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Authenticity Assessment of Estragole and Methyl Eugenol by On-Line Gas Chromatography−Isotope Ratio Mass Spectrometry

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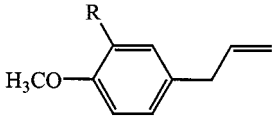
On-line capillary gas chromatography−isotope ratio mass spectrometry was used in the combustion (HRGC−C−IRMS) and the pyrolysis (HRGC−P−IRMS) modes to determine $\delta^{13}C_{PDB}$, $\delta^{2}H_{SMOW}$, and $\delta^{18}O_{SMOW}$ data of estragole (**1**) and methyl eugenol (**2**) originating from various sources. For **1**, similar $\delta^{13}C$ values, i.e., ranging from −35.4 to −29.9 ‰ and from −36.4 to −28.8 ‰ for the product of synthetic and natural origins, respectively, were found. The $\delta^{2}H$ values ranged from −155 to −3 ‰ for synthetic **1** and from −193 to −105 ‰ for **1** from natural origin, whereas the determination of $\delta^{18}O$ data gave values from +1.8 to +24.8 ‰ and from +2.7 to +18.7 ‰ for **1** from synthetic and natural origins, respectively. As synthetic **2** is produced by methylation of natural eugenol, the IRMS techniques did not allow differentiation of synthetic **2** from the product of natural origin. The recorded data ranges were nearly identical, i.e., $\delta^{13}C$ = −37.4 to −35.0 ‰ and −41.1 to −32.2 ‰; $\delta^{2}H$ = −155 to −126 ‰ and − 217 to −107 ‰; $\delta^{18}O$ = +5.5 to +6.6 ‰ and +2.7 to +6.9 ‰, each for **2** from synthetic and natural origins, respectively.

KEYWORDS: Authenticity profiles; origin assessment; multi-element stable isotope ratio analysis; estragole; methyl eugenol; tarragon oil; sweet basil oil; pimento oil; laurel leaf oil; *Tagetes lucida*; HRGC−C/P−IRMS

INTRODUCTION

The structurally related *p*-allylalkoxybenzene derivatives estragole (4-methoxyallylbenzene, **1**), and methyl eugenol (3,4-dimethoxyallylbenzene, **2**), occur naturally in a variety of traditional foods, particularly in spices such as tarragon, basil, fennel, star anise, and anise.



R = H, estragole

R = OCH_3, methyl eugenol

In 2000, it was reported that chronic oral intake of high dose levels of methyl eugenol was associated with increased incidence of hepatotoxicity and liver and stomach neoplasms in F344/N rats and B6C3F1 mice (*1*). The results of this study, together with recent data on the pharmacokinetics, metabolism, toxicity, and genotoxicity for **1** and **2**, as well as those of other allylalkoxybenzenes, have been evaluated most recently (*2*). This evaluation revealed that present exposure to **1** and **2** resulting from consumption of food, mainly spices and added as such, does not pose a significant cancer risk. Nonetheless, chemically synthesized **1** and **2** were banned for food use most recently by the German government; however, their use as constituents of natural tissues, such as spices or essential oils, remains permitted (*3*). At present, similar regulation is under discussion by the EU authorities. Thus, there is a strong interest in the ability to differentiate analytically the origin of these allylalkoxybenzenes. For this purpose, the well-established analytical techniques based on enantiodifferentiation cannot be applied, as they are limited to chiral structures. However, mass spectrometrical measurement of isotope ratios has been demonstrated to be an effective tool for flavor authenticity assessment (*4*−*6*). In this paper, we describe first studies of origin assessment of **1** and **2** using the on-line coupling of gas chromatography and multi-element stable isotope ratio mass spectrometry (HRGC−IRMS).

MATERIALS AND METHODS

Chemicals and Essential Oils. Commercial synthetic and natural samples of **1** and **2** were from Acros, Geel, Belgium; Aldrich, Steinheim, Germany; Fluka, Deisenhofen, Germany; Lancaster, Mülheim, Germany; and Roth, Karlsruhe, Germany. In addition, **1** and **2**

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were self-prepared from turpentine oil according to reference *7*, as well as by methylation of eugenol according to reference *8*, respectively.

Essential oils were from Adrian, Marseille, France; Ayus, Bühl, Germany; BFA, Le Cannet, France; Handa, Nottingham, UK; Roth, Karlsruhe, Germany; and Vieille, Vallauris, France. *Tagetes lucida* oil was a gift from Prof. Dr. C. Bicchi, Torino, Italy.

Sample Preparation. Synthetic and natural samples of **1** and **2**, as well as essential oils, were dissolved (0.2−2.0 mg/mL) in diethyl ether, and the solutions were directly analyzed by HRGC−MS and HRGC−C/P−IRMS.

The volatiles from dried basil (*Ocimum basilicum*) and estragon (*Artemisia dracunculus*) herbs, and from pimento (*Pimenta racemosa*) fruits, all purchased from Klenk, Schwebheim, Germany, were separated by simultaneous distillation extraction (SDE) (3 h) using pentane/diethyl ether mixture (1 + 1, v/v). The extract obtained from each of 50 g was dried over anhydrous sodium sulfate, filtered, and carefully concentrated to an appropriate volume using a Vigreux column (40 °C). As shown by model experiments carried out with **1** and **2**, the influence of SDE on the IRMS data was negligible (data not presented). The same experience has been previously reported (*9*).

Gas Chromatography−Isotope Ratio Mass Spectrometry (HRGC−IRMS). A Finnigan Delta plus XL isotope ratio mass spectrometer coupled by an open-split via a combustion/pyrolysis (C/P) interface to an HP 6890 gas chromatograph (GC) was used. The GC was equipped with a J & W DB-Wax fused silica capillary column (60 m × 0.32 mm i.d.; df = 0.25 μm). The following conditions were employed: 1 μL splitless injection (250 °C); temperature program, raised from 50 to 220 °C at 5 °C/min; helium flow, 2 mL/min and 3 mL/min for $\delta^{18}O$ as well as $\delta^{13}C$ and $\delta^{2}H$ determinations, respectively.

Interfaces. $^{13}C/^{12}C$: combustion by oxidation reactor (Al_2O_3, 0.5 mm i.d., 1.5 mm o.d., 320 mm) with Cu, Ni, Pt (each 240 mm × 0.125 mm) to CO_2 at 960 °C; water separation by Nafion membrane.

Pyrolysis. (i) $^{2}H/^{1}H$: pyrolysis in the reactor (Al_2O_3; 1.5 mm o.d., 320 mm) to H_2 at 1440 °C. (ii) $^{18}O/^{16}O$: pyrolysis in the reactor (Al_2O_3; 1.5 mm o.d., 320 mm; Pt, Ni) to CO at 1250 °C, using auxiliary ("magic-mix") gas, 1% hydrogen in helium; flow 0.7 mL/min.

In addition, an open-split coupling of the IRMS to elemental analyzers (EA) ($^{13}C/^{12}C$ Euro Vector EA 3000, Milano, Italy; temperature, 1000 °C; $^{2}H/^{1}H$ and $^{18}O/^{16}O$ HT Sauerstoff-Analysator, HEKATech, Wegberg, Germany; temperature, 1460 °C) was realized for off-line control determinations of reference substances.

System stability checks were carried out routinely by measuring an International Atomic Energy Agency (IAEA) standard.

The isotope ratios are expressed in per mil (‰) deviation relative to the PDB and SMOW international standards. For $^{13}C/^{12}C$ and $^{2}H/^{1}H$ determinations the mass spectrometer was calibrated against reference CO_2 and H_2 gasses, respectively, (Messer Griesheim, Frankfurt, Germany) with defined $^{13}C/^{12}C$ and $^{2}H/^{1}H$ content ($\delta^{13}C_{PDB}$: −24.9 ‰; $\delta^{2}H_{SMOW}$: −200 ‰). Results are expressed in $\delta^{13}C_{PDB}$ values as

$$\delta^{13}C_{PDB}\ [‰] = \left(\frac{R_{Sample} - R_{PDB}}{R_{PDB}}\right) \times 1000 \qquad (a)$$

where R is the isotope ratio $^{13}C/^{12}C$. The same calculation was used for the determination of $\delta^{2}H_{SMOW}$ values.

$\delta^{18}O$ determinations were performed by secondary standardization. As SMOW could not be directly analyzed under the described HRGC−P−IRMS conditions, (noncertified) CO gas (Messer Griessheim, Frankfurt, Germany) was used as working standard. Owing to the parallel connection of a pyrolysis EA to the IRMS, the $\delta^{18}O_{SMOW}$ value of the CO gas could be determined by measuring the international standard $BaCO_3$ (IAEA-CO-9) (with a known $\delta^{18}O_{PDB}$ value of −15.3 ± 0.1 ‰). By means of the equilibration (b)

$$\delta^{18}O_{SMOW} = 1.03091 \times \delta^{18}O_{PDB} + 30.91 \qquad (b)$$

(*10*), the $\delta^{18}O_{SMOW}$ value of $BaCO_3$ was calculated to be +15.14 ± 0.1 ‰.

The calculation of $\delta^{18}O(CO)_{SMOW}$ is then performed according to (c)

$$\delta^{18}O(CO)_{SMOW}\ [‰] = \left[\frac{\left(\frac{\delta^{18}O(BaCO_3)_{SMOW}}{1000} + 1\right)}{\left(\frac{\delta^{18}O(BaCO_3)_{CO}}{1000} + 1\right)} - 1\right] \times 1000 \qquad (c)$$

and, thus, indirect calculation of $\delta^{18}O_{SMOW}$ values of substances to be determined can be realized.

As mean value for the CO reference gas, $\delta^{18}O_{SMOW}$ = +6.8 ± 0.2 ‰ was calculated. Control analyses performed daily using the IAEA standard $BaCO_3$ revealed high stability of the CO gas within this standard deviation, thus permitting secondary standardization of $^{18}O/^{16}O$ ratios.

In general, 6-fold determinations were carried out, and standard deviations were calculated. Additional control of peak recognition was performed by reference compounds and HRGC−MS registered under identical separation conditions as samples.

Gas Chromatography−Mass Spectrometry (HRGC−MS). A Fisons GC 8000 series gas chromatograph with split injection (220 °C; 1:20) was directly coupled to a Fisons Instruments MD 800 mass spectrometer. The same type of J&W DB-Wax fused silica column was used under conditions identical to those mentioned above (HRGC−IRMS). The temperature of the ion source was 210 °C, and that of the connecting parts was 230 °C. The electron energy for the EI mass spectra was 70 eV, and the cathodic current was 4.1 mA.

RESULTS AND DISCUSSION

Whereas HRGC−C−IRMS is a well-established technique in authenticity studies of volatiles (*4*−*6*), information about $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios determined by HRGC−P−IRMS is rather scarce. Fundamental technical information (*11*−*13*) has been extended recently by application to several flavor compounds (*9*), including vanillin (*14*), benzaldehyde (*15*), and citral (*16*, *17*).

In previous studies we have observed that with our instrumental setup the pyrolysis shows structurally dependent kinetics, and, consequently, it is necessary to determine the range of sample amounts, in which not only reproducibility but also linearity of data can be obtained (*9*, *15*, *17*). Thus, first, the $\delta^{2}H$ and $\delta^{18}O$ values of defined references of **1** and **2** were reproducibly determined off-line via the equipped elemental analyzer (EA). In subsequent HRGC−P−IRMS analyses using reference compounds **1** and **2** the kinetic of pyrolysis was checked. The results of these fundamental experiments are summarized as follows: The $\delta^{2}H_{SMOW}$ data were −20 ± 3‰ and −3 ± 4‰ as well as −104 ± 3‰ and −133 ± 5‰ for **1** and **2** determined by EA−P−IRMS and HRGC−P−IRMS, respectively. The $\delta^{18}O_{SMOW}$ values were +13.9 ± 0.5‰ and +13.3 ± 0.3‰ as well as +6.7 ± 0.3‰ and +6.3 ± 0.2 ‰ for **1** and **2** measured by EA−P−IRMS and HRGC−P−IRMS, respectively. Reproducible linearity was observed at amounts of >1 μg (on-column) in $\delta^{2}H$ determinations, whereas in $\delta^{18}O$ measurements linearity was given at amounts ranging from 0.6 to 2 μg (on-column) of **1** and **2**, respectively.

Thus, both the $\delta^{2}H$ and $\delta^{18}O$ data agreed sufficiently with those measured off-line by EA analysis. The observed differences from the off-line recorded values might be explained by the quite high impurities (1−3%) in the samples influencing the isotope ratios analyzed by the EA technique. As additionally shown from the data, however, the low dynamic of the system is a crucial, hampering fact as only in a small range of concentrations is linearity of data observed.

Estragole 1. The multi-element data of **1** measured in various samples by HRGC−C/P−IRMS are summarized in **Table 1**.

Table 1. $\delta^{13}C_{PDB}$, $\delta^{2}H_{SMOW}$, and $\delta^{18}O_{SMOW}$ Values of Estragole (**1**) from Various Origins Determined by HRGC–C/P–IRMS[a]

origin	$\delta^{13}C_{PDB}$ [‰]	$\delta^{2}H_{SMOW}$ [‰]	$\delta^{18}O_{SMOW}$ [‰]
commercial			
nature identical ($n = 4$)	–32.8 to –29.9	–155 to –3	+13.3 to +24.8
produced from turpentine oil	–35.4	–19	+1.8
natural/essential oils			
commercial "natural" ($n = 2$)	–29.6 to –28.8	–125 to –110	+12.5 to +18.7
commercial basil oil ($n = 7$)	–34.7 to –32.2	–177 to –141	+13.2 to +15.1
commercial tarragon oil ($n = 4$)	–36.4 to –34.7	–193 to –173	+2.7 to+8.5
Ocimum basilicum	–33.6	–105	+10.2
Tagetes lucida	–35.9	–185	+3.5

[a] Mean values (or ranges of mean values) from six determinations with standard deviations of 0.1–0.2, 1–3, and 0.2–0.6 for $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$, respectively; n = number of samples.

Table 2. $\delta^{13}C_{PDB}$, $\delta^{2}H_{SMOW}$, and $\delta^{18}O_{SMOW}$ Values of Methyl Eugenol (**2**) from Various Origins Determined by HRGC–C/P–IRMS[a]

origin	$\delta^{13}C_{PDB}$ [‰]	$\delta^{2}H_{SMOW}$ [‰]	$\delta^{18}O_{SMOW}$ [‰]
commercial			
nature identical ($n = 5$)	–37.4 to –35.0	–155 to –126	+5.5 to +6.6
methylation of eugenol	–40.2	–114	+3.8
natural/essential oils			
commercial pimento oil ($n = 3$)	–35.2 to –32.2	–162 to –157	+4.1 to + 7.0
commercial laurel oil	–33.6	–107	+2.7
Pimenta racemosa	–32.5	–135	+8.9
Artemisia dracunculus	–41.1	–217	+4.5
Ocimum basilicum	–35.2	–123	+6.9
Tagetes lucida	–34.8	–179	+3.9

[a] Mean values (or ranges of mean values) from six determinations with standard deviations of 0.1–0.2, 1–4, and 0.2–0.6 for $\delta^{13}C$, $\delta^{2}H$, and $\delta^{18}O$, respectively; n = number of samples.

As expected, similar $\delta^{13}C$ data were recorded for synthetic ($\delta^{13}C_{PDB} = -35.4$ to -29.9 ‰) and natural samples ($\delta^{13}C_{PDB} = -36.4$ to -28.8 ‰) (*4*). The $\delta^{2}H$ values ranged from -155 to -3 ‰ for synthetic **1** and from -193 to -105 ‰ for the product from natural origin, whereas $\delta^{18}O$ data ranging from $+1.8$ to $+24.8$ ‰ and from $+2.7$ to $+18.7$ ‰ for **1** from synthetic and natural origins, respectively, were determined. Owing to the biochemical origin and precursors of **1**, $\delta^{18}O_{SMOW}$ values ranging from $+6$ to $+12$‰ can be expected (*18*). This range was surpassed, in part, by a few commercial samples under study declared to be natural, rendering their natural status questionable. However, the results suffer from the limited number of samples and their uncertified declaration, hampering unambiguous judgment. The $\delta^{18}O$ values determined for the nature-identical samples corresponded to the information recently provided on ^{18}O patterns of flavor compounds (*18*).

Methyl Eugenol 2. Only limited literature information about the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ isotope ratios of eugenol determined by IRMS is available (*4*, *18*, *19*). For authenticity assessment purposes, the $\delta^{2}H$ and $\delta^{18}O$ data of eugenol are comparable to that of **2**, as no discrimination by methylation in the course of biogenesis has been observed (*4*). The multi-element data of various samples of **2** determined by HRGC−C/P−IRMS in the present study are summarized in **Table 2**. As synthetic **2** is produced by methylation of natural eugenol (i.e., clove oil), the IRMS techniques did not allow differentiation of synthetic **2** from the product of natural origin. Similar data ranges were recorded, i.e., $\delta^{13}C = -37.4$ to -35.0 ‰ and -41.1 to -32 ‰; $\delta^{2}H = -155$ to -126 ‰ and -217 to -107 ‰; $\delta^{18}O = +5.5$ to $+6.6$ ‰ and $+2.7$ to $+8.9$ ‰, each for **2** from synthetic and natural origins, respectively. The biochemically expected area of $\delta^{18}O_{SMOW}$ pattern of **2** has been described to be around $+6$‰ (*18*). Thus, the recorded data fit well to the biogenetically related assumptions.

In summary, despite the limited number of samples under study, this investigation illustrates the potential and the limits of multi-element IRMS techniques. The recently introduced HRGC−P−IRMS mode is a helpful first step in the development of on-line techniques expected in the future to overcome the problems of low dynamics and limited ranges of linearity, both of which still require laborious sample treatment.

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