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Determination of $^{2}H/^{1}H$ and $^{13}C/^{12}C$ Isotope Ratios of (*E*)-Methyl Cinnamate from Different Sources Using Isotope Ratio Mass Spectrometry

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For the authenticity assessment of (*E*)-methyl cinnamate from different origins, combustion/pyrolysis−isotope ratio mass spectrometry (C/P-IRMS) was used by an elemental analyzer (EA) and on-line capillary gas chromatography coupling (HRGC-C/P-IRMS). For that reason, (*E*)-methyl cinnamate self-prepared from synthetic, natural, and semisynthetic educts was analyzed in comparison to the commercial synthetic and natural ester. In addition, (*E*)-methyl cinnamate from basil extract and a number of commercial natural aromas was investigated. The data of self-synthesized synthetic (*E*)-methyl cinnamate, i.e., $\delta^{13}C_{V-PDB} = -33.8‰$ and $\delta^{2}H_{V-SMOW} = +349‰$, corresponded with that found for the commercial synthetic samples (−29.5 to −31.4‰ and +328 to +360‰ for $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$, respectively). The ester produced from natural educts by acid as well as *Candida antarctica* catalysis revealed $\delta^{13}C_{V-PDB} = -25.6$ and −30.1‰ as well as $\delta^{2}H_{V-SMOW} = -162$ and −169‰, respectively. Acid-catalyzed semisynthetic products differed in their $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$ values depending on the origin of their educts. For the ester from synthetic methanol and natural cinnamic acid, −27.3 and −126‰ were determined for $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$, respectively, whereas for the ester produced from natural methanol and synthetic acid $\delta^{13}C_{V-PDB} = -30.6‰$ and $\delta^{2}H_{V-SMOW} = +287‰$ were found. Basil extract showed −28.9 and −133‰ for $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$, respectively. Commercial aromas declared to be natural revealed $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$ data ranging from −25.7 to −28.5‰ as well as −85 to −191‰, respectively, indicating, in part, incorrect declaration.

KEYWORDS: Methyl cinnamate; isotope ratio mass spectrometry; IRMS; HRGC-C/P-IRMS; $^{13}C/^{12}C$ ratio; $^{2}H/^{1}H$ ratio

INTRODUCTION

Methyl cinnamate, one of the common substances used in higher amounts in the flavor industry, has been reported to be present in commercially interesting amounts in Kokila (*Cinnamomum cecidodaphne*) from Nepal and *Eucalyptus campanulata* from Australia (*1*, *2*). It further occurs in various essential oils such as, e.g., *Alpinia malaccensis* and *Gastrochilus panduratum* Ridl. and Galgant rhizomes, *Ocimum canum* Sims., *Ocimum basilicum* L., and cinnamon leaves, *Narcissus jonquilla* L. flower as well as Peru balsam (*3*). The ester has also been found in avocado, beli (*Aegle marmelos*), bourbon vanilla, camembert cheeses, cloudberry, cocoa, cranberry, guava, loquat, pineapple, plum and plum brandy, prune, rhubarb, starfruit, and strawberry fruit and jam (*4*). With its fruity, balsamic odor similar to strawberry and a corresponding sweet taste, methyl cinnamate ranges among the major attractive industrial flavor compounds (*5*).

Information about the isotope ratios of methyl cinnamate is scarce. In their review on stable isotope ratio analysis, Schmidt et al. (*6*) reported for the ester declared to be natural −29.1 and −147‰ for $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$, respectively. Previously, a $\delta^{13}C_{V-PDB}$ value of 29.18‰ has been published for a not further specified sample of methyl cinnamate (*7*). The successful application of recently introduced multielement isotope ratio mass spectrometry (IRMS) for the authenticity assessment of flavor substances (*8*−*11*) has encouraged us to apply this technique also to the analysis of methyl cinnamate. In this paper, we present $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$ data of (*E*)-methyl cinnamate from various sources, i.e., commercial synthetic and natural reference samples, self-prepared synthetic, semisynthetic and natural products, basil (*O. basilicum*) extracts, and a number of commercial aromas declared to be natural.

MATERIAL AND METHODS

Samples and Chemicals. Synthetic methanol (from methane) and natural samples were from BASF (Ludwigshafen, Germany), Sigma-Aldrich (Steinheim, Germany), Fluka (Deisenhofen, Germany), Roth (Karlsruhe, Germany), and Symrise (Holzminden, Germany), respectively. Synthetic cinnamic acid was from Aldrich (Steinheim, Germany) and Avocado (Karlsruhe, Germany). Natural cinnamic acid was purchased from Aldrich and was self-prepared by diethyl ether extraction

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from Peru balsam (Caelo, Hilden, Germany). Benzaldehyde was from Fluka, and acetic anhydride was from Grüssing (Filsungen, Germany). All other chemicals were purchased from Sigma-Aldrich. Solvents were redistilled before use.

Methyl cinnamate reference samples were purchased from Fluka, Sigma-Aldrich, Acros (Geel, Belgium), and ABCR (Karlsruhe, Germany). Natural samples were kindly provided by SAM GmbH (Mannheim, Germany).

Basil extracts came from Flavex (Rehlingen, Germany). Samples of galgant rhizomes were from Caelo and Klenk (Schwebheim, Germany). Strawberries were harvested in the Würzburg area from June to July. Carambola fruits (*Averrhoa carambola*) from Malaysia were purchased at local supermarkets. Cinnamon extracts from China and Sri Lanka and cinnmon bark, cinnamon leaf extracts, and cinnamon oil were purchased from local drugstores. Commercial samples of natural aromas type "strawberry" and "blueberry" were kindly provided by SAM GmbH.

Sample Preparation. Synthetic and natural reference samples as well as essential oils/extracts were dissolved (1 mg/mL) in diethyl ether, and the solutions were directly analyzed by on-line capillary gas chromatography coupling (HRGC-MS) and HRGC-combustion/pyrolysis (C/P)-IRMS or directly via an elemental analyzer (EA).

After they were homogenized, fruits were subjected to simultaneous distillation/extraction (SDE) for 2 h using a pentane + diethyl ether mixture (1 + 1, v/v). Model experiments comprised SDE (pentane + diethyl ether, 1 + 1, v/v) of methyl cinnamate (100 mg in 800 mL of water, respectively). All extracts were dried over anhydrous sodium sulfate, filtered, and carefully concentrated to approximately 1 mL using a Vigreux column (45 °C).

Syntheses and Hydrolyses. Chemical ester synthesis was performed in mol scale according to textbook prescription (*12*) using *p*-toluene sulfonic acid as the catalyst. For enzymatic esterification, lipase from *Candida antarctica* (Sigma) was used as described by Fonteyn et al. (*13*). Perkin synthesis of cinnamic acid was performed according to textbook prescription (*14*). All esters were distilled and stored at 4 °C on molecular sieves (3 Å, Fluka). Their purity was checked by refraction index and HRGC-MS analyses. Ester hydrolysis was carried out with 15% sodium hydroxide according to textbook prescription (*12*).

HRGC-MS. An HP Agilent 6890 Series gas chromatograph with split injection (220 °C; 1:20) was directly coupled to an HP Agilent 5973 Network mass spectrometer (Agilent Technologies Inc., CA). The flavor compounds were separated on a J&W DB-Wax fused silica capillary column (30 m × 0.25 mm; $d_f = 0.25$ μm). The temperature program was as follows: 3 min isothermal at 50 °C and then raised at 4 °C/min. Identification was performed by comparison of linear retention indices and mass spectral data of sample constituents with that of authentic reference compounds.

HRGC-IRMS. A Finnigan Delta plus XL isotope ratio mass spectrometer coupled by an open-split via a C/P interface to an HP 6890 GC was used. The GC was equipped with an J&W DB-Wax fused silica capillary column (60 m × 0.32 mm i.d.; $d_f = 0.25$ μm). The conditions were employed as follows: 1 μL splitless injection (250 °C); temperature programs: 50−220 °C at 5 °C/min; helium flow, 2 mL/min.

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

Pyrolysis. $^2H/^1H$: The effluent from the GC passes through a ceramic tube (Al_2O_3; 0.5 mm i.d., 320 mm) for pyrolysis to H_2 at 1440 °C.

In addition, coupling elemental analyzers (EAs) ($^{13}C/^{12}C$, Euro Vector EA 3000, Milano, Italy; temperature, 1000 °C; $^2H/^1H$, HT Sauerstoff-Analysator, HEKATech, Wegberg, Germany; temperature, 1460 °C) to the IRMS were realized for off-line control determination of reference samples.

Daily system stability checks were carried out by measuring reference samples with known $^{13}C/^{12}C$ and $^2H/^1H$ ratios. A stability check of the used reference gases was continuously performed by measuring International Atomic Energy Agency (IAEA, Vienna, Austria) standards with defined $^{13}C/^{12}C$ and $^2H/^1H$ ratios (for $^{13}C/^{12}C$ IAEA-CH-7 and for $^2H/^1H$ IAEA-CH-7 and NBS 22 oil).

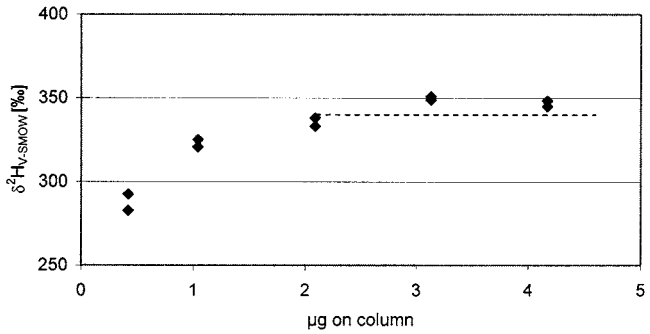

Figure 1. Linearity check of δ^2H_{V-SMOW} determination of (*E*)-methyl cinnamate (synthetic reference).

The isotope ratios are expressed in per mil (‰) deviation relative to the V-PDB and V-SMOW international standards. For $^{13}C/^{12}C$ determinations, the mass spectrometer was calibrated against reference CO_2 gas (Messer Griesheim, Frankfurt, Germany) with a defined $\delta^{13}C_{V-PDB} = -24.9‰$. Results are expressed in $\delta^{13}C_{V-PDB}$ values as:

$$\delta^{13}C_{V-PDB}\,(‰) = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{V-PDB}} - 1\right] \times 1000$$

The isotope ratios for $^2H/^1H$ are expressed in per mil (‰) deviation relative to the Vienna standard mean ocean water (V-SMOW) international standard. The mass spectrometer was calibrated against reference H_2 gas (Messer Griesheim) with a defined $\delta^2H_{V-SMOW} = -270 \pm 10‰$. Results are expressed in δ^2H_{V-SMOW} units as:

$$\delta^{2}H_{V-SMOW}\,(‰) = \left[\frac{(^{2}H/^{1}H)_{sample}}{(^{2}H/^{1}H)_{V-SMOW}} - 1\right] \times 1000$$

In general, 6-fold determinations were carried out, and standard deviations were calculated. The latter were ±0.1 and ±5‰ for $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} determinations, respectively (all data given below refer to these standard deviations). Additional peak recognition was performed by reference compounds and HRGC-MS registered under identical separation conditions as samples.

To determine the δ^2H_{V-SMOW} values of (*E*)-methyl cinnamate, the system reliability had to be proven by measuring commercial references "off-line" via the equipped EA. Comparison of the data recorded by EA-C/P-IRMS, e.g., +328‰ (for the synthetic reference from Fluka), revealed good agreement with that determined by HRGC-C/P-IRMS analysis (+344‰). The area of linearity for the δ^2H_{V-SMOW} determination was given from 2 to >4 μg (on column) (**Figure 1**).

The influence of sample preparation on the $^2H/^1H$ isotope ratio checked by model SDE separation was found to be negligible ($\delta^2H_{V-SMOW} = +347‰$). Standard deviations are ±0.1 and ±5‰ for $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW}, respectively.

RESULTS AND DISCUSSION

The results of isotope ratios determined off-line, i.e., via EA, and on-line, i.e., via HRGC, by IRMS in the C/P modes for synthetic and natural (*E*)-methyl cinnamate reference samples, commercial natural aromas, and basil extracts are represented in **Figure 2** (in a number of plant tissues, such as galgant rhizomes, Peru balsam, strawberries, carambola (*A. carambola*), and cinnamon extracts and oils, (E)-methyl cinnamate was not found in amounts sufficient for IRMS analysis).

As to synthetic methyl cinnamate references ($n = 3$), $\delta^{13}C_{V-PDB}$ data ranged from −29.5 to −31.4‰ and δ^2H_{V-SMOW} values ranged from +328 to +360‰. For two natural methyl cinnamate samples, −28.2 and −29.8‰ for $\delta^{13}C_{V-PDB}$ as well as −123 and −176‰ for δ^2H_{V-SMOW} were measured. Basil extracts gave data in the range of natural references ($\delta^{13}C_{V-PDB}$: −28.9 to −29.0‰; δ^2H_{V-SMOW}: −133 to −126‰). The isotopic

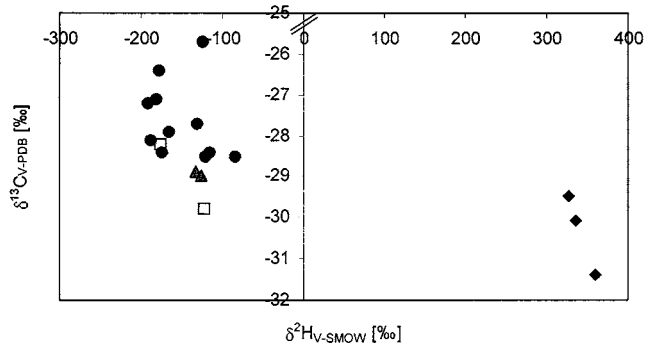

Figure 2. Correlation of $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} values (‰) of (*E*)-methyl cinnamate from synthetic reference (◆), natural reference (□), basil extracts (▲), and commercial aromas declared to be natural (●). Standard deviations: ±0.1 and ±5‰ for $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} determinations, respectively.

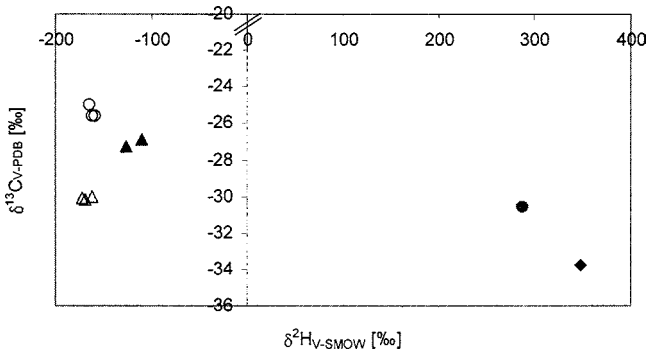

Figure 3. Correlation of $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} values (‰) of (*E*)-methyl cinnmate synthesized from synthetic educts (◆) and synthesized from natural educts using lipase from *C. antarctica* (△) and *p*-toluenesulfonic acid (○) catalysis. Semi-synthetic (*E*)-methyl cinnamate from synthetic cinnamic acid and "natural" methanol (●) as well as from "natural" cinnamic acid and synthetic methanol (▲). Standard deviations: ±0.1 and ±5‰ for $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} determinations, respectively.

ratios for commercial natural aromas type strawberry and blueberry were found to range from −25.7 to −28.5‰ and −85 to −191‰ for $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW}, respectively. In part, these data differed from that recorded for the natural methyl cinnamate references and basil extracts.

Syntheses of (*E*)-methyl cinnamate involved both acid (*p*-toluene sulfonic acid) and lipase catalysis. To achieve isotopic ratios for synthetic (*E*)-methyl cinnamate, synthetic methanol (originating from methane; $\delta^{13}C_{V-PDB} = -42.0$ ‰; $\delta^2H_{V-SMOW} = -44$‰) and synthetic cinnamic acid ($\delta^{13}C_{V-PDB} = -30.8$‰; $\delta^2H_{V-SMOW} = +421$‰), the latter self-produced via Perkin synthesis, were used. The formed (*E*)-methyl cinnamate gave $\delta^{13}C_{V-PDB} = -33.8$‰ and $\delta^2H_{V-SMOW} = +349$‰ as shown in **Figure 3**. This result was in good agreement with the data recorded for commercially available synthetic samples (**Figure 2**).

Besides syntheses of synthetic (*E*)-methyl cinnamate, acid catalysis with *p*-toluene sulfonic acid was also used to prepare semisynthetic esters. For that reason, synthetic methanol (see above) was employed with natural cinnamic acid ($\delta^{13}C_{V-PDB} = -25.6$‰; $\delta^2H_{V-SMOW} = -124$‰) and, vice versa, natural methanol ($\delta^{13}C_{V-PDB} = -30.0$ ‰; $\delta^2H_{V-SMOW} = -227$‰) with commercially available synthetic cinnamic acid ($\delta^{13}C_{V-PDB} = -29.9$‰; $\delta^2H_{V-SMOW} = +427$‰). In addition, cinnamic acid was isolated from a natural source, namely, Peru balsam. The recorded data, i.e. −25.8‰ for $\delta^{13}C_{V-PDB}$ and −156‰ for δ^2H_{V-SMOW}, were in good agreement with that of the commercially available natural cinnamic acid used for synthesis.

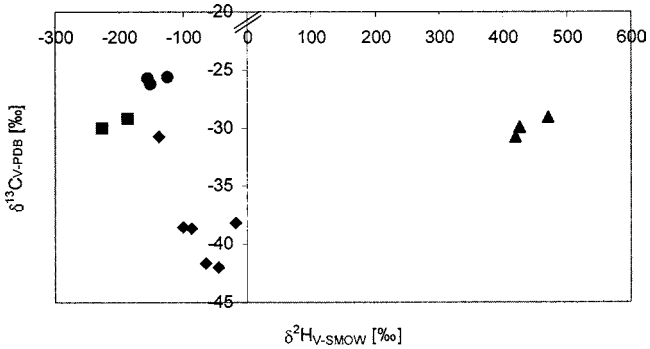

Figure 4. Correlation of $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} values (‰) of synthetic methanol from gas (◆), natural methanol (■), natural cinnamic acid (●), and synthetic cinnamic acid (▲). Standard deviations: ±0.1 and ±5‰ for $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} determinations, respectively.

The data for the synthesized semisynthetic samples of (*E*)-methyl cinnamate are summarized in **Figure 3**. For the ester obtained from synthetic methanol and natural cinnamic acid, $\delta^{13}C_{V-PDB} = -26.9$ to −27.3‰ and $\delta^2H_{V-SMOW} = -110$ to −126‰ were determined, whereas the ester produced from natural methanol and synthetic acid exhibited $\delta^{13}C_{V-PDB} = -30.6$‰ and $\delta^2H_{V-SMOW} = +287$‰.

IRMS data of commercially available samples of methanol and cinnamic acid are represented in **Figure 4**. For synthetic methanol ($n = 6$), $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW} values ranged from −30.8 to −42.0‰ and from −18 to −138‰, respectively. For natural methanol ($n = 2$), data ranging from −29.2 to −30.0‰ and from −188 to −227‰ for $\delta^{13}C_{V-PDB}$ and δ^2H_{V-SMOW}, respectively, were found. Differences were also found between synthetic ($n = 3$; $\delta^{13}C_{V-PDB}$ from −29.1 to −30.8‰ and δ^2H_{V-SMOW} from +421 to +472‰) and natural cinnamic acid ($n = 3$; $\delta^{13}C_{V-PDB}$ from −25.6 to −26.2‰ and δ^2H_{V-SMOW} from −124 to −156‰). As synthetic methyl cinnamate is known to be produced by oxidation of benzyldehyde (produced from toluene), these data are in good agreement with that of synthetic benzaldehyde (*15*).

The IRMS results obtained for the self-prepared esters reveal the minor influence of methanol on the δ^2H_{V-SMOW} data of the produced (*E*)-methyl cinnamate. Using synthetic methanol and natural cinnamic acid, the $^2H/^1H$ ratios were still negative for the ester, and employing synthetic methanol ($\delta^{13}C_{V-PDB}$ −42.0‰), no depletion of $^{13}C/^{12}C$ ratios was observed.

Syntheses with natural educts were performed in two different ways of catalysis. Both natural methanol (with $\delta^{13}C_{V-PDB} = -30.0$‰ and $\delta^2H_{V-SMOW} = -227$‰) and cinnamic acid ($\delta^{13}C_{V-PDB} = -25.6$‰; $\delta^2H_{V-SMOW} = -124$‰) were subjected to ester synthesis catalyzed by *p*-toluene sulfonic acid as well as *C. antarctica* lipase. Interestingly, the enzymatically catalyzed esterification led to depletion in carbon isotope ratio from −25.0 (acid catalysis) to −30.2‰ (lipase catalysis) (**Figure 3**). Further work is in progress to check the variability of data to be expected for various esterification methods.

A few years ago, Schmidt's group (*6*, *16*) proposed to assess the authenticity of esters by $^{18}O/^{16}O$ analysis of their alcohol moiety liberated by hydrolysis. Recently, we have confirmed the efficiency of this procedure and were able to demonstrate that in addition to the $^{18}O/^{16}O$ data also the $^2H/^1H$ ratios can be successfully used (*17*). Applying this technique to analytically distinguish between the various self-synthesized samples of (*E*)-methyl cinnamate, clear-cut differentiation of the self-prepared esters under study was obtained. As shown from the data

Table 1. $\delta^{13}C_{V-PDB}$ and $\delta^{2}H_{V-SMOW}$ Values (‰) of (*E*)-Methyl Cinnamate (Synthesized by Acid Catalysis a and Lipase b from Educts of Synthetic s[a] and Natural n Origin [s/s, n/s, s/n, n/n]), the Educts Methanol, Cinnamic Acid, and Methanol Obtained after Ester Hydrolysis (Methanol h)[b]

compound	origin	type of catalysis	$\delta^{13}C_{V-PDB}$	$\delta^{2}H_{V-SMOW}$
methanol	s		–42.0	–66
cinnamic acid	s1		–30.8	+421
methyl cinnamate		a	–33.8	+349
methanol h			–33.0	–48
methanol	n		–30.0	–227
cinnamic acid	s		–29.9	+427
methyl cinnamate		a	–30.6	+287
methanol h			–30.5	–161
methanol	s		–42.0	–66
cinnamic acid	n		–25.6	–124
methyl cinnamate		a	–27.3	–126
methanol h			–42.0	–83
methanol	n		–30.0	–227
cinnamic acid	n		–25.6	–124
methyl cinnamate		a	–25.6	–162
methanol h			–27.6	–171
methanol	n		–30.0	–227
cinnamic acid	n		–25.6	–124
methyl cinnamate		b	–30.1	–169
methanol h			–28.2	–178

[a] From three companies, additionally self-prepared (s1), thus slightly differing in their IRMS data. [b] Each esterification was repeated twice; because of the high reproducibility of data (variations within ±0.2 and ±5‰ for $\delta^{13}C_{V-PDB}$ and $\delta\delta^{2}H_{V-SMOW}$, respectively), only one representative example is shown.

summarized in **Table 1**, a distinct isotope discrimination between the $^{2}H/^{1}H$ ratios of methanol used as educt and the alcohol liberated from each of the synthesized esters was observed; however, the differences between the data of the synthetic and natural alcohols remained high enough to allow analytical distinction.

Despite the limited number of samples, the presented data stress the efficiency of the IRMS techniques described. Ester hydrolysis and subsequent $^{2}H/^{1}H$ analysis of the liberated alcohol allow doubtless origin control. However, this procedure is limited to cases in which sufficient substance is available, e.g., in the course of the industrial control of raw materials. Future work will be done to enrich the amounts of (*E*)-methyl cinnamate from natural plant sources, in particular, strawberry, to be able to extend the actual IRMS database.

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