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Original article

Improved detection of honey adulteration by measuring differences between $^{13}C/^{12}C$ stable carbon isotope ratios of protein and sugar compounds with a combination of elemental analyzer - isotope ratio mass spectrometry and liquid chromatography - isotope ratio mass spectrometry ($\delta^{13}C$-EA/LC-IRMS)*

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Abstract – The detection of honey adulteration with invert sugar syrups from various C3 and C4 plant sources was realized by coupling an isotope ratio mass spectrometer both to an elemental analyzer and to a liquid chromatograph (EA/LC-IRMS). For 451 authentic honeys measured, the individual $\delta^{13}C$ values of bulk honey, its protein fraction, fructose, glucose, and di- and trisaccharides ranged from –22.5 to –28.2‰ and did not show differences ($\Delta\delta^{13}C$) of more than ± 0.9‰ (average), with a maximum standard deviation of 0.7‰. The $\Delta\delta^{13}C$ (fructose – glucose) value was significantly lower (0 ± 0.3‰). Based on the obtained results and considering a confidence level of 99.7%, the following limits for $\Delta\delta^{13}C$ values of authentic honey are proposed: $\Delta\delta^{13}C$ max.: ± 2.1‰ (maximum difference between all measured $\delta^{13}C$ values); $\Delta\delta^{13}C$ fru – glu: ± 1.0‰; $\Delta\delta^{13}C$ (‰) protein - honey: ⩾ – 1.0‰. The newly developed EA/LC-IRMS method and the purity criteria defined represent a significant improvement compared to existing methods.

honey / adulteration / LC-IRMS / EA-IRMS / $\delta^{13}C$ values / C3 sugar / C4 sugar

1. INTRODUCTION

Honey is considered a value-added product of natural origin and genuine purity. Authenticity testing of honey is one of the most important and challenging issues in the field of honey analysis (Bogdanov and Martin, 2002) because of the high costs of production, the great consumer demand (especially in Europe), and the low margin of profit as the market prices continue to fall. Therefore, the detection of sugar adulteration is of major importance in order to protect consumers against fraud and to reveal fraudulent use of invert sugar syrups in the food industry.

Testing honey adulteration can be done by analyzing different physicochemical parameters like melissopalynological pattern, sensory analysis, sugar profile, amino acid profile, enzyme activities (diastase, invertase), hydroxymethylfurfural, and proline (Cotte et al., 2003, 2004; IHC, 2006). The comparison of the results with the naturally occurring values (Bogdanov et al., 2003; Doner, 1977; Lipp, 1994; Persano Oddo and Piro, 2004; Swallow and Low, 1994; Von der Ohe et al., 1991; White, 1992b; White and Siciliano, 1980) can hint at a possible adulteration. However, this procedure is time-consuming and only

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Table I. Typical $\delta^{13}C$ values of C4 and C3 plants and their sugar products compared to carbon dioxide from ambient air ([a] Winkler and Schmidt, 1980; [b] White and Doner, 1978a, b, [c] own unpublished data), and the ability of the EA-IRMS method to determine the addition of certain sugars to honey. [1] $\delta^{13}C$ (‰) = $[(^{13}C/^{12}C_{\text{sample}} - {}^{13}C/^{12}C_{\text{VPDB}}) \times 1000] \div {}^{13}C/^{12}C_{\text{VPDB}}$; VPDB: Vienna Pee Dee Belemnite (reference standard material); [2] EA-IRMS: elemental analyzer – isotope ratio mass spectrometry, determination of C4 sugars in honey by measuring the $\delta^{13}C$ values of honey and protein (method AOAC 998.12).

Carbon source	Typical $\delta^{13}C$ values (‰)[1]	EA-IRMS[2] detection/quantification
CO_2 from ambient air[a]	–7 to –9	
C4 plants[a]	–8 to –16	
- maize[a]	–8 to –13	
- maize hydrolysates[a]	–9.5 to –12.5	yes/qualified
- high fructose corn syrup[b]	–9.5 to –9.8	yes/yes
- sucrose from sugar cane[a]	–10.3 to –12.2	yes/qualified
C3 plants[a]	–22 to –32	
- wheat[a]	–23.5 to –26.5	
- sucrose from beet[a]	–24.3 to –26.4	no/no
- rice syrup[c]	–26.1 to –27.4	no/no
- fructose from chicory[c]	–26.3	no/no
- high fructose syrup[c]	–25.4 to –25.9	no/no
- bee feeding syrup[c]	–24.2	no/no

sensitive enough to detect adulterations of simple nature.

The introduction of stable carbon isotope ratio mass spectrometry (SCIRA, EA-IRMS) analysis was a milestone in the detection of more sophisticated adulterations with syrups imitating the sugar profile of honey (White and Doner, 1978a, b). The principle of this method is based on the differences in the metabolic enrichment of the ^{13}C isotope due to the different photosynthetic pathways of the so-called C3 plants, representing the nectar providing sources for the bees, and the C4 plants (e.g. maize and sugar cane) from which invert sugar syrups are produced (Padovan et al., 2003; Winkler and Schmidt, 1980). As a result, the slower reacting $^{13}CO_2$ is depleted to a larger extend in C3 plants than in C4 plants during the CO_2 fixation (kinetic isotope effect). Thus, it is possible to detect the addition of cheap C4 sugar because of its different $\delta^{13}C$ value (i.e., the $^{13}C/^{12}C$ isotope ratio related to Vienna Pee Dee Belemnite as reference standard material, expressed in ‰), which averages –9.7‰ compared to honey with an average $\delta^{13}C$ value of –25.4‰. The typical $\delta^{13}C$ values of C3 and C4 plants and the sugar products thereof are listed in Table I.

A substantial improvement of this method was achieved by using the isolated honey protein as an internal standard which enhanced sensitivity and thus lowered the limit of detection for C4 sugars from ca. 20 to 7% (White and Winters, 1989; White, 1992a). This new procedure was validated for a worldwide application (White et al., 1998) and is still considered as the analytical reference method for the determination of C4 sugar adulteration of honey (AOAC, 1999; Kerkvliet and Meijer, 2000; Lees, 2003; Ruoff and Bogdanov, 2004). However, using the protein value as internal standard also has its drawbacks in some cases, e.g. a higher measurement uncertainty for honeys with a low protein content like acacia or lavender, or the risk of altered $\delta^{13}C$ values of protein for honeys containing a high amount of yeast or remainder of bee feeding supplement.

As a conclusion, there is still the need for additional and more precise techniques to cope with honey adulteration. Long-time experience in the authenticity testing of honey shows that adulteration with invert sugar syrups produced from C4 plants decreased to a low and constant level, but adulteration with other types of sugar syrups (e.g. from C3 plants like beet and rice, see Tab. I), not

detectable by the existing methods, is increasing over the last years. The development of liquid chromatography hyphenated to stable carbon isotope ratio mass spectrometry (LC-IRMS) has opened new perspectives for the $^{13}C/^{12}C$ carbon isotope ratio analysis of honey (Krummen et al., 2004). LC-IRMS allows for a one-step separation of the individual sugar components of honey and the online determination of their $\delta^{13}C$ values, avoiding the disadvantages of offline methods as well as offering a real alternative to highly sophisticated techniques like gas-chromatography-combustion-IRMS or SNIF-NMR which have not achieved the desired acceptance for routine high-throughput analysis of honey adulteration (Cotte et al., 2007; Meier-Augenstein, 1999).

By applying LC-IRMS for the determination of the $\delta^{13}C$ values of fructose, glucose, and sucrose in honey, and calculating the differences ($\Delta\delta^{13}C$) between these values, it could be shown that both the adulteration with C4 and C3 sugars can be detected with a sensitivity of 1 to 10%, depending on the type of sugar syrup (Cabañero et al., 2006).

In this work, the application of a further developed EA/LC-IRMS method is presented. The results of the measured $\delta^{13}C$ values of bulk honey, isolated protein, fructose, glucose, disaccharides, and trisaccharides in authentic honeys and the natural occurring differences between these values calculated herefrom ($\Delta\delta^{13}C$ values) were used to set up a valuable tool for a state-of-the-art analysis of sugar adulteration of honey.

2. MATERIALS AND METHODS

2.1. Reagents

Ultrapure water (18.2 MΩ) was produced using a NanoPure Diamond system from Werner GmbH, Leverkusen, Germany. Crystalline phosphoric acid (puriss. p.a. ≥ 99%, Fluka 79622), sodium peroxodisulfate (purum p.a. ≥ 99%, Fluka 71890), and sodium tungstate dihydrate (puriss. p.a. ≥ 99%, Fluka 72070) were purchased from Sigma-Aldrich, Munich, Germany. Sulfuric acid (p.a. 98%, Merck 1.12080.1000) was purchased from VWR, Darmstadt, Germany. He 5.7 (carrier gas), CO_2 4.5 (working standard reference gas) and O_2 4.5 (flash combustion gas) were purchased from Air Products, Bremen, Germany.

The chemical oxidation reagents, 0.5 M phosphoric acid and 0.5 M peroxodisulfate solution, were prepared in brown glass bottles using an ultrasonic bath and a water-jet pump for vacuum degassing (removal of dissolved carbon dioxide).

2.2. Standards

D-(-)-fructose (Riedel-de-Haen 15760), D-(+)-glucose monohydrate (Riedel-de-Haen 16301), D-(+)-sucrose (Riedel-de-Haen 16104), D-(+)-maltose monohydrate (Fluka 63419), D-(+)-turanose (Fluka 93760), isomaltose (Sigma-Aldrich I-7253), D-(+)-trehalose (Fluka 90208), erlose (Sigma-Aldrich E-1896), D-(+)-melezitose hydrate (Sigma-Aldrich M-5375), maltotriose (Fluka 63430), and D-(+)-raffinose pentahydrate (Sigma-Aldrich 14701KA) were all purchased from Sigma-Aldrich, Munich, Germany. As carbon stable isotope reference standard, sucrose IAEA-CH6 ($\delta^{13}C$ value: –10.4‰), was obtained from the IAEA, Vienna, Austria.

2.3. Instrumentation and measurement conditions

For protein isolation and drying, a model 5804 centrifuge (10000 rcf) and a model S301 vacuum concentrator from Eppendorf, Hamburg, Germany were used.

EA-IRMS (determination of $\delta^{13}C$ values of protein and bulk honey): a Thermo-Electron Flash EA 1112 elemental analyzer was coupled via a Thermo-Finnigan ConFlo III Interface (He pressure: 0.8 bar, CO_2 pressure: 2 bar) to a Thermo-Electron Delta V Advantage Isotope Ratio Mass Spectrometer. The elemental analyzer was operated in the NC modus and controlled by the Finnigan Eager 300 Ver. 1.02 software. The oxidation and reduction reactors were heated to 900 °C and 650 °C, respectively. The GC separation column was temperated to 45 °C. The He carrier gas flow was approx. 110 mL/min. The O_2 purge for flash combustion was 4 s at a flow rate of 175 mL/min per sample. The CO_2 reference gas pulse was introduced two times (20 s each) at the beginning of each run. 100–200 μg of each honey or protein were

weighed in small tin capsules using an MX-5 ultramicrobalance from Mettler-Toledo, Giessen, Germany. Each honey or protein was measured three times. The results were accepted and the average value was calculated, if the difference between the three measured values was $\leqslant 0.2‰$. One run took approx. 8 min.

LC-IRMS (determination of $\delta^{13}C$ values of fructose, glucose, di-and trisaccharides): a LC system consisting of a bio-compatible Knauer Smartline P1000 Pump, a Knauer Smartline Manager 5000 gradient module, a Merck-Hitachi AS2000A autosampler, a Jetstream 2 Plus column oven (operated at 55 °C) and a Phenomenex Rezek RCM (Ca^{2+}) 300 × 8 mm separation column was coupled via a Finnigan LC IsoLink Interface to a Thermo-Finnigan Delta+ Advantage Isotope Ratio Mass Spectrometer. Sample injection volume was 10 μL. The eluent was 100% ultrapure water (18.2 MΩ) at a flow rate of 0.3 mL/min. The chemical oxidation reagents were mixed to the eluent via a t-piece at a flow rate of 0.05 mL/min each by the IsoLink interface pumps. The eluent and reagent bottles were kept under constant He purge to prevent CO_2 contamination from ambient air. The pressure gauges were adjusted to 0.5 bar for He carrier gas, 1.2 bar for helium purge gas and 1.2 bar for CO_2 reference gas. Two in-line filters (5 μm) were placed between autosampler and column as well as between t-piece and oxidation reactor in order to prevent plugging of the oxidation reactor capillary or damage of the CO_2 membrane separation unit of the LC IsoLink interface. The temperature of the oxidation reactor was set at 99.9 °C. Two dilutions were measured for each sample, 0.8 g/L (fructose and glucose) and 4g/L (di- and trisaccharides). The CO_2 reference gas pulse was introduced three times (20 s each) at the beginning of each run. One run took approx. 45 min.

Thermo Electron Isodat version 2.38 was used as data processing software and calculation of $\delta^{13}C$ values for both instrument setups.

2.4. Samples

All honeys used as authentic reference samples were either delivered by honey traders or directly from beekeepers and underwent an additional confirmative authenticity analysis (botanical and geographical origin, humidity, HMF, pH-value, free acids, sugar profile, SCIRA test, enzyme activities, sensory analysis, proline, ion chromatographic analysis of cations and anions). Honeys from the following geographical regions of commercial honey production were included: Argentina, Austria, Bulgaria, Canada, China, Czech Republic, El Salvador, France, Germany, Great Britain, Greece, Hungary, India, Italy, Japan, Malaysia, Mexico, Romania, Slovakia, Slovenia, Spain, Turkey, Ukraine, Uruguay, Vietnam.

The honeys tested for adulteration were pretrade honeys for analysis or packed honeys collected from supermarkets (geographical origins: Southeastern Europe, Asia and Latin America). The criteria for suspecting an adulteration were unusual values or properties in the above mentioned analytical parameters, barely meeting the currently established legal requirements or reference values cited in the relevant scientific literature.

Sugar syrups were either purchased from sugar producers, in beekeeping shops, collected at food and beekeeping fairs or received as samples of suspected adulterants from third parties.

2.5. Sample preparation

Sample preparation for EA-IRMS was carried out according to AOAC method 998.12 (AOAC, 1999). Sample preparation for LC-IRMS was done by diluting 200 mg of honey to the appropriate concentrations (see measurement conditions, 2.3) with ultrapure water and filtered through a 0.45 μm membrane filter. The sample solutions have to be prepared freshly every day.

2.6. Calibration and result calculation

The CO_2 reference gas used to calculate the $\delta^{13}C$ values in each analytical run was calibrated with a laboratory working standard (glucose monohydrate, $\delta^{13}C$ value: –26.4 ± 0.2‰) which was calibrated against the IAEA-CH6 stable carbon reference standard. The reason for this procedure is the limited availability of the IAEA standard. The laboratory working standard was measured in each sequence of sample measurements at least once. Additionally, a quality control sample of a chosen honey was measured in each LC-IRMS measurement sequence. The $^{13}C/^{12}C$ carbon isotope ratios were reported as $\delta^{13}C$ values related to Vienna Pee Dee Belemnite (VPDB) according to the AOAC method (AOAC, 1999; see also Tab. I).

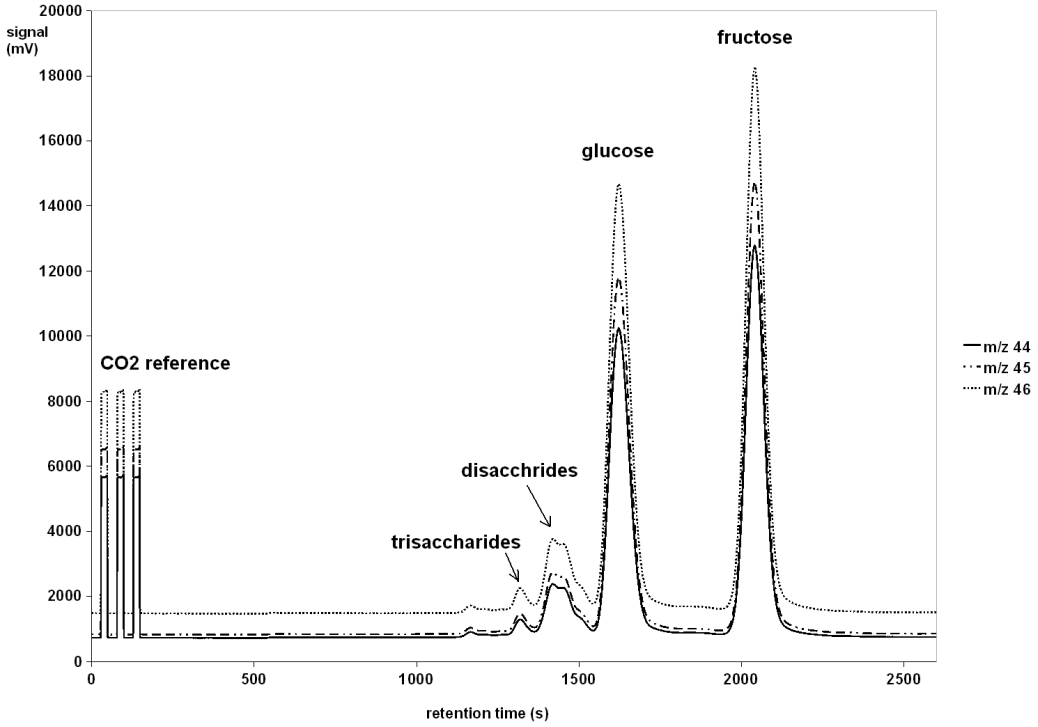

Figure 1. LC-IRMS chromatogramm of authentic polyfloral honey (sample 2a, Tab. VII). $\delta^{13}C$ values: fructose –26.0‰, glucose –25.9‰, disaccharides –26.8‰, trisaccharides –26.1‰.

3. RESULTS AND DISCUSSION

3.1. Liquid chromatographic separation of honey sugars for $\delta^{13}C$ measurement

The common determination of the sugar profile of honey using an amino column and a mixture of water and acetonitrile as eluent (IHC, 2006) cannot be used in connection with stable carbon isotope ratio analysis which is very sensitive to any background levels of organic and inorganic substances containing carbon. Polymeric styrene-divinylbenzene columns loaded with cations (Ca^{2+}, Pb^{2+}, Ag^{+}, H^{+}) and operated with ultrapure water as eluent turned out to be a good alternative. In this type of chromatography, the sugars are separated by ligand-exchange and size exclusion mechanisms. Compared to reversed phase chromatography, large columns (300 × 8 mm), low flow rates (< 0.6 mL/min), and elevated temperatures have to be used to achieve a good separation. In order to prevent partial hydrolysis of sucrose and other higher sugars which may cause peak deformation and unreliable $\delta^{13}C$ values, a column heating temperature of 55 °C was found to be the optimum without loosing significantly peak resolution. A column loaded with Ca^{2+} ions was finally chosen for the experiments, giving the best baseline separation (Fig. 1) for the peaks of fructose, glucose, disaccharides (including sucrose, turanose, maltose, isomaltose and trehalose) and trisaccharides (including erlose, melezitose, maltotriose and raffinose). Although there were slight differences between the tested columns loaded with Ca^{2+}, Pb^{2+}, Ag^{+} or H^{+} ions, none of them was able to separate the di- and trisaccharides itself. These columns can only separate higher sugars by their grade of polymerisation (DP-2, DP-3, etc.). The samples were measured at two concentrations, 0.8 g/L for fructose and glucose,

Table II. Reproducibility data of the EA/LC-IRMS method; results of the quality control sample (real life sample of an adulterated honey); n = 79 (one measurement on each of 79 different days, within 5 subsequent months); δ^{13}C: explanation see Table I; $\Delta\delta^{13}$C (‰) p – h: difference δ^{13}C protein – δ^{13}C honey; ave.: average value; s. d.: standard deviation; * apparent C4 sugar content, calculated according to method AOAC 998.12: C4 sugar (%) = [(δ^{13}C protein – δ^{13}C honey) × 100] ÷ [δ^{13}C protein – (–9.7)].

Parameter	ave.	s. d.
δ^{13}C (‰) protein (p)	–25.2	0.2
δ^{13}C (‰) honey (h)	–24.5	0.2
$\Delta\delta^{13}$C (‰) p – h	–0.7	0.2
C4 sugar (%) *	4.5	0.2
δ^{13}C (‰) fructose (fru)	–23.4	0.1
δ^{13}C (‰) glucose (glu)	–26.0	0.1
δ^{13}C (‰) disaccharides (ds)	–25.6	0.3
δ^{13}C (‰) trisaccharides (ts)	–25.0	0.5
fru/glu ratio	1.49	0.1
ds (area %)	5.9	0.5
ts (area %)	1.7	0.3
retention time shift (%, for all peaks)	–	0.3

and 4 g/L for di- and trisaccharides, respectively, in order to measure the δ^{13}C values within the linear range of the IRMS.

3.2. Measurement precision of δ^{13}C EA/LC-IRMS (reproducibility of results)

A quality control honey was measured in every sample sequence within a time range of 5 months (one measurement per day, determined on 79 different days). The standard deviations are shown in Table II. Compared to the conventional EA-IRMS method (AOAC, 1999) with a measurement uncertainty of ± 0.2‰ for the δ^{13}C values of protein and honey, the standard deviations of the δ^{13}C values of fructose, glucose, di- and trisaccharides determined by the LC-IRMS method are comparable and within the range of a similar method previously reported (Cabañero et al., 2006). As expected, the standard deviations for the δ^{13}C values of di- and trisaccharides were somewhat higher compared to those of fructose and glucose due to the considerably lower amounts present in the honey and their detection as a peak sum. Considering the additional factors contributing to a rise in measurement uncertainty (LC system modules, chromatographic separation, chemical oxidation, CO_2 gas separation unit), the determined standard deviations underline the suitability of the LC-IRMS method for the routine analysis of honey.

3.3. δ^{13}C values of authentic honey samples measured by δ^{13}C EA/LC-IRMS

The results of the 451 authentic honeys analyzed by the newly developed EA/LC-IRMS method are listed in Tables III and VI. The measured δ^{13}C values are in very good agreement to previously published data of δ^{13}C values for protein, honey, fructose, glucose and sucrose (AOAC, 1999; Cabañero et al., 2006; Kerkvliet and Meijer, 2000; Padovan et al. 2003; White and Doner, 1978a, b; White and Winters, 1989; White, 1992a; White et al., 1998). For disaccharides other than sucrose and trisaccharides, published data was not available in the literature, so that these values are reported for the very first time. When comparing the measured δ^{13}C values, it becomes quite clear that there are only small differences between the average δ^{13}C values of protein, honey, fructose, glucose, di- and trisaccharides (Tab. III). The isotopic shift of di- and trisaccharides is presumably due to kinetic isotope effects in the biochemical pathways of the sugars. Since the standard deviations of the individual δ^{13}C values did not exceed 1‰, it was concluded that the method is suitable for the detection of honey adulteration by sugar addition using the differences between the individual δ^{13}C values ($\Delta\delta^{13}$C values).

3.4. Purity criteria for honey based on differences between δ^{13}C values measured by δ^{13}C EA/LC-IRMS

The results for the $\Delta\delta^{13}$C values of the authentic honeys tested are listed in Table IVa. All $\Delta\delta^{13}$C values were below ± 1‰ with a

Table III. EA/LC-IRMS results of authentic honey samples (n = 451); $\delta^{13}C$: explanation see Table I; $\Delta\delta^{13}C$ (‰) p – h: difference $\delta^{13}C$ protein – $\delta^{13}C$ honey; ave.: average value, s. d.: standard deviation, range: minimum to maximum value observed; * apparent C4 sugar content (calculation see Tab. II).

Parameter	ave.	s. d.	range
$\delta^{13}C$ (‰) protein (p)	–25.2	0.7	–22.7 to –26.7
$\delta^{13}C$ (‰) honey (h)	–25.5	0.7	–23.0 to –27.3
$\Delta\delta^{13}C$ (‰) p - h	0.3	0.4	–0.9 to 1.5
C4 sugar (%) *	0.3	0.9	0 to 5.7
$\delta^{13}C$ (‰) fructose (fru)	–25.5	0.7	–23.2 to –27.5
$\delta^{13}C$ (‰) glucose (glu)	–25.5	0.7	–22.7 to –27.2
$\delta^{13}C$ (‰) disaccharides (ds)	–25.8	1.0	–22.5 to –28.2
$\delta^{13}C$ (‰) trisaccharides (ts)	–24.7	1.0	–22.6 to –27.5
fru/glu ratio	1.30	0.21	0.92 to 1.82
ds (area %)	6.8	2.4	1.2 to 14.1
ts (area %)	1.8	1.1	0.0 to 8.0
oligosaccharides (area %)	< 0.7	–	–

Table IVa. Differences in the $\delta^{13}C$ values ($\Delta\delta^{13}C$) of authentic honey samples from Table III (n = 451); $\delta^{13}C$: explanation see Table I; ave.: average value, s. d.: standard deviation, max. d.: absolut value of maximum difference observed. Abbreviations of sugars and protein according to Table III.

Parameter	ave.	s. d.	max. d.
$\Delta\delta^{13}C$ (‰) fru – glu	0.0	0.3	1.0
$\Delta\delta^{13}C$ (‰) fru – ds	0.3	0.7	2.0
$\Delta\delta^{13}C$ (‰) fru – ts	–0.8	0.7	2.0
$\Delta\delta^{13}C$ (‰) fru – p	–0.3	0.4	1.5
$\Delta\delta^{13}C$ (‰) glu – ds	0.3	0.7	1.9
$\Delta\delta^{13}C$ (‰) glu – ts	–0.7	0.7	2.0
$\Delta\delta^{13}C$ (‰) glu – p	–0.3	0.4	1.6
$\Delta\delta^{13}C$ (‰) ds – ts	–0.9	0.6	2.1
$\Delta\delta^{13}C$ (‰) ds – p	–0.6	0.7	2.0
$\Delta\delta^{13}C$ (‰) ts – p	0.5	0.7	2.1

Table IVb. Proposed limits for the $\Delta\delta^{13}C$ values as purity criteria for honey. Statistical certainty (confidence level) 99.7%. Abbreviations of sugars and protein according to Table III; $\Delta\delta^{13}C$ (‰) max.: maximum difference between all measured $\delta^{13}C$ values.

Parameter		Proposed limit
$\Delta\delta^{13}C$ (‰) fru – glu		±1.0
$\Delta\delta^{13}C$ (‰) fru – ds $\Delta\delta^{13}C$ (‰) fru – ts $\Delta\delta^{13}C$ (‰) fru – p $\Delta\delta^{13}C$ (‰) glu – ds $\Delta\delta^{13}C$ (‰) glu – ts $\Delta\delta^{13}C$ (‰) glu – p $\Delta\delta^{13}C$ (‰) ds – ts $\Delta\delta^{13}C$ (‰) ds – p $\Delta\delta^{13}C$ (‰) ts – p	subsumed as $\Delta\,\delta^{13}C$ (‰) max.	±2.1
$\Delta\delta^{13}C$ (‰) p – h		$\geqslant -1.0$

Table V. Check for compliance with the established purity criteria (see Tab. IVb) by analysis of varying mixtures of two authentic honeys with very different properties; honey a: polyflora honey (Latin America); honey b: acacia honey (Europe); %); δ^{13}C: explanation see Table I; $\Delta\delta^{13}$C (‰) p – h: difference δ^{13}C protein – δ^{13}C honey; $\Delta\delta^{13}$C fru – glu: difference δ^{13}C fru – δ^{13}C glu; $\Delta\delta^{13}$C (‰) max. (abs.): maximum difference (absolute) between all measured δ^{13}C values.

honey a honey b	0% 100%	10% 90%	25% 75%	50% 50%	75% 25%	90% 10%	100% 0%
δ^{13}C (‰) protein (p)	–24.4	–24.5	–24.7	–24.9	–25.0	–25.3	–25.2
δ^{13}C (‰) honey (h)	–24.7	–24.9	–25.1	–25.5	–25.7	–26.0	–26.3
$\Delta\delta^{13}$C (‰) p – h	0.3	0.4	0.4	0.6	0.7	0.7	1.1
C4 sugar (%)	0	0	0	0	0	0	0
δ^{13}C (‰) fru	–24.8	–25.0	–25.2	–25.5	–25.7	–26.0	–26.4
δ^{13}C (‰) glu	–24.7	–24.9	–25.2	–25.6	–25.8	–26.0	–26.3
δ^{13}C (‰) ds	–24.6	–24.8	–24.9	–25.3	–25.5	–25.9	–26.0
δ^{13}C (‰) ts	–23.4	–23.5	–23.8	–23.9	–24.1	–24.5	–24.5
$\Delta\delta^{13}$C (‰) fru – glu	–0.1	–0.1	0	0.1	0.1	0	–0.1
$\Delta\delta^{13}$C (‰) max. (abs)	1.4	1.4	1.4	1.7	1.7	1.5	1.9
fru/glu ratio	1.60	1.56	1.48	1.37	1.32	1.22	1.15
ds (area %)	10.0	10.0	9.5	8.8	8.6	8.1	7.1
ts (area %)	3.1	3.0	2.6	2.2	2.0	1.7	1.2
EA-IRMS	pass	pass	pass	pass	pass	pass	pass
EA/LC-IRMS	pass	pass	pass	pass	pass	pass	pass

standard deviation $\leqslant$ 0.7‰. Compared to previous findings (Cabañero et al., 2006), $\Delta\delta^{13}$C (fructose – glucose) values were exactly the same (0 ± 0.3‰), but for the maximum range the double value (± 1.0‰ instead of ± 0.5‰) was observed. This can be explained by the larger number of authentic reference honeys measured (451 compared to 54 samples). The highest differences observed did not exceed 2.1‰ in any case. This is also in excellent agreement with the results of Cabañero et al.

Taking these findings into account, simple purity criteria for honey can be defined (Tab. IVb). A statistical certainty (confidence level) of 99.7% was considered by multiplicating the standard deviation of 0.3‰ for $\Delta\delta^{13}$C (fructose – glucose) and the highest standard deviation of 0.7‰ (Tab. IVa) for the other $\Delta\delta^{13}$C values (subsumed as "$\Delta\delta^{13}$C max.") with a factor of 3. Since most packed honeys are blends of several honeys, it was ensured that these blends will meet the proposed limits. As an example, the $\Delta\delta^{13}$C values of mixtures of acacia and polyflora honey (Tab. V) which have very different physicochemical properties and geographical origins still fulfil the proposed purity criteria.

3.5. Detecting honey adulteration using the δ^{13}C-EA/LC-IRMS method and the defined purity criteria

The described EA/LC-IRMS method and the defined purity criteria for the $\Delta\delta^{13}$C values were applied to 684 honey samples suspected of being adulterated with sugar. Using the AOAC method (AOAC, 1999), adulteration was detected in 3% of all cases. However, applying the new EA/LC-IRMS method, 34% of the samples were found to be adulterated. It has to be taken into account that these figures are not representative for the whole honey market as such, because only samples

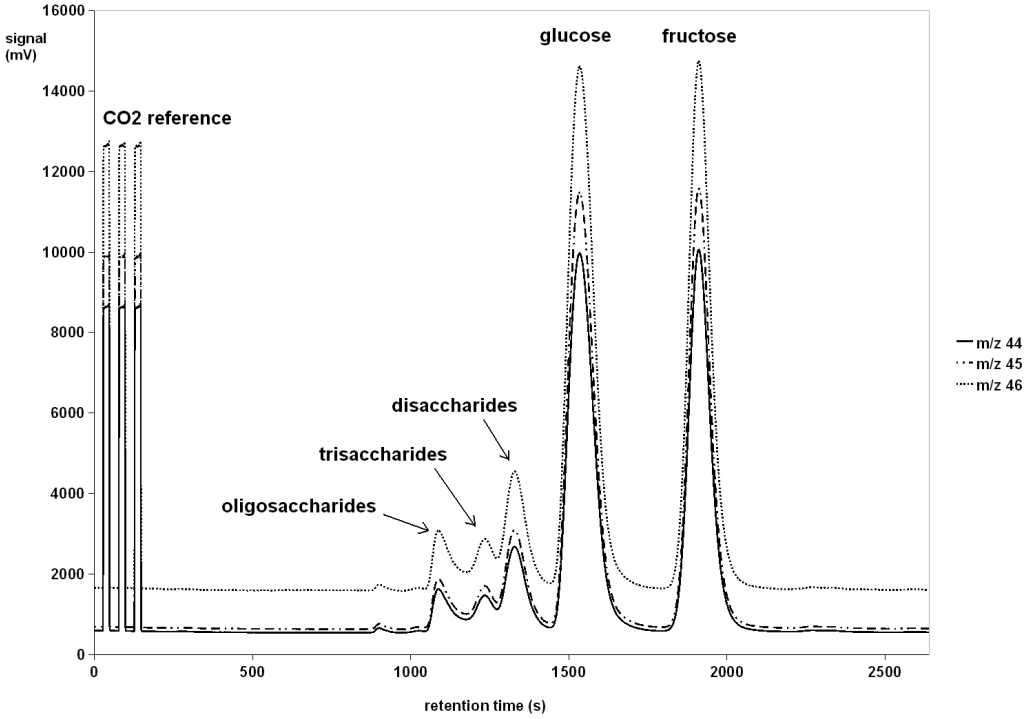

Figure 2. LC-IRMS chromatogramm of adulterated polyfloral honey (sample 2d, Table VII). $\delta^{13}C$ values: fructose –27.4‰, glucose –27.0‰, disaccharides –26.4‰, trisaccharides –24.3‰, oligosaccharides –26.7‰. Adulteration: ca. 11% rice syrup.

with a concrete suspicion of adulteration were analyzed. So, in real life, the proportion of adulterated honeys on the market is significantly lower. However, the findings show that sugar adulteration of honey is still a problem the honey sector has to cope with. Also, it demonstrates the enormous potential of the new EA/LC-IRMS technique having an increased sensitivity and the ability to detect different kinds of sugar adulteration previously not revealed. In order to illustrate the practicability of the EA/LC-IRMS method, real life examples are given in the following.

Figure 1 shows a LC-IRMS chromatogram of an authentic polyflora honey. The differences between individual $\Delta\delta^{13}C$ values of fructose, glucose, di- and trisaccharides are within 0.9‰ (see also Tab. VII, sample 2a). In contrast, a LC-IRMS chromatogram of an adulterated polyflora honey sample is shown in Figure 2. An oligosaccharide peak at approx. 1050 seconds appears which must not be present in authentic honeys (limit of detection: 0.7 area%). Therefore, the $\delta^{13}C$ value of the oligosaccharide peak represents the approximate $\delta^{13}C$ value of the added sugar which must come from a C3 plant source. Additionally, the $\Delta\delta^{13}C$ max. value of 3.1‰ (see also Tab. VII, sample 2(d)) is outside the naturally occurring range. Further investigations revealed that this particular sample was adulterated with approx. 11% rice syrup, confirmed by artificially adulterating an authentic polyflora honey with the corresponding amount of rice syrup and obtaining the same chromatographic appearance and $\delta^{13}C$ values. It has to be mentioned that the oligosaccharide peak cannot be observed using the common LC-RI method (IHC, 2006).

Table VII lists further results for different types of honeys (acacia, polyflora, lime, honeydew) which were both measured by the

Table VI. Comparison of $\delta^{13}C$ (‰) values (explanation see Tab. I) of different types of authentic honey and some high fructose invert sugar syrups (HFISS) and other syrups available on the market; ave.: average value, s. d.: standard deviation, range: minimum to maximum value observed.

$\delta^{13}C$ (‰) value of:	ave.	s. d.	range
all honeys (n = 451)	–25.5	0.7	–23.0 to –27.3
acacia honey (n = 65)	–24.9	0.5	–23.9 to –25.8
rape honey (n = 51)	–26.1	0.4	–25.4 to –26.7
orange honey (n = 8)	–24.9	0.3	–24.4 to –25.2
polyflora honey (n = 125)	–26.0	0.4	–24.7 to –27.2
Yucatan honey (n = 33)	–25.4	0.6	–24.0 to –26.6
lime honey (n = 5)	–25.5	0.1	–25.3 to –25.7
forest honey (n = 7)	–25.7	0.6	–24.7 to –27.0
HFISS 1 (C3/C4 sugar mix)	–19.8	0.2	–
HFISS 2 (C3 sugar)	–26.4	0.2	–
HFISS 3 (C4 sugar)	–11.3	0.2	–
bee feeding syrup	–24.2	0.2	–
rice syrup	–26.1	0.2	–

official AOAC method (EA-IRMS) and the newly developed EA/LC-IRMS method. All honeys passed the AOAC test. However, only the samples marked with an (a) can be considered as authentic. The other honeys, marked with (b), (c) or (d) had $\Delta\delta^{13}C$ values outside the naturally occurring range of ± 1‰ for $\Delta\delta^{13}C$ (fructose – glucose) and ± 2.1‰ for $\Delta\delta^{13}C$ (‰) max., respectively.

3.6. Method sensitivity: Newly developed δ^{13}C-EA/LC-IRMS vs. conventional δ^{13}C-EA-IRMS

It has been shown already that sugar additions to honey can be detected reliably at levels between 1% (C4 sugars) and 10% (C3 sugars) using LC-IRMS (Cabañero et al., 2006). This is confirmed by the results obtained with the newly developed EA/LC-IRMS method regarding the significantly lower detection limit for C4 sugar adulterations compared to EA-IRMS (e.g. samples 1(c) and 2(c) in Tab. VII) and the ability to detect adulterations with C3 sugar, e.g. rice syrup which mainly occurs in Chinese honeys (sample 2(d) in Tab. VII and Fig. 2).

Table VI also lists the $\delta^{13}C$ values of three different kinds of high fructose invert sugar syrup (HFISS) and a bee feeding syrup from Europe. It is evident that the syrups (except HFISS 3) cannot be detected at all using only the difference $\Delta\delta^{13}C$ (protein – honey). In this respect, the earlier discussed results for the di- and trisaccharides of the samples 1(c), 2(c), 2(d), 3(b) and 4(b) in Table VII clearly demonstrate the importance of these minor sugars as marker molecules for the detection of sugar adulteration. This is illustrated by the following simple experiment: HFISS 1 (see Tab. VI), which is a mixture of C3 and C4 plant sugars and available on the market, was used to artificially adulterate authentic acacia honey with amounts of 5, 10, 20 and 50% of invert sugar syrup. The results are shown in Table VIII. The AOAC method can only detect adulteration with this type of syrup at levels > 27%. Using the newly developed EA/LC-IRMS method, the limit of detection can be lowered to ca. 8%. Moreover, the usual calculation of the C4 sugar content according to the AOAC method causes a misleading result interpretation. For example, the adulteration with 20% HFISS 1 simulated a C4 sugar content of 5.5% which would have not been objected.

4. CONCLUSION

As a logical consequence of the findings presented, it is proposed that the detection

Table VII. Real life honey samples: (1) acacia, (2) polyflora, (3) lime, (4) honeydew, (a) authentic, (b, c, d) adulterated; fru: fructose; glu: glucose, ds: disaccharides, ts: trisaccharides, os: oligosaccharides, n.a.: not analyzable, n.d.: not detected (< 0.7 area %); $\delta^{13}C$: explanation see Table I; $\Delta\delta^{13}C$ (‰) p – h: difference $\delta^{13}C$ protein – $\delta^{13}C$ honey; $\Delta\delta^{13}C$ fru – glu: difference $\delta^{13}C$ fru – $\delta^{13}C$ glu; $\Delta\delta^{13}C$ (‰) max. (abs.): maximum difference (absolute) between all measured $\delta^{13}C$ values. Conspicuous values bold.

sample	1(a)	1(b)	1(c)	2(a)	2(b)	2(c)	2(d)	3(a)	3(b)	4(a)	4(b)
$\delta^{13}C$ (‰) protein (p)	–24.6	–25.2	–24.3	–25.6	–25.2	–24.6	–26.4	–25.7	–25.6	–25.6	–25.3
$\delta^{13}C$ (‰) honey (h)	–24.7	–24.6	23.9	–26.0	–25.0	–23.8	–27.0	–25.6	–25.7	–25.8	–25.6
$\Delta\delta^{13}C$ (‰) p – h	0.1	–0.6	–0.4	0.4	–0.2	–0.8	0.6	–0.1	0.1	0.2	0.3
C4 sugar (%)	0	3.9	2.7	0	1.3	5.4	0	0.6	0	0	0
$\delta^{13}C$ (‰) fru	–24.7	**–23.8**	–25.0	–26.0	–24.4	–24.7	–27.4	–25.5	–26.0	–25.7	–26.1
$\delta^{13}C$ (‰) glu	–24.8	–26.0	–24.6	–25.9	**–25.7**	–24.2	–27.0	–25.5	–25.7	–25.7	–25.7
$\delta^{13}C$ (‰) ds	–24.8	–25.7	**–22.6**	–26.8	–24.8	**–17.3**	–26.4	–25.8	**–23.9**	–26.5	**–23.8**
$\delta^{13}C$ (‰) ts	–23.9	–25.0	**–18.7**	–26.1	–24.5	**–16.2**	**–24.3**	–25.5	–24.4	–26.1	**–23.8**
$\delta^{13}C$ (‰) os	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	–26.7	n.a.	n.a.	n.a.	n.a.
$\Delta\delta^{13}C$ (‰) fru – glu	0.1	2.2	–0.4	–0.1	1.3	–0.5	–0.4	0	–0.3	0	–0.4
$\Delta\delta^{13}C$ (‰) max. (abs.)	0.9	2.2	6.3	1.2	1.3	8.5	3.1	0.3	2.1	0.8	2.3
fru/glu ratio	1.56	1.60	1.53	1.17	1.27	1.28	0.86	1.39	1.27	1.24	1.37
ds (area %)	9.6	5.3	10.8	4.8	8.1	8.1	8.5	9.9	8.3	7.5	18.3
ts (area %)	2.8	1.4	4.5	1.2	3.8	0.9	3.1	4.1	1.4	4.7	2.4
os (area %)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.0	n.d.	n.d.	n.d.	n.d.
EA-IRMS	pass	pass	pass	pass	pass	pass	pass	pass	pass	pass	pass
EA/LC-IRMS	pass	fail	fail	pass	fail	fail	fail	pass	fail	pass	fail

of sugar adulteration of honey should rather be determined by calculating $\Delta\delta^{13}C$ values than calculating the amount of adulteration, which is only applicable in the case of adulteration with syrups of which the $\delta^{13}C$ values are known. In all other cases this procedure can cause misinterpretations. If mixtures of different sugar syrups are used for adulteration, a quantification of the amount of added sugar is almost impossible. Since the sugars added to honey are chemically identical to those of the honey, they cannot be distinguished. Therefore, a convenient way to circumvent this problem is to look at the $\Delta\delta^{13}C$ values falling outside the naturally occurring range. As presented in this work, this principle is working very well. When applied routinely in laboratories dealing with the issue of sugar adulteration of honey, it is believed that the proposed EA/LC-IRMS method can be a suitable succession of the AOAC method, providing enhanced sensitivity and the ability to detect more adulterations of honey with different types of sugar syrups produced from C4 and C3 plant sources than ever before.

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Amélioration de la détection de la falsification du miel en mesurant les différences entre les rapports C^{13}/C^{12} des isotopes stables de carbone des composés protéiniques et glucidiques en combinant la spectrométrie de masse des rapports

Table VIII. Addition of high fructose invert sugar syrup 1 (HFISS 1) to an authentic acacia honey. Limits of detection: EA-IRMS ca. 27%, EA/LC-IRMS ca. 8%. δ^{13}C: explanation see Table I; $\Delta\delta^{13}$C (‰) p – h: difference δ^{13}C protein – δ^{13}C honey; $\Delta\delta^{13}$C fru – glu: difference δ^{13}C fru – δ^{13}C glu; $\Delta\delta^{13}$C (‰) max. (abs.): maximum difference (absolute) between all measured δ^{13}C values.

HFISS 1 acacia honey	0% 100%	5% 95%	10% 90%	20% 80%	50% 50%	100% 0%
δ^{13}C (‰) protein (p)	–24.3	–24.3	–24.3	–24.3	–24.3	n.a.
δ^{13}C (‰) honey (h)	–24.4	–24.3	24.1	–23.5	–22.3	–19.8
$\Delta\delta^{13}$C (‰) p - h	0.1	0	–0.2	–0.8	–2.0	n.a.
C4 sugar (%)	0	0	1.4	5.5	13.7	n.a.
δ^{13}C (‰) fru	–24.4	–24.2	–24.3	–24.2	–23.7	–22.8
δ^{13}C (‰) glu	–24.5	–24.6	–24.0	–23.0	–20.8	–10.9
δ^{13}C (‰) ds	–24.2	–24.1	–23.5	–22.3	–19.7	–12.4
δ^{13}C (‰) ts	–23.6	–23.1	–22.1	–20.6	–19.1	–11.4
δ^{13}C (‰) os	n.a.	n.a.	n.a.	–12.3	13,0	–12,1
$\Delta\delta^{13}$C (‰) fru - glu	0.1	0.4	–0.3	–1.2	–2.9	–11.9
$\Delta\delta^{13}$C (‰) max. (abs.)	0.9	1.5	2.2	12.0	11.3	8.5
fru/glu ratio	1.48	1.48	1.67	1.87	2.46	5.61
ds (area %)	8.2	9.2	9.8	9.7	8.9	4.1
ts (area %)	2.8	2.9	3.2	3.4	2.9	2.2
os (area %)	n.d.	n.d.	n.d.	0.8	1.7	3.2
EA-IRMS	pass	pass	pass	pass	fail	fail
EA/LC-IRMS	pass	pass	fail	fail	fail	fail

isotopiques couplée à un analyseur élémentaire et la spectrométrie de masse des rapports isotopiques couplée à la chromatographie en phase liquide (δ^{13}C-EA/LC-IRMS).

miel / falsification / LC-IRMS / EA-IRMS / valeur δ^{13}C / sucre en C3 / sucre en C4

Zusammenfassung – Verbesserter Nachweis von Honigverfälschungen durch Messung der Differenzen zwischen den Kohlenstoff-Stabilisotopenverhältnissen $^{13}C/^{12}C$ des Proteins und der Zuckerkomponenten mit einer Kombination aus Elementaranalysator – Isotopenverhältnismassenspektrometrie und Flüssigchromatographie – Isotopenverhältnismassenspektrometrie (δ^{13}C-EA/LC-IRMS). Der Authentizitätsnachweis von Honig, welcher aufgrund seiner natürlichen Herkunft und seiner ursprünglichen Reinheit als hochwertiges Produkt angesehen wird, ist nach wie vor eine der anspruchsvollsten Aufgaben im Bereich der Honiganalytik. Trotz der Vielzahl an analytischen Methoden zur Bestimmung von Honigverfälschungen gibt es weiterhin Bedarf an zusätzlichen und genaueren Messtechniken. Aufgrund langjähriger Erfahrung lässt sich feststellen, dass die klassische Verfälschung mit Invertzuckersirupen hergestellt aus C4-Pflanzen auf ein konstant niedriges Niveau gesunken ist, aber die Verfälschung mit anderen Zuckersirupen, z.B. hergestellt aus C3-Pflanzen wie Zuckerrübe oder Reis (Tab. I), welche mit den existierenden Methoden nicht nachgewiesen werden können, in den letzten Jahren zugenommen hat. In dieser Arbeit wurde eine weiterentwickelte EA/LC-IRMS Methode, basierend auf der kürzlich vorgestellten LC-IRMS Technik, verwendet, um die δ^{13}C-Werte des Honigs, des isolierten Proteins, der Fructose, der Glucose, der Di- und Trisaccharide von 451 authentischen Honigmustern zu messen. Die ermittelte Präzision der Methode war bemerkenswert gut (Tab. II). Die absoluten δ^{13}C-Werte der authentischen Honige lagen im Bereich von –22,5 ‰ bis –28,2 ‰ (Tab. III und VI). Die mittleren δ^{13}C-Werte von Protein, Honig, Fructose, Glucose, Di- und Trisacchariden waren sehr ähnlich und die jeweiligen Standardabweichungen lagen unter 1 ‰ (Tab. III). Deshalb wurde die EA/LC-IRMS Methode als geeignet erachtet, um Honigverfälschungen anhand der

Differenzen ($\Delta\delta^{13}C$) zwischen den einzelnen $\delta^{13}C$-Werten nachzuweisen. Die mittleren $\Delta\delta^{13}C$-Werte lagen unter ± 1 ‰, mit Standardabweichungen ⩽ 0,7 ‰ (Tab. IVa). Die höchsten beobachteten Differenzen überschritten in keinem Fall 2,1 ‰. Basierend auf diesen Befunden konnten Maximalwerte für die $\Delta\delta^{13}C$-Werte als Kriterium für die Unverfälschtheit von Honig definiert werden (Tab. IVb), wobei eine statistische Sicherheit von 99,7 % berücksichtigt wurde. Da es sich bei den meisten im Handel befindlichen Honigen um Mischungen verschiedener Honige handelt, wurde sichergestellt, dass die aufgestellten Grenzwerte auch auf solche Mischhonige anwendbar sind (Tab. V). Mit der beschriebenen EA/LC-IRMS Methode und den damit aufgestellten Kriterien für die Unverfälschtheit von Honig wurden 684 Honigmuster, bei denen ein Verdacht auf Verfälschung bestand, untersucht. 34 % dieser Proben wurden mit dieser Methode als verfälscht klassifiziert, wogegen mit der herkömmlichen AOAC Methode lediglich 3 % der Proben als verfälscht erkannt wurden. Die Ergebnisse von Realproben sind in Tabelle VII und Abbildungen 1–2 detailliert aufgeführt. Darüber hinaus wird anschaulich dargestellt, dass das neue Prinzip der Beurteilung anhand von $\Delta\delta^{13}C$-Werten im Vergleich zur Berechnung der Menge an zugesetztem Zuckersirup, wie in der AOAC Methode beschrieben, vorteilhafter ist, da es zu Fehlinterpretationen bei Zuckersirupen oder Mischungen von Zuckersirupen mit unbekannten $\Delta\delta^{13}C$-Werten kommen kann (Tab. VIII). Die neu entwickelte EA/LC-IRMS Methode erwies sich als ein wertvolles Hilfsmittel zur Analyse von Honigverfälschungen mit Invertzuckersirupen gemäß dem aktuellen Stand der Analysentechnik, verbunden mit einer höheren Empfindlichkeit, der Möglichkeit Verfälschungen mit verschiedenen C3- und C4-Zuckersirupen nachzuweisen, ihrer Eignung als Routinemethode in der Hochdurchsatzanalyse kommerzieller Honigproben und dem Potential, sich als zukünftige Standardmethode zum Nachweis von Zuckerverfälschung von Honig zu etablieren.

Honig / Verfälschung / LC-IRMS / EA-IRMS / $\delta^{13}C$-Werte / C3-Zucker / C4-Zucker

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