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Carbon isotope analysis in apple nectar beverages

Análise isotópica do carbono em néctar de maçã

Ricardo FIGUEIRA[1]*, Waldemar Gastoni VENTURINI FILHO[1], Carlos DUCATTI[2]

Abstract

The aims of this study were to use the isotope analysis method to quantify the carbon of C_3 photosynthetic cycle in commercial apple nectars and to determine the legal limit to identify the beverages that do not conform to the safety standards established by the Brazilian Ministry of Agriculture, Livestock and Food Supply. These beverages (apple nectars) were produced in the laboratory according to the Brazilian legislation. Adulterated nectars were also produced with an amount of pulp juice below the permitted threshold limit value. The $\delta^{13}C$ values of the apple nectars and their fractions (pulp and purified sugar) were measured to quantify the C_3 source percentage. In order to demonstrate the existence of adulteration, the values found were compared to the limit values established by the Brazilian Law. All commercial apple nectars analyzed were within the legal limits, which enabled to identify the nectars that were in conformity with the Brazilian Law. The isotopic methodology developed proved efficient to quantify the carbon of C_3 origin in commercial apple nectars.

Keywords: *legislation; adulteration; IRMS.*

Resumo

Os objetivos deste trabalho foram utilizar o método de análise isotópica para quantificar o carbono do ciclo fotossintético C_3 em néctares de maçã comerciais e mensurar o limite de legalidade para identificar as bebidas em inconformidade com o Ministério da Agricultura, Pecuária e Abastecimento. Essa bebida foi produzida em laboratório, conforme a legislação brasileira. Também foram produzidos néctares adulterados com quantidade de suco polposo abaixo do permitido. Os $\delta^{13}C$ dos néctares de maçã e de suas frações (polpa e açúcar purificado) foram mensurados para quantificar a porcentagem de fonte C_3. Para determinar a existência de adulteração, foi calculado o limite de legalidade de acordo com a legislação brasileira. Todos os néctares comerciais de maçã analisados foram classificados como legais. O limite de legalidade possibilitou identificar as bebidas que estavam em conformidade com a legislação brasileira. A metodologia desenvolvida provou ser eficiente para quantificar o carbono de origem C_3 em néctares de maçã comerciais.

Palavras-chave: *legislação; adulteração; IRMS.*

1 Introduction

Adulteration in food and beverages is a great challenge for the world market (ANTOLOVICH; LI; ROBARDS, 2001). In the fruit beverage industries, the addition of fruit juice in amounts below the limits established by legislation is a known practice. To identify such adulteration, the isotope analysis is the most sophisticated and specific technique used in the food and beverages area (REID; O'DONNELL; DOWNEY, 2006). Stable isotope techniques have been used by official institutions in the quality control of beverages as an instrument of tax assessment for fraudulent products (KELLY, 2003).

The methodology for the determination of the carbon isotope ratio ($^{13}C/^{12}C$) is based on a mixture of compounds produced from plants of the C_3 photosynthetic cycle (apple, grape, orange, etc.) and C_4 (sugarcane, corn, etc.). The C_3 vegetables have relative isotope enrichment ($\delta^{13}C$) from -22.00 to –34.00 per mil (‰). In C_4 vegetables, the $\delta^{13}C$ varies from 9.00 to –16.00‰. This difference between C_3 and C_4 plants is also found in their products and derivatives enabling the accurate determination of the carbon botanical source (KOZIET; ROSSMANN; MARTIN, 1993; ROSSMANN, 2001).

Most isotope techniques requires the use of the database of isotope values of raw materials (fruit juice and sugar) as a reference for comparison to estimate the composition of the products to be analyzed. However, the database can be substituted for isotope analysis of an internal standard. The use of an internal component as a reference isotope decreases errors of measurement depending on the isotope variability of raw materials (KELLY, 2003). For the apple nectar, the insoluble solids (pulp) can be used as internal standards. Sugar has no internal reference; therefore, there is the need to adopt a fixed isotope value from a database of various types of sugars (DONER, 1995).

According to the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA), apple nectar is a non-fermented beverage obtained by the dilution of the edible portion of vegetal matter and sugars in potable water (BRAZIL,

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[1] *Laboratório de Bebidas, Faculdade de Ciências Agronômicas, Universidade Estadual Paulista – UNESP, CP 237, CEP 18603-970, Botucatu, SP, Brazil, e-mail: ricardofigueira@hotmail.com*
[2] *Centro de Isótopos Estáveis Ambientais, Instituto de Biociências, Universidade Estadual Paulista – UNESP, Botucatu, SP, Brazil*
**Corresponding author*

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2009). According to the Brazilian Law, there is no specific identification or quality standards for this product with regard to the percentage of pulp in apple nectar. Thus, MAPA determines that the nectar, whose minimum quantity of pulp from a determined fruit has not been fixed by any specific technical regulation, must contain, at least 30% (m/m) of its respective pulp (BRAZIL, 2003).

Despite the standards established by Brazilian legislation, currently conventional physicochemical analyses do not measure the quantity of pulpy juice (C_3 source) added to nectar formulations. Consequently, monitoring the product to verify whether this aspect meets the standards demanded by law has not been done properly.

The aims of this study were to use the isotope analysis method to quantify the carbon of C_3 photosynthetic cycle in commercial apple nectars and to identify the beverages that do not conform to the MAPA in terms of the legal limit established by the Brazilian legislation.

2 Materials and methods

The raw materials were supplied by Brazilian apple beverage companies. Two samples of pulpy concentrated apple juice and twenty samples of sugarcane were obtained.

The value of relative isotopic enrichment of carbon ($\delta^{13}C$) was obtained by the Isotope Ratio Mass Spectrometer (IRMS) (Delta S Finnigan Mat) with an analytical error of 0.2‰. The $^{13}C/^{12}C$ ratio in relation to the international standard Pee Dee Belemnite (PDB) was calculated using Equation 1, where: $\delta^{13}C$ = relative isotopic enrichment of the sample in relation to standard PDB (adimensional); R = isotopic ratio $^{13}C/^{12}C$ of the sample and the standard (adimensional) (BARRIE; PROSSER, 1996).

$$\delta^{13}C(Sample, PDB) = \left[\frac{\left(R_{Sample} - R_{Standard}\right)}{R_{Standard}}\right] * 10^3 \tag{1}$$

Since there are only two isotopically different sources (pulpy apple juice - C_3 source; and sugarcane - C_4 source), stable isotopes of the chemical element carbon were used (^{13}C and ^{12}C) to quantify the participation of the C_3 and C_4 sources. This measurement is obtained using Equations 2 and 3, whose value of relative isotopic enrichment of the product reflects the proportion of carbon-13 from each source. The symbols of Equations 2 and 3 are: δa, δb, and $\delta product$ = relative isotopic enrichment of C_3, C_4 carbon source, and the product, respectively (adimensional); A and B = relative proportion of C_3 and C_4 source in product, respectively (adimensional).

$$\delta a * A + \delta b * B = \delta p \tag{2}$$

$$A + B = 1 \tag{3}$$

2.1 Laboratory production of apple nectars

Apple nectars were produced in the laboratory using raw materials (concentrated pulpy apple juice, sugarcane, and water) in duplicate, according to Brazilian legislation. Adulterated nectars were also produced an amount of pulp juice below the permitted limited value established by MAPA. Thus, apple nectars were produced at the concentration of 12 °Brix (the lowest value found in commercial nectars). These products were manufactured with values from 0, 5, 10 up to 80% (m/m) of pulpy juice at 10.5 °Brix (lowest proportion of soluble solids permitted for apple juice according to Brazilian Law - BRAZIL, 2003) using the mass balance for soluble solids (Equation 4). In these beverages, the theoretical amount of C_3 source (%C_3) was calculated using Equation 5, where: *°Brix* = soluble solids content of the *pulpy*, *sugar* (100 °Brix), *water* (0 °Brix), and *nectar*; *M* = mass of the *pulpy*, *sugar*, *water,* and *nectar*.

$$^{\circ}Brix_{Pulpy} * M_{Pulpy} + {}^{\circ}Brix_{Sugar} * M_{Sugar} + {}^{\circ}Brix_{Water} * M_{Water} = {}^{\circ}Brix_{Nectar} * M_{Nectar} \tag{4}$$

$$\%C_3 = \frac{^{\circ}Brix_{Pulpy} * M_{Pulpy}}{^{\circ}Brix_{Pulpy} * M_{Pulpy} + {}^{\circ}Brix_{Sugar} * M_{Sugar}} * 100 \tag{5}$$

2.2 Isotopic analysis of pulp extracted from nectars manufactured in the laboratory and from commercial apple nectars - δa

The pulp was extracted according to the method proposed by Rossmann et al. (1997). Each pulp sample (0.50 mg), in duplicate, was packed into tin capsules and combusted into an Elemental Analyzer (EA 1108 – CHN – Fisons Elemental Analyzer) at 1020 °C to produce CO_2. This gas was compared with standard CO_2 (PDB) to determine the relative isotopic enrichment by IRMS.

2.3 Isotopic analysis of sugarcane - δb

The liquid sugarcane samples were diluted with distilled water to a concentration of 10 °Brix. The solid sugar samples were ground in a cryogenic grinder with liquid nitrogen (Spex CertiPrep 6750 Freezer/Mill) for three minutes at –196 °C to obtain a fine homogeneous texture (≤65 µm). Each sample was placed in tin capsules (0.35 µL – liquid samples; 0.03 mg – solid samples) and placed into the Elemental Analyzer.

2.4 Isotopic analysis of commercial and laboratory-manufactured nectars - δp

For the isotopic analysis of the apple nectars produced commercially and those produced in the laboratory, 0.35 µL of sample was placed in tin capsules and placed into the Elemental Analyzer.

2.5 Isotopic analysis of purified sugar extracted from nectars produced in the laboratory and from commercial nectars - δp

For sugar purification, the method proposed by Koziet, Pichlmayr and Rossmann (1995) was used. A solution of purified sugar (sugar C_3 + sugar C_4) obtained at the end of the procedure was packed into a tin capsule and placed into the Elemental Analyzer.

2.6 Definition of the parameter δp in the isotopic analysis of apple nectars produced in the laboratory

The isotopic analysis of the nectar produced in the laboratory was carried out in the nectar itself (δp) and in its fractions of pulp (δa) and purified sugar (δp). The isotopic value of sugarcane (δb) was obtained from the database. Since the value of δp can be derived from the nectar or from its purified sugar, two values of the C_3 percentage are obtained (Equation 2).

In order to determine the best combination, the practical results (IRMS) were subtracted from the theoretical C_3 source concentration (item 2.1). The *Errors* obtained (|theoretical C_3 source concentration - practical C_3 source concentration|) were submitted to Covariance Analysis ($\alpha = 0.05$) using the SAS software program (STATISTICAL…, 2003-2008) according to Equation 6, where: y_{ij} = a noticed *Error* of combination *i* and level *j* of *x*; α_i = effect of i^{th} treatment, β = parameter of the linear regression, x_{ij} = concentration level *j* of sugar, and e_{ij} = random error (ZAR, 1999).

$$y_{ij} = \mu + \alpha_j + \beta_{xi} + e_{ji} \tag{6}$$

The value of *F* test is statiscally significant ($p < 0.05$). the *Errors* of each combination were compared using the Tukey Test ($\alpha = 0.05$) (ZAR, 1999) and the SAS software program (STATISTICAL…, 2003-2008). Moreover, the mean and standard deviation of *Errors* were calculated. Based on statistical analysis and the mean of *Errors*, the combination with the practical result closest to the theoretical result was determined. The chosen combination was used to quantify the carbon concentration from C_3 source in the next stages of the development of the method.

2.7 Legal limit for apple nectars

To determine whether the commercial apple nectars were adulterated or unadulterated, it was necessary to determine the legal limit to verify the conformity or nonconformity of beverages with Brazilian standards. The legal limit specifies the minimum concentration of C_3 source that the apple nectar must present to be considered legal under Brazilian Law.

The legal limit was obtained by the theoretical measurement of the quantity of minimum C_3 source calculated using the mass balance for soluble solids (°Brix) in apple nectars with concentrations of soluble solids from 10.5; 11.0; 11.5 up to 15.0 °Brix with the addition of 30% pulpy apple juice (m/m) at 10.5 °Brix. The minimum amounts of C_3 source were related to the respective concentrations of soluble solids (10.5; 11.0; 11.5 up to 15.0 °Brix). The resultant curve originated the legal limit.

2.8 Concentration of C_3 source and assessment of conformity of commercial apple nectars

To assess the conformity of commercial apple nectars, a range of C_3 source concentrations was calculated for each commercial product using Equations 2 and 3. The isotopic value of pulp extracted from commercial nectar was used for δa, whereas the lightest and heaviest isotopic values for sugarcane were used for δb; for δp, the isotopic value of the commercial nectar or its purified sugar (item 2.6) were used. In Equations 2 and 3, the isotopic value of pulp (δa) was combined with the lightest and heaviest isotopic values of δb and the isotopic value of δp to obtain, respectively, the maximum and minimum quantifications of C_3 source. Therefore, a C_3 source concentration range was established for each commercial product.

The values of the C_3 source concentration range in commercial nectars were plotted on a dispersion graph and related to °Brix of commercial samples. On this same graph, the legal limit values were included. When C_3 source concentration range surpassed the legal limit, the product was considered legal. When the range was below this threshold, the product was defined as adulterated. When the concentration range values matched the legal threshold values, this product was classified as legally uncertain.

3 Results and discussion

3.1 Sugarcane isotopic analysis

The mean isotopic value of sugars used by the apple nectar manufacturers was 12.72 ± 0.16 per mil (‰). The lightest isotopic value was –13.06‰, and the heaviest was –12.51‰. Koziet, Pichlmayr and Rossmann (1995) obtained the isotopic value of –11.23 ± 0.20‰ from sugarcane. The lightest isotope value was –11.51‰, and the heaviest was –10.76‰. The isotope values reported in this article are smaller than those reported by Koziet, Pichlmayr and Rossmann (1995). Environmental (radiation, soil moisture, soil salinity, etc.) and biological factors (photosynthetic capacity, genetic variation, competition, etc.) can influence the carbon isotope composition in C_3 and C_4 plants (BOUTTON, 1996).

3.2 Isotopic analysis and definition of δp in apple nectars produced in the laboratory

In the nectars and in fractions of purified sugar, the increase of pulpy juice up to 10.5 °Brix in the formulations produced enriched the samples of C_3 source). For the pulp, since it is the internal standard, this variation in relative isotopic enrichment did not occur (Table 1), an observation also reported by Jamin et al. (1998) and Kelly (2003), who investigated the internal standard of juices without quantifying the sugar used in adulteration.

In samples 21 to 28, the addition of up to 35% pulpy juice (m/m) in the formulation of nectars did not incorporate pulp into the beverage in an amount sufficient to be extracted (Table 1).

Using the isotopic value of pulp extracted from nectars produced in the laboratory (Table 1) in δa, Crystal sugar (–12,64‰) in δb, and nectar or its purified sugar (Table 1) in δp, the practical quantifications of C_3 source percentage (Table 2) were obtained.

In the covariance analysis, the *F* Test values were not significant ($p > 0.05$). Therefore, there was no statistical difference between the combinations. Since combination 2 had

Table 1. Relative isotopic enrichment of carbon-13 in nectars produced in the laboratory and in their purified sugar and pulp fractions.

Nº	Juice (%)[1]	δa (δ‰)		δp (δ‰)		δp (δ‰)	
		Pulp	Average deviation	Nectar	Average deviation	Purified Sugar	Average deviation
21	0	I.Q.P.	-	−12.64	0.01	−12.88	0.07
22	5	I.Q.P.	-	−13.32	0.01	−13.47	0.07
23	10	I.Q.P.	-	−13.88	0.07	−14.05	0.07
24	15	I.Q.P.	-	−14.69	0.15	−14.57	0.01
25	20	I.Q.P.	-	−15.08	0.06	−15.15	0.17
26	25	I.Q.P.	-	−15.75	0.04	−15.60	0.04
27	30	I.Q.P.	-	−16.42	0.04	−16.09	0.08
28	35	I.Q.P.	-	−17.08	0.05	−16.68	0.06
29	40	−27.63	0.05	−17.81	0.04	−17.89	0.04
30	45	−27.60	0.11	−18.51	0.07	−18.55	0.11
31	50	−27.61	0.01	−19.16	0.04	−19.19	0.01
32	55	−27.67	0.05	−19.85	0.01	−19.88	0.09
33	60	−27.60	0.04	−20.42	0.04	−20.45	0.02
34	65	−27.60	0.02	−21.11	0.02	−21.16	0.03
35	70	−27.70	0.05	−21.86	0.11	−21.88	0.02
36	75	−27.71	0.07	−22.50	0.08	−22.55	0.04
37	80	−27.65	0.11	−23.12	0.04	−23.16	0.01

[1] % pulpy juice added to 10.5ºBrix (m/m); I.Q.P. = Insufficient amount of Pulp extracted from nectars to proceed to isotopic analysis.

Table 2. Comparison between the theoretical and practical values of C_3 source and error estimate in combinations of δa and δp in the apple nectar produced in the laboratory.

Nº	Juice (%)[1]	Theoretical %C_3[2]	1		2	
			δ_P *vs.* δ_N	*Error* (%)[3]	δ_P *vs.* δ_{AP}	*Error* (%)
29	40	35.00	34.54	0.46	35.08	0.08
30	45	39.38	39.28	0.10	39.55	0.17
31	50	43.75	43.56	0.19	43.76	0.01
32	55	48.13	48.01	0.12	48.20	0.07
33	60	52.50	52.02	0.48	52.26	0.24
34	65	56.88	56.65	0.23	56.98	0.10
35	70	61.25	61.21	0.04	61.38	0.13
36	75	65.63	65.45	0.18	65.78	0.15
37	80	70.00	69.83	0.17	70.10	0.10
Average (%)				0,22a[4]		0,12a
Standard deviation				0,15		0,07

[1] % pulpy juice added to 10.5 °Brix (m/m); [2] % theoretical C_3 source obtained by mass balance for soluble solids (item 2.1.); [3] |% theoretical C_3 source – % practical C_3 source|; δ_P *vs.* δ_N - pulp extracted from nectar produced in the laboratory (δa) *vs.* nectar (δp) produced in the laboratory; δ_P *vs.* δ_{AP} - pulp extracted from the nectar produced in the laboratory (δa) *vs.* purified sugar extracted from the nectar (δp) produced in the laboratory; [4] Tukey Test (a = 0,05).

Table 3. Mass balance for apple nectars produced with 30% pulpy apple juice (m/m) at 10.5 °Brix.

Nº	Juice (%)[1]	°Brix Juice	Juice (g)	Sugar (°Brix)	Sugar (g)	Water (°Brix)	Water (g)	Nectar (°Brix)	Nectar (g)	Legal limit (%C_3 Source)
38	30	10.5	75	100	18.37	0	156.62	10.5	250	30.00
39	30	10.5	75	100	19.62	0	155.37	11.0	250	28.64
40	30	10.5	75	100	20.87	0	154.12	11.5	250	27.39
41	30	10.5	75	100	22.12	0	152.87	12.0	250	26.25
42	30	10.5	75	100	23.37	0	151.62	12.5	250	25.20
43	30	10.5	75	100	24.62	0	150.37	13.0	250	24.23
44	30	10.5	75	100	25.87	0	149.12	13.5	250	23.33
45	30	10.5	75	100	27.12	0	147.87	14.0	250	22.50
46	30	10.5	75	100	28.37	0	146.62	14.5	250	21.72
47	30	10.5	75	100	29.62	0	145.37	15.0	250	21.00

[1] %pulpy juice added at 10.5 °Brix (m/m).

Table 4. Relative isotopic enrichment of carbon-13 of pulp and of purified sugar extracted from commercial apple nectars.

Nº	°Brix	δa (δ‰)		δp (δ‰)	
		Pulp	Average deviation	Sugar purified	Average deviation
48	12.90	−28.49	0.05	−17.62	0.06
49	13.60	−29.29	0.15	−19.55	0.01
50	12.50	−27.60	0.04	−18.51	0.01
51	12.50	−28.05	0.01	−17.76	0.07
52	12.40	−27.88	0.04	−17.76	0.03
53	12.60	−27.51	0.08	−17.52	0.07

the lowest mean *Errors*, it was chosen to measure the C_3 source concentration in pulpy apple juice (Table 2).

Queiroz et al. (2007) verified the conformity of orange juice sold in the Brazilian market through carbon isotope analysis. In this study, the best result for the C_3 source quantification was also obtained using the isotope values of purified sugar extracted from orange juices (δp).

3.3 Legal limit for apple nectars

To identify the conformity of commercial nectar, it was necessary to determine a legal limit. This calculation was performed in apple nectars with concentrations of soluble solids from 10.5; 11.0; 11.5 up to 15.0 °Brix with the addition of 30% pulpy juice (m/m) at 10.5 °Brix (Table 3).

Using Equation 5, it was possible to calculate the minimum C_3 source percentage of nectars produced with 30% pulpy juice (m/m) at 10.5 °Brix (Table 3).

3.4 Concentration of C_3 source and determination of conformity of commercial apple nectars

Six samples of apple nectars were collected from the Brazilian market. In these beverages, the isotopic analyses of the pulp and purified sugar were carried out. The isotopic values varied from -27.51 to −29.29‰ for the pulp, and from −17.52 to −19.55‰ for the purified sugars (Table 4).

To calculate the C_3 source concentration range of commercial apple nectars, the isotopic value of the pulp was used as the standard for δa (Table 4), two isotopic values of commercial sugars were used for δb, and one isotopic value of purified sugar was used for δp (Table 4). The isotopic value of −13.06‰ was used as the lightest standard for δb, and −12.51‰ was used as the heaviest standard for sugarcane samples (item 3.1). The results of the C_3 source concentration range are shown in Figure 1.

Comparing the concentration range of C_3 source (Figure 1) with the °Brix of commercial nectars (Table 4) and the legal limit values (Table 3), it was observed that all samples were above the legal limit, and thus in accordance with the Brazilian Law (Figure 1).

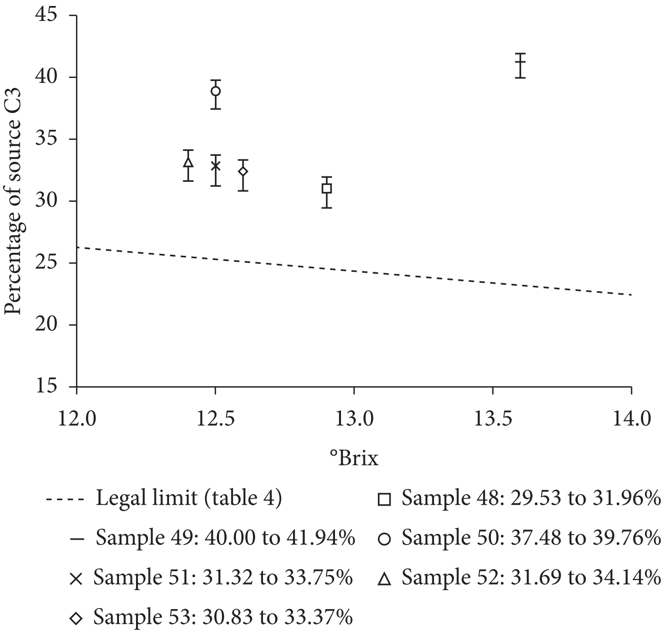

Figure 1. Percentage of source C3 in commercial apple nectars.

4 Conclusions

Legal limit was an important methodological innovation that enabled to identify the nectars that were in conformity with the Brazilian Law. The isotopic method developed proved efficient to measure the amount of carbon originating from C_3 in commercial apple nectars and to identify the beverages that are in conformity with the Brazilian Law. This method can be applied to verify the conformity of other pulpy fruit beverages.

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