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Detection of adulteration of commercial honey samples by the $^{13}C/^{12}C$ isotopic ratio

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Abstract

It is very difficult to detect adulteration by conventional laboratory methods. However, differences in stable carbon isotope ratio ($^{13}C/^{12}C = ‰$), between honey and its protein fraction give a qualitative and quantitative indication of honey adulteration. Forty honey samples from Brazil and eight imported from Argentina, Canada and the USA, were analyzed to detect possible adulteration by gas chromatography/mass spectrometry. Honey was adulterated with sucrose solution and high fructose corn syrup (HFCS) to determine detection limits. The $^{13}C/^{12}C$ value for honey and its protein fraction should not differ more than 1‰ for delta (δ). The range of values found for bee-produced honey was −21.96‰ to −30.47‰ for C3 plants and −11.82‰ to −19.00‰ for C4 plants (produced near cut sugar cane and not considered to be flower honey). For cane sugar it was −11.33‰ to −11.78‰, and −9.70‰ to −9.78‰ for HFCS. Adulteration was found in six Brazilian honey samples.

Keywords: Isotope ratio; Gas chromatograph mass spectrometry; Honey adulteration; Brazil

1. Introduction

Honey is a valued sweet and viscous substance produced by bees from flower nectar or from honeydew. Floral honey is composed mainly of the carbohydrates, fructose and glucose; but these sugars can be artificially added to falsify honey. Chemical techniques have been developed for the detection of honey adulteration. The most widely used is high performance liquid chromatography (HPLC) (Földhazi, 1994), but this methodology does not detect low levels of adulteration nor is it adequate for the more sophisticated falsifications (Europa Scientific-Application Note 1998; Smith & Epstein 1971). The gas chromatograph/mass spectrometry (GC/MS) technique (Doner & White, 1977; White & Winters, 1989, White, 1992) 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 (including cane and corn sugar), when compared to dicotyledons (most flowering plants from which bees collect nectar). The different ratios of carbon isotopes are produced by different photosynthesis cycles (Bender, 1971; Smith & Epstein, 1971). Plants with the Calvin–Benson photosynthetic cycle (C3) have $^{13}C/^{12}C = δ‰$ values from −21‰ to −32‰ and plants with the Hatch-Slack photosynthetic cycle (C4) have values from −12‰ to −19‰ of $^{13}C/^{12}C = δ‰$; C4 plants have high ^{13}C when compared to C3 plants (Calvin & Bassham, 1962; Hatch & Slack, 1979; Hatch et al., 1967). This stable carbon isotope ratio analysis (SCIRA) has been used to detect adulteration in honey. Honey that has $δ^{13}C$ values less negative than −23.5‰ is considered suspect (White & Winter, 1989). Companies that produce adulterated honey adapted to this new technique by blending artificial sweeteners with honeys that had $δ^{13}C$ ($^{13}C/^{12}C$) 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, a laboratory can determine if the honey was adulterated, and can estimate the percentage of adulteration by the difference in the $^{13}C/^{12}C$ ratios between the sugar in the honey and its protein. The% adulteration can be calculated by the formula: % adulteration = [(δ‰ Prot. −δ‰ Honey)/(δ‰ Prot. −δ‰ sweetener)] × 100. This method is called internal

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standard isotope ratio analysis (ISCIRA) (White & Winters, 1989).

Addition of corn or cane sugars from C4 plants to honey in amounts that result in a delta value (δ ^{13}C) more negative than-23.5‰ for the mixture can not be detected by the original SCIRA procedure (White & Doner, 1978). Such adulteration however is detected by the ISCIRA procedure from the delta value of the carbon in the protein contained in the honey, which shows the isotopic composition of honey before the addition of C4 plant sugars. Forty Brazilian honey samples and eight imported samples were analyzed on an ANCA 20–20-IRMS on-line Mass Spectrometer from Europa Scientific (England). Pure honey samples were adulterated

Table 1
Stable isotope ratios in honeys and their protein fractions,% adulteration and honey quality

Sample no.	Country	$^{13}C/^{12}$ δ ‰ honey	$^{13}C/^{12}$ δ ‰ protein	% Adulteration	Honey quality
1	USA	−27.12±0.14	−26.52±0.16	0	Pure
2	USA	−28.06±0.10	−26.99±0.12	0	Pure
3	USA	−26.12±0.11	−26.03±0.10	0	Pure
4	Argentina	−27.64±0.14	−27.32±0.16	0	Pure
5	Canada[a]	−27.39±0.11	−25.33±0.12	0	Pure
6	Canada[b]	−27.39±0.11	−25.09±0.14	0	Pure
7	Canada[a]	−15.61±0.16	−24.81±0.16	69.8	** Adulterated
8	Canada[b]	−15.61±0.16	−24.49±0.15	69.1	** Adulterated
9	Brazil	−24.48±0.12	−24.59±0.10	0.74	*Pure
10	Brazil	−27.12±0.12	−26.25±0.12	0	Pure
11	Brazil	−23.08±0.15	−22.68±0.18	0	Pure
12	Brazil	−24.82±0.12	−24.06±0.13	0	Pure
13	Brazil	−26.66±0.12	−26.73±0.12	1.1	*Pure
14	Brazil	−26.12±0.10	−25.74±0.12	0	Pure
15	Brazil	−25.57±0.12	−22.97±0.15	0	Pure
16	Brazil	−23.22±0.21	−25.39±0.17	15.8	Adulterated
17	Brazil	−28.70±0.10	−26.26±0.11	0	Pure
18	Brazil	−23.61±0.09	−22.49±0.10	0	Pure
19	Brazil	−26.71±0.10	−24.18±0.10	0	Pure
20	Brazil	−28.29±0.08	−24.45±0.10	0	Pure
21	Brazil	−28.86±0.12	−25.84±0.18	0	Pure
22	Brazil	−26.57±0.20	−24.87±0.21	0	Pure
23	Brazil	−28.86±0.21	−25.04±0.20	16.3	Adulterated
24	Brazil	−21.96±0.18	−22.95±0.16	8.8	Adulterated
25	Brazil	−19.01±0.15	−20.19±0.12	13.8	Adulterated
26	Brazil	−25.23±0.12	−24.49±0.12	0	Pure
27	Brazil	−27.27±0.11	−23.93±0.13	0	Pure
28	Brazil	−17.11±0.22	−23.34±0.24	53.2	Adulterated
29	Brazil	−20.85±0.10	−20.06±0.12	0	Pure
30	Brazil	−26.08±0.11	−22.91±0.10	0	Pure
31	Brazil	−25.45±0.10	−26.09±0.10	4.4	*Pure
32	Brazil	−24.25±0.10	−23.52±0.11	0	Pure
33	Brazil	−17.78±0.10	−22.80±0.10	44.9	Adulterated
34	Brazil	−25.15±0.11	−20.71±0.12	0	Pure
35	Brazil	−27.17±0.12	−24.08±0.11	0	Pure
36	Brazil	−25.86±0.12	−24.08±0.12	0	Pure
37	Brazil	−28.19±0.16	−22.68±0.17	0	Pure
38	Brazil	−30.47±0.21	−25.90±0.19	0	Pure
39	Brazil	−29.47±0.05	−25.38±0.04	0	Pure
40	Brazil	−27.65±0.14	−26.49±0.25	0	Pure
41	Brazil	−27.80±0.10	−26.26±0.11	0	Pure
42	Brazil	−23.61±0.09	−22.49±0.10	0	Pure
43	Brazil	−26.71±0.10	−24.18±0.10	0	Pure
44	Brazil	−28.29±0.08	−24.45±0.10	0	Pure
45	Brazil	−11.82±0.18	−11.47±0.20	0	Pure
46	Brazil	−19.00±0.12	−19.13±0.18	0	Pure
47	Brazil	−15.01±0.20	−15.06±0.21	0	Pure
48	Brazil	−23.67±0.14	−22.97±0.12	0	Pure

Samples marked with *Pure means that they were adulterated by sweeteners, but under the pre-established limit ($\delta = 1$ ‰). ** Those samples were deliberately adulterated.

[a] The protein fraction was extracted in the Canadian laboratory.

[b] The protein fraction was extracted in our laboratory.

with 1, 2, 5 or 10% cane sugar sucrose solution (60% concentration) or with 20, 50 or 90% high fructose corn syrup (HFCS, 55% concentration) to see how precisely we could detect low to high concentrations of these added sweeteners.

2. Materials and methods

2.1. Reagents

(a) Tungstic acid, sodium salt—10% solution (Carlo Erba-Italy). Weigh 10 grams of tungstic acid and dilute with pure water to 100 ml.
(b) Sulfuric acid—2/3 N (Merck-Germany). Add 1.88 ml HS_2O_4 to pure water and dilute to 100 ml.

2.2. Samples

This study was conducted with 40 samples of honeys of various botanical origins produced in Brazil, and eight imported samples, one from Argentina, three from the USA, and four from Canada. Four samples (5–8) were received from a honey analysis laboratory in Canada. Samples 7 and 8 (Table 1) had been purposely adulterated; they had 70% HFCS. We were not previously informed about the percentage adulteration. The other two samples 5–6 were pure. Samples 5 and 7 already had the protein fractions extracted in the Canadian laboratory. Samples 6 and 8 were the same as samples 5 and 7, respectively, but their protein fraction was extracted in our laboratory to compare the results. The other honey samples were provided by beekeepers, bought in supermarkets or at road stands. All of them were stored in glass jars and kept at 4–5 °C until analysis. Two pure samples were purposely adulterated with sweeteners. An Argentine honey (no. 4) was adulterated with cane sugar syrup (60% concentration), at levels of 1, 2, 5 and 10% and a pure Brazilian honey (no. 47) was adulterated with HFCS (55% concentration), at levels of 20, 60 and 90% (Table 3).

Table 2
Stable carbon isotope ratios in high fructose corn syrup (HFCS) and cane-sugar sucrose syrup samples

Sample	$^{13}C/^{12}$ (δ ‰) cane sugar	$^{13}C/^{12}$ (δ ‰) HFCS
1	−11.71	−9.75
2	−11.49	−9.78
3	−11.33	−9.70
4	−11.78	−9.72
5	−11.78	−9.70
6	−11.74	−9.76
Mean	−11.63±0.18	−9.73±0.06

2.3. Sample preparation for $^{13}C/^{12}C$ (δ ‰)

Honey samples (40 ml) were placed in glass tubes and centrifuged at 1500×*g*, to remove solid material and then passed through a 100–150 mesh strainer to remove heavy and insoluble material. A 10-g sample of honey was placed in a clear 50-ml centrifuge tube and 4 ml of distilled water added and mixed on a vortex; 2-μl aliquots were taken to determine the $^{13}C/^{12}C$ ratio, in triplicate. 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 agitated in a water bath at 80 °C until visible flocks were formed, after about 3–4 h. The samples were then centrifuged at 1500×*g* and the supernatant removed with a Pasteur pipette. The precipitate was washed by adding 5 ml of distilled water and agitating on a vortex; the precipitate was then separated. This procedure was repeated at least five times, until the supernatant was clear. The precipitated protein was transferred to a small (1.5 ml) Eppendorff tube and centrifuged, and the supernatant was removed. These samples were dried on a freeze dryer or in a speed vacuum. After drying, 2–4 mg was placed into tin capsules in triplicate and run for $^{13}C/^{12}C$; Pee Dee Bee *Belemintella americana* fossil limestone from the National Institute of Standards and Technology in Gaithersburg, Maryland, USA was used as a standard.

Table 3
Stable carbon isotope ratios in adulterated honeys and their protein fractions

Sample no.	% Sweetener added	$^{13}C/^{12}$ δ ‰ Honey	$^{13}C/^{12}$ δ ‰ Protein	% Adulteration	Honey quality
04	0% Sucrose syrup	−27.64±0.14	−27.32±0.16	0	Pure
04	1% Sucrose syrup	−27.43±0.18	−27.64±0.18	0.5	*Pure
04	2% Sucrose syrup	−27.11±0.19	−27.60±0.18	1.3	*Pure
04	5% Sucrose syrup	−26.21±0.18	−27.70±0.19	4.0	*Pure
04	10% Sucrose syrup	−25.04±0.19	−27.70±0.20	7.0	*Pure
48	0% HFCS	−23.67±0.14	−22.97±0.12	0	Pure
48	20% HFCS	−21.67±0.04	−22.94±0.06	11.2	Adulterated
48	60% HFCS	−19.48±0.15	−22.97±0.06	30.6	Adulterated
48	90% HFCS	−17.49±0.11	−22.96±0.06	48.2	Adulterated

*Pure = considered pure by the limit given by δ = 1 ‰. Sucrose syrup—60% sugar cane sucrose solution. HFCS—55% High fructose corn syrup.

3. Results and discussion

If less than 13% sweeteners are added, the traditional HPLC methodology is unable to detect the adulteration; however the GC/MS–ISCIRA is able to detect very low percentages of adulteration, because the $^{13}C/^{12}C$ is so strongly different in honey, when compared to cheap sweeteners. The internal standard $^{13}C/^{12}C$ isotope ratio method (ISCIRA) is a sensitive, fast and precise methodology to detect honey adulteration with these sweeteners. The values found for pure honey were −21.96‰ to −30.47‰ for C3 plants and −11.82‰ to −19.00‰ for C4 plants (Table 1). The cane sugar syrup values were −11.33‰ to −11.78‰, mean −11.63‰ (sd ± 0.18), and for HFCS they were −9.72‰ to −9.78‰, mean −9.73 ‰ (S.d. ± 0.06) (Table 2). The value for the $^{13}C/^{12}C$ ratio (δ ^{13}C) of the honey and its protein should not differ more than 1‰ for delta (δ), which would correspond to 7% added corn or cane sugar. Values above this indicate adulteration. This is the practical limit for considering the sample pure. Samples from the USA, Argentina and Canada were pure, and one lab sample from Canada was about 70% adulterated. Though the protein fraction of samples 5 and 7 was separated in Canada by a different method, than the corresponding samples (no. 6 and 8), precipitated in our laboratory with the method of White and Winters (1989), the results for the honey carbohydrates and protein were nearly identical (Table 3).

Among the 20 Brazilian samples that came from Bahia state, six were found to be adulterated (Table 1). All those that came from the other Brazilian states were pure. Three samples (45, 46 & 47) were produced by C4 plants, and the others came from C3 plant nectars. The C4 plant "honey" apparently had been collected from honey bee colonies placed near recently cut sugar cane, and the bees collected the sap that oozed from the cut cane stems. This is not considered honey in Brazilian markets.

All of the samples that were deliberately contaminated had a detectably lower δ ^{13}C ratio in the sugar fraction than in the protein fraction of the honey (Table 3). The honey samples were only considered "officially" adulterated when the δ in the sugar fraction compared to the protein was above 1‰, which would correspond to 7% adulteration. Sweetener solutions with concentrations of 55% (HFCS) and 60% (cane sugar), were used which is less than the approximately 82% found in honey. A correction for this difference would make our detection values (Table 3) closer to the quantities of foreign sugar that we added to the honey samples. For example, 11.2% adulteration was detected when 20% of 55% HFCS was added. The correction for sugar concentration in the two solutions would give 82% sugar in honey/55% sugar in the HFCS = 1.49 × 11.2% adulteration = 16.7% adulteration, which is close to the 20% HFCS added.

Adulteration in known and unknown honey samples was detected by determining the $^{13}C/^{12}C$ ratio of the carbohydrates and the protein of the honey. Honey from apiaries placed near cut sugar cane was apparently pure by the internal protein standard method, however the $^{13}C/^{12}C$ ratio was above the values accepted for pure honey.

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