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Presence of C4 Sugars in Honey Samples Detected by The Carbon Isotope Ratio Measured by IRMS

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Abstract

Honey is occasionally adulterated with low-cost sugars. The $^{13}C/^{12}C$ ratio is –22 to –33δ‰, in honey from C3 plants, –10 to –20δ‰ in honey from C4 plants, and –11 to –13.5δ‰ in honey from Crassulacean Acid Metabolism plants. When C4 sugar is added to pure honey, the $^{13}C/^{12}C$ ratio will be altered, whereas it corresponding $^{13}C/^{12}C$ ratio protein extract will remains constant. The difference accepts in $^{13}C/^{12}C$ results between honey and its associated protein extract is -1δ‰ deviation, which provides the international benchmark of 7% of C4 sugar added. This is the international tolerated limit established to consider the honey pure or not. In the present study, 30 imported honey samples were analyzed by ISCIRA mass spectrometry. Twelve of them came from country A, 10 from B, 4 from C, and 4 from D. Six of the samples from country A (50%) and 4 of the samples from country B (40%), were adulterated, whereas none of the samples from countries C and D were adulterated. We also analyzed exogenous sugars, (cane sugar, and corn sugar) and the mean of ten replicates was –11.6 and –10.5δ‰ deviation respectively; as well the two pure honey and the pure honey were proposal adulterated with exogenous sugars, by serial dilution as: 0; 0.5; 1; 2; 5; 10; 15; 20; 50 and 70%.

Key words: Honey adulteration; carbon isotope ratio; exogenous sugars.

1. Introduction

Honey is produced by bees that collect nectar from different types of plants and basically consists of a mixture of several sugars, especially fructose and glucose (Anass Terrab, José M. Vega-Perez, Maria J. Díez, Francisco J. Heredia 2001 and Miguel Ordonez Y.,Echazarreta C.M., Mora-Escobedo R.2005). Because it especial flavor and attractive price, low-cost sugars from sugar cane syrup or corn syrup glucose are occasionally used to adulterated honey (White, 1992). The addition of these sugars to pure honey has become an international problem

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and many laboratories all over the world are trying to monitor this adulteration using different analytical techniques to determine the purity or the adulteration of honey. However, the chromatography tests and others analytical procedures used are not so sensitive enough to detect very low concentration of adulterating sugars (White, 1992). Mass spectrometry of the stable carbon isotope ratio (SCIRA), was the first mass spectrometry method accept by the Association of Official Analytical Chemistry (AOAC, 1990), as the official method for the detection of the addition of these sugars. SCIRA detects the natural abundance of the stable carbon isotopes and provide the isotope ratio between ^{12}C, the most abundant in nature (99%) and its isotope ^{13}C, with low abundance 1%. This ratio reflects the photosynthetic cycle concerning the utilization and fixation of CO2 by different plants. The process of CO2 fixation by plants occurs according to one of the following three pathways respectively, described by Hatch, Slack & Johnson (1967, 1979): the Calvin and Benson cycle or C3 cycle, occurring in the most of the plants, which a $^{13}C/^{12}C$ isotope ratio range from –22 to –33 δ‰ deviation, the Hatch Slack cycle or C4 cycle which occurs in a more reduced number of plants with a $^{13}C/^{12}C$ ratio of –10 to – 20 δ‰, and the cycle for Crassulacean Acid Metabolism plants (CAM), such as cactus, pineapple etc, which are able to perform both cycles of CO_2 fixation, with a δ deviation ranging from –11 to –13.5 δ‰.

Bees collect the nectar most from flowers of C3 plants cycle, and to a lesser extent from the flowers of C4 and CAM plants, which have different $^{13}C/^{12}C$ ratio. The mean of $^{13}C/^{12}C$ value determined by SCIRA has been established at –23.5 δ‰, (White & Winters, 1989), however, the analysis of the carbon isotope ratio yields different $^{13}C/^{12}C$ values for honeys obtained from different floral sources. If bees collect more nectar from C4 such as sugar cane exudates (after the plant is cut-out), or from CAM plants compared to C3 plants, the $^{13}C/^{12}C$ of honey determined by SCIRA will be higher than –23.5 δ‰, but this does not means that the honey is adulterated, as can be determined by measuring the $^{13}C/^{12}C$ of honey and it associated protein extract, by ISCIRA, (White, 1992). The addition of corn or cane sugar syrups to pure honey will change its carbon isotope ratio composition, but not its protein composition (White & Winters, 1989 and White 1992). Bees produce all protein in honey by reactions between enzymes and the nectar and therefore the isotope ratio of the honey and its protein will have very close values when honey is pure. Therefore, the difference between the carbon isotope ratio of the protein extract and of honey will provide an exact measure of any minimum level of adulteration. The difference accepts in $^{13}C/^{12}C$, between honey and its associated protein extract is -1δ‰, (one delta per mil, deviation), which provides the international benchmark of 7% sugar added, according to White and Winters, 1989. This was the international tolerated limit pre-established to consider honey pure or not and this is the accepts interval defined by White and Winters 1989, by reject criteria for internal standard isotope ratio where honey will be reject above 4s.

Over the last few years the practice of adulteration of honey with low-cost sugars has become commonplace in many countries (White, 1992).

However, the chromatography tests and others analytical procedures used are not so sensitive enough to detect very low concentration of adulterating sugars (White, 1992), so we are showing the sensibility of this methodology at low % of C4 sugars, added to the pure honey, (Table 1).

Table 1: The δ‰ value of exogenous sugar added simulating an adulteration for sample 1 and 2

Sample 1				
% of C4 sugar	δ‰ Honey± sd	δ‰ Prot ± sd	% A	% E
0	-27.2±0.12	-26.9±0.16	**0**	-
0.5	-26.8±0.20	-26.9±0.16	**0.6**	20
1.0	-26.7±0.17	-26.9±0.16	**0.9**	- 10
2.0	-26.6±0.19	-26.9±0.16	**2.0**	0
5.0	-26.1±0.28	-26.9±0.16	**5.2**	4
10	-25.2±0.34	-26.9±0.16	**10.9**	9
15	-24.5±0.27	-26.9±0.16	**15.8**	5.3
20	-23.7±0.36	-26.9±0.16	**20.6**	3
50	-19.2±0.44	-26.9±0.16	**50.5**	1
70	-16.1±0.40	-26.9±0.16	**70.4**	0.6
Sample 2				
% of C4 sugar	δ‰ Honey± sd	δ‰ Prot ± sd	% A	% E
0	-27.6±0.22	-26.7±0.39	**0**	-
0.5	-26.7±0.65	-26.7±0.39	**0.5**	0
1.0	-26.6±0.39	-26.7±0.39	**1.1**	10
2.0	-26.4±0.58	-26.7±0.39	**2.2**	2
5.0	-26.0±0.34	-26.7±0.39	**4.8**	- 4
10	-25.2±0.32	-26.7±0.39	**10.3**	3
15	-24.5±040	-26.7±0.39	**14.7**	- 2
20	-23.5±0.48	-26.7±0.39	**21.1**	5.5
50	-18.9±0.52	-26.7±0.39	**51.9**	3.8
70	-15.9±0.62	-26.7±0.39	**71.9**	2.7

% A: Percentage of adulteration founded in honey samples
% E: Theoretical adulteration value in relation to the founded value

In the present study, 30 imported honey samples were analyzed by ISCIRA mass spectrometry. Twelve of them came from country A, 10 from B, 4 from C, and 4 from D. Six of the samples from country A (50%) and 4 of the samples from country B (40%), were adulterated, whereas none of the samples from countries C and D were adulterated. We also analyzed cane sugar and corn sugar solution (exogenous sugars) and the average of ten

replicates was –11.6 and –10.5 δ‰ deviation respectively. The two pure honey (sample 1 and 2), were deliberately adulterated with exogenous sugars solution by serial dilution as: 0; 0.5; 1; 2; 5; 10; 15; 20; 50 and 70%, to determine whether similar levels of C4 sugar added to them, could be detected in this study, and the results are presented in Table 1.

2. Materials and Methods

2.1. Reagents

A 2/3 sulfuric acid solution, from Merck, Germany, and a 10% sodium tungstate solution, from Carlo Erba Italy), was prepared.

2.2. Honey Samples

Thirty commercial imported samples were sent to our laboratory by the Minister of Agriculture of the city of Lara, state of Rio Grande do Sul, Brazil. They were left at room temperature until the preparation for analysis by mass spectrometer in order to determine the composition of the $^{13}C/^{12}C$ isotope ratio.

2.3. Preparation of the honey samples for the determination of the carbon isotopic ratio.

An aliquot of each honey sample was filtered through a 150-mesh sieve to remove insoluble material and impurities. Ten mL of each honey sample were placed in a 15 mL centrifuge tube with a screw cap, 4 mL of distilled water were added, and the mixture was shaken in a Vortex for 1 minute. One mL was then transferred to Eppendorf tube, and three 4 μl in three replicates of each samples were used to determine the $^{13}C/^{12}C$ ratio, with the remaining material being used for the protein extraction and purification.

2.4. Precipitation of the protein in the honey samples, for carbon isotopic ratio analyses.

Two mL of a 10% tungstate solution were mixed with 2 mL of 2/3 N sulfuric acid solution, in a tube and shaken and the mixture was added to a 10 mL honey sample. The tubes were shaken in a Vortex mixer for 1 minute and then placed in a water bath at 70°C, with constant shaking for 4 hours until flakes were visualized. The samples were centrifuged at 2000 rpm for 10 minutes and the supernatant was removed with a Pasteur pipette. The precipitate was washed with the 5 mL of distilled water, shaken in a Vortex mixer and centrifuged again. This procedure was repeated four more times, until the supernatant became clear and free of sugars. The final protein precipitate was transferred to a 1.5mL Eppendorf tube, and lyophilized. About 1-2 mg protein was obtained and 200 μg placed in tin capsules in three replicates and the $^{13}C/^{12}C$ ratio (δ‰ deviation), was then determined for each sample. The TS Limestone, provided by the Atomic Energy Commission, Vienna, was used for isotope determination as reference

standard. The purity or adulteration of each honey sample was calculated according to the formula described by White and Winters 1989: [(- δ‰ Prot) – (δ‰ Honey)]*100 / (δ‰ Prot.) – (δ‰ Sweetener).

2.5. Equipment

The following equipments were used for the determinations: a lyophilizer from Labconco, from USA, a model Anca 20-20, mass spectrometer from Europa Scientific, from England, a model MR 1812 centrifuge Juan from USA, a model INFORS AG HT heated shaker, from Germany, and a model AP 56 Vortex Phoenix tube shaker from Brazil.

3. Results and Discussion

The $^{13}C/^{12}C$ value detected in an endogenous sugar after 10 replicates was since –11.5 to –11.8 δ‰ deviations, with a mean –11.6 δ‰, and this value was used in the formula for the calculation of the percent adulteration of the two pure honey samples: for honey one the value founded was -27.2 and -27.6 δ‰ deviation for honey two, and their associated protein extracts were -26.9 and -26.7δ‰ deviation respectively. The $^{13}C/^{12}C$ for corn sugar was –10.5 to –10.6 δ‰ deviation, with a mean -10.5 δ‰. To calculate the purity or adulteration of the imported honey we used the average value, between sugar cane and corn sugar analyzed -11.1δ‰ deviation. Thirty supposed pure honey samples, and their corresponding proteins extracts were analyzed in three replicates for the $^{13}C/^{12}C$ and the results are presented in table 2. We detected 10 honey samples adulterated with C4 sugars, with isotope ratio ranging from –19.3 to –27.2 δ‰ and from –21.2 to –27.9 δ‰ deviation for their associated protein, while the isotope ratio ranged from –23.7 to –29.9 δ‰ for pure honey samples and from –22.5 to –27.8 δ‰ deviation for their proteins extracts. The analyses results of the two pure honeys and their serial dilution with exogenous sugars, (Table 1), is showing a sensitive enough to detect low or high concentration of adulterating by C4 sugars. The % of the error (founded value*100/ theoretical value), for sample 1 varied from – 10 to 20, and for sample 2, was since -4 to 10.

Of 30 samples analyzed on the basis of the $^{13}C/^{12}C$ ratio (Table 2), presented a level of adulteration with C4 sugars above the international established and accepted limit of 7%. Sample 1A, was adulterated with 1.34% and sample 19B was adulterated with 3.91% of C4 sugar, but were accepted as pure honey because the value was below the established limit. Adulteration above 7% was detected in 5 of the 12 samples from country A (42%) and in a 3 of the samples from country B (30%), whereas no adulteration was detected in samples from country C and country D. So, mass spectrometry of isotope ratios is a precise and effective method for the evaluation of the quality of honey, permitting a precise determination of the addition C4 ingredients, (sugar cane and corn sugar).

Table 2: The δ‰ values for the honey samples and their pair protein

Sample No	δ‰ Honey (± sd)	δ‰ Prot (± sd)	% A	Honey Quality
1A	-24.3±0.18	-24.5±0.32	1.3	**A**
2A	-24.2±0.24	-23.0±0.32	0	**P**
3A	-25.4±0.09	-28.2±0.30	16.0	**A**
4A	-24.9±0.02	-23.9±0.37	0	**P**
5A	-29.9±0.16	-27.8±0.27	0	**P**
6A	-26.0±0.23	-25.5±0.03	0	**P**
7A	-26.6±0.11	-26.4±0.37	0	**P**
8A	-25.1±0.04	-24.1±0.11	0	**P**
9A	-19.3±0.05	-21.2±0.22	18.6	**A**
10A	-25.34±0.09	-28.2±0.65	16.7	**A**
11A	-25.2±0.09	-26.3±0.39	7.3	**A**
12A	-24.9±0.08	-26.2±0.58	8.1	**A**
13B	-26.9±0.05	-23.6±0.34	0	**P**
14B	-24.1±0.24	-23.0±0.32	0	**P**
15B	-23.7±0.10	-22.5±0.40	0	**P**
16B	-24.8±0.13	-26.3±0.39	10.1	**A**
17B	-26.0±0.07	-27.8±0.46	10.9	**A**
18B	-25.8±0.10	-27.1±0.14	8.3	**A**
19B	-27.2±0.18	-27.9±0.38	3.9	**A**
20B	-27.7±0.10	-27.2±0.36	0	**P**
21B	-25.6±0.30	-24.5±0.50	0	**P**
22B	-26.2±0.15	-25.1±0.24	0	**P**
23C	-26.7±0.15	-24.2±0.35	0	**P**
24C	-25.6±0.05	-25.1±0.41	0	**P**
25C	-28.7±0.03	-24.0±0.51	0	**P**
26C	-28.8±0.12	-22.9±0.62	0	**P**
27D	-25.3±0.59	-22.8±0.38	0	**P**
28D	-24.3±0.10	-23.5±0.08	0	**P**
29D	-23.9±0.19	-23.2±0.12	0	**P**
30D	-25.3±0.17	-22.6±0.40	0	**P**

Samples: 1 to 12 from country A, 13 to 22 from country B, 23 to 26 from C, and 27 to 30 from country D.

A= Adulterated by the carbon isotope ratio analysis

P= Pure determined by the carbon isotope ratio analysis

% A = % of adulteration founded by $^{13}C/^{12}C$

When a pure honey sample is adulterated with sugar cane syrup or corn syrup glucose, the $^{13}C/^{12}C$ of sugars in honey varies but the $^{13}C/^{12}C$ of it associated protein remains unchanged. So this indicating that there is an interaction between these two parameters, in such a way that, the larger the amount of added sugars, the more distant they will be, so, the protein extract is used as an internal standard for the corresponding honey, as showing in Fig.1.

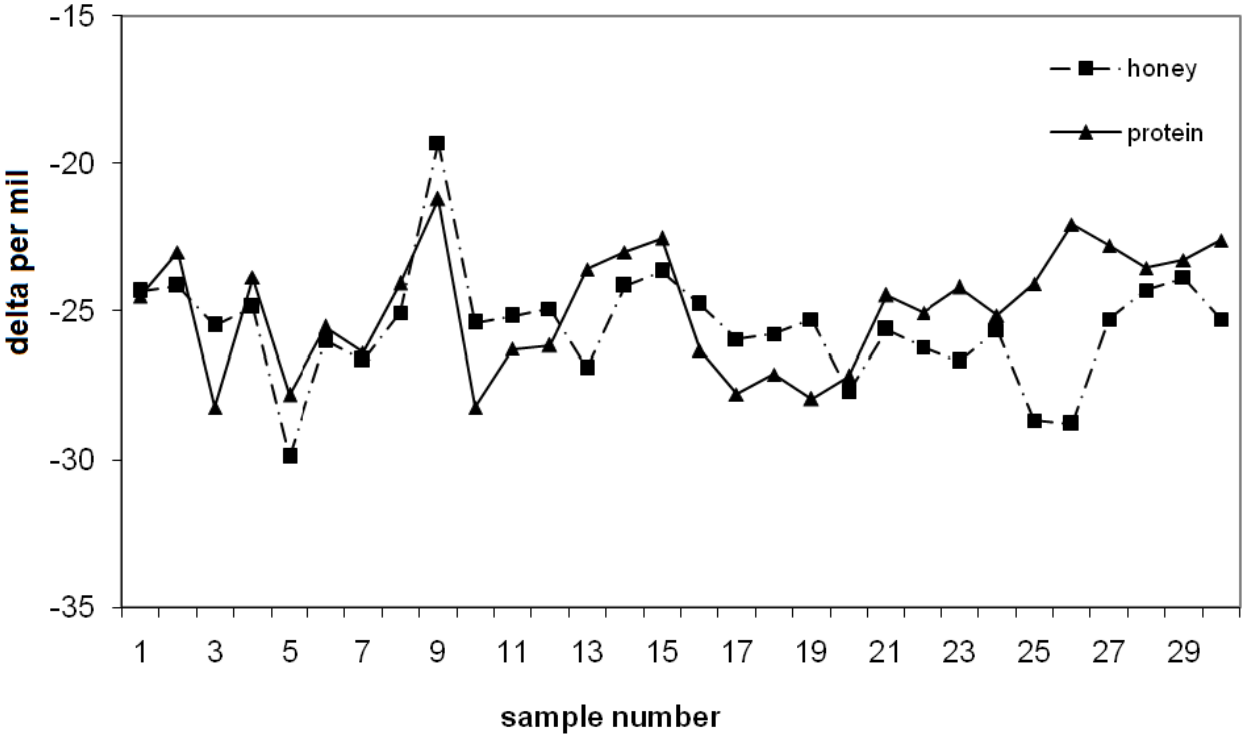

Fig.1 Comparison the delta per mil values by honeys and their pair proteins

After the $^{13}C/^{12}C$ analyses, we can see that only the sample 9A, would be considered adulterated when tested by SCIRA (value established at –23.5 δ‰), while the remaining 9 adulterated samples would be accepted as pure, whereas all the 8 samples would be considered to be adulterated when tested by ISCIRA, except for sample 1A and 19B, which would be accepted as pure by being below the 7% limit. This demonstrates the importance of the ISCIRA method to detect the purity or the adulteration of commercialized honey when is performed with sugars from a C4 plants.

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