

$^2\text{H}/^1\text{H}$ and $^{13}\text{C}/^{12}\text{C}$ Isotope Ratios of *trans*-Anethole Using Gas Chromatography–Isotope Ratio Mass Spectrometry

STEFFI BILKE AND ARMIN MOSANDL*

Institut für Lebensmittelchemie, Biozentrum J.W. Goethe-Universität, Marie Curie Strasse 9,
 D-60439 Frankfurt/Main, Germany

Authenticity assessment of *trans*-anethole is deduced from $^2\text{H}/^1\text{H}$ and $^{13}\text{C}/^{12}\text{C}$ isotope ratios, determined by gas chromatography–isotope ratio mass spectrometry (GC–IRMS). For that purpose, self-prepared anise and fennel oils, and synthetic and “natural” samples of *trans*-anethole, as well as commercially available anise and fennel oils have been investigated. Authenticity ranges of $^2\text{H}/^1\text{H}$ and $^{13}\text{C}/^{12}\text{C}$ isotope ratios of *trans*-anethole were defined. Scope and limitations of the applied online GC–IRMS techniques are discussed.

KEYWORDS: GC–P–IRMS; $^2\text{H}/^1\text{H}$ isotope ratio analysis; GC–C–IRMS; $^{13}\text{C}/^{12}\text{C}$ isotope ratio analysis; *trans*-anethole; fennel oil; anise oil

INTRODUCTION

Enantioselectivity and isotope discrimination during biosynthesis are phenomena which may serve as endogenous parameters in the authenticity control of flavor compounds. Whereas enantioselective analysis by enantio-multidimensional gas chromatography–mass spectrometry (enantio-MDGC–MS) is a well-established analytical technique (1), new on-line techniques in isotope ratio mass spectrometry (IRMS) have been developed recently (2). The on-line determination of $^{13}\text{C}/^{12}\text{C}$ isotope ratios by gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) is widely used in flavor analysis (1, 3). Gas chromatography, online coupled with isotope mass spectrometry via a pyrolysis reactor (GC–P–IRMS) is a relatively new method to determine $^2\text{H}/^1\text{H}$ isotope ratios (2, 4, 5). This method has also been applied in authenticity control of flavor compounds (6–9). Multi-element isotopic analysis by means of GC–IRMS provides a new approach to authenticity assessment, in particular for nonchiral flavor compounds such as *trans*-anethole. Martin et al. (10) first demonstrated the application of site-specific natural isotopic fractionation–nuclear magnetic resonance (SNIF–NMR) for origin identification of *trans*-anethole. Balabane et al. (11) described a linear relationship between the $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ isotope ratios of natural *trans*-anethole isolated from anise and fennel oils.

Culp et al. (12) determined the $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ isotope ratios of synthetic and natural *trans*-anethole via off-line IRMS. The determined $\delta^2\text{H}_{\text{V-SMOW}}$ values of natural *trans*-anethole were more negative than those of synthetic *trans*-anethole, and the $\delta^{13}\text{C}_{\text{V-PDB}}$ values were more positive in the natural samples, however, including more or less overlapping ranges.

In this investigation we have determined online the $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ isotope ratios of synthetic and natural *trans*-anethole,

and *trans*-anethole of self-prepared anise and fennel oils, as well as commercially available anise and fennel oils. These results demonstrate the benefit of multi-element isotopic analysis in the authenticity assessment of flavor compounds using online GC–IRMS techniques.

MATERIALS AND METHODS

Chemicals and Essential Oils. Synthetic and “natural” trade qualities of *trans*-anethole were obtained from Fluka (Buchs, Switzerland), Sigma-Aldrich (Seelze, Germany), Merck Eurolab (Darmstadt, Germany), Acros Organics (Geel, Belgium), and Kaders GmbH (Hamburg, Germany). Commercial anise and fennel oils were obtained from Kaders GmbH (Hamburg, Germany), Sigma-Aldrich (Seelze, Germany), Morgentau (Johann-Upfingen, Germany), and Dr. Motschall (Fredenbeck, Germany). Anise and fennel seed samples were obtained from Bornträger & Schlemmer (Offstein, Germany), Carl Sperling & Co (Lüneburg, Germany), Conrad Appel (Darmstadt, Germany), N. L. Chrestensen (Erfurt, Germany), and Bauer GmbH (Vestenbergsgreuth, Germany).

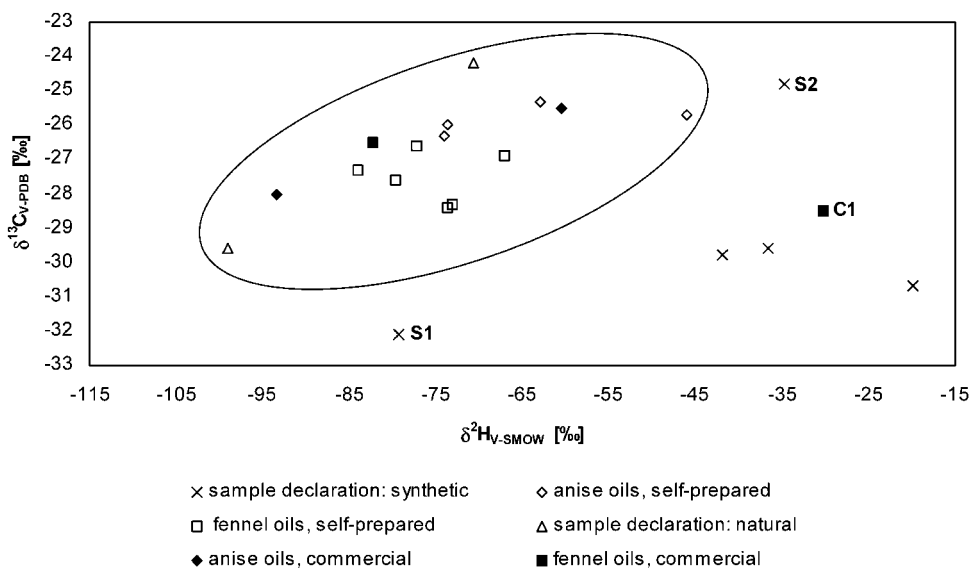
Sample Preparation. The anise and fennel seeds were hydrodistilled for 2 h using the official DAB distillation apparatus according to DAB 9 (13).

High-Temperature Conversion/Elemental Analyzer–Isotope Ratio Mass Spectrometry (TC/EA–IRMS). Determination of the $^2\text{H}/^1\text{H}$ isotope ratios of the tertiary standards was carried out using an elemental analyzer (TC/EA), coupled to a Deltaplus XL isotope ratio mass spectrometer via a ConFlo III-Interface (ThermoFinnigan MAT, Bremen, Germany). The following conditions were employed: reactor temperature 1450 °C, GC column temperature 110 °C, flow (helium) 120 mL/min. The isotope ratios are expressed in per mill (‰) versus V-SMOW (Vienna standard mean ocean water). The reference gas (Messer-Griesheim, Frankfurt, Germany) was calibrated against the standards IAEA-CH 7 ($\delta^2\text{H}_{\text{V-SMOW}} = -100.3 \pm 2.0$ ‰) and NBS 22 ($\delta^2\text{H}_{\text{V-SMOW}} = -118.5 \pm 2.8$ ‰). The $\delta^2\text{H}_{\text{V-SMOW}}$ values of five tertiary standards (5-nonanone, menthol, *trans*-anethole, 4-octanolide, and 4-decanolide) were determined via TC/EA–IRMS (number of measurements 10–15, standard deviation < 3‰). The reproducibility

* Corresponding author. Phone: +4969-79829202/203. Fax: +4969-79829207. E-mail: Mosandl@em.uni-frankfurt.de.

Table 1. Range of $\delta^2\text{H}_{\text{V-SMOW}}$ and $\delta^{13}\text{C}_{\text{V-PDB}}$ Values of *trans*-anethole

sample	$\delta^2\text{H}_{\text{V-SMOW}}$ range [‰]	$\delta^{13}\text{C}_{\text{V-PDB}}$ range [‰]	number of samples
synthetic	-20 to -79	-24.8 to -32.1	5
natural	-71 to -99	-24.2 to -29.6	2
anise oils, self-prepared	-46 to -74	-25.3 to -26.3	4
fennel oils, self-prepared	-67 to -84	-26.6 to -28.3	6
anise and fennel oils, commercial	-30 to -93	-25.5 to -28.5	4

Figure 1. Correlation of $\delta^{13}\text{C}_{\text{V-PDB}}$ values and $\delta^2\text{H}_{\text{V-SMOW}}$ values of *trans*-anethole.

and the accuracy of the measurements via GC–P–IRMS were routinely checked by means of these tertiary standards.

Gas Chromatography–Pyrolysis–Isotope Ratio Mass Spectrometry (GC–P–IRMS). The on-line determination of the $^2\text{H}/^1\text{H}$ isotope ratios was performed with an HP 6890 gas chromatograph (GC), coupled to a Delta^{plus} XL isotope ratio mass spectrometer via a pyrolysis reactor (ceramic tube (Al_2O_3), 0.5 mm i.d., length 320 mm, reactor temperature 1440 °C) and an open split (ThermoFinnigan MAT, Bremen, Germany). The GC was equipped with a 30 m \times 0.25 mm i.d., 0.5- μm film VB 5 column (ValcoBond, Gig Harbor, WA). The following conditions were employed: splitless injection (injector temperature 240 °C); temperature program, starting from 40 °C, isothermal for 15 min, increasing by 5 °C/min to 240 °C; carrier gas and flow, He 0.8 mL/min.

The pyrolysis reactor was regularly conditioned by leading methane through the reactor in backflush mode (at operating temperature for a period of 5 min) (14).

Fivefold determinations were carried out; the standard deviations (s) of the GC–P–IRMS measurements ranged between 1 and 3‰.

Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry (GC–C–IRMS). On-line determination of the $^{13}\text{C}/^{12}\text{C}$ isotope ratios was performed with a Siemens SiChromat 2-8 gas chromatograph (GC), connected to a Delta S isotope ratio mass spectrometer via an oxidation reactor (ceramic tube (Al_2O_3), 0.5 mm i.d., length 320 mm, reactor temperature 960 °C) and a combustion interface II (reduction reactor temperature, 600 °C) (ThermoFinnigan MAT, Bremen, Germany). The GC was equipped with a 60 m \times 0.25 mm i.d., 0.5- μm film Rtx 5 column (Restek, Bad Homburg, Germany). The following conditions were employed: split injection (injector temperature 240 °C, split flow 20 mL/min); temperature program, starting from 80 °C, isothermal for 5 min, increasing by 5 °C/min to 240 °C; carrier gas and flow, He 1.8 mL/min.

Fivefold determinations were carried out; the standard deviations (s) of the GC–P–IRMS measurements were $\leq 0.3\%$.

The isotope ratios are expressed in per mill (‰) versus V-PDB (Vienna peedee belemnite).

The reference gas (Messer-Griesheim, Frankfurt, Germany) was calibrated against five tertiary standards (5-nonanone, menthol, 4-oc-tanolide, *trans*-anethole, and 4-decanolide) with well-known $\delta^{13}\text{C}_{\text{V-PDB}}$ values.

RESULTS AND DISCUSSION

To determine authenticity ranges, the isotope ratios of *trans*-anethole in self-prepared anise and fennel oils were investigated. The influence of the hydrodistillation on the determined values was checked by distilling a synthetic reference using conditions the same as those used for the anise and fennel seed samples. The determined $^2\text{H}/^1\text{H}$ isotope value corresponded well in view of the standard deviation ($\delta^2\text{H}_{\text{V-SMOW}} = -34\% \pm 3\%$ and $\delta^2\text{H}_{\text{V-SMOW}} = -30\% \pm 2\%$, respectively). Consequently, sample preparation does not affect the evaluation of $^2\text{H}/^1\text{H}$ isotope ratios. Furthermore, the $\delta^2\text{H}_{\text{V-SMOW}}$ and $\delta^{13}\text{C}_{\text{V-PDB}}$ values of commercial *trans*-anethole samples, labeled as natural or synthetic, were determined.

The $\delta^2\text{H}_{\text{V-SMOW}}$ and $\delta^{13}\text{C}_{\text{V-PDB}}$ values of the *trans*-anethole samples from different origins are summarized in Table 1. The $\delta^2\text{H}_{\text{V-SMOW}}$ values of *trans*-anethole from self-prepared anise and fennel oils are within the range of -46 to -84‰. The values of the commercial samples labeled as “natural” are in the same range ($\delta^2\text{H}_{\text{V-SMOW}} = -71\%$ and $\delta^2\text{H}_{\text{V-SMOW}} = -99\%$, respectively). The values of most of the synthetic samples are clearly more positive than those of the authentic samples, except from sample S1 with $\delta^2\text{H}_{\text{V-SMOW}}$ value = -79‰ (Figure 1).

The $\delta^{13}\text{C}_{\text{V-PDB}}$ values of the authentic samples (self-prepared fennel and anise oils) were within a range of -25.3 to -28.3‰. Whereas the values of most of the synthetic samples were well below ($\delta^{13}\text{C}_{\text{V-PDB}} = -29.6$ to -32.1‰) those of the authentic samples. The value of one of the synthetic samples (sample S2) was much more positive and in the same range as the

authentic samples. The *trans*-anethole samples which are labeled as "natural" showed totally different $\delta^{13}\text{C}_{\text{V-PDB}}$ values. The value of one sample ($\delta^{13}\text{C}_{\text{V-PDB}} = -24.2\%$) was in the range of the authentic samples, whereas the value of the other sample ($\delta^{13}\text{C}_{\text{V-PDB}} = -29.6\%$) was close to the values of the synthetic samples.

As the reported data show, it is possible to define authenticity ranges for *trans*-anethole in fennel and anise oils. However, the values determined for the synthetic samples do not allow them to be identified as synthetic on the basis of either the $\delta^2\text{H}_{\text{V-SMOW}}$ values or the $\delta^{13}\text{C}_{\text{V-PDB}}$ values. The $\delta^2\text{H}_{\text{V-SMOW}}$ value of sample S1 was in the same range as that of the authentic samples, whereas the $\delta^{13}\text{C}_{\text{V-PDB}}$ value indicated the sample as synthetic. The contrary situation was found for sample S2: the $\delta^{13}\text{C}_{\text{V-PDB}}$ value was in the range of the authentic samples whereas the $\delta^2\text{H}_{\text{V-SMOW}}$ value indicated the sample as synthetic. Synthetic *trans*-anethole is prepared by isomerization of estragol; this is why the $\delta^{13}\text{C}_{\text{V-PDB}}$ value of the resulting *trans*-anethole is identical to that of estragol (15). The synthetic *trans*-anethole samples with a more negative $\delta^{13}\text{C}_{\text{V-PDB}}$ value may be synthesized from anisole. The differences in the $\delta^{13}\text{C}_{\text{V-PDB}}$ values of the synthetic samples are caused by various syntheses (16).

In **Figure 1** the $\delta^{13}\text{C}_{\text{V-PDB}}$ values of *trans*-anethole are plotted against $\delta^2\text{H}_{\text{V-SMOW}}$ values. In this plot a characteristic authenticity area for *trans*-anethole can be defined. The synthetic samples are all clearly separated from the authentic samples. The samples S2 and S1 are identified as synthetic compounds because of their $\delta^2\text{H}_{\text{V-SMOW}}$ (S2) and $\delta^{13}\text{C}_{\text{V-PDB}}$ (S1) values, respectively. The values of *trans*-anethole of the commercial oils are within the authentic area, whereas one commercial fennel oil (sample C1) is identified as synthetic because of its $\delta^2\text{H}_{\text{V-SMOW}}$ value. In summary, the presented data show that $\delta^2\text{H}/\delta^{13}\text{C}$ analysis by GC-IRMS provides a new technique for authenticity assessment of flavor compounds.

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