



Authentication of the naturalness of wintergreen (Gaultheria genus) essential oils by gas chromatography, isotope ratio mass spectrometry and radiocarbon assessment

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1 **Authentication of the naturalness of wintergreen (*Gaultheria* genus) essential**
2 **oils by gas chromatography, isotope ratio mass spectrometry and**
3 **radiocarbon assessment**

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15

Abstract

The essential oil of wintergreen, which is extracted by steam distillation from *Gaultheria* genus leaves, is mainly used in aromatherapy. However, due to its adulteration easiness with synthetic material, it is necessary to control samples naturalness with accuracy. The purpose of this work was to develop a methodology to authenticate the essential oil of wintergreen. Wintergreen essential oil is composed of more than 99% methyl salicylate. This aromatic ester can be easily synthesized and used to adulterate wintergreen essential oil. Authentic wintergreen essential oil can be distinguished from adulterated oils by examining their compositions. The detection of methyl salicylate synthetic marker compounds (methyl 4-hydroxybenzoate, dimethyl 4-hydroxyisophthalate or dimethyl 2-hydroxyisophthalate) or the absence of several naturally occurring minor secondary metabolites (ethyl salicylate and vitispirane) contribute to the authentication. Isotopic values of bulk wintergreen essential oil have also been determined using an isotope ratio mass spectrometer (IRMS): the $\delta^{13}\text{C}$ values of authentic samples range from -36.78 to -33.36‰, the $\delta^2\text{H}$ values range from -173 to -115‰ and the $\delta^{18}\text{O}$ values range from -1.3 to 5.7‰. However, these analytical methods cannot account for the natural variability in the essential oils. To determine the boundaries of the natural isotopic values, ^{14}C radioactive isotope activity assessment was undertaken, which allows for the determination of the genuineness of samples that are not assessed using multistable isotope approaches. Additional studies evaluating ^{14}C activity of a noncompliant sample using IRMS identified a ^{14}C -labeled synthetic methyl salicylate adulteration.

Keywords

Wintergreen essential oil, Methyl salicylate, Chemical composition, Isotope ratio mass spectrometry, Authentication, Radiocarbon activity

1. Introduction

Natural raw materials such as essential oils are obtained from the extraction of vegetal products and are currently widely used in food products, cosmetics (including fragrances), pharmaceuticals, and

41 aromatherapy products due to their medicinal and olfactory properties. These products are
42 sometimes susceptible to fraud: essential oils sold to consumers do not always possess their original
43 compositions, despite being labeled 100% pure and natural. Suppliers resort to adulterating their
44 products by adding low-cost products to their natural materials to improve the product quality, to
45 increase the raw material volume, or for economic reasons. One of the main adulterations of
46 essential oils is the addition of less expensive synthetic compounds. Thus, it is necessary to develop
47 methods of authentication to identify such practices in order to empower consumers to be sure of
48 the natural quality of their purchases and to enable suppliers to prove the authenticity of their
49 products.

50 The essential oil of wintergreen can be obtained by the steam distillation of leaves from different
51 species of the genus *Gaultheria*. These essential oils are produced mainly in high altitude locations in
52 North America (Canada), China, and Nepal, and the harvest period usually lasts from May until
53 September. The botanical sources of *Gaultheria* that are most commonly used to produce
54 wintergreen essential oils are *procumbens* and *fragrantissima*, because these species yield a high
55 concentration of methyl salicylate. However, wintergreen essential oils obtained from other species
56 of *Gaultheria* such as *yunnanensis* and *hispidula* are commercialized as well. The steam distillation
57 yield of leaves is less than 2% (Bantawa et al., 2011). The quantity of essential oils produced in China
58 is estimated to be between 20 and 70 Mg. Farmers are interested in growing the essential oil plants
59 only where prices are high enough to make the crop economically viable and falling prices will quickly
60 lead to a change to the cultivation of other more valuable crops.

61 Due to its very high concentration in the aromatic ester methyl salicylate, wintergreen is notable of
62 the field of essential oils. Methyl salicylate represents more than 99% of the composition of most
63 wintergreen species after a bidistillation process (Cong et al., 2015; Nikolić et al., 2013; Singh et al.,
64 2018). The aromatic and herbaceous wintergreen fragrance can be used in perfumery and in the
65 cosmetic and food industries, but the main uses are in aromatherapy, oral care, and natural

preservatives for cosmetics. Many medical ointments used in aromatherapy are composed of wintergreen essential oil (Mukhopadhyay et al., 2016) because of its analgesic and anti-inflammatory properties (Delplancq, 2015; Liu et al., 2013; Michel et al., 2014; Park et al., 2011), which efficiently relieve pain caused by muscle fever (Ribnicky et al., 2003). Antispasmodic, vasodilators, and liver stimulant properties of the essential oil have also been reported (Franchomme et al., 2012). Wintergreen possesses other properties that are useful for various applications. For example, wintergreen essential oil is used as a biopesticide (Dayan et al., 2009) in food crops and food packaging because of its antifeedant properties (Jeyasankar, 2012; Prakash and Prakash, 2015), and it is used as a natural preservative in cosmetic products because of its antioxidant and antibacterial activities (Pandey et al., 2017).

Suppliers desiring to increase their profits illegally might be inclined to adulterate their natural product by adding synthetic materials that are less expensive. For the 2018 harvest, the price of essential oil from China was between 50 and 65 euros per kilogram. The most common method of wintergreen essential oil adulteration is the addition or substitution of synthetic methyl salicylate. This synthetic molecule can be prepared through several different chemical pathways (Fig. 1.). The most common pathway starts with salicylic acid that has been obtained by conducting a Kolbe Schmitt reaction (Luo et al., 2016) using phenol, which can be synthesized from petroleum products using the Hock process (also known as cumene process). The methylation of salicylic acid leads to synthetic methyl salicylate (D'Souza and Nagaraju, 2007; Iijima and Yamaguchi, 2008; Su et al., 2009). Another previously reported method to obtain salicylic acid employs the Lowig oxidation process with salicylaldehyde as the starting material. Salicylaldehyde can be obtained in several ways, such as via the Reimer Tiemann reaction, but salicylaldehyde also occurs naturally in meadowsweet flowers (Olennikov et al., 2016). This process is more expensive than the classical salicylic acid approach, and an additional step is required. The most economical current method to synthesize methyl salicylate is the hydrolysis of aspirin (acetylsalicylic acid), which is industrially produced from salicylic acid (Hartel and Hanna, 2009; Montes et al., 2006).

Chiral analysis cannot be performed on methyl salicylate to guarantee the natural authenticity of the product because the methyl salicylate molecule does not possess an asymmetrical carbon. Previous studies used isotope ratio mass spectrometry (IRMS) to obtain pertinent isotopic data for methyl salicylate. Synthetically produced methyl salicylate possesses $\delta^{13}\text{C}$ values ranging from -28.32 to -30.84‰ and $\delta^2\text{H}$ values from -51 to -97‰, whereas natural essential oil values obtained from birch range from -33.11 to -33.66‰ for $\delta^{13}\text{C}$ and from -56 to -163‰ for $\delta^2\text{H}$ (Culp and Noakes, 1992). An authentication method for methyl salicylate in black tea using ^{13}C GC-C-IRMS was published in 1999 (Weinert et al., 1999). The most recently developed naturalness authentication method used ERETIC analysis via SNIF-NMR (Le Grand et al., 2005).

The objective of this study was to develop an accurate methodology to ensure the naturalness authentication of the wintergreen essential oil. Samples from various providers were studied and compared with commercially produced and laboratory-synthesized methyl salicylate. Different studies were carried out including a characterization of the essential oils, a determination of the synthetic precursors and impurities, the analysis of stable isotope ratios of methyl salicylate, and an assessment of the radiocarbon activity.

2. Material and Methods

2.1. *Samples information*

Twenty-one *Gaultheria procumbens* L. essential oils samples were provided by Albert Vieille (Vallauris, France), originating from China and Nepal. Thirty samples of *Gaultheria procumbens* L., *Gaultheria fragrantissima* Wall., and *Gaultheria leucocarpa* var. *yunnanensis* (Franch.) T.Z. Hsu & R.C. Fang. essential oils were obtained from different suppliers and originated from China and Canada. Eleven commercial samples were purchased from a pharmacy. Two authentic samples of *Gaultheria fragrantissima* Wall. and *Gaultheria leucocarpa* var. *yunnanensis* (Franch.) T.Z. Hsu & R.C. Fang. essential oils, originating in China (Yunnan province) and Nepal, were supplied by Young Living (Lehi, UT, USA). Five synthetic methyl salicylate commercial samples were purchased from several suppliers

(Sigma Aldrich (Saint Louis, MO, USA), Thermo Fisher Scientific (Waltham, MA, USA), and TCI (Tokyo, Japan)). Several methyl salicylates were made in our laboratory via different synthetic pathways. One sample was obtained from aspirin (Sigma Aldrich), three were synthesized with salicylic acid (TCI and Cooper, Melun, France) as a starter material, and one sample was made from salicylaldehyde (Sigma Aldrich).

2.2. Gas chromatography (GC-FID) analysis

GC analyses were carried out using a gas chromatograph 6890N (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID), with the detector temperature set at 250°C. Separation of the volatile compounds was performed on a HP-1 capillary column (50 m*0.320 mm*0.52 µm, Agilent Technologies) and a HP-Innowax capillary column (60 m*0.320 mm*0.50 µm). The GC conditions for the HP-1 column were programmed as follows: 1 µL of pure sample was injected into the GC in the split mode (120:1) with the injector temperature set at 250°C. The carrier gas was helium at a constant flow rate of 1.5 mL/min. The initial oven temperature was held at 80°C for 2 min, ramped to 220°C at a rate of 2°C/min, and then to 295°C at a rate of 10°C/min and held for 30 min. The GC conditions for the Innowax column were programmed as follows: 1 µL of pure sample was injected into the GC in the split mode (100:1) with the injector temperature set at 250°C. The carrier gas was helium at a constant flow rate of 1.3 mL/min. The initial oven temperature was held at 60°C for 2 min, ramped to 245°C at a rate of 2°C/min and held for 35 min. Identification of the volatile compounds was achieved using the Kovats Index determined with relative retention indices from C5 to C30 alkanes.

2.3. Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analyses were performed on a gas chromatograph 6890N GC System (Agilent Technologies) interfaced with a 5975 inert XL MSD mass spectrometer with electron impact ionization (70 eV). Each 1 µL pure sample was injected into the GC in the split mode (120:1), with the injector temperature set at 250°C. Helium was used as the carrier gas with a constant flow rate of 1.2 mL/min. Separation

of the volatile compounds was achieved using a HP-1 capillary column (50 m*0.200 mm*0.50 μ m, Agilent Technologies). The initial oven temperature was held at 60°C for 2 min and then ramped at a rate of 3°C/min to reach a final temperature of 320°C and then held for 10 min. The mass scanning range was from 35 to 350 m/z in TIC. The SIM parameters were as follows: the targeted mass ions (m/z) were 91 (tropylium ion), 121 (M - CH₃O) and 152 (molecular ion) from 36 to 44 min to detect methyl 4-Hydroxybenzoate and 120 (ortho rearrangement), 147 (double loss of CH₃OH (methanol) and CH₃O), 178 (loss of CH₃OH), and 210 (molecular ion) from 44 to 50 min to detect dimethyl 4-hydroxyisophthalate and dimethyl 2-hydroxyisophthalate. NIST library and lab-made library, combined with retention time, retention indices, literature references, and standards injections were used to identify the molecules.

2.4. Elemental Analysis –Isotope Ratio Mass Spectrometry (EA-IRMS)

High purity carbon dioxide (CO₂) 99.998% (4.8 Messer), hydrogen (H₂) 99.999% (5.0 Messer), and carbon monoxide (CO) 99.997% (4.7 Messer) were used as the working reference gases. Helium flow at 99.999% was used as the carrier gas.

International standards IAEA CH7 (polyethylene foil: $\delta^{13}\text{C}$ (‰) -31.82, $\delta^2\text{H}$ (‰) -100.3), USGS40 (L-glutamic acid: $\delta^{13}\text{C}$ (‰) -26.39), and IAEA-601 (Benzoic acid: $\delta^{18}\text{O}$ (‰) +23.3) supplied by the IAEA (International Agency of Atomic Energy, Vienna, Austria) were used for the calibration of all the studied samples. All sample values were obtained in duplicate and were verified by bracketing the analytical sequence with the international standards every sixth analysis.

The carbon isotopic analyses ($\delta^{13}\text{C}$ value) of the synthetic standards were carried out using a Flash HT elemental analyzer connected to a Delta V Plus via a Conflo V interface (Thermo Fisher Scientific, Bremen, Germany). The samples were weighted in tin capsules (5 X 8, Sántis Analytical AG, Teufen, Switzerland) and dropped into the combustion unit, which was maintained at 915°C in a stream of helium with a flow rate of 90 mL.min⁻¹ and pulsed with oxygen. The flash combustion (approximately 1800°C) caused the complete oxidation of carbon to CO₂. The gases flowed through the reduction

furnace, which was held at 600°C and included a copper wire where the excess oxygen was trapped. The reduction furnace was followed by a water trap containing anhydrous magnesium perchlorate. The Flash HT elemental analyzer was connected to the isotope ratio mass spectrometer Thermo Delta V Plus via an online interface (Conflo IV). This intermediary device provided for automatic adjustment of the sample dilution and generation of the reference gas pulses, which facilitated the isotopic determination.

The $\delta^2\text{H}$ and $\delta^{18}\text{O}$ measurements of the synthetic standards were carried out using a TC-EA elemental analyzer linked to a Delta V Plus via a Conflo V interface (all Thermo Fisher Scientific). Samples weighed in silver capsules (3.3 X 5 mm, Sántis Analytical AG, Teufen, Switzerland) were dropped into the pyrolysis unit, which was held at 1400°C. This high-temperature reactor consisted of an inner glassy carbon tube half-filled with glassy carbon granules integrated into an external ceramic tube. Hydrogen and oxygen were converted to H_2 and CO and separated on a gas chromatography column prior to introduction into the IRMS via the same interface (Conflo IV). The interface, as previously described, provided steady and regular reference gas pulses.

The $\delta^{13}\text{C}$ values (‰) were calibrated relative to Vienna Pee Dee Bee Belemnite (VPDB) using two multipulse injections of the CO_2 reference gas. H_2 and CO reference gases were used in the same way for the determination of the $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values (‰), and the results were calibrated to Standard Mean Ocean Water (SMOW).

Isotopic results are expressed in delta unit (δ) according to the following formula:

$$\delta(^{13}\text{C}/^{12}\text{C}) = \left[\frac{R(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{R(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right]$$

where the $\delta^{13}\text{C}$ values are given within a maximum standard deviation (SD) of $\pm 0.21\text{‰}$, $\delta^2\text{H}$ within $\text{SD}=\pm 5\text{‰}$, and $\delta^{18}\text{O}$ within $\text{SD}=\pm 0.6\text{‰}$.

2.5. Radiocarbon analysis via Liquid Scintillation Counting (LSC)

All liquid samples were burned in a pyrocombustion mineralization device (Mineralyseur ERALY) to obtain CO₂. This device consists of a quartz combustion tube and two furnaces in series. An automatic syringe pump type device makes the injection of liquid samples possible. The first furnace, mounted at 400°C, is swept by a flow of nitrogen and triggers the vaporization of the liquid produced. The second furnace, maintained at 750°C, is crossed by an oxygen flow and ensures the oxidation of the gas resulting from the vaporization. The carbon dioxide formed is collected and chemically transformed into benzene (C₆H₆). It is in this liquid form that its carbon-14 content will be measured by counting in liquid scintillation. The samples are measured in liquid scintillation counters with very low background with TRI-CARB 1500, 2000CA/LL, 2260XL, 2560TR/XL, 3170TR/SL, and 2900TR Packard and Perkin Elmer counters. A sample is counted four or five times during 1000 min in different counters each time. The counting rate of the background noise of the counter is subtracted (net count rate) from the counting rate of the sample. The ¹⁴C activity is obtained by normalizing the net count rate from the count rate of the reference standard (NBS Oxalic Acid). All the values of modern carbon are in percent modern and are corrected from isotopic fractionation, using the data of stable isotopes (ratio ¹³C/¹²C) measured on CO₂ obtained from the combustion of the sample. Values are given within a maximum SD of ± 0.6%.

3. Results and discussion

3.1. Essential oil composition via GC-FID and GC-MS

The compositions of wintergreen essential oils and commercial and laboratory-made synthetic methyl salicylates are listed in Table 1 with relative Kovats indices. The major compounds are also represented in Fig. 2.

The synthetic methyl salicylates exhibited a high purity, nearly 99%, but several impurity peaks can be seen in their compositions, which can be attributed to precursors and secondary products of the methyl salicylate synthesis. Seven compounds were identified. Phenol (**16**) was the initial synthesis precursor of the Kolbe Schmitt reaction. Ethyl salicylate (**2**), methyl o-cresotinate (**18**), methyl 2-

215 methoxybenzoate (**17**), and methyl 4-hydroxybenzoate (**13**) were all produced during the
216 methylation of salicylic acid. Dimethyl 4-hydroxyisophthalate (**15**) and dimethyl 2-
217 hydroxyisophthalate (**14**) were secondary products of a Kolbe Schmitt reaction impurity. The other
218 minor compounds were primarily from residual solvents. The ex-salicylaldehyde sample was the only
219 synthetic methyl salicylate without the presence of dimethyl 4-hydroxyisophthalate, which was the
220 second major component in the other six samples. The absence of this compound was expected,
221 since it did not come from the carboxylation of phenol, which was identified in trace amounts in the
222 ex-salicylaldehyde sample.

223 In the set of wintergreen essential oils, a total of 62 compounds were identified, covering more than
224 99% of the total peak area of the 64 samples analyzed. The methyl salicylate represented, among the
225 natural essential oils, the majority of the volatile compounds and represented 97.36 to 99.71% of the
226 total amount. Most of the other components were found in trace amounts and were not
227 systematically present in the wintergreen oils. Natural variability, due to botanical varieties and
228 geographical origins, induces important differences among natural wintergreen compositions. The
229 minority compounds that were the most prevalent were limonene (**1**) (Mean value \pm SD= 0.04 \pm
230 0.06%), ethyl salicylate (**2**) (0.09 \pm 0.07%), linalool (**3**) (0.05 \pm 0.02%), alpha-pinene (**4**) (0.01 \pm 0.02%),
231 geraniol (**5**) (0.02 \pm 0.02%), benzyl alcohol (**6**) (0.02 \pm 0.01%), vitispirane (**7**) (0.02 \pm 0.01%), eugenol
232 (**8**) (0.02 \pm 0.02%), beta pinene (**9**) (0.01 \pm 0.01%), saffrole (**10**) (0.01 \pm 0.04%), para cymene (**11**) (0.01
233 \pm 0.01%), trans-beta caryophyllene (**12**) (0.01 \pm 0.01%), and phenol (**16**) (0.01 \pm 0.00%).

234 Methyl 4-hydroxybenzoate, dimethyl 4-hydroxyisophthalate, and dimethyl 2-hydroxyisophthalate
235 have only been identified in several adulterated essentials oils, in which many secondary metabolites
236 found in authentic wintergreens are in low abundance, such as ethyl salicylate (0.02 \pm 0.02%) and
237 vitispirane (0.00 \pm 0.01%), whereas these samples have similar methyl salicylate concentrations
238 compared with those of natural oils. These three molecules can potentially be used as synthesis
239 marker compounds, but they have not always been detected, probably because of an addition of

impurity-free methyl salicylate or another remedy. A single-ion monitoring (SIM) method was developed for GC-MS in order to monitor trace amounts of these adulteration marker components in all the essential oils in this study.

3.2. Stable-Isotopes analysis

The determination of minor peaks establishing that the process of production is synthesis is not so easy to show, when natural wintergreen is partly diluted. In this scheme, IRMS will be relevant to conclude. The isotopic measurement is performed in 5 min allowing fast and cheap analyses when the assessment of raw materials need to be done.

The $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\delta^{18}\text{O}$ isotopic values of the 64 wintergreen essential oils samples are summarized in Table 2 and shown in a 3D-plot representation (Fig. 3.). Because methyl salicylate constitutes more than 99% of the wintergreen essential oil, isotopic measurements have been conducted using an elemental analyzer connected to an isotope ratio mass spectrometer (EA-IRMS) for bulk stable isotope analysis (BSIA).

The natural methyl salicylates exhibited significantly different values of the three isotope ratios investigated, $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\delta^{18}\text{O}$, compared with those of the synthetic samples. The 3D plot of the isotopic values clearly shows two distinct areas: one for the authentic wintergreen samples and the other one for the synthetic methyl salicylates and adulterated wintergreens. The samples with at least one identified synthetic marker compound demonstrated values close to those of the synthetic methyl salicylates. Conversely, the essential oils without markers possessed isotopic deltas in the same range as those of the natural essential oils. Only two samples were found in the synthetic range that did not have any synthetic markers, but they were located far away from the natural essential oils: an adulteration was clearly demonstrated. All three isotopes were highly correlated, as shown in Fig. 4.

According to Culp et al. (1992), natural methyl salicylates have δ -values, measured in five samples, ranging between -33.11 and -33.66‰ for ^{13}C and from -56 to -163‰ for ^2H . The results obtained in this study of 41 authentic wintergreen oils indicated that the authentic range is tighter for $\delta^2\text{H}$, with an average value of -135‰ and a maximal value of -115‰, and different $\delta^{13}\text{C}$ natural values, with an upper mean value of -34.98‰. The synthetic normal range of $\delta^2\text{H}$ is wider than the deltas of the other isotopes, notably depending on the synthetic origin of the methyl salicylates, as the $\delta^2\text{H}$ isotopic fractionation is larger. Clear differentiation is distinguished between natural and synthetic δ -values: synthetic methyl salicylates show more positive δ -values, especially on the $\delta^{18}\text{O}$, which is consistent with previous work on isotopic patterns of phenylpropanoids (Schmidt et al., 2001). Isotopic ratios of adulterated essential oils have been determined to be between natural and synthetic ones.

3.3. Radiocarbon activity assessment

3.3.1. Analysis of the wintergreen ^{14}C activity

The analysis of the ^{14}C radiocarbon activity was conducted to determine the percentages of biosourced carbon in the samples. A natural product is composed of 100% biosourced carbon, which is equivalent to an activity of 13.7 ± 0.1 dpm/gC in 2017 (Disintegrations per minute per gram of carbon), whereas a synthetic compound from petrochemistry does not have any ^{14}C activity. An adulteration with synthetic methyl salicylate will result in a mixture of natural and synthetic products, which will result in an intermediate radiocarbon activity. This analysis can be carried out with liquid scintillation counting (LSC) or with accelerator mass spectrometry (AMS) methods.

The determination of the percentage of biosourced carbon accurately establishes the percentage of adulteration in analyzed samples. Furthermore, this percentage helps confirm the naturalness of samples in order to verify the results from GC-FID, GC-MS, and EA-C/P-IRMS and to determine more precise authenticity ranges.

Twenty samples from the analytical panel were chosen to be analyzed with LSC. Biosourced carbon percentages of the 20 samples are listed in Table 3. The naturalness range of the analyzed samples was large, from the 100% synthetic-free to the 100% nonnatural. The synthetic commercial methyl salicylate (Sample 20) had no ^{14}C activity, as expected, because it is usually prepared from petroleum products. Wintergreen essential oils that have a higher activity than natural products are probably a mixture of branches and leaves of the *Gaultheria*, because woody tissues are generally older vegetal raw materials, resulting in a higher ^{14}C activity due to higher radioactivity in the past years. These values have been rounded to 100%. Surprisingly, Sample 17 showed 100% biosourced carbon, whereas stable isotopic data classified this sample in the synthetic origin category. Further investigations were carried out to explain this anomaly.

3.3.2. Correlation study

A linear correlative study (pairwise correlation) was conducted to determine which variables from the 3 isotopes and the 73 secondary metabolites that constituted the wintergreen essential oils were the best correlated with the percentage of biosourced carbon. Sample 17 was excluded from this statistical test.

Results determined for the 20 samples analyzed in LSC show that $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ are the isotope values that best described the naturalness of the samples with a Pearson correlation coefficient greater than 0.95 in absolute value. Although $\delta^2\text{H}$ is also strongly correlated with ^{14}C activity, its higher measurement uncertainty and its more significant isotopic dispersion in the various synthetic samples resulted in a lower linear correlation coefficient. Fig. 5. shows the correlation between $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ and the biosourced carbon contents.

The metabolite study confirmed that the presence of synthetic impurities is correlated with the naturalness of the samples. Methyl 4-hydroxybenzoate and dimethyl 4-hydroxyisophthalate both had a Pearson correlation coefficient higher than 0.90 in absolute value. Furthermore, some alternative metabolites were directly correlated with the percentage of biosourced carbon (with $r > |0.85|$). A

small amount of vistispirane (<0.20%) and ethyl salicylate (<0.06%) could potentially indicate the addition of a synthetic material. However, the concentration of these compounds can be extremely variable from one wintergreen to another, as geographical origin and variety affect the composition of essential oils.

3.3.3. ¹⁴C-labeled-compound addition

Sample 17 demonstrated a natural radiocarbon activity, despite being clearly adulterated, as its $\delta^{13}\text{C}$, ^2H and ^{18}O values were in the synthetic area. One hypothesis is that this sample had been adulterated with a ¹⁴C marked compound. ¹⁴C labeled phenol, methanol, salicylic acid, and methyl salicylate are available on the market. The methyl salicylate molecule is composed of 8 carbons. Each of these carbons can potentially be ¹⁴C labeled. To demonstrate this adulteration, a saponification was conducted to remove the methyl and produce salicylic acid. This chemical reaction was carried out on samples 1 and 17 with the addition of sodium hydroxide and methanol by heating under reflux. After methanol removal and acidification, a white powder of salicylic acid was obtained. Radiocarbon activity was assessed on both salicylic acids and compared with the parent wintergreen. The ex-sample 1 (1S) salicylic acid ¹⁴C activity was not altered by the saponification, whereas ex-sample 17 (17S) salicylic acid exhibited a biosourced carbon percentage greater than 115%. These observations indicate that the removed carbon did not have any radioactive activity and that the produced salicylic acid had a higher radioactive activity than any natural material. Therefore, this wintergreen essential oil was indeed adulterated with the addition of synthetic methyl salicylate and some ¹⁴C-labeled methyl salicylate. A 1 mL solution of 250 microcurie 6C-ring labeled methyl salicylate can mimic approximately 40500 kg of 100% synthetic methyl salicylate to appear as a 100 % biosourced content, despite its synthetic origin. Use of ¹⁴C-labeled salicylic acid or methanol is also a feasible approach to adulterating synthetic methyl salicylate during the Kolbe Schmitt reaction. This fraud could not be detected with radiocarbon activity measurement methods, and only the use of IRMS highlighted this adulteration possibility.

337 **4. Conclusion**

338 The multianalytical approach presented in this study demonstrated several methods to clearly
339 authenticate the naturalness of wintergreen essential oils.

340 An abnormally low concentration of some secondary metabolites in wintergreen, such as ethyl
341 salicylate or vitispirane, can indicate that adulteration has occurred. However, this marker is only an
342 indicator, as compositions differ between each species. The detection of resultant impurities from
343 the synthesis of methyl salicylate (methyl 4-hydroxybenzoate, dimethyl 4-hydroxyisophthalate, and
344 dimethyl 2-hydroxyisophthalate) is another indicator of fraud. The presence of these impurities is
345 proof of the addition of synthetic material.

346 Multielement isotopic analysis with IRMS can also assess the compliance of wintergreen. The
347 advantages of this method are its rapidity and its inexpensive price. The minimal values of
348 acceptability of the 3 isotope deltas were determined by applying an empirical law ($\text{mean} + 3 \times \text{SD}$)
349 considering that the values of the 41 authentic essential oils followed a unimodal normal
350 distribution. The results of these calculations showed that 99.87% of the authentic essential oils
351 exhibited $\delta^{13}\text{C}$, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ isotopic values lower than -32.87‰, -101‰, and 5.9‰, respectively.
352 Wintergreen essential oils exhibiting a higher value for at least one stable isotope are likely to have
353 been adulterated with synthetic material. However, natural isotopic variation is important, because
354 the authenticity area of one isotope can be obscured by slightly adulterated essential oils.

355 Consequently, multielement isotopic analysis is needed to ensure the naturalness of the product.

356 The ^{14}C radioactive activity is a powerful tool to precisely assess the authenticity of natural products,
357 but this study also demonstrated the complementarity of using both stable and radioactive isotope
358 techniques to authenticate wintergreen essential oils, as ^{14}C assessment cannot detect ^{14}C -labeled
359 product additions, which have been only detected with IRMS.

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Table 1. Chemical compositions of presumed authentic wintergreen essential oils, adulterated ones, synthetic and lab-made methyl salicylates

No	Compounds	CAS	KI HP1 ^a	KI Innowax ^b	% Total peak area		
					Authentic wintergreen essential oils (n=41)	Synthetic methyl salicylate (n=10)	Adulterated wintergreen essential oils (n=23)
					Min-Max	Min-Max	Min-Max
	Ethanol	64-17-5	480	937	0.00-1.37	0.00-0.03	0.00-2.74
	Acetone	67-64-1	489	827	0.00-0.03	0.00-0.02	0.00-0.02
	Hexanal	66-25-1	772	1097	0.00-0.01	nd	0.00-tr
	(E)-2-Hexenal	6728-26-3	822	1241	0.00-0.01	nd	0.00-tr
	(Z)-3-Hexenol-1	928-96-1	831	1397	0.00-0.09	0.00-tr	0.00-0.04
	(E)-2-Hexenol	928-95-0	840	1415	0.00-0.01	nd	0.00-0.01
	1-Hexanol	11-27-1	843	1360	0.00-0.04	0.00-tr	0.00-0.01
	Heptanal	11-71-7	875	1206	0.00-0.01	nd	0.00-tr
	2-Heptanol	543-49-7	879	1320	0.00-0.04	nd	0.00-0.02
	Benzaldehyde	100-52-7	929	1546	0.00-0.01	0.00-tr	0.00-tr
(4)	α -Pinene	80-56-8	932	1037	0.00-0.05	0.00-tr	0.00-0.59
	Camphene	79-92-5	945	1088	0.00-0.01	nd	0.00-0.01
	1-Heptanol	111-70-6	948	1463	0.00-tr	nd	0.00-0.02
(16)	Phenol	108-95-2	950	1981	0.00-0.02	0.00-0.12	0.00-0.06
	Sabinene	3387-41-5	966	1138	0.00-0.01	nd	0.00-0.03
(9)	β -Pinene	127-91-3	972	1129	0.00-0.02	nd	0.00-0.15
	Myrcene	123-35-3	981	1177	0.00-0.01	nd	0.00-0.01
	(Z)-3-Hexenyl Acetate	3681-71-8	984	1334	0.00-0.01	nd	0.00-tr
	α -Phellandrene	99-83-2	999	1185	0.00-0.01	nd	0.00-tr
(6)	Benzyl Alcohol	100-51-6	1004	1881	tr-0.04	nd	0.00-0.02
	α -Terpinene	99-86-5	1011	1202	0.00-0.01	nd	0.00-0.01
(11)	p-Cymene	99-87-6	1013	1295	0.00-0.05	nd	0.00-0.05
(1)	Limonene	138-86-3	1023	1219	0.00-0.33	0.00-tr	0.00-0.80
	1,8-Cineole + β - Phellandrene	470-82-6 555-10-2	1023	1229	0.00-0.01	nd	Nd
	γ -Terpinene	99-85-4	1050	1269	0.00-0.03	nd	0.00-0.01
	1-Octanol	111-87-5	1051	1565	0.00-0.06	nd	0.00-0.01
	Terpinolene	586-62-9	1080	1308	0.00-0.01	nd	0.00-0.01
(3)	Linalool	78-70-6	1083	1561	tr-0.11	nd	0.00-0.46
	Camphor	76-22-2	1121	1556	0.00-0.01	nd	0.00-0.02
	(E)-Pinocarveol	1674-08-4	1124	1683	0.00-0.01	nd	0.00-0.01
	Isopulegol	7786-67-6	1131	1598	0.00-0.01	nd	0.00-0.02
	Ethyl Benzoate	93-89-0	1144	1688	0.00-0.01	nd	0.00-0.18
	Terpinen-4-ol	562-74-3	1162	1622	0.00-0.02	nd	0.00-tr
	Methyl Salicylate	119-36-8	1169	1809	97,36-99.71	96,36-99.93	94.94-99.76
	β -Cyclocitral	432-25-7	1199	1640	0.00-0.01	nd	0.00-0.01
	Nerol	106-25-2	1210	1810	0.00-0.05	nd	0.00-0.01
	Neral	106-26-3	1215	1708	0.00-0.08	nd	0.00-0.03
(5)	Geraniol	106-24-1	1233	1866	0.00-0.06	nd	0.00-0.01
	Geranial	141-27-5	1242	1760	0.00-0.06	nd	0.00-tr
(2)	Ethyl Salicylate	118-61-6	1245	1821	0.05-0.50	0.00-0.04	0.00-0.05
(10)	Safrole	94-59-7	1263	1908	0.00-0.16	nd	0.00-0.01
(18)	Methyl o-Cresotinate	23287-26-5	1267	-	0.00-tr	0.00-tr	0.00-0.01
	Bornyl acetate	200-694-4	1270	1612	0.00-0.08	nd	Nd
(7)	Vitispirane	65416-59-3	1271	1504	0.02-0.03	nd	0.00-0.01
	Isobornyl acetate	125-12-2	1274	1618	0.00-0.02	nd	0.00-0.01

	Nagina Ktone (Dehydro Elshotzia Ketone)	6138-88-1	1276	1884	0.00-0.01	nd	Nd
(17)	Methyl 2-Methoxybenzoate	606-45-1	1295	2070	0.00-0.01	0.00-0.08	0.00-0.01
(8)	Eugenol	97-53-0	1327	2196	0.00-0.12	nd	0.00-0.04
	α -Terpenyl Acetate	80-26-2	1333	1724	0.00-0.03	nd	0.00-tr
	Methyl 2,4-Dihydroxybenzoate	2150-47-2	1346	-	0.00-0.01	0.00-0.02	0.00-tr
	α -Copaene	223-364-4	1377	1517	0.00-0.01	nd	0.00-tr
(13)	Methyl 4-Hydroxybenzoate	202-785-7	1410	-	Nd	0.00-0.06	0.00-0.04
(12)	(E)- β -Caryophyllene	87-44-5	1419	1628	0.00-0.04	nd	0.00-0.10
	Aromadendrene	25246-27-9	1439	1634	0.00-0.05	nd	0.00-tr
	α -Humulene	6753-98-6	1452	1704	0.00-0.01	nd	0.00-0.01
	Eugenyl Acetate	93-28-7	1493	2287	0.00-0.03	nd	0.00-0.01
	Δ -Cadinene	478-76-1	1515	1785	0.00-0.03	nd	0.00-0.02
	(Z)-3-Hexenyl Benzoate	3681-71-8	1542	2160	0.00-0.01	nd	0.00-tr
(15)	Dimethyl 4-Hydroxyisophthalate	5985-24-0	1573	2468	Nd	0.00-0.19	0.00-0.68
(14)	Dimethyl 2-Hydroxyisophthalate	36669-06-04	1611	2583	Nd	0.00-tr	0.00-0.03
	Benzyl Salicylate	118-58-1	1834	2757	0.00-0.05	nd	0.00-0.04
	Total identified				99.89 \pm 0.20	99.06 \pm 1.33	99.85 \pm 0.39

Compositions of essential oils have been determined with GC-FID and GC-MS analysis. Compounds are listed in order of their elution from HP1 column. Total identification data are reported as mean \pm standard deviation.

^a KI: Kovats Index on HP1 column

^b KI: Kovats Index on HP-Innowax column

n: Number of samples, nd: Non-detected, tr: Traces (Compounds detected below 0.01%)

Table 2. $\delta^{13}\text{C}$, $\delta^2\text{H}$, and $\delta^{18}\text{O}$ values of methyl salicylate from various origins determined by EA-C/P-IRMS

Isotopic values	Authentic wintergreen essential oils (n=41)				Synthetic methyl salicylate (n=10)				Adulterated wintergreen essential oils (n=23)			
	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)
$\delta^{13}\text{C}$	-36,78	-33,36	-34.98	0.70	-32,09	-27,71	-29.49	1.49	-32.37	-27,45	-29.42	1.36
$\delta^2\text{H}$	-173	-115	-135	11	-87	63	-54	44	-99	7	-56	28
$\delta^{18}\text{O}$	-1,3	5.7	1.3	1.5	19,5	32.0	23.3	4.0	8.6	29,2	19.0	4.4

n: Number of samples

SD: Standard deviation

EA-C/P-IRMS: Elemental Analysis – Combustion / Pyrolysis – Isotope Ratio Mass Spectrometry

Table 3. Percentage of bio-sourced carbon determined by LSC, along with $\delta^{13}\text{C}$, $\delta^2\text{H}$, $\delta^{18}\text{O}$ values determined by EA-C/P-IRMS, and GC-FID relative peak area percentage of ethyl salicylate, vitispirane, methyl 4-hydroxybenzoate and dimethyl 4-hydroxyisophthalate on 19 samples of wintergreen essential oils (Sample numbers 1 to 19) and one commercial methyl salicylate (Sample number 20). Pairwise correlation values between bio-sourced carbon % and other variables are also referenced. Percentage of bio-sourced carbon determined by LSC on salicylic acid samples (1S and 17S) obtained from samples 1 and 17.

Sample number	Product	LSC	EA-C/P-IRMS			GC-FID (% relative peak area)			
		Biosourced carbon (%)	$\delta^{13}\text{C}$ (‰)	$\delta^2\text{H}$ (‰)	$\delta^{18}\text{O}$ (‰)	Ethyl salicylate	Vitispirane	Methyl-4-hydroxybenzoate	Dimethyl 4-hydroxyisophthalate
1	Wintergreen	92.9	-34.63	-133	1.6	0.09	0.02	0.00	0.00
1S	Salicylic Acid	92.8	-	-	-	-	-	-	-
2	Wintergreen	96.4	-34.99	-119	0.7	0.09	0.02	0.00	0.00
3	Wintergreen	36.2	-29.80	-92	15.0	0.03	0.01	0.01	0.17
4	Wintergreen	100.0	-34.95	-134	0.0	0.06	0.02	0.00	0.00
5	Wintergreen	0.0	-29.18	-43	20.4	0.01	0.00	0.03	0.23
6	Wintergreen	7.4	-29.39	7	20.0	0.02	0.00	0.02	0.14
7	Wintergreen	14.0	-29.75	-13	17.9	0.02	0.00	0.03	0.24
8	Wintergreen	49.1	-31.89	-59	12.8	0.03	0.01	0.02	0.15
9	Wintergreen	28.7	-30.10	-23	16.5	0.02	0.00	0.03	0.21
10	Wintergreen	0.0	-28.41	-46	19.8	0.01	0.01	0.02	0.11
11	Wintergreen	93.5	-34.16	-128	4.4	0.06	0.02	0.00	0.00
12	Wintergreen	100.0	-34.39	-129	1.0	0.10	0.02	0.00	0.00
13	Wintergreen	14.6	-29.17	-17	18.9	0.02	0.00	0.03	0.24
14	Wintergreen	26.8	-30.26	-30	17.1	0.02	0.00	0.02	0.11
15	Wintergreen	27.9	-30.18	-34	17.0	0.02	0.00	0.02	0.12
16	Wintergreen	100.0	-35.63	-137	2.8	0.08	0.02	0.00	0.00
17	Wintergreen	104.7	-28.29	-78	20.0	0.01	0.00	0.00	0.00
17S	Salicylic Acid	116.5	-	-	-	-	-	-	-
18	Wintergreen	100.0	-35.84	-135	3.0	0.12	0.02	0.00	0.00
19	Wintergreen	100.0	-34.65	-135	1.2	0.15	0.02	0.00	0.00
20	Methyl Salicylate	0.0	-28.33	-71	21.7	0.01	0.00	0.02	0.19
Pairwise correlation (Pearson correlation coefficient <i>r</i>)			-0.99	-0.90	-0.99	0.89	0.93	-0.92	-0.90

LSC: Liquid Scintillation Counting

EA-C/P-IRMS: Elemental Analysis – Combustion / Pyrolysis – Isotope Ratio Mass Spectrometry

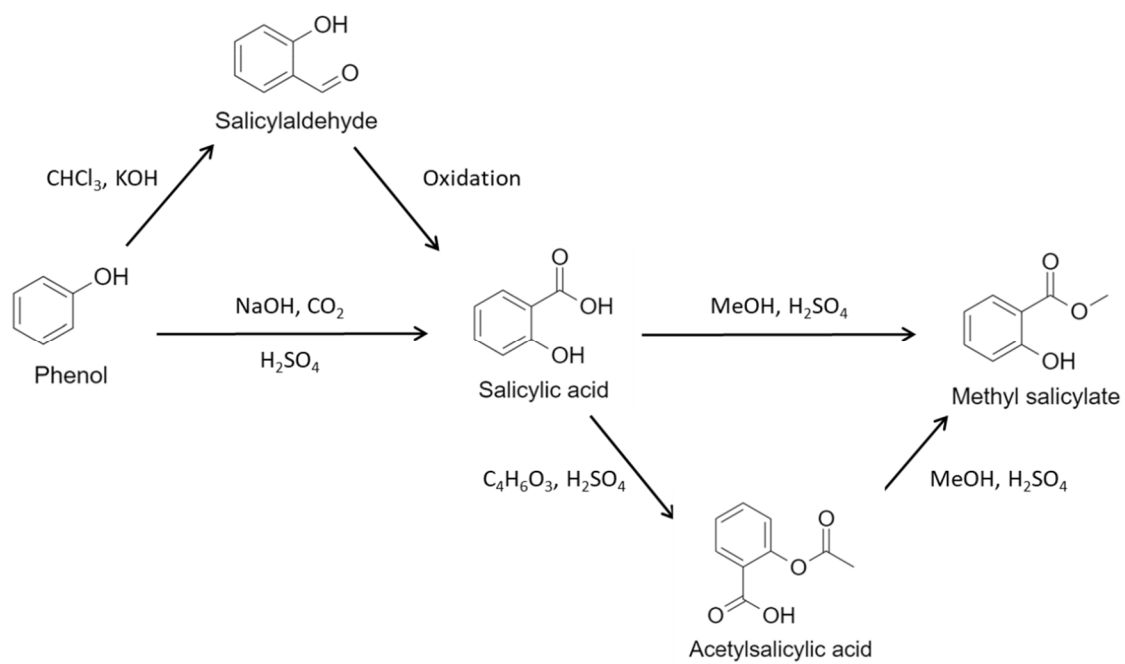


Fig. 1. Principal synthetic paths to synthesize methyl salicylate

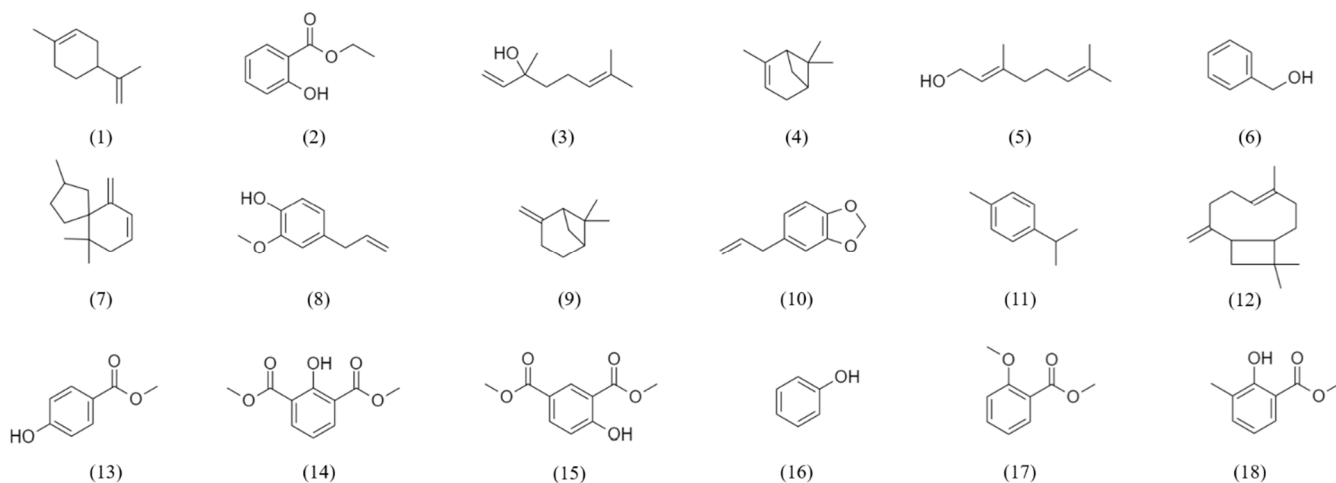


Fig. 2. Structure of major compounds of the essential oil of wintergreen and of resultant impurities from methyl salicylate synthesis. (1) Limonene; (2) ethyl salicylate; (3) linalool; (4) α -pinene; (5) geraniol; (6) benzyl alcohol; (7) vitispirane; (8) eugenol; (9) β -pinene; (10) safrole; (11) p-cymene; (12) (E)- β -caryophyllene; (13) methyl 4-hydroxybenzoate; (14) dimethyl 2-hydroxyisophthalate; (15) dimethyl 4-hydroxyisophthalate; (16) phenol; (17) methyl 2-methoxybenzoate; (18) methyl o-cresotinate

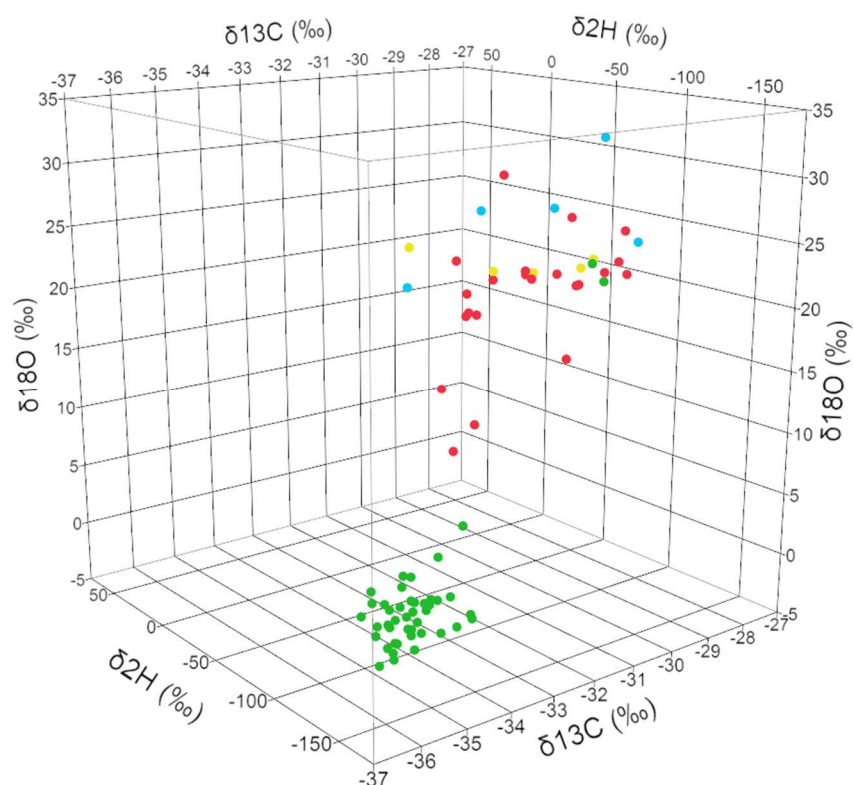


Fig. 3. EA-C/P-IRMS (Elemental Analysis – Combustion / Pyrolysis – Isotope Ratio Mass Spectrometry) multi-element isotopic analysis of methyl salicylate from different origins: lab-made methyl salicylates (in blue); commercial methyl salicylates (in yellow); wintergreen essential oils with at least one synthetic marker compound (in red); wintergreen essential oils with no synthetic impurities (in green). Each sample has been analyzed at least in duplicate: δ values are a mean result of both determined results.

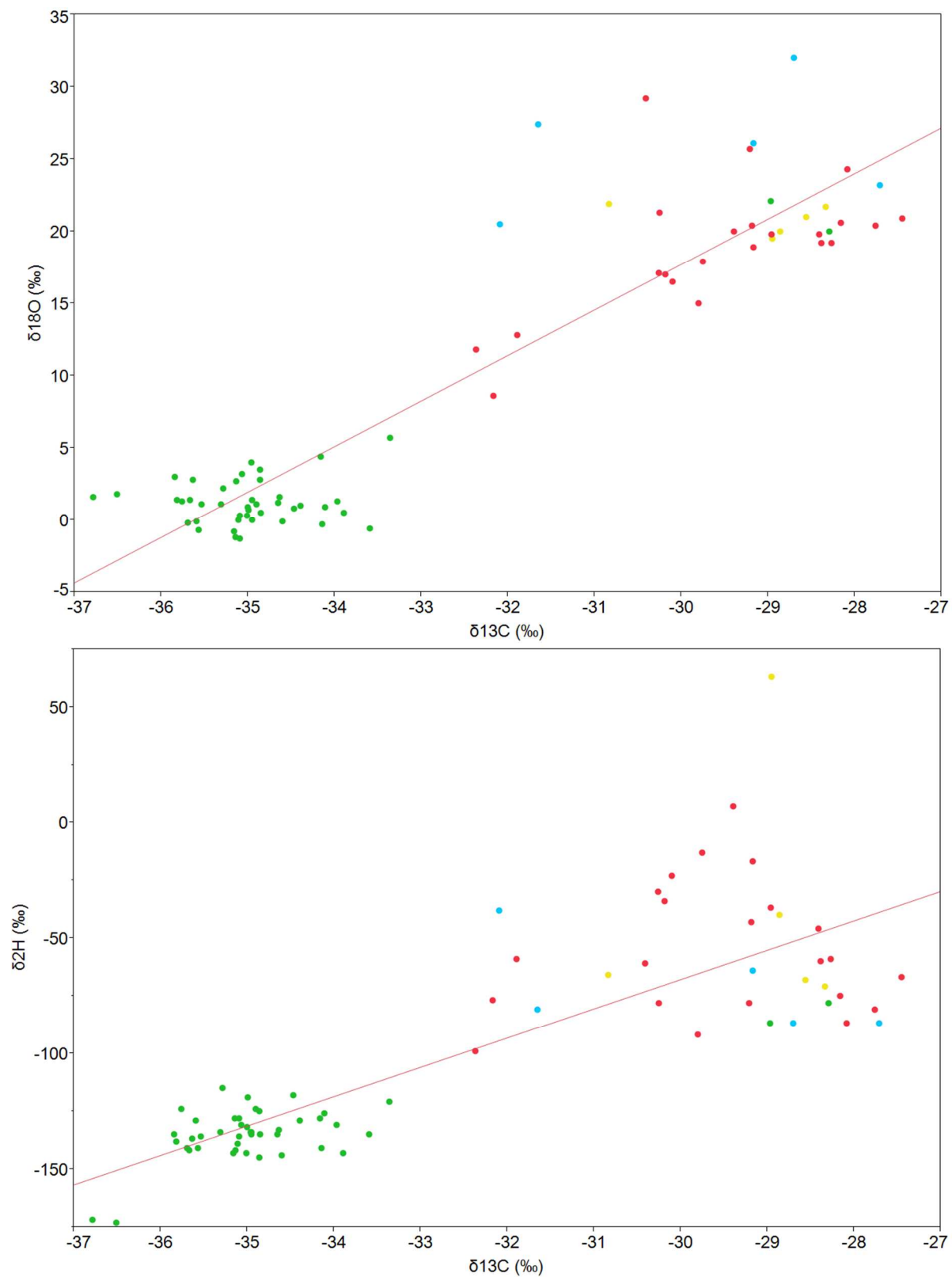


Fig. 4. Correlation between $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values ($r^2=0.85$) and between $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values ($r^2=0.67$) of methyl salicylates from different origins: lab-made methyl salicylates (in blue); commercial methyl

493 salicylates (in yellow); wintergreen essential oils with at least one synthetic marker compound (in
494 red); wintergreen essential oils with no synthetic impurities (in green).

495

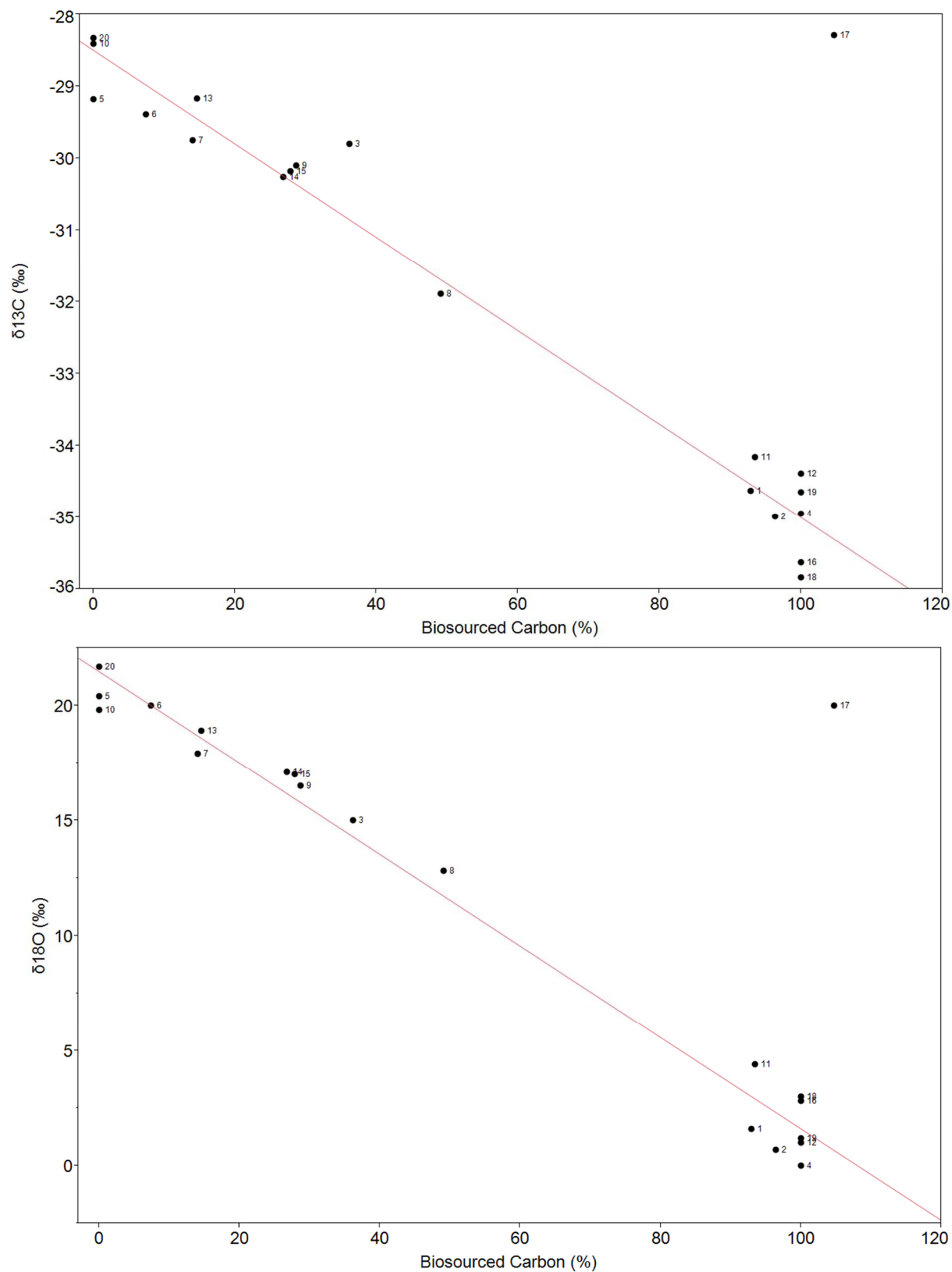


Fig. 5. Bivariate fit of $\delta^{13}\text{C}$ (‰) and $\delta^{18}\text{O}$ (‰) by % of bio-sourced carbon measured on 19 wintergreen essential oils and one commercial synthetic methyl salicylate (Sample 20). In red: Linear regression fit of the data (excluding sample 17). Determination coefficient: $r^2=0.97$ and $r^2=0.98$.

