JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Liquid Chromatography Coupled to Isotope Ratio Mass Spectrometry: A New Perspective on Honey Adulteration Detection

ANA I. CABAÑERO,* JOSE L. RECIO, AND MERCEDES RUPÉREZ

Laboratorio arbitral agroalimentario del Ministerio de Agricultura, Pesca y Alimentación, Crta. Coruña km 10,700, 28023 Madrid, Spain

A new procedure to determine individual sugar (sucrose, glucose, and fructose) $^{13}C$ isotope ratios, using liquid chromatography−isotope ratio mass spectrometry (HPLC-IRMS), has been developed to improve isotopic methods devoted to the study of honey authenticity. For this purpose 79 commercial honey samples from various origins were analyzed. Values of $\delta^{13}C_{honey}$ ranged from −14.2 to −27.2‰, and $\delta^{13}C_{protein}$ ranged from −23.6 to −26.9‰. A very strong correlation is observed between the individual sugar $^{13}C$ ratios, which are altered in the event of sugar addition, even at low levels. The use of $\Delta\delta^{13}C$ [fruct-glu], $\Delta\delta^{13}C$ [fruct-suc], and $\Delta\delta^{13}C$ [gluc-suc] systematic differences as an authenticity criterion permits the sugar addition [$C_3$, beet sugar; or $C_4$, cane sugar, cane syrup, isoglucose syrup, and high-fructose corn syrup (HFCS)] to be reliably detected (DL = 1−10%). The new procedure has advantages over existing methods in terms of analysis time and sensitivity. In addition, it is the first isotopic method developed that allows beet sugar addition detection.

**KEYWORDS: Honey; carbon isotope; adulteration detection; HPLC-IRMS**

## INTRODUCTION

Honey consumption has increased during the past decade due to consumers' preference for natural and pure products with no additives or preservative addition. Nowadays the honey industry is an important economic and social growth activity. In addition, honey is a high-volume product, governed by market rules of supply and demand.

During the past decades Varroa and other illnesses have been responsible for honey shortages. This has caused serious problems in the honey industry and forced governments to establish stricter controls on imported products to prevent fraud.

In addition, whenever price differences exist, there is a potential for a dishonest trader to attempt to make money by passing off a cheaper product as a more expensive one or by adding low-commodity components such as sweeteners. As a consequence, although the adulteration of honey is not injurious to health, problems of honey fraud negatively influence market growth by damaging consumer confidence.

Several analytical chemical techniques have been developed for the detection of honey adulteration (*1*). The most widely used is high-performance liquid chromatography (HPLC) (*2*), but this methodology does not detect low levels of adulteration, nor is it adequate for the more sophisticated falsifications (*3*, *4*). Up to now, several novel techniques/methods have been developed to determine total honey adulteration as mid-infrared (MIR) spectroscopy or high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD); however, they have not been able to detect unambiguously low levels of honey adulteration by several sugars by a unique method (*5*, *6*).

On the other hand, the isotope ratio mass spectrometry (IRMS) technique (*7*−*9*) is a precise methodology that can detect low to high levels of adulteration. It is determined by the $^{13}C/^{12}C$ isotope ratio, which is different in monocotyledonous plants ($C_4$, cane and corn), when compared to dicotyledons ($C_3$, most flowering plants from which bees collect nectar). The different ratios of carbon isotopes are produced by different photosynthesis cycles (*4*, *10*). Plants with the Calvin−Benson photosynthetic cycle ($C_3$) have $^{13}C/^{12}C$ ($\delta$) values from −23 to −28‰ (*11*), and plants with the Hatch−Slack photosynthetic cycle ($C_4$) have values from −9 to −15‰ of $^{13}C/^{12}C$ ($\delta$) (*12*); $C_4$ plants have high $^{13}C$ when compared to $C_3$ plants. As a result, stable carbon isotope ratio analysis (SCIRA) was used to detect adulteration in honey (*13*); honeys with $\delta^{13}C$ values less negative than −23.5‰ were considered to be suspect (*8*).

Companies that produce adulterated honey adapted to this new technique by blending artificial sweeteners with honeys that had $\delta^{13}C$ ($^{13}C/^{12}C$) lower than −23.5‰. However, by comparing the carbon isotope ratios in the protein and the sugars of honey, which should be the same if they come from the same source (*8*, *14*, *15*), it is determined if the honey is adulterated, and the percentage of adulteration can be estimated by the difference in the $^{13}C/^{12}C$ ratios between the sugar in the honey and its protein.

Therefore, existing methodologies for honey analyses are able to detect several frauds. However, as defrauders become more sophisticated both in the type of fraud perpetrated and in the

* Author to whom correspondence should be addressed (e-mail labcoris@mapya.es; telephone + 34 913474971).

10.1021/jf062067x CCC: $33.50 
Published on Web 11/22/2006

ways used to cover it up, more powerful tools such as separative techniques coupled to IRMS are required, and it is thus vital to combat and prevent fraud.

Whereas the coupling of isotope ratio mass spectrometry (IRMS) to gas chromatography (GC) is a well-established technique, the combination of IRMS with liquid chromatography (LC) remains a challenge.

Many compounds of nutritional interest are not volatile and have to be derivatized to be analyzed by GC (*16*); however, with LC the addition of large numbers of exogenous carbons from derivatization to generate the volatile species that would be required for GC-IRMS analysis can be avoided. On the other hand, obtaining a reliable and reproducible conversion of organic molecules into $CO_2$ is relatively straightforward when GC is coupled to IRMS, but coupling LC to IRMS is much more complicated because the $CO_2$ has to be generated in, and extracted from, the liquid phase.

To date, several approaches for producing $CO_2$ from organic compounds and its application to LC-IRMS have been developed (*17*−*19*); however, they were characterized by isotope fractionation of low molecular weight compounds, low recoveries, and low sensitivity. A new interface for LC-IRMS based on chemical oxidation has been developed by Krummen et al. (*20*) and is currently commercially available (Finnigan LC Isolink). In this interface the sample is oxidized while still in the mobile phase, and afterward the $CO_2$ is separated from the liquid phase for isotopic analysis. This process is shown to be quantitative and without isotopic fractionation.

To improve the currently available methodologies for honey adulteration detection and, thus, prevent honey fraud, a new method based on the separation of the main sugars of honey and later isotopic characterization by HPLC-IRMS employing the new interface has been developed. This new procedure, its validation, the results obtained on commercial honey, and finally its practical application to the authenticity control of honey are described in this paper.

## MATERIALS AND METHODS

**Reagents.** All reagents were of analytical grade and were used without further purification. Milli-Q water (Millipore, Bedford, MA) was used in all experiments.

For protein isolation, tungstic acid (sodium salt, 10%) (Carlo Erba, Italy) and 2/3 N sulfuric acid (Merck, Darmstadt, Germany) were used.

For HPLC-IRMS studies, 0.5 M orthophosphoric acid (Fluka, Buchs, Switzerland) and 0.5 M sodium peroxodisulfate (Fluka) were prepared.

All saccharide standards were purchased from Merck.

Carbon dioxide from Air Liquid (quality N-48) was used as working reference gas.

**Instrumentation.** A Carlo Erba (Milan, Italy) NC 2500 elemental analyzer (EA) was coupled to a Delta Plus isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) and served for total $\delta^{13}C$ of honey and proteins.

An LC Isolink interface (Thermo Electron, Bremen, Germany) was coupled to an isocratic liquid chromatographic system and to a Delta-Plus Advantage isotope ratio mass spectrometer (Thermo Electron) without any modifications to evaluate the $\delta^{13}C$ of individual sugars of honey. The eluent was delivered with a Surveyor LC pump (Thermo Electron). The LC pump and the chromatography column were connected to a Rheodyne 7125 injection valve equipped with a 25 $\mu$L loop. For sugars separation a ligand-exchange column (HyperREZ Carbohydrate $H^+$, 30 cm, 8 mm) (Thermo, Chesire, U.K.) was used.

To connect the LC column to the Surveyor pump and the manufactured interface, PEEK tubing and nuts having 0.25 mm i.d. were used. The tubes connecting the mobile phase bottles to the pump itself were manufactured of 'No-Ox' material ($^1/_8'' \times 1.5''$, Socochim, Lausanne, Switzerland) to avoid "re-gassing" of the eluent. An in-line filter of 0.25 mm (Vici, Schmidlin Labor, Switzerland) was also placed after the LC column to avoid any particles passing into the interface.

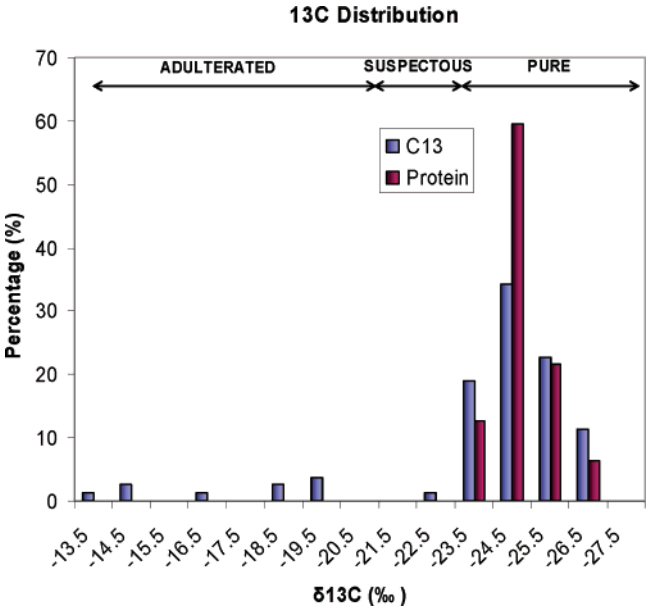


**Figure 1.** Stable carbon isotope ratio distribution of honey and its protein.

The isotope ratio mass spectrometer, data acquisition system, and Surveyor pump were controlled by a PC running under Microsoft (Redmond, WA) Windows XP Professional. The IRMS instrument and Surveyor pump were also controlled using Isodat 2.0 SP 1.43 (Thermo Electron). The EA was also controlled using the same Isodat software.

A Beckman Coulter (Fullerton, CA) centrifuge TJ-25 was used for the separation of the supernatant after protein extraction from honey samples.

**Samples.** This study was conducted with 79 samples of honey of various botanical and geographical origins. Five pure samples of guaranteed origin were purposely adulterated with cane sugar, cane sugar syrup, beet sugar, isoglucose syrup, and high-fructose corn syrup (HFCS), at levels of 1, 2, 5, 10, 20, or 30%.

**Sample Preparation.** *a. For Total $^{13}C/^{12}C$ Determination of Honey and Protein.* Honey: 1 mg of honey sample, approximately, was placed into tin capsules.

Protein: a 10−12 g sample of honey was placed in a clear 50 mL centrifuge tube, and 4 mL of distilled water added and mixed.

In another tube, 2 mL of 10% sodium tungstate solution was mixed thoroughly with 2 mL of 2/3 N sulfuric acid and then added to and mixed with the honey/water solution. The tubes were swirled in a water bath at 80 °C until visible flocs were formed and clear supernatant was observed.

If no visible floc forms, or if the supernatant remains turbid, acid should be added in 2 mL increments with heating repeated between additions.

The tubes were filled with water, the contents mixed, the tubes centrifuged at 1500*g*, and the supernatants removed. The precipitate was washed by adding 50 mL of distilled water and agitating; the precipitate was then separated. This procedure was repeated at least five times, until the supernatant was clear. The precipitated protein was dried in an oven (75 °C) during at least 3 h. Next, 1 mg approximately was placed into tin capsules.

*b. For $^{13}C/^{12}C$ Determination of Individual Sugars.* The honey samples were diluted with Milli-Q water, filtered through 0.45 $\mu$m filters, placed into glass vials, and analyzed by HPLC coupled to IRMS.

**$^{13}C/^{12}C$ Measurements.** *a. Honey and Protein by EA-IRMS.* The analysis was carried out according to official methods 978.17 and 991.41 of the AOAC, using a mass spectrophotometer for stable carbon isotope ratio analysis coupled to an elemental analyzer. The equipment was operated using a flow of helium of 100 mL $min^{-1}$, at a temperature of 1020 °C in the oxidation tube and 650 °C in the reduction tube and 40 °C in the chromatographic column.

**Table 1.** Study of the Repeatability of $\delta^{13}C$ Results

| | | HPLC-IRMS | | | | | | EA-IRMS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | repetitions per sample | mean $\delta^{13}C$ sucrose (‰) | SD (‰) | mean $\delta^{13}C$ glucose (‰) | SD (‰) | mean $\delta^{13}C$ fructose (‰) | SD (‰) | mean $\delta^{13}C$ sucrose (‰) | SD (‰) | mean $\delta^{13}C$ glucose (‰) | SD (‰) | mean $\delta^{13}C$ fructose (‰) | SD (‰) |
| sugar measurement (standards) | 10 | –25.7 | 0.2 | –10.7 | 0.2 | –24.1 | 0.2 | –25.6 | 0.2 | –10.9 | 0.2 | –24.3 | 0.2 |
| honey measurement (sample) | 5 | –27.3 | 0.2 | –25.7 | 0.2 | –25.7 | 0.2 | | | | | | |

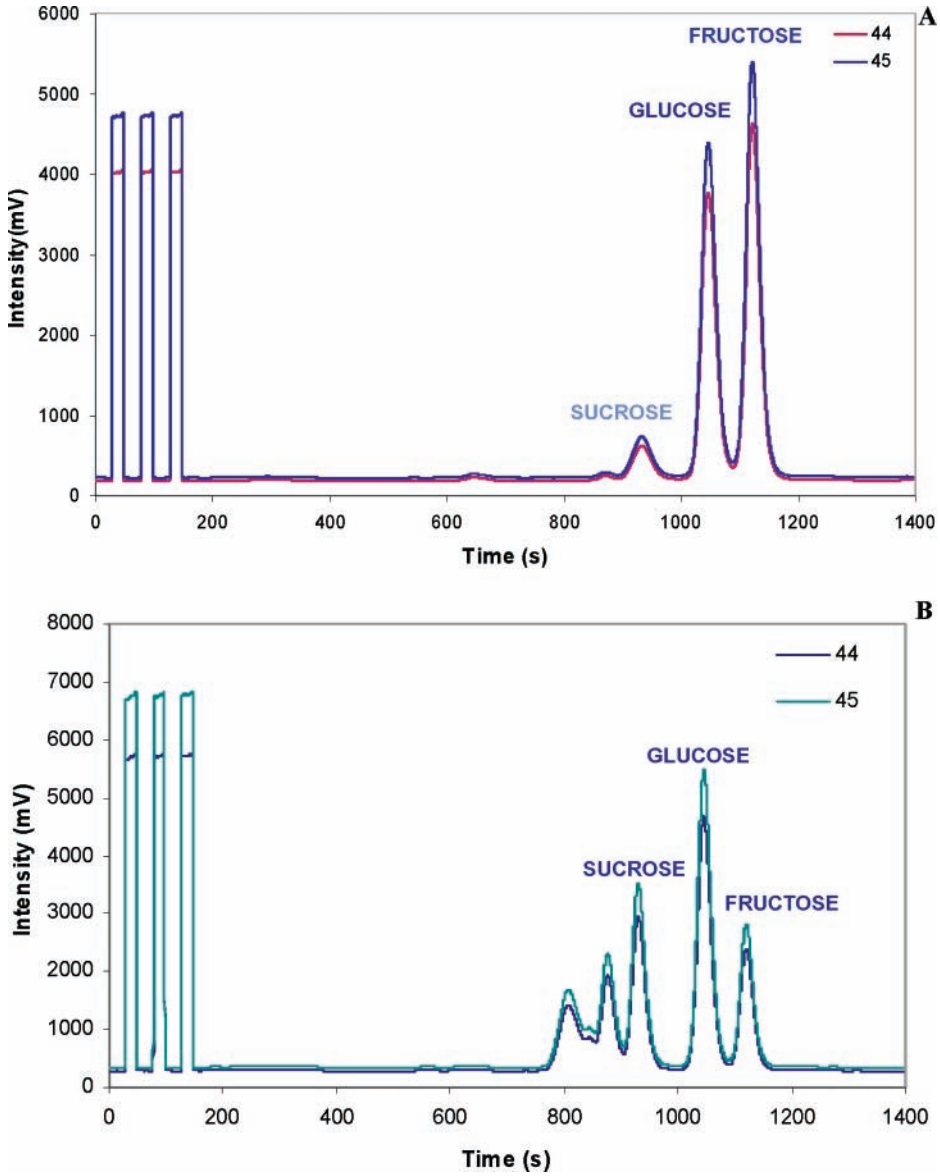


**Figure 2.** Chromatogram of (**A**) pure honey and (**B**) adulterated honey. Column: HyperREZ Carbohydrate H+ (300 mm × 8 mm). Conditions: HPLC flow, 400 $\mu$L min$^{-1}$ water; oxidation and acid reagent flow, 50 $\mu$L min$^{-1}$, 25 °C.

The instrument was equipped with an autosampler for solid samples; the cycle time for one complete determination was 400 s.

Suitable control references were included in each batch.

*b. Sugars by HPLC-IRMS.* The analysis was carried out using a mass spectrophotometer for stable carbon isotope ratio analysis coupled to liquid chromatography. The spectrophotometer was operated at an accelerating voltage of 10 kV. The ion source was held at a pressure of $2.3 \times 10^{-6}$ mbar, and ions were generated by electron impact at 70 eV. Three Faraday cup detectors monitored simultaneously and continuously the $CO_2^{\bullet+}$ signals for the three major ions at *m*/*z* 44 ($^{12}CO_2$), *m*/*z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$), and *m*/*z* 46 ($^{12}C^{18}O^{16}O$). To obtain comparable signal outputs, the preamplifier feedback resistors were selected to be $3 \times 10^8$, $3 \times 10^{10}$ and $1 \times 10^{11}$ Ω for *m*/*z* 44, 45, and 46, respectively.

The LC flow rate of the eluent (Milli-Q water) was 400 $\mu$L min$^{-1}$, and the flow rate of the acid and oxidant reagents in the LC interface was 50 $\mu$L min$^{-1}$ each. The temperatures of the interface reactor and the column were set at 99.9 and 25 °C, respectively. The helium flow rate of the separation unit was set at 1 mL min$^{-1}$.

In addition, the reagent bottles were degassed with helium during the complete chromatographic run. The pump heads of the oxidant and

**Table 2.** Stable Carbon Isotopic Characterization of the Main Sugars of Pure Honey[a]

| isotopic parameter | $\delta^{13}$C sucrose (‰) | $\delta^{13}$C glucose (‰) | $\delta^{13}$C fructose (‰) | $\delta^{13}$C total HPLC (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\Delta\delta^{13}$C [fruct-gluc] (‰) | $\Delta\delta^{13}$C [fruct-suc] (‰) | $\Delta\delta^{13}$C [gluc-suc] (‰) |
|---|---|---|---|---|---|---|---|---|---|
| $x \pm$ SD | −26.3 ± 1.0 | −25.0 ± 1.0 | −25.1 ± 1.0 | −25.2 ± 1.0 | −25.1 ± 0.9 | −25.1 ± 0.7 | 0.0 ± 0.3 | 1.2 ± 0.4 | 1.3 ± 0.4 |
| range | −24.3 to −28.8 | −23.2 to −27.3 | −23.0 to −27.3 | −23.4 to −27.4 | −23.3 to −27.2 | −22.8 to −26.6 | −0.5 to 0.5 | 0.6–2.1 | 0.5–2.0 |

[a] $n = 54$.

acid pumps were rinsed with water at least twice a day to avoid crystallization of buffers used at high concentration.

Suitable control references were included in each batch.

**Calibration and Isotopic Calculation.** At the beginning of each run, three pulses of $CO_2$ reference gas were admitted into the inlet system for about 20 s. The constant flow rate during this period gives these peaks a flat-top appearance. A level of $CO_2$ corresponding to 5 V at $m/z$ 44 was used to calibrate the system. Isotope ratios are expressed relative to international standards, rather than being reported as absolute isotope values. The $^{13}C/^{12}C$ abundance ratio was expressed as $\delta^{13}C$ values calibrated against the international standard (Vienna Pee Dee Belemnite, VPDB).

The delta notation is defined as

$$\delta^{13}C_{sample} = [(R_s/R_{st}) - 1] \times 1000$$

where $R_s$ is the ratio of $^{13}C/^{12}C$ in the sample and $R_{st}$ is the ratio of the international standard used. The result of this calculation is a relative $\delta$ calibrated against the international standard.

The working standard was sucrose with an analytical uncertainty of measurement of $-10.2 \pm 0.5$‰ for carbon. The working standard was calibrated versus the official reference material BCR 657 glucose (Bureau of Reference of European Commission) with a $\delta^{13}C$ value of $-10.76 \pm 0.04$‰. Samples were analyzed in duplicate, and standards were analyzed in four repetitions and the values averaged.

**Statistical Analysis.** A one-factor analysis of variance was applied to detect possible differences in $\delta^{13}C$ total by AOAC methods and the proposed HPLC method. A significance level of $p < 0.05$ was adopted for the comparisons. Statgraphics Plus version 4.0 (Statistical Graphics) was used for the statistical analysis.

## RESULTS AND DISCUSSION

**Isotopic Characterization of Honey (EA-IRMS).** Honey sugar and honey protein of all of the samples ($n = 79$) were isotopically characterized by $\delta^{13}C$ analysis (**Figure 1**).

The carbon isotope ratios of all samples ranged from −14.2 to −27.2‰, with a mean of −24.3 ± 2.6‰.

Considering the AOAC 978.71 official method, from the total number of samples, 87% ($n = 69$) had $\delta^{13}C$ isotopic values more negative than −23.5‰ and, so, were considered to be pure. Samples that had values less negative than −21.5‰ totaled 11% ($n = 9$) and were classified as adulterated.

According to this method, carbon values between −23.5 and −21.5‰ are described as questionable. In our case, 2% of the samples ($n = 1$), one sample, was in the range of −23.5 to −21.5‰ and therefore considered to be suspect of adulteration.

Considering the AOAC 991.4 method, the value for the $^{13}C/^{12}C$ ratio ($\delta^{13}C$) of the honey and its protein should differ by no more than 1‰ for delta ($\delta$), which would correspond to 7% added corn or cane sugar. Values above this indicate adulteration.

If the $\delta^{13}C$ values of both the honey and the protein of all the samples are taken into consideration, the adulteration of two samples, with values more negative than −23.5‰, by adding $C_4$ sugars can be confirmed. Thus, using the difference in stable carbon isotope ratio between a honey and its protein fraction permits the detection of an adulteration that cannot be detected by the previous method: $C_4$ sugar addition to honey in amounts that produce a mixture with a $\delta^{13}C$ value more negative than −23.5‰. In addition, this method led to the classification of the suspicious result previously stated to be pure, pointing out the importance of using the protein as an internal reference.

In conclusion, the adulterated honeys ranged from −14.2 to −24.6‰ ($\delta^{13}C_{honey}$) and from −24.4 to −26.6‰ ($\delta^{13}C_{protein}$);

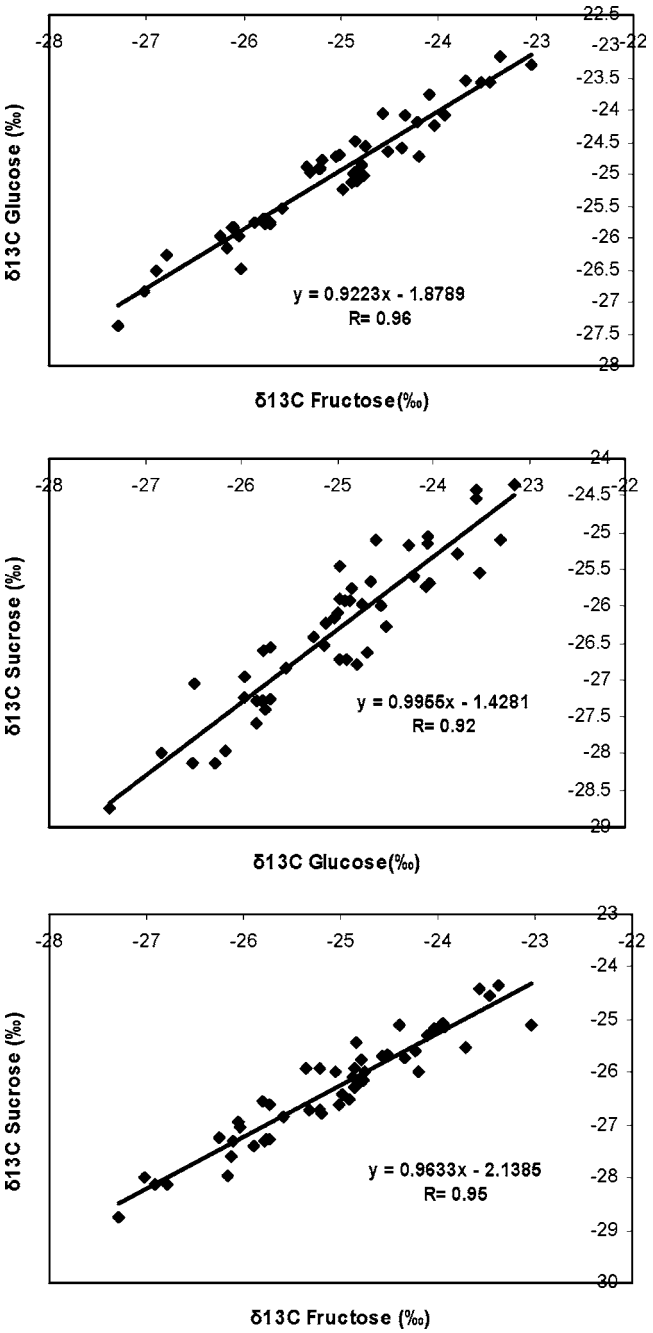


**Figure 3.** Stable carbon isotope ratios of glucose, sucrose, and fructose in pure honeys.

**Table 3.** Stable Carbon Isotopic Characterization of the Main Sugars of Adulterated Honeys

| sample | $\delta^{13}$C sucrose (‰) | $\delta^{13}$C glucose (‰) | $\delta^{13}$C fructose (‰) | $\delta^{13}$C total HLPC (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\Delta\delta^{13}$C [fruct-gluc] (‰) | $\Delta\delta^{13}$C [frutc-suc] (‰) | $\Delta\delta^{13}$C [gluc-suc] (‰) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | −18.7 ± 0.2 | −20.4 ± 0.2 | −20.9 ± 0.2 | −20.5 ± 0.2 | −20.2 | −24.6 | −0.5 | −2.2 | −1.7 |
| 2 | −18.7 ± 0.1 | −19.2 ± 0.3 | −19.5 ± 0.4 | −19.3 ± 0.4 | −19.4 | −24.5 | −0.3 | −0.9 | −0.6 |
| 3 | −18.2 ± 0.1 | −20.5 ± 0.1 | −20.9 ± 0.1 | −20.5 ± 0.1 | −20.3 | −24.7 | −0.4 | −2.7 | −2.3 |
| 4 | −13.8 ± 0.3 | −14.0 ± 0.2 | −20.9 ± 0.2 | −15.5 ± 0.2 | −14.2 | −24.6 | −6.7 | −6.8 | −0.1 |
| 5 | −17.5 ± 0.2 | −20.1 ± 0.2 | −20.5 ± 0.3 | −20.0 ± 0.2 | −19.9 | −24.4 | −0.5 | −3.0 | −2.6 |
| 6 | −16.8 ± 0.3 | −13.0 ± 0.4 | −22.1 ± 0.1 | −15.7 ± 0.1 | −15.3 | −25.3 | −9.1 | −5.3 | 3.7 |
| 7 | −14.3 ± 0.1 | −13.6 ± 0.1 | −22.6 ± 0.1 | −16.1 ± 0.1 | −15.2 | −24.9 | −9.0 | −8.3 | 0.7 |
| 8 | −24.3 ± 0.4 | −25.2 ± 0.1 | −24.9 ± 0.1 | −25.0 ± 0.1 | −24.6 | −25.9 | 0.3 | −0.6 | −0.9 |
| 9 | −13.1 ± 0.4 | −25.4 ± 0.4 | −28.1 ± 0.3 | −22.4 ± 0.4 | −18.8 | −26.6 | −2.7 | −15.0 | −12.3 |
| 10 | −14.7 ± 0.3 | −17.3 ± 0.1 | −16.6 ± 0.1 | −16.8 ± 0.1 | −16.7 | −25.4 | 0.7 | −2.0 | −2.6 |
| 11 | −24.1 ± 0.1 | −24.2 ± 0.1 | −23.9 ± 0.1 | −24.0 ± 0.4 | −23.7 | −25.3 | 0.4 | 0.2 | −0.2 |
| 12[a] | −25.3 ± 0.2 | −26.6 ± 0.2 | −26.6 ± 0.2 | −26.3 ± 0.2 | −26.5 | −26.0 | 0.0 | −1.3 | −1.3 |
| 13[a] | −27.0 ± 0.1 | −27.0 ± 0.1 | −27.0 ± 0.1 | −27.0 ± 0.1 | −27.1 | −26.9 | −0.1 | −0.1 | 0.0 |
| 14[a] | −23.4 ± 0.1 | −23.2 ± 0.1 | −23.1 ± 0.1 | −23.2 ± 0.1 | −23.5 | −24.5 | 0.0 | 0.3 | 0.2 |

[a] Adulterated honey not detected by AOAC methods.

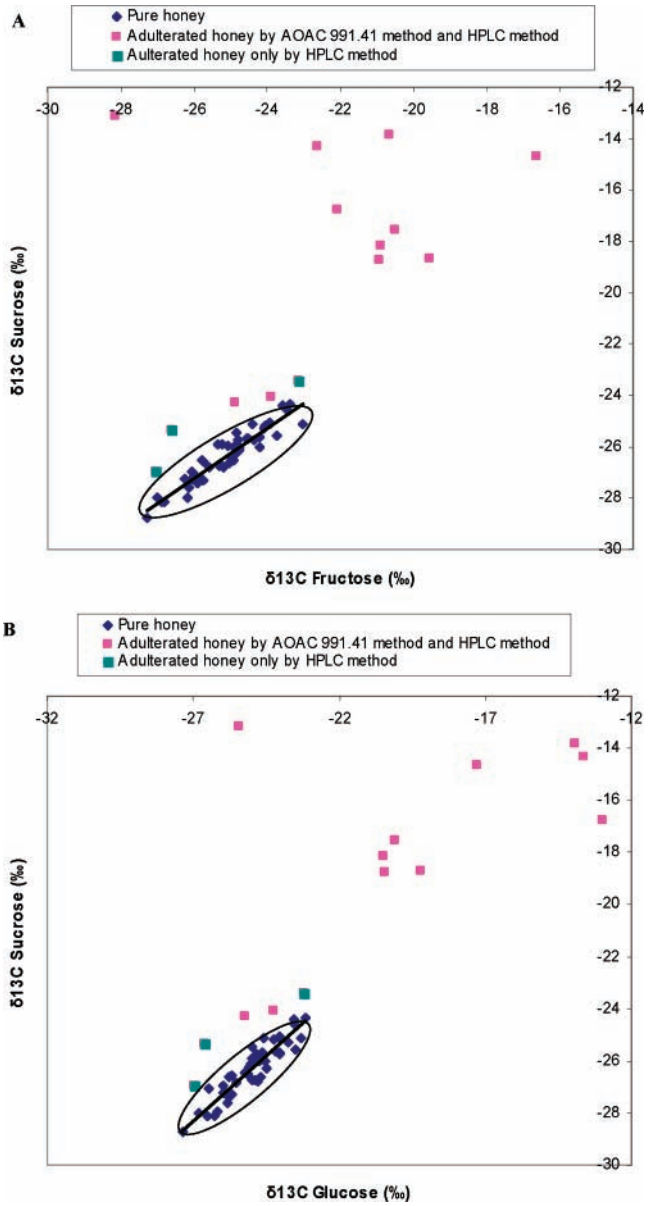


**Figure 4.** Bidimensional plot of the carbon isotopic ratios of (**A**) sucrose and fructose and (**B**) sucrose and glucose of commercial honeys.

and for the pure honeys $\delta^{13}C_{honey}$ ranged from −23.3 to −27.2‰ and $\delta^{13}C_{protein}$ from −23.6 to −26.9‰.

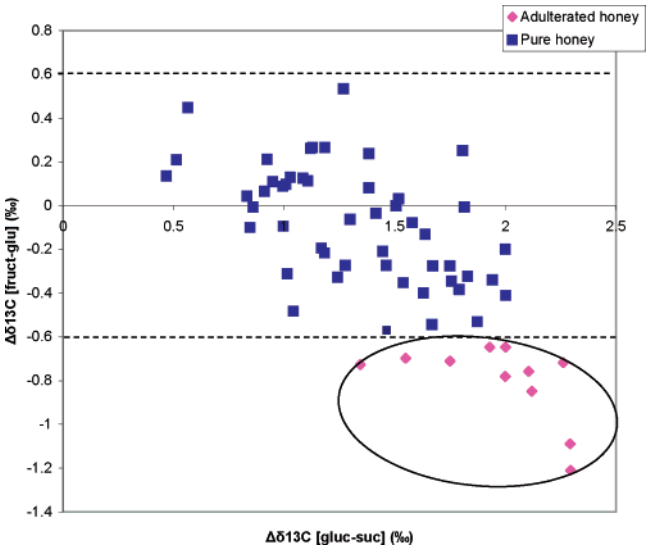


**Figure 5.** Plot of $\Delta\delta^{13}$C [fruct-gluc] versus $\Delta\delta^{13}$C [gluc-suc] values of commercial honeys.

**Isotopic Characterization of Main Sugars of Honey (HPLC-IRMS).** *Reliability of the Method.* The use of appropriate methods of chromatographic separations is of key importance in IRMS. A pivotal analytical challenge for coupling HPLC to IRMS is to find HPLC conditions that allow baseline separation of analytes within a given mixture. Furthermore, when studying $^{13}C/^{12}C$ ratios, one should not rule out the possibility that the different steps of the method employed may cause isotopic fractionation.

The optimization of those parameters affecting the process such as column, temperature, mobile phase, and flow rates (oxidant, acid, and mobile phase) was evaluated. The best results were found working under the conditions described under Materials and Methods.

The precision of the measurement for sucrose, glucose, and fructose was determined by repeating the analysis 10 times on the same sample (0.020 ng $L^{-1}$ of each carbohydrate in a 10 $\mu$L loop size) under repeatability conditions (**Table 1**). The precision of the overall procedure was tested by repeating the whole process five times on a honey sample.

The overall reproducibility of the complete process has been determined by performing five independent analyses on the same honey sample on three different days.

**Table 4.** Stable Carbon Isotope Ratios in Adulterated Honeys and Their Protein Fractions

| | pure honey | | 10% beet sugar added | | 20% beet sugar added | | 5% cane sugar added | | 10% cane sugar added | | 5% cane syrup added | | 10% cane syrup added | | 10% HFCS added | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sample | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) | $\delta^{13}$C total EA (‰) | $\delta^{13}$C protein (‰) |
| 1 | −25.3 | −24.9 | −25.1 | −24.8 | −25.2 | −24.8 | −24.5 | −24.9 | −23.7 | −24.9 | −24.6 | −24.7 | −24.0 | −24.6 | −23.9 | −24.9 |
| 2 | −24.4 | −24.3 | −24.3 | −24.2 | −24.4 | −24.2 | −23.6 | −24.3 | −22.6 | −24.3 | −23.8 | −24.1 | −23.2 | −23.9 | −23.3 | −24.2 |
| 3 | −25.5 | −25.3 | −25.3 | −25.2 | −25.2 | −25.2 | −24.6 | −25.3 | −23.9 | −25.3 | −24.8 | −24.9 | −24.0 | −24.7 | −24.3 | −25.3 |
| honey quality | | | | | | | | | | | | | | | | |
| AOAC method | pure[a] | | pure | | pure | | pure | | adulterated | | pure | | pure | | adulterated | |
| HPLC-IRMS | pure | | adulterated | | adulterated | | adulterated | | adulterated | | adulterated | | adulterated | | adulterated | |

[a] Pure = considered to be pure by the limit given by $\delta$ = 1‰.

Because at the 95% confidence level no significant differences were detected between the certified value (BCR 657 glucose) and the experimental one, the methods used were considered to be accurate for $\delta^{13}$C determination.

*$\delta^{13}$C Values of Sugars from Honey*. Next the developed method was applied to all honey samples.

The samples previously classified as pure by AOAC methods 978.17 and 991.41 were processed through the chromatographic column and later measured by IRMS to study the isotopic values of the main sugars of honey (sucrose, glucose, and fructose).

Sixty-eight percent of the pure samples ($n$ = 54) showed an appropriate chromatographic profile (baseline separation of analyte peaks) and isotopic parameters (according to $C_3$ plants) (**Figure 2A**).

Results of the mean values of sucrose, fructose, glucose, and total C of these samples are summarized in **Table 2**. No differences were found in $\delta^{13}$C among different methods ($p$ > 0.05).

As can be seen in **Figure 3** the $\delta^{13}$C values of glucose, fructose, and sucrose are strongly correlated and are in the expected range of a $C_3$-based product. Therefore, a characteristic pattern of $^{13}$C in authentic honeys can be built.

In addition, the $\delta^{13}$C values of glucose and fructose are 1.2 and 1.3‰, respectively, higher than that of its precursor (sucrose). This enrichment is the result of specific kinetic isotope effects during biosynthesis.

In sugars from natural honeys a constant $\Delta\delta^{13}$C between fructose and glucose (0.0 ± 0.3‰), between fructose and sucrose (1.2 ± 0.4‰), and between glucose and sucrose (1.3 ± 0.4‰) has also been observed. This implies that the simultaneous determination of the $^{13}$C/$^{12}$C ratio of the main sugars in honey leads to a characteristic carbon-13 fingerprint of authentic honey, and, therefore, this feature might be used as a means to test for illegal addition of sweeteners to honey.

On the other hand, 100% of the samples ($n$ = 11) considered to be adulterated by official methods were processed through the HPLC system, and the $\delta^{13}$C values of sucrose, glucose, and fructose (**Figure 2B**) were measured.

The results of these samples are presented in **Table 3** and show that $\Delta\delta^{13}$C [fruct-suc] and [gluc-suc] values fall outside the range of the pure samples, which indicates the undeclared presence of sugar and agrees with the AOAC prediction.

In addition, three samples (**Table 3**, samples 12−14) previously considered as pure by official methods showed $\delta^{13}$C values similar to the isotopic pattern of adulterated samples. Therefore, these samples could also be addressed as adulterated by $C_4$ sugar addition.

**Figure 4** shows $\delta^{13}$C values of sucrose versus fructose and glucose for pure and adulterated samples. The disparity between values for natural and added sugars is obvious, so that it seems possible to detect adulteration rapidly using these isotopic values apart from comparing protein and honey values, lowering the detection limit established for the routine methods employed so far.

Finally, low $\Delta\delta^{13}$C [fruct-gluc] values (from −0.6 to −1.2‰) were measured for 11 additional samples previously considered as pure by AOAC methods. **Figure 5** shows the values for each sample. The $\delta^{13}$C values fall outside the natural honey range ($x$ ± 2$s$ = 0 ± 0.6‰), indicating that these samples have also been manipulated. It should be noted that this manipulation was not detected on the basis of the $^{13}$C values of the whole sugar fraction.

**Honey Adulteration with Cane Sugar and Cane Syrup.** The ability of the developed method to detect an addition of exogenous sweeteners was further confirmed by spiking five samples deliberately with known amounts of cane sugar and cane syrup (1, 2, 5, 10, and 20%). The spiked samples were analyzed by EA-IRMS and HPLC-IRMS using the procedures described above.

Analysis of the samples according to the official method (AOAC 991.41) (**Table 4**) showed that the addition of 5% of cane sugar and 5 and 10% of cane syrup does not result in a significant change of the $\delta^{13}$C value of the honey and its protein ([$\delta^{13}C_{honey} - \delta^{13}C_{protein}$] < 1) and, therefore, this method is not able to detect this adulteration at these levels, at was expected.

On the other hand, the results of HPLC-IRMS experiments are presented in **Figure 6A**,**B**, showing a decrease of the $\Delta\delta^{13}$C [fruct-suc] and [gluc-suc] values with increasing amounts of sugar or syrup.

Thanks to the complementary information brought by the multicomponent $^{13}$C profile (sucrose, glucose, and fructose) of the sample and taking into account the values of **Figure 6A**,**B**, the detection limits of the newly developed method lie around 1 and 2% for cane sugar and cane syrup addition detection, respectively. Thus, the combined use of sucrose, glucose, and fructose $^{13}$C/$^{12}$C ratios allows a satisfactory detection of exogenous $C_4$ sugar addition, providing a significant improvement of the detection limit of official methods, which represents a powerful way to detect sugar addition in honey.

**Honey Adulteration with Beet Sugar.** Bees collect nectar mostly from dicotyledons plants (Calvin−Benson photosynthetic metabolism). In addition, beet sugar shows a $C_3$ plant metabolism. Therefore, both honey and sugar have similar $^{13}$C/$^{12}$C values. Because of this, the use of stable carbon isotope ratio analysis might demonstrate HFCS and cane sugar presence in honey; however, it has not been able to detect $C_3$

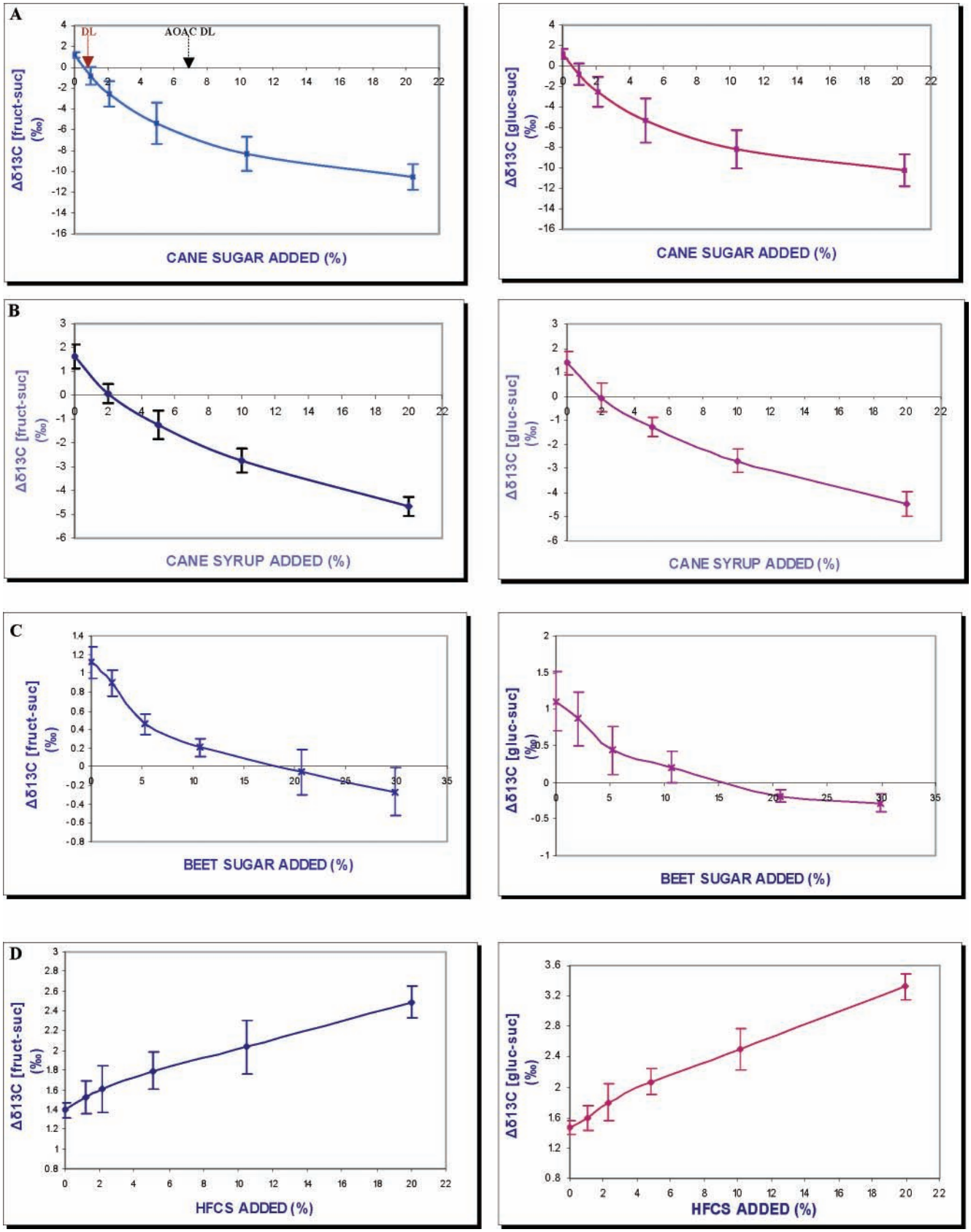


**Figure 6.** Plot of $\Delta\delta^{13}$C [fruct-suc] or $\Delta\delta^{13}$C [gluc-suc] values versus percentage of (**A**) cane sugar, (**B**) cane syrup, (**C**) beet sugar, and (**D**) HFCS addition.

sugar addition. Therefore, beet sugar presence in honey is undetectable by any isotopic analytical procedures available at this time.

In an attempt to show the ability of the proposed method to detect beet sugar addition, five samples were spiked with 2, 5, 10, 20, and 30% of beet sugar deliberately. The spiked samples

were analyzed by EA-IRMS and HPLC-IRMS using the procedures described above.

Analysis of the samples according to the official method (AOAC 991.41, **Table 4**) showed that the addition of beet sugar, even in large amounts, will not result in a significant change of the $\delta^{13}C$ value of the honey and its protein ($[\delta^{13}C_{honey} - \delta^{13}C_{protein}] < 1$) and, therefore, it is not able to detect this adulteration as was previously pointed. On the other hand, the $^{13}C$ values of individual sugars changed as expected, and the rules established previously (**Table 2**: $\Delta\delta^{13}C$ [fruct-suc] $= 1.2 \pm 0.4$ and $\Delta\delta^{13}C$ [gluc-suc] $= 1.3 \pm 0.4$) are no longer respected (**Figure 6C**). Therefore, the measurement of $^{13}C/^{12}C$ ratios of the individual sugars is more sensitive for detecting sugar additions than the whole sugar fraction. In fact, the newly developed method is the only one able to detect beet sugar addition.

In conclusion, the combined use of $\Delta\delta^{13}C$ [fruct-suc] and [gluc-suc] allows a satisfactory detection of exogenous $C_3$ sugar addition, providing a low detection limit (5−10%), which represents an advantage over the official methods employed routinely for honey analysis that are not able to detect this kind of sugar addition.

**Honey Adulteration with HFCS and Isoglucose Syrup.** Inexpensive HFCS and isoglucose syrup superficially resembling honey in their monosaccharide composition became available four decades ago and have been widely used since then. These syrups are low cost and pass undetected by traditional analytical techniques.

To test further the performance of the newly developed procedure, five samples were spiked with 1, 2, 5, 10, and 20% of HFCS and isoglucose syrup. All of the spiked samples were analyzed according to the procedures described above by EA-IRMS and HPLC-IRMS.

Analysis of the samples by AOAC official method detects the presence of HFCS in honey when the adulteration is ≥10% (**Table 4**). On the other hand, and considering the HPLC-IRMS results, an opposite tendency has been observed compared to the previous adulterations (**Figure 6A−C**). The results show a decrease of the $\Delta\delta^{13}C$ [fruct-glu] and an increase of $\Delta\delta^{13}C$ [fruct-suc] and $\Delta\delta^{13}C$ [gluc-suc] (**Figure 6D**) with increasing amounts of syrup.

This behavior has been also previously observed in 11 commercial honeys (**Figure 5**). These samples were previously classified as pure by AOAC method; however, using the developed method it can be confirmed that the honey has been altered by the addition of small amounts (<7%) of HFCS or isoglucose.

In conclusion, values of $\Delta\delta^{13}C$ [fruct-glu] lower than −0.6 and/or values of $\Delta\delta^{13}C$ [gluc-suc] higher than 2 reflect an undeclared presence of sugar not detected by the method employed routinely.

According to the literature, HFCS is one of the most frequently used sweeteners (*21*); however, the isotopic pattern of most adulterated honeys (sucrose, glucose, and fructose) does not reflect this kind of adulteration (**Figure 4**). Therefore, we can state that honey has been altered by the addition of a sweetener other than HFCS. This fact is of great significance, because all previous studies and conclusions about honey adulteration were made considering this sweetener to be the main exogenous sugar added to honey.

Further investigation of alternative sweeteners can provide useful information about real honey adulteration situation/status.

From the results of this study it can be concluded that an accurate and sensitive method based on the main sugars of honey (sucrose, glucose, and fructose), separation by HPLC, and later IRMS detection has been found to be a fast and simple method for honey adulteration (by $C_3$ or $C_4$ sugar addition) detection.

Furthermore, the newly developed method provides several additional benefits over other isotopic traditional methods: speed (reducing substantially the operating time required from at least 6 h for the traditional method to 20 min), lack of sample preparation, reduced consumption of reagents, and simplicity of the operative procedure. As a result, the method described here is expected to be widely used in the future.

## LITERATURE CITED


(1) Padovan, G. J.; De Jong, D.; Rodrigues, L. P.; Marchini, J. S. Detection of adulteration of commercial honey samples by the 13C/12C isotopic ratio. *Food Chem.* **2003**, *82*, 633−636.

(2) Földhazzi, G. Analysis and quantification of sugars in honey of different botanical origin using high performance liquid chromatography. *Acta Aliment.* **1994**, *23*, 299−311.

(3) Europe Scientific-Application Note, 1998. Authentication of honey; pp 1−3 (28/07/98).

(4) Smith, B. N.; Epstein, S. Two categories of 13C/12C ratios for higher plants. *Plant Physiol.* **1971**, *47*, 380−384.

(5) Kelly, J. D.; Petisco, C.; Downey, G. Application of fourier transform midinfrared spectroscopy to the discrimination between Irish artisanal honey and such honey adulterated with various sugar syrups. *J. Agric. Food Chem.* **2006**, *54*, 6166−6171.

(6) Cordella, C.; Militao, J. S. L. T.; Climent, M. C.; Drajnudel, P.; Carbol-Bass, D. Detection and quantification of honey adulteration via direct incorporation of sugar syrups or bee-feeding: preliminary study using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and chemometrics. *Anal. Chim. Acta* **2005**, *531*, 239−248.

(7) Doner, L. W.; White, J. W. $^{13}C/^{12}C$ ratio relatively uniform among honeys. *Science* **1977**, *197*, 891−892.

(8) White, J. W.; Winters, K. Honey protein as internal standard for stable carbon isotope ratio detection of adulteration honey. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 907−911.

(9) White, J. W. Internal standard stable carbon isotope ratio method for determination of C4 plant sugars in honey: collaborative study and evaluation of improved protein preparation procedure. *J. Assoc. Off. Anal. Chem.* **1992**, *75*, 543−548.

(10) Bender, M. M. Variationin the 13C/12C ratios of plants in relation to the pathway of photosynthetic carbon dioxide fixation. *Phytochemistry* **1971**, *10*, 1239−1244.

(11) Bricout, J.; Fontes, J. C.; Merlivat, L. Detection of synthetic vanillin in vanilla extracts by isotopic analysis. *J. Assoc. Off. Anal. Chem.* **1974**, *57*, 713−715.

(12) Schmidt, H. L. Food quality control and studies on human-nutrition by mass-spectrometric and nuclear-magnetic-resonance isotope ratio determination. *Fresenius' Z. Anal. Chem.* **1986**, *324*, 760−766.

(13) AOAC official method 978.17. Corn and cane sugar products in honey. *AOAC Official Methods of Analysis. Sugars and Sugar Products*; AOAC: Arlington, VA, 1995; Chapter 44, pp 27−29.

(14) AOAC official method 991.41. $C_4$ plant sugars in honey. *AOAC Official Methods of Analysis. Sugars and Sugar Products*; AOAC: Arlington, VA, 1995; Chapter 44, pp 29−31.

(15) White, J. W.; Winters, K.; Martin, P.; Rossmann, A. Stable carbon isotope ratio analysis of honey: validation of internal standard procedure for worldwide application *J. AOAC Int.* **1998**, *81*, 610−619.

(16) Meier-Augenstein, W. Gas chromatography coupled to isotope ratio mass spectrometry. *J. Chromatogr. A* **1999**, *842*, 351−371.

(17) Caimi, R. J.; Brenna, J. T. High-precision liquid chromatography−combustion isotope ratio mass spectrometry. *Anal. Chem.* **1993**, *65*, 3497−3500.
(18) Abrambson, F. P.; Markey, S. P. Mass spectrometric analysis of sulfur in microgram quantities of biological macromolecules using a reaction interface. *Biomed. Environ. Mass Spectrom.* **1986**, *13*, 411−415.
(19) Brand, W. A.; Dobberstein, P. Microcombustion of ng amounts of carbon in non-volatile materials for isotope ratio evaluation. *Isotopes Environ. Health Stud.* **1996**, *32*, 255−262.
(20) Krummen, M.; Hilkert, A. W.; Juchelka, D.; Duhr, A.; Schlüter, H.; Persch, R. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2260−2266.
(21) Moguel-Ordoñez, Y.; Echazarreta, C. M.; Mora-Escobedo, R. $^{13}C$ isotopic index of honeys produced in the Yucatan peninsula, Mexico. *J. Apic. Res.* **2005**, *44*, 49−53.

**Received for review July 21, 2006. Revised manuscript received October 3, 2006. Accepted October 4, 2006.**

JF062067X