

# Stable Isotope Analysis of Flavor Compounds

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The authenticity of natural flavor compounds is an important topic for the flavor industry. Over the last decade stable isotope analysis has become the most important tool for authenticity testing of non-chiral flavor compounds like vanillin and benzaldehyde.<sup>1</sup> Our work deals with multi-element isotope ratio mass spectrometry using a new online IRMS-technique in combination with <sup>2</sup>H-NMR.

The origin of organic molecules can be determined by the examination of their stable isotope ratios. Isotope ratio mass spectrometry (IRMS) is well established for analysis of the isotope ratios of H, C, N, O, and S. Additionally, <sup>2</sup>H-NMR, also called SNIF-NMR (Site-Specific Natural Isotope Fractionation - Nuclear Magnetic Resonance),<sup>2</sup> is used for the evaluation of site-specific hydrogen isotope ratios in a molecule. IRMS, as well as <sup>2</sup>H-NMR, can be used to determine the authenticity of flavor and fragrance compounds.<sup>3</sup>

Most common is the analysis of the stable isotope ratio of <sup>13</sup>C/<sup>12</sup>C by IRMS. The measured ratio is expressed as δ-value, which is defined as follows:

$$\delta^{13}\text{C} [‰] = \left[ \frac{[\text{C}_{13}\text{sample}] / [\text{C}_{12}\text{sample}]}{[\text{C}_{13}\text{standard}] / [\text{C}_{12}\text{standard}]} - 1 \right] \cdot 1000$$

As an international standard the carbonate, Vienna-PeeDee-Belemite, is used. This is provided by the International Atomic Energy Agency (IAEA) in Vienna. For the analysis of stable isotope ratios, the molecule of interest has to be converted to certain gases, which can be detected by the IRMS. For the analysis of δ<sup>13</sup>C-values, for example, a conversion to CO<sub>2</sub> has to be performed first, due to the type of mass spectrometer which suits this kind of analysis.<sup>4</sup> The quantitative pyrolysis of compounds is achieved by using elemental analyzers or special pyrolysis devices, such as the so-called combustion interface. The combustion interface permits a gas chromatograph to be coupled to the IRMS (i.e. the combustion of the compounds is performed online without any preparative step in between).<sup>5</sup> However, this online technique was first used for the evaluation of δ<sup>13</sup>C values, due to the lack of proper online combustion interfaces for other elements. The biggest advantage of the technique is the opportunity to connect the IRMS to a gas chromatography system (GC/IRMS).

## Experiment Notes

For the IRMS experiments a Delta<sup>plus</sup>XL-IRMS from Finnigan MAT GmbH (Bremen, Germany) was used. The δ<sup>13</sup>C values were measured by coupling the IRMS to a HP6890 GC (60m RTX-WAX column) equipped with a combustion interface (Finnigan MAT GmbH, Bremen) operated at approximately 900°C. The δ<sup>2</sup>H and δ<sup>18</sup>O values of compounds in complex mixtures were analyzed by the same GC/IRMS, but with the corresponding TC interfaces (Finnigan MAT GmbH, Bremen) operated at approx. 1400°C. Samples were diluted in tert-butyl methyl ether before injection (approximately 10µL substance in 0.3mL solvent). Alternatively, δ<sup>2</sup>H and δ<sup>18</sup>O values of pure compounds were determined by using the same IRMS coupled to an TC/EA-interface (Finnigan MAT GmbH, Bremen) operated at approximately 1400°C. Injections were performed by liquid injection of the pure samples (approximately 0.05µL). As international standards V-SMOW (for δ<sup>2</sup>H and δ<sup>18</sup>O) and V-PDB (for δ<sup>13</sup>C) were used.

Vanilla extracts were obtained by using the AOAC-method,<sup>13</sup> followed by a silica gel chromatography to remove resinoids (15g silica gel, 70-230 mesh, with diethyl ether as solvent). Benzaldehyde extractions were performed by using the SAFE-technique.<sup>12</sup> Previous tests did not show any isotope fractionation due to silica gel chromatography or SAFE-technique.

For extraction, apricot kernels were ground and added to n-pentane and diethyl ether (1:2) and finally extracted by using the SAFE-technique.<sup>12</sup>

Special NMR-analyses were performed using <sup>2</sup>H-NMR experiments on a Varian VXR-400S spectrometer at a temperature of 298 K. The spectra were recorded at 61.34 MHz using a specific 10 mm (o.d.) probe. The molar fractions of the monodeuterated isotopomers, f<sub>i</sub>, were directly calculated from the signal area.

Table 1. Plant categories regarding CO<sub>2</sub>-fixation during photosynthesis

CO <sub>2</sub> -fixation	Example	δ <sup>13</sup> C (‰)
C <sub>3</sub> plants	Wheat, barley, sugar beet, rice	-24...-32
CAM plants	Succulents, orchids (e.g. Vanilla planifolia), pineapple	-12...-30
C <sub>4</sub> plants	Sugar cane, corn, sorghum	-10...-16

Over the last few years, combustion interfaces for the online determination of deuterium/hydrogen<sup>6</sup> and oxygen-18/oxygen-16 ratios,<sup>7</sup> have been developed. Of these is the High Temperature Conversion Interface (TC) from Finnigan MAT GmbH (Bremen, Germany). Similar interfaces were built for the analysis of pure compounds, in order to achieve a higher sample throughput without using GC separation. Moreover, compounds like carbohydrates cannot be analyzed directly by GC. For the determination of δ<sup>2</sup>H- and δ<sup>18</sup>O-values of pure compounds, a TC/EA interface can be used (High Temperature Conversion/Elemental Analysis Interface;

major pathways are known for the CO<sub>2</sub>-fixation (Table 1).

Examination of δ<sup>13</sup>C-values can give information about the natural or synthetic origin of a molecule. This is especially true for flavor compounds in which it is necessary to distinguish between plant origin and synthetic origin (see results and discussion below). Sometimes, a one-dimensional data set (δ<sup>13</sup>C) is not sufficient for this purpose. Then a multi-element analysis<sup>9</sup> with additional measurement of δ<sup>2</sup>H- and δ<sup>18</sup>O-values is necessary. The two- or even three-dimensional data sets enable an identification of the origin.

## Results and Discussion

**Vanillin:** Vanillin is one of the most important flavor ingredients. It can be obtained synthetically, via the fermentation of various substrates, or by extraction from vanilla beans. Depending on its origin, the price of vanillin varies considerably. Therefore, a powerful tool for authenticity testing of vanillin is necessary. A very popular method is the determination of δ<sup>13</sup>C-values, the I.O.F.I. information letter 775, originally issued by the French authorities, refers to δ<sup>13</sup>C-values > -21‰.<sup>10</sup> However, analyzing just the δ<sup>13</sup>C-values might not be sufficient to determine the origin of vanillin. This study employed the multi-element approach and analyzed δ<sup>13</sup>C and δ<sup>2</sup>H-values of vanillin from different origins. The above-mentioned online IRMS technique was used. We were also able to analyze vanilla bean extracts by a GC/IRMS coupling using the TC interface from Finnigan MAT GmbH. Finally, we analyzed vanillin from vanilla beans harvested in 1999 from different geographical origins and different biochemical and synthetic processes (Table 2).

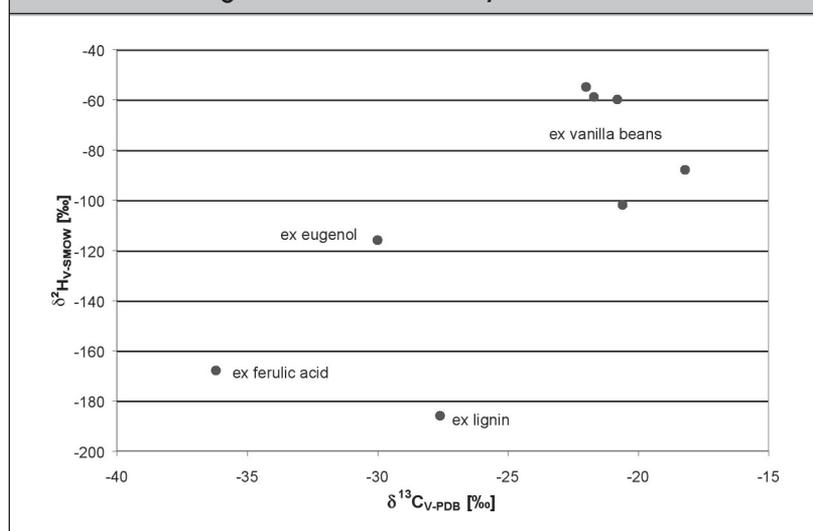
Figure 1 shows a two-dimensional plot of the isotopic ratios of different samples of vanillin, and it is obvious that vanillin ex vanilla beans can clearly be distinguished from the other vanillin qualities.

**Butanol:** Another important natural flavor compound is butanol. Samples of this compound (from various sources) were analyzed by IRMS. Again, the multi-element approach was used, but this time the δ<sup>2</sup>H, δ<sup>13</sup>C, and δ<sup>18</sup>O-values of different samples were analyzed. The

Table 2. δ<sup>2</sup>H- and δ<sup>13</sup>C-values of vanillin of different origins

Vanillin origin	δ <sup>13</sup> C (‰) <sub>V-PDB</sub> ± 0.2 (‰)	δ <sup>2</sup> H (‰) <sub>V-SMOW</sub> ± 5 (‰)
1 ex eugenol	-30.3	-116
2 ex ferulic acid (fermentation)	-36.4	-168
3 ex lignin	-27.6	-186
4 ex beans (Madagascar)	-22.0	-55
5 ex beans (Indonesia)	-20.6	-102
6 ex beans (Tahiti)	-18.2	-88
7 ex beans (Mexico)	-20.8	-60
8 ex beans (Comoro Islands)	-21.7	-59

Figure 1. Multielement analysis of vanillin



combination of the values gives a characteristic three-dimensional fingerprint of substances from different origins.

Table 3 shows the stable isotope ratios of different butanol samples. Figure 2 shows a two-dimensional plot with  $\delta^{13}\text{C}$ - and  $\delta^{18}\text{O}$ -values of those butanol samples. Our samples were from the following origins: two different natural origins (A and B, cf. Table 3), probably from plants with

C3-metabolism (e.g. potato, rice, soybean) and C4-metabolism (e.g. corn, sugar cane, sorghum) and nature-identical butanol from a petrochemical origin. The origins can easily be distinguished by comparing their  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$  values. Comparison with Table 1 shows that samples 1-3 might be derived from C3-plants, while samples 4-6 have originated from C4-plants. Sample 7 is the nature-identical butanol.

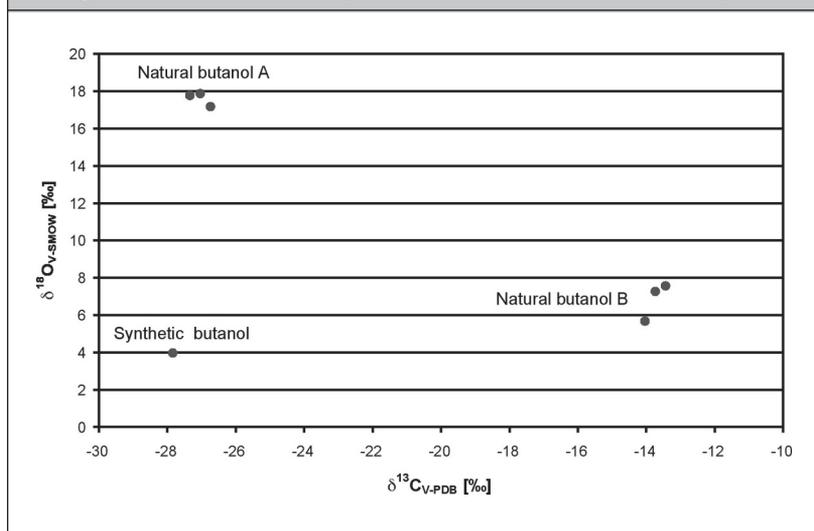
The  $\delta^{18}\text{O}$ -values were recorded by using two different methods: a) GC/IRMS with a GC/TC interface; and b)

**Table 3.  $\delta$ -values of butanol of different origins**

Butanol origin	$\delta^{13}\text{C}$ (‰) <sub>V-PDB</sub>	$\delta^{18}\text{O}$ (‰) <sub>V-SMOW</sub>	$\delta^{18}\text{O}$ (‰) <sub>V-SMOW</sub>	$\delta^2\text{H}$ (‰) <sub>V-SMOW</sub>
	± 0.2 (‰)	± 1.5 (‰) (GC/TC)	± 1.5 (‰) (TC/EA)	± 5 (‰)
1 Natural A	-27.3	22.2	17.1	-228
2 Natural A	-27.3	22.2	17.1	-222
3 Natural A	-28.3	22.2	17.8	-155
4 Natural B	-13.3	11.6	5.6	-290
5 Natural B	-14.0	13.2	7.2	-290
6 Natural B	-13.7	11.9	7.5	-274
7 Petrochemical	-27.8	8.8	3.9	-138

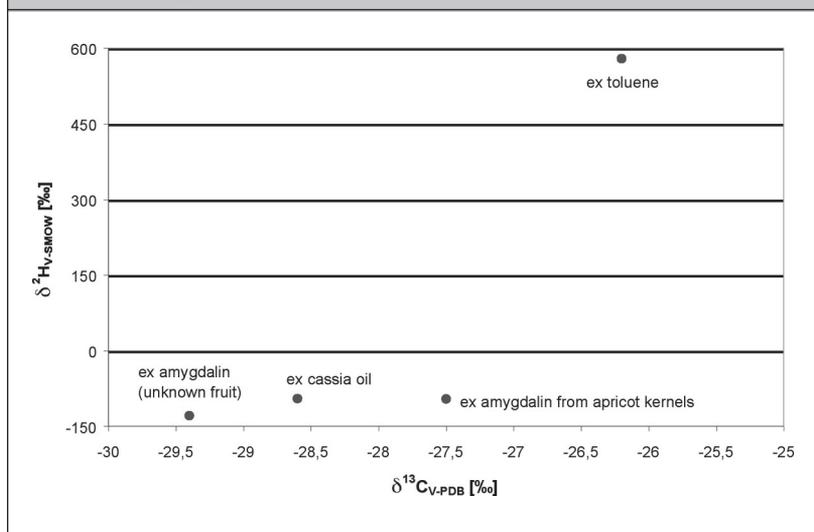
IRMS analysis without GC using a TC/EA interface (direct inlet). Interestingly, comparison of the two methods revealed a constant shift of the values (approximately 4.8 percent). Due to the completely different origins of the samples, impurities cannot be the reason for this shift. Further studies regarding the system parameters are under investigation. Regardless of which method was used, the obtained values can easily be used to distinguish the origins of the samples.

**Figure 2. Multielement analysis of butanol ( $\delta^{18}\text{O}$  from TC/EA analysis)**



Benzaldehyde: A very popular flavor compound for cherry flavors is benzaldehyde (some results regarding benzaldehyde were previously presented at the 22<sup>nd</sup> ASI-Meeting in Göttingen, Germany in 1999). Naturally occurring benzaldehyde is the main compound of the essential

**Figure 3. Multielement analysis of benzaldehyde**



**Table 4.  $\delta$ -values of benzaldehyde**

Benzaldehyde origin	$\delta^{13}\text{C}$ (‰) <sub>V-PDB</sub> ± 0.2 (‰)	$\delta^2\text{H}$ (‰) <sub>V-SMOW</sub> ± 5 (‰)
1 ex cassia oil	-28.6	-95
2 ex amygdalin (commercial source)	-29.4	-129
3 ex apricot kernels from China (own extraction)	-27.5	-96
4 ex toluene	-26.2	+579

oils from kernels of bitter almonds, apricots, peaches, plums and cherries. In those fruits, benzaldehyde is present as the glycoside amygdalin. Synthetic sources for benzaldehyde are the chemical oxidation of toluene and the hydrolysis of benzal chloride. Another source of benzaldehyde is the retro-aldol degradation of natural cinnamic aldehyde from cassia oil. The evaluation of a two-dimensional data set ( $\delta^2\text{H} / \delta^{13}\text{C}$ ) from different benzaldehydes can clearly distinguish between synthetic benzaldehyde (ex toluene) and natural benzaldehyde (cf. Table 4 and Figure 3).

Additional  $^2\text{H}$ -NMR analysis can even distinguish between benzaldehyde ex cassia oil and benzaldehyde ex amygdalin, because benzaldehyde ex cassia oil has different molar fractions of the isotopomers compared to the molar fractions of other benzaldehyde qualities (especially at the ortho- and meta-position, cf. Table 5).

Additionally, we analyzed possible changes of the isotope ratios of benzaldehyde in food matrices. It was demonstrated by Kempe et al.<sup>11</sup> that enzymes present in dairy products can cause changes in the  $\delta^{13}\text{C}$ -value of vanillin. To detect any isotope shifts of benzaldehyde we analyzed pure benzaldehyde, the same benzaldehyde in two different cherry flavors and finally the benzaldehyde of those flavors in two different food matrices (cherry soft drink and yogurt). For enrichment of benzaldehyde before GC/IRMS analysis, a flavor extraction was performed using solvent assisted flavor extraction (SAFE).<sup>12</sup>

The results shown in Table 6 demonstrate that only minor isotopic changes are observed. In order to study the influence of other food matrices or certain storage conditions further experiments have to be performed.

**Conclusion**

We have established the online IRMS technique for the analysis of  $\delta^2\text{H}$ ,  $\delta^{13}\text{C}$  and  $\delta^{18}\text{O}$ -values in our lab, and are therefore able to determine the isotope ratios of flavor compounds even in complex mixtures. The multi-element IRMS approach in combination with  $^2\text{H}$ -NMR allows, in many cases, an unambiguous identification of the origin of a certain flavor compound. Our results demonstrate the value of multi-element IRMS and  $^2\text{H}$ -NMR for the determination of the authenticity of vanillin, butanol and benzaldehyde. Further relevant flavor compounds are under investigation and will be reported on in detail in the near future. ■

**References**

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**Table 5.  $^2\text{H}$ -NMR measurements of the molar fractions from different benzaldehydes**

Sample	$f(\text{D-C=O})$	$f(\text{ortho})$	$f(\text{meta})$	$f(\text{para})$
Benzaldehyde (synthetic ex toluene)	0.510 $\pm 0.002$	0.193 $\pm 0.001$	0.206 $\pm 0.001$	0.091 $\pm 0.003$
Benzaldehyde (ex cassia oil)	0.168 $\pm 0.001$	0.353 $\pm 0.001$	0.293 $\pm 0.001$	0.186 $\pm 0.001$
Benzaldehyde (ex apricot kernels)	0.166 $\pm 0.001$	0.321 $\pm 0.001$	0.320 $\pm 0.003$	0.194 $\pm 0.004$

All results obtained are in accordance with values published by Schmidt et al.<sup>1</sup>.

**Table 6.  $\delta$ -values of benzaldehyde in different matrices**

Sample	Matrix	$\delta^{13}\text{C} (\text{‰})_{\text{V-PDB}}$ $\pm 0.2 (\text{‰})$	$\delta^2\text{H} (\text{‰})_{\text{V-SMOW}}$ $\pm 5 (\text{‰})$
1	Pure natural benzaldehyde	-30.3	-96
2	Cherry flavor concentrate for yogurt, containing sample 1	-30.1	-101
3	Cherry flavor concentrate for soft drinks, containing sample 1	-30.2	-96
4	Yogurt, containing sample 2	-30.3	-101
5	Soft drink, containing sample 3	-30.3	-96

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