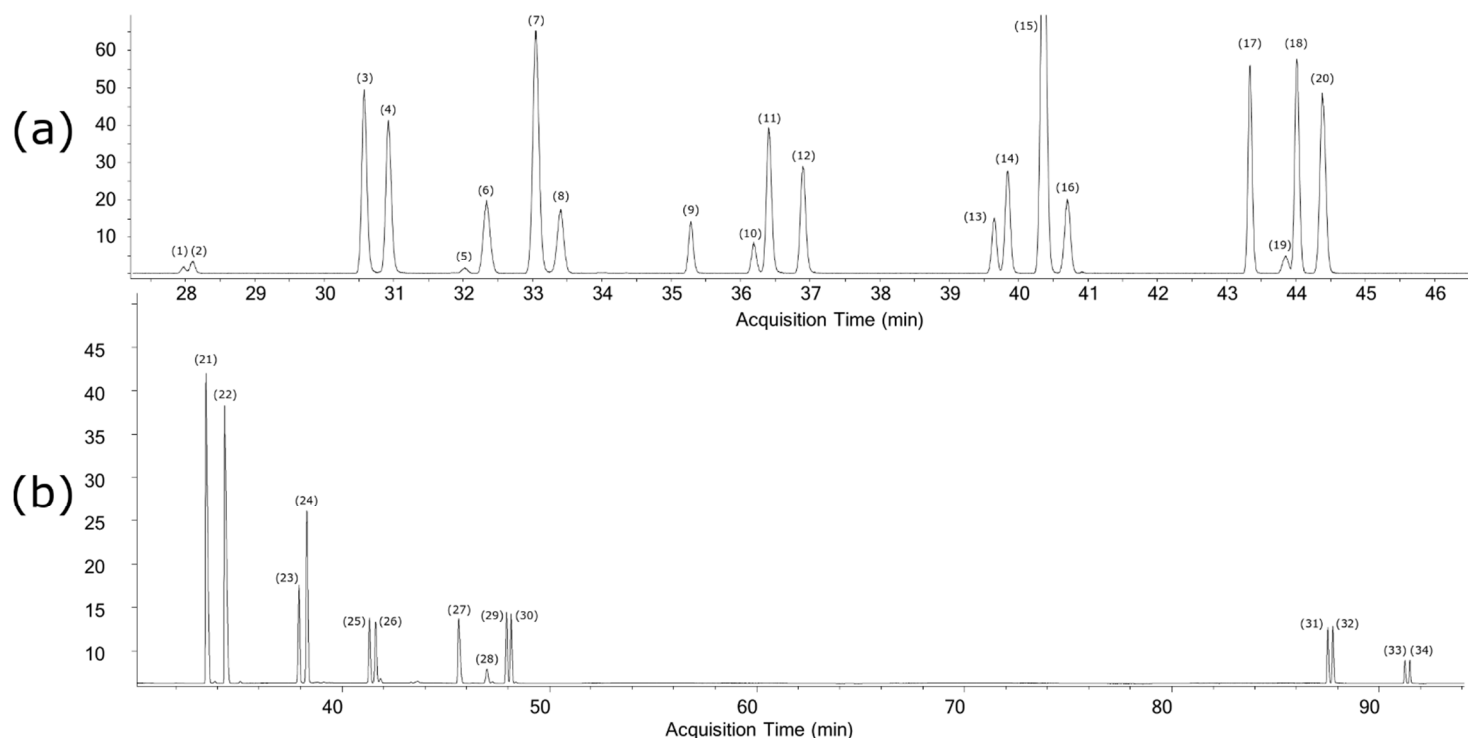
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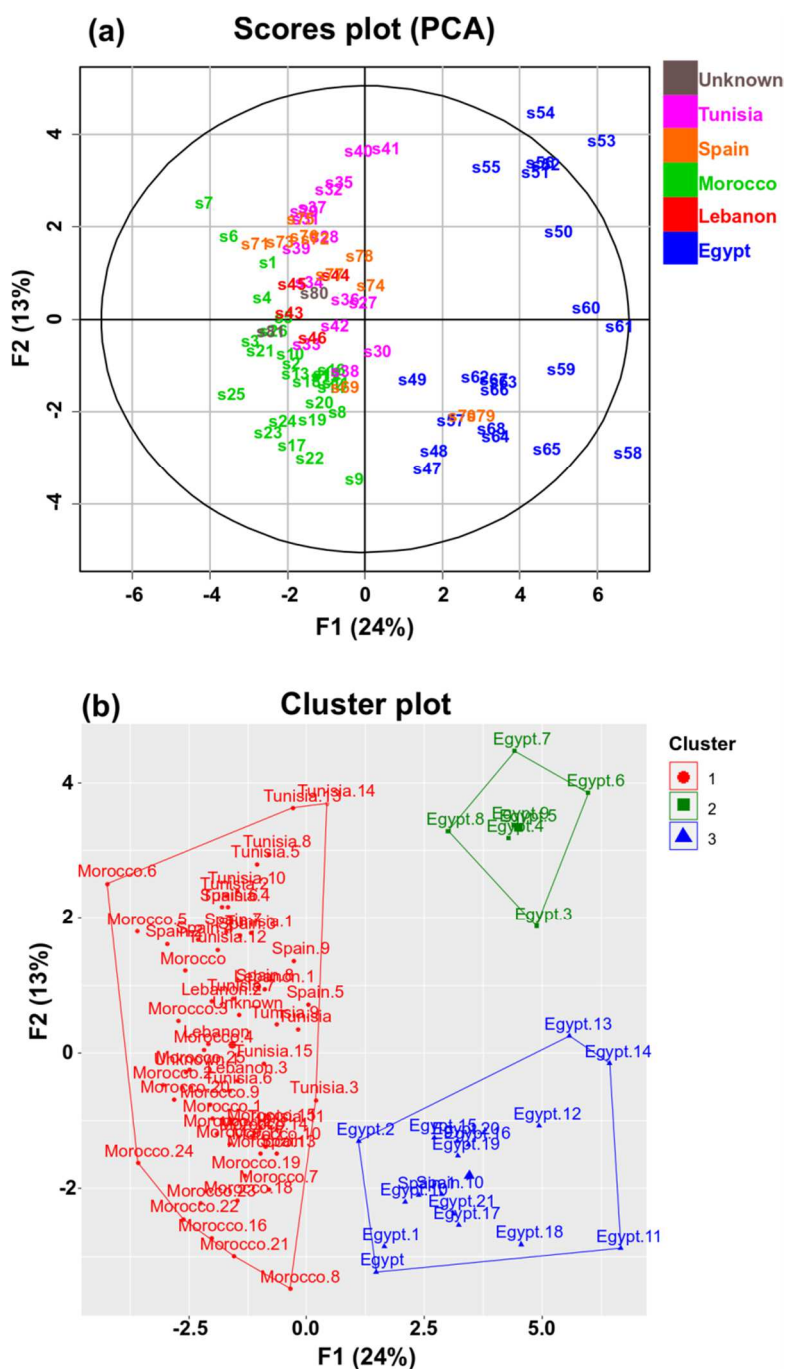
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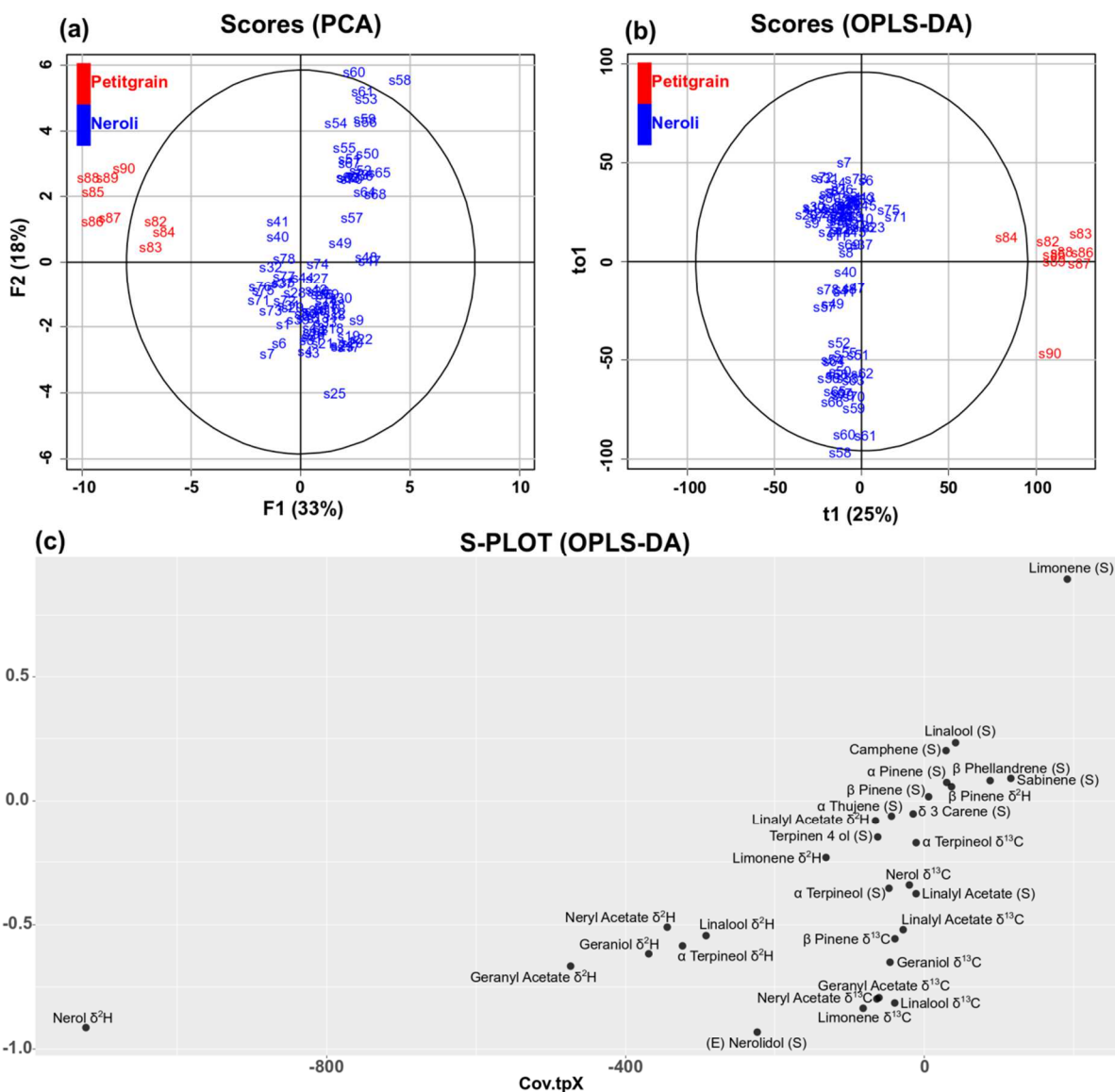
528 **Figure 1. (a)** Second dimension chromatographic profile obtained with MDGC (Columns HP-1
529 15m*0.200mm*0.5um. combined by Dean Switch system with a second column Cyclosil β
530 30m*0.250mm*0.25um). This method enables the separation of 10 chiral metabolites: α -
531 thujene 1(+), 2(-); α -pinene 3(-), 4(+); α -fenchene 5(+), 6(-); camphene 7(-), 8(+); sabinene 9(+),
532 10(-); β -pinene 11(+), 12(-); α -phellandrene 13(-), 14(+); δ -3-carene 15(+), 16(-); limonene 17(-
533), 18(+); β -phellandrene 19(-), 20(+). **(b)** Second dimension chromatographic profile obtained
534 with MDGC (HP-1 column 15m*0.200mm*0.5um, combined by Dean Switch system, with a
535 second column DEtTBuSilBetaCDX PS086 25m*0.25mm*0.25 μ m). This method enables the
536 separation of 7 chiral metabolites: linalool 21(+), 22(-); borneol 23(-), 24(+); terpinen-4-ol
537 25(+), 26(-); α -terpineol 27(-), 28(+); linalyl acetate 29(+), 30(-). (Z)-nerolidol 31(+), 32(-); (E)-
538 nerolidol 33(-), 34(+).

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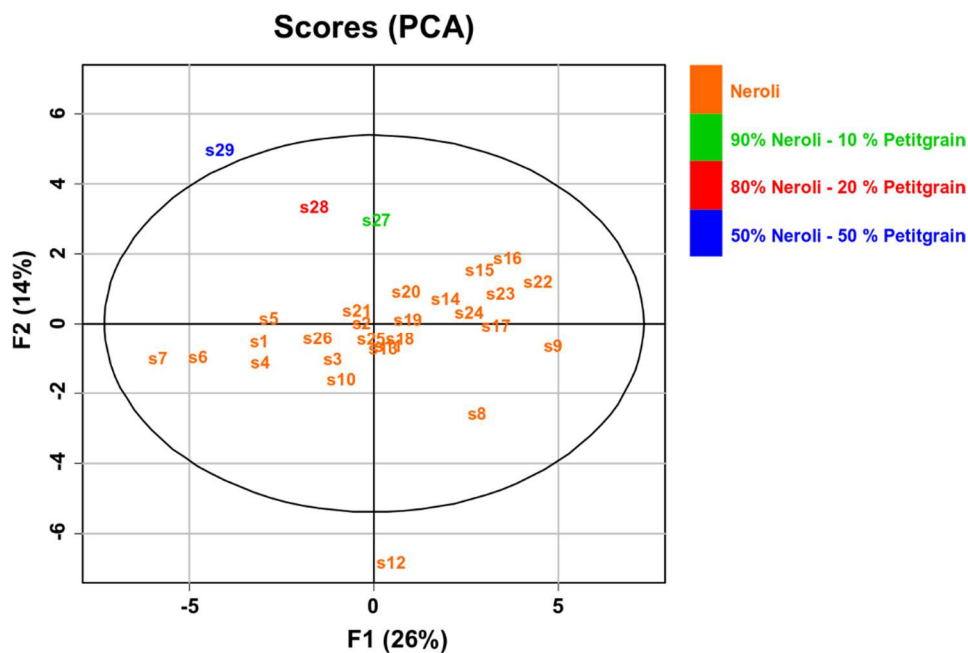


540
 541 **Figure 2. (a)** Score plot of principal component F1 (24%) and F2 (13%) of Principal Component
 542 Analysis (PCA) model developed from enantiomeric ratio percentages and $\delta^{13}\text{C}$ and $\delta^2\text{H}$
 543 compound specific isotope analysis (CSIA) values determined on neroli essential oils from
 544 different geographical origins: Morocco (in green), Egypt (in blue), Tunisia (in pink), Lebanon
 545 (in red), and Spain (in orange). Samples with unknown geographical origins are represented in
 546 grey. Clusters are represented via **(b)** hierarchical cluster plot. Cluster 1 (in red) includes

- 547 Moroccan, Lebanese, Tunisian, and most Spanish samples. Cluster 2 (in green) includes only
- 548 Egyptian samples. Cluster 3 (in blue) comprises some Egyptian and two Spanish samples.



549
 550 **Figure 3. (a)** Score plot of principal component t1 (33%) and t2 (18%) of Principal Component
 551 Analysis (PCA) model developed from enantiomeric ratio percentages and $\delta^{13}\text{C}$ and $\delta^2\text{H}$
 552 compound specific isotope analysis (CSIA) values determined on neroli (in blue) and petitgrain
 553 (in red) essential oils. **(b)** Orthogonal Partial Least Square – Discriminant Analysis (OPLS-DA)
 554 score plot of enantiomeric ratio percentages and $\delta^{13}\text{C}$ and $\delta^2\text{H}$ CSIA values. **(c)** OPLS-DA
 555 loadings S-Plot. This representation shows the correlation against the covariance.



556

557 **Figure 4.** Principal Component Analysis representation of the addition of petitgrain in neroli
 558 essential oil. Three levels of adulteration were undertaken: 10% (green), 20% (red), and 50%
 559 (blue) additions of petitgrain in 100% pure and natural Moroccan neroli essential oils (Samples
 560 represented in orange).

561

Table 1: Enantiomeric distribution of chiral secondary metabolites of neroli and petitgrain essential oils from different geographical origins: Morocco, Tunisia, Lebanon, Egypt, Spain, Madagascar, and Paraguay.

| Essential oil type | Geographical origin | Number of Samples | Enantiomeric distribution (%) | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|--------------------|---------------------|-------------------|-------------------------------|------|------------------------|-----|---------------------|-----|----------------|------|-----------------------|------|---------------------------|------|----------------|-----|-----------------------------|------|----------------|-----|---------------------|------|---------------------------|-----|-----------------------|-----|-----------------|-----|------|----|
| | | | α -Thujene S-(+) | | α -Pinene S-(-) | | Camphene 1S, 4R-(-) | | Sabinene S-(-) | | β -Pinene S-(-) | | δ -3-Carene 1S-(+) | | Limonene S-(-) | | β -Phellandrene S-(+) | | Linalool S-(+) | | Terpinen-4-ol S-(+) | | α -Terpineol S-(-) | | Linalyl Acetate S-(+) | | (E)-Nerolidol S | | | |
| | | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Neroli | Morocco | 26 | 0.0 | 0.0 | 87.9 | 2.5 | 98.6 | 1.0 | 93.3 | 6.9 | 99.2 | 1.9 | 0.6 | 2.9 | 1.9 | 1.0 | 25.8 | 10.8 | 26.7 | 1.4 | 35.7 | 1.8 | 30.8 | 1.3 | 1.9 | 0.9 | 98.6 | 0.4 | | |
| Neroli | Tunisia | 16 | 3.3 | 6.9 | 88.9 | 3.1 | 97.9 | 1.3 | 79.5 | 16.4 | 99.1 | 1.0 | 9.6 | 16.6 | 2.6 | 0.9 | 23.6 | 14.0 | 23.0 | 1.3 | 40.4 | 5.2 | 31.9 | 0.9 | 1.2 | 0.4 | 98.2 | 0.4 | | |
| Neroli | Lebanon | 4 | 0.0 | 0.0 | 90.8 | 2.6 | 100.0 | 0.0 | 95.4 | 0.8 | 99.8 | 0.1 | 11.3 | 22.7 | 1.7 | 0.3 | 27.3 | 15.2 | 20.7 | 1.2 | 36.6 | 1.4 | 30.0 | 0.4 | 1.4 | 0.4 | 98.8 | 0.2 | | |
| Neroli | Egypt | 22 | 23.4 | 26.7 | 65.9 | 9.6 | 94.3 | 6.1 | 24.9 | 20.8 | 94.6 | 17.3 | 0.3 | 1.1 | 2.5 | 0.6 | 53.1 | 18.1 | 18.6 | 4.0 | 59.8 | 6.2 | 32.5 | 6.2 | 0.7 | 0.1 | 97.5 | 1.5 | | |
| Neroli | Spain | 11 | 12.0 | 26.7 | 86.5 | 9.5 | 97.7 | 3.5 | 77.1 | 30.9 | 99.5 | 0.8 | 0.0 | 0.0 | 2.2 | 0.8 | 40.0 | 18.3 | 28.2 | 3.4 | 42.7 | 9.9 | 29.7 | 2.5 | 1.4 | 0.3 | 98.4 | 1.0 | | |
| Petitgrain | Madagascar | 3 | 0.0 | 0.0 | 92.7 | 3.0 | 100.0 | 0.0 | 92.0 | 0.6 | 98.2 | 0.9 | 0.0 | 0.0 | 9.2 | 2.8 | 18.3 | 16.7 | 24.4 | 4.3 | 30.9 | 7.8 | 27.6 | 1.2 | 0.4 | 0.3 | 81.0 | 5.5 | | |
| Petitgrain | Paraguay | 6 | 5.5 | 6.1 | 81.0 | 4.4 | 100.0 | 0.0 | 73.9 | 27.7 | 98.4 | 0.6 | 0.9 | 0.8 | 24.0 | 2.3 | 53.5 | 4.9 | 28.1 | 3.4 | 42.3 | 10.2 | 27.0 | 0.2 | 0.4 | 0.2 | 77.0 | 6.2 | | |

Table 2: Stable isotope $\delta^{13}\text{C}$ distribution of secondary metabolites of neroli and petitgrain essential oils from different geographical origins: Morocco, Tunisia, Lebanon, Egypt, Spain, Madagascar, and Paraguay.

| Essential oil type | Geographical origin | Number of Samples | Isotopic Distribution (‰) | | | | | | | | | | | | | | | | | | | |
|--------------------|---------------------|-------------------|---------------------------------------|------|--------------------------------|------|--------------------------------|------|---------------------------------------|------|---|------|-------------------------------------|------|---------------------------------------|------|-----------------------------|------|--------------------------------|------|-------------------------------------|------|
| | | | β -Pinene $\delta^{13}\text{C}$ | | Limonene $\delta^{13}\text{C}$ | | Linalool $\delta^{13}\text{C}$ | | Linalyl Acetate $\delta^{13}\text{C}$ | | α -Terpineol $\delta^{13}\text{C}$ | | Neryl Acetate $\delta^{13}\text{C}$ | | Gernayl Acetate $\delta^{13}\text{C}$ | | Nerol $\delta^{13}\text{C}$ | | Geraniol $\delta^{13}\text{C}$ | | (E) Nerolidol $\delta^{13}\text{C}$ | |
| | | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Neroli | Morocco | 26 | -26.32 | 0.97 | -26.77 | 1.20 | -25.47 | 0.95 | -25.08 | 1.19 | -26.64 | 1.90 | -27.96 | 0.88 | -26.77 | 0.98 | -26.35 | 1.13 | -26.58 | 0.78 | -29.64 | 2.05 |
| Neroli | Tunisia | 16 | -26.99 | 2.32 | -27.33 | 1.02 | -26.03 | 0.47 | -26.15 | 1.23 | -26.99 | 2.19 | -29.30 | 2.01 | -28.87 | 1.31 | -27.39 | 1.36 | -28.09 | 2.33 | -30.65 | 2.45 |
| Neroli | Lebanon | 4 | -26.99 | 1.08 | -27.83 | 0.63 | -26.37 | 0.65 | -25.54 | 0.35 | -28.10 | 1.69 | -28.41 | 0.42 | -27.38 | 0.60 | -27.18 | 0.51 | -27.19 | 0.75 | -29.44 | 0.65 |
| Neroli | Egypt | 22 | -26.71 | 1.57 | -27.54 | 1.19 | -25.96 | 0.88 | -26.35 | 0.84 | -27.88 | 1.54 | -28.68 | 0.77 | -27.67 | 0.89 | -26.45 | 1.70 | -27.02 | 1.00 | -29.03 | 0.73 |
| Neroli | Spain | 11 | -27.29 | 0.87 | -28.31 | 0.94 | -26.85 | 0.35 | -27.54 | 1.24 | -28.39 | 0.72 | -28.99 | 0.72 | -28.42 | 0.61 | -27.17 | 0.80 | -28.63 | 1.48 | -27.98 | 1.17 |
| Petitgrain | Madagascar | 3 | -28.01 | 1.31 | -35.52 | 0.60 | -29.73 | 0.21 | -29.43 | 0.36 | -28.31 | 0.08 | -33.80 | 1.14 | -33.38 | 0.82 | -27.98 | 3.07 | -30.77 | 0.47 | N/A | N/A |
| Petitgrain | Paraguay | 6 | -31.51 | 2.92 | -34.21 | 2.97 | -29.48 | 0.23 | -28.40 | 0.57 | -28.21 | 1.68 | -34.20 | 0.51 | -32.89 | 0.85 | -28.62 | 1.37 | -31.99 | 1.16 | N/A | N/A |

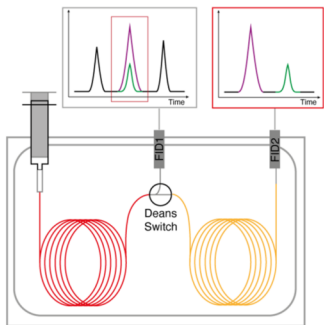
Table 3: Stable isotope $\delta^2\text{H}$ distribution of secondary metabolites of neroli and petitgrain essential oils from different geographical origins: Morocco, Tunisia, Lebanon, Egypt, Spain, Madagascar, and Paraguay.

| Essential oil type | Geographical origin | Number of Samples | Isotopic Distribution (‰) | | | | | | | | | | | | | | | | | | | |
|--------------------|---------------------|-------------------|------------------------------------|----|-----------------------------|----|-----------------------------|----|------------------------------------|----|--|----|----------------------------------|----|------------------------------------|----|--------------------------|----|-----------------------------|----|----------------------------------|-----|
| | | | β -Pinene $\delta^2\text{H}$ | | Limonene $\delta^2\text{H}$ | | Linalool $\delta^2\text{H}$ | | Linalyl Acetate $\delta^2\text{H}$ | | α -Terpineol $\delta^2\text{H}$ | | Neryl Acetate $\delta^2\text{H}$ | | Geranyl Acetate $\delta^2\text{H}$ | | Nerol $\delta^2\text{H}$ | | Geraniol $\delta^2\text{H}$ | | (E) Nerolidol $\delta^2\text{H}$ | |
| | | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Neroli | Morocco | 26 | -213 | 12 | -221 | 12 | -210 | 7 | -223 | 24 | -224 | 12 | -210 | 9 | -203 | 14 | -196 | 11 | -201 | 11 | -102 | 8 |
| Neroli | Tunisia | 16 | -214 | 12 | -223 | 8 | -210 | 6 | -237 | 17 | -228 | 15 | -208 | 11 | -204 | 10 | -189 | 9 | -204 | 15 | -106 | 8 |
| Neroli | Lebanon | 4 | -218 | 4 | -229 | 5 | -209 | 7 | -227 | 3 | -222 | 5 | -202 | 8 | -203 | 16 | -198 | 7 | -205 | 4 | -103 | 4 |
| Neroli | Egypt | 22 | -208 | 32 | -216 | 17 | -199 | 16 | -217 | 15 | -217 | 14 | -187 | 16 | -182 | 9 | -177 | 15 | -188 | 9 | -84 | 11 |
| Neroli | Spain | 11 | -211 | 6 | -212 | 21 | -210 | 16 | -220 | 32 | -223 | 7 | -214 | 11 | -205 | 12 | -198 | 17 | -194 | 10 | -101 | 14 |
| Petitgrain | Madagascar | 3 | -212 | 15 | -217 | 3 | -219 | 5 | -212 | 6 | -239 | 3 | -221 | 3 | -229 | 2 | -271 | 14 | -220 | 1 | N/A | N/A |
| Petitgrain | Paraguay | 6 | -205 | 4 | -239 | 10 | -239 | 5 | -240 | 8 | -255 | 4 | -240 | 5 | -241 | 4 | -291 | 12 | -232 | 17 | N/A | N/A |

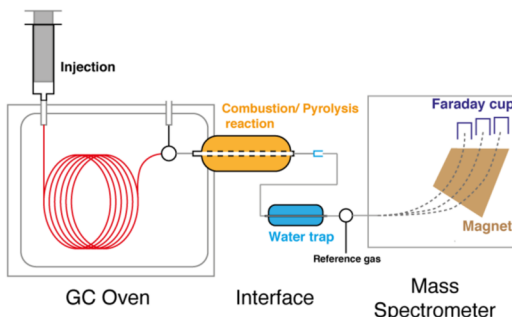
Neroli essential oil sample



Enantiomeric and Stable Isotope Ratio Fingerprints



Enantio-MDGC

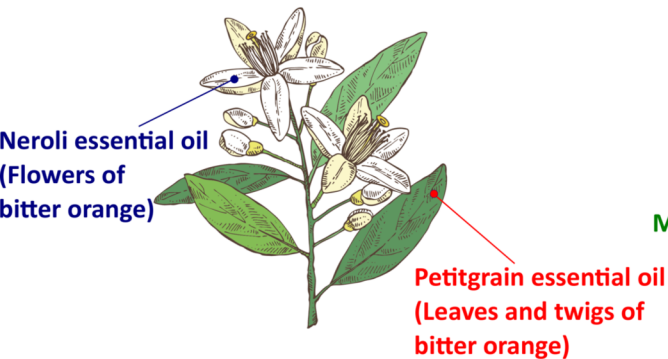


GC-C/P-IRMS



Chemometric models

Purity authentication



Geographical differentiation

