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Original

Isotope analysis ($\delta^{13}C$) of pulpy whole apple juice

Análise isotópica ($\delta^{13}C$) em suco integral de maçã com polpa

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Abstract

The objectives of this study were to develop the method of isotope analysis to quantify the carbon of C_3 photosynthetic cycle in pulpy whole apple juice and to measure the legal limits based on Brazilian legislation in order to identify the beverages that do not conform to the Ministry of Agriculture, Livestock and Food Supply (MAPA). This beverage was produced in a laboratory according to the Brazilian law. Pulpy juices adulterated by the addition of sugarcane were also produced. The isotope analyses measured the relative isotope enrichment of the juices, their pulpy fractions (internal standard) and purified sugar. From those results, the quantity of C_3 source was estimated by means of the isotope dilution equation. To determine the existence of adulteration in commercial juices, it was necessary to create a legal limit according to the Brazilian law. Three brands of commercial juices were analyzed. One was classified as adulterated. The legal limit enabled to clearly identify the juice that was not in conformity with the Brazilian law. The methodology developed proved efficient for quantifying the carbon of C_3 origin in commercial pulpy apple juices.

Keywords: *internal standard; isotope; carbon; IRMS; adulteration; quality.*

Resumo

Os objetivos deste trabalho foram desenvolver o método de análise isotópica para quantificar o carbono do ciclo fotossintético C_3 em sucos com polpa de maçã integral e mensurar o limite de legalidade, baseado na legislação brasileira, para identificar as bebidas que não estão em conformidade com o Ministério da Agricultura, Pecuária e Abastecimento (MAPA). Esta bebida foi produzida em laboratório, conforme a legislação brasileira. Também foram produzidos sucos polposos adulterados com adição de açúcar de cana. Nas análises isotópicas foi mensurado o enriquecimento isotópico relativo dos sucos e de suas frações polpa (padrão interno) e açúcar purificado. Com estes resultados foi estimada a quantidade de fonte C_3 pela equação da diluição isotópica. Para determinar a existência de adulteração nos sucos comerciais foi necessária a criação do limite de legalidade de acordo com a legislação brasileira. Três marcas de sucos comerciais foram analisadas. Uma foi classificada como adulterada. O limite de legalidade possibilitou identificar claramente o suco que estava em inconformidade com a legislação brasileira. A metodologia desenvolvida provou ser eficiente para quantificar o carbono de origem C_3 em sucos polposos de maçã comerciais.

Palavras-chave: *padrão interno; isótopo; carbono; IRMS; adulteração; qualidade.*

1 Introduction

A commonly known practice among manufacturers of natural fruit juices is the addition of sugar derived from sugarcane (*Saccharum officinarum* L.). The addition of sugar reduces product cost, thus causing economic disadvantages for producers that obey the law (ROSSMANN, 2001).

Isotope analysis is the most sophisticated and specific technique that is widely used in the food and beverage area (REID; O'DONNELL; DOWNEY, 2006) to identify this adulteration. The stable isotope techniques have been used by official institutions for the quality control of beverages as an instrument of tax assessment for fraudulent products (KELLY, 2003).

The methodology for the determination of the carbon isotopic ratio ($^{13}C/^{12}C$) is based on a mixture of compounds produced from C_3 (apple, grape, orange, etc.) and C_4 photosynthetic cycle plants (sugarcane, corn, etc.). The C_3 vegetables have relative isotope enrichment ($\delta^{13}C$) from -22.00 to -34.00 per thousand (‰). 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 require 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 by the isotope analysis of an internal standard. The use of an internal component as a reference isotope reduces measurement errors depending on the isotope variability of raw materials (KELLY, 2003). The insoluble solids (pulp) of whole apple juice can be used as internal standard. Sugar has no internal reference. Therefore, there it is necessary to adopt a fixed isotope value which comes from a database of various types of sugars (DONER, 1995).

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In Brazil, the Ministry of Agriculture, Livestock and Supply (MAPA) defines whole apple juice as an unfermented, undiluted beverage obtained from the edible portion of an apple (*Malus domestica*, B.) using an adequate technological process (BRASIL, 2000). In this beverage, the addition of sugar is prohibited (BRASIL, 2009). Furthermore, the concentration of soluble solids must be at least 10.5 °Brix (BRASIL, 2000).

Even with the standards currently codified by the Brazilian law, conventional physicochemical analyses do not measure the amount of sugarcane (C_4source) added into the formulation of whole pulpy juices (C_3source). Consequently, the product enforcement, which must verify whether those beverages conform to the standards demanded by law, is impaired.

The objectives of this study were to develop the method of isotope analysis to quantify the carbon of C_3 photosynthetic cycle in pulpy whole apple juice and to measure the legal limits based on Brazilian legislation in order to identify the beverages that do not conform to the MAPA.

2 Materials and methods

The value of relative isotope enrichment of carbon ($\delta^{13}C$) was obtained by Isotope Ratio Mass Spectrometry (IRMS) (Delta S Finnigan Mat). The ratio $^{13}C/^{12}C$ in relation to the international standard Pee Dee Belemnite (PDB) was calculated by Equation 1 where: δC = relative isotope enrichment of the sample in relation to PDB (adimensional); R = isotope ratio $^{13}C/^{12}C$ of the sample and the standard (adimensional), respectively:

$$\delta^{13}C(Sample, PDB) = \left[\frac{(R_{Sample} - R_{S\tan dard})}{R_{S\tan dard}}\right] \times 10^3 \quad (1)$$

Since there are two different isotope sources (whole apple juice - C_3 source; sugarcane- C_4 source), stable isotopes of carbon were utilized (^{13}C and ^{12}C) to quantify the participation of the C_3 and C_4 sources. This measurement is obtained by Equations 2 and 3, in which the value of relative isotope enrichment of the product reflects the proportion of carbon-13 in each source. The symbols δa, δb, and $\delta product$ are the relative isotope enrichment of C_3 and C_4 carbon sources and the product, respectively (adimensional); A and B are the relative proportion of C_3 and C_4 sources in the product, respectively (adimensional).

$$\delta a \times A + \delta b \times B = \delta p \quad (2)$$

$$A + B = 1 \quad (3)$$

The raw materials supplied by Brazilian apple beverage companies included two samples of concentrated pulpy apple juice, seventeen samples of sugarcane, and three samples of additives permitted by the Brazilian law (citric acid, ascorbic acid, and xanthan gum).

2.1 Laboratory production of pulpy apple juices

Starting from the raw materials (concentrated pulpy apple juice, sugarcane, and water), pulpy apple juices were produced in the laboratory according to the Brazilian law. Adulterated juices were also produced by adding sugarcane. Pulpy juices adulterated by the addition of sugarcane were also produced. Thus, juices were produced at the concentration of soluble solids of 10.5 °Brix, with increasing quantities of sugarcane: 0; 2.5; 5.0, and 20.0%, mass per mass. The theoretical quantity of C_3source in the juices was calculated by the balance of mass of soluble solids (Equation 4).

$$°Brix = \frac{Mass\ of\ Soluble\ Solids}{Mass\ of\ Solution} \times 100 \quad (4)$$

2.2 Isotope analysis of pulp extracted from juices manufactured in the laboratory and from commercial apple juices - δa

Pulp extraction (internal standard) was conducted according to the methodology proposed by Rossmann et al. (1997). Each pulp sample (0.50mg), in duplicate, was added into a tin capsule, packed, and placed into an Elemental Analyzer (EA 1108 – CHN – Fisons Elemental Analyzer) to be burned at 1020 °C and release CO_2. This gas was compared with the standard CO_2 (PDB) for the determination of the relative isotope enrichment in the IRMS.

2.3 Isotope 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 homogeneous fine texture (≤ 65 μm). Each sample was placed into tin capsules (0.35 μL – liquid samples; 0.03 mg – solid samples) and inserted into the Elemental Analyzer.

2.4 Isotope analysis of laboratory-manufactured and commercial pulpy apple juices - δp

For the isotope analysis of the pulpy juices manufactured in the laboratory and commercial pulpy apple juices, 0.35 microliter (μL) of the sample was placed into tin capsules and inserted into the Elemental Analyzer.

2.5 Isotope analysis of purified sugar extracted from laboratory-manufactured and commercial pulpy apple juices - p

For the purification of sugar, the method proposed by Koziet et al. (1995) was utilized. The solution of purified sugar (C_3sugar + C_4sugar) obtained at the end of the procedure was placed into in a tin capsule and introduced into the Elemental Analyzer.

2.6 Isotope analysis of additives utilized in laboratory used in the production of pulpy apple juices

The liquid and granulated samples of the additives utilized in the manufacturing of commercial pulpy apple juices followed the methodology described in item 2.3.

2.7 Definition of the parameter δp in the isotope analysis of laboratory-manufactured pulpy apple juices

The isotope analysis of pulpy juices manufactured in the laboratory was accomplished in the juice itself (δp), in its pulp

fractions (δa), and purified sugar (δp). The isotope value of sugarcane (δb) was obtained from the databank. Since the value of δp can be derived from the juice or from its purified sugar, two C_3 percentage values were obtained (Equation 2 and 3).

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 (α = 0.05) by using the SAS software according to Equation 5, where: *yij* = a noticed *Error* of combination; *i* and level *j* of *x*; αi = effect of i^{th} treatment; β = parameter of the linear regression; *xij* = concentration level *j* of sugar; and *eij* = random error (ZAR, 1999).

$$y_{ij} = \mu + \alpha_i + \beta x_j + e_{ij} \quad (5)$$

Since the value of *F* test is statistically significant ($p < 0.05$), the *Errors* of each combination were compared using the Tukey test (α = 0.05) (ZAR, 1999). Moreover, the mean and standard deviation of *Errors* were measured. Based on statistical analysis and the mean of *Errors*, the combination that had the practical result closest to the theoretical results was determined. The chosen combination was used to quantify the carbon concentration of C_3 source in the next stages of method development.

2.8 Comparison between pulpy apple juices manufactured with and without additive

The inclusion of some additives can distort the isotope result in commercial products. To estimate the difference that those additives can cause in the measurements of C_3source, pulpy apple juices were manufactured with additives and compared statistically with pulpy apple juices produced without additives. This comparison was accomplished by utilizing the *t*-test for paired samples with $\alpha < 0.05$ (ZAR, 1999).

The additives used in the apple juices and their concentrations are: citric acid (0.23% - mass/mass), ascorbic acid (0.01% - mass/mass), and xanthan gum (0.04% – mass/mass).

2.9 Legal limit for whole pulpy apple juices

In order to determine whether or not commercial apple juices are adulterated, it was necessary to create a legal limit with the aim of identifying the beverages in conformity with Brazilian laws. The legal limit specifies the minimum concentration of C_3 source that an apple juice must present to be considered legal according to the Brazilian legislation.

2.10 Concentration of C_3source and determination of conformity of whole commercial apple juices

To determine the conformity of whole commercial apple juices, a concentration range of C_3source was calculated for each commercial product using Equations 2 and 3. The isotope value of pulp extracted from commercial juice was utilized for δa. For δb, the lightest and heaviest isotope values of sugarcanes were used. For δp, the isotope value of commercial juice or its purified sugar was used (item 2.7). In Equations 2 and 3, the isotope value of the pulp (δa) was combined with the lightest and heaviest isotope values of δb together with the isotope value of δp to obtain, respectively, the maximum and minimum quantifications of C_3 source, thus establishing a concentration range of C_3 source for each commercial product.

The concentration range values of C_3source of whole commercial juices were compared with the legal limit. When the C_3 source concentration range surpassed or matched the legal limit values, the juice was considered legal. When the concentration range was below, the juice was considered adulterated.

3 Results and discussion

3.1 Isotope analysis of sugarcane and additives

Table 1 displays the results of the isotope analyses of the sugars utilized by manufacturers of pulpy apple juices. In those samples, the mean isotope enrichment was -12.72 ± 0.16‰. The lightest isotope value was -13.06‰ (sample 14), while the heaviest was -12.51‰ (sample 15). Koziet, Pichimayr and Rossmann (1995), analyzing the isotope composition of sugars, found the mean isotope value of -11.23 ± 0.20‰ for sugarcane. The lightest isotope value was -11.51‰ and

Table 1. Relative isotope enrichment of carbon-13 in sugarcanes. Database for δb.

N°	Sugars	Mean (δ‰)	Average deviation
1	Crystal	-12.63	0.01
2	Crystal	-12.92	0.07
3	Crystal	-12.71	0.04
4	Crystal	-12.69	0.08
5	Crystal	-12.89	0.08
6	Crystal	-12.56	0.04
	Mean	-12.73[a1]	-
	S.D.[2]	0.14	-
7	Refined	-12.62	0.02
8	Refined	-12.80	0.11
9	Refined	-12.78	0.01
	Mean	-12.73[a]	-
	S.D.	0.10	-
10	Liquid	-12.69	0.03
11	Liquid	-12.56	0.01
12	Liquid	-12.88	0.02
13	Liquid	-12.54	0.15
14	Liquid	-13.06	0.11
	Mean	-12.75[a]	-
	S.D.	0.22	-
15	Inverted	-12.51	0.07
16	Inverted	-12.69	0.01
17	Inverted	-12.79	0.01
	Mean	-12.66[a]	-
	S.D.	0.14	-
	General mean	-12.72	-
	S.D.	0.16	-

[1]Teste *t* (α = 0,05); [2]standard deviation.

the heaviest was -10.76‰. The isotope values reported in Table 1 are lighter than those cited in the reference. Environmental (radiation, soil moisture, soil salinity, etc.) and biological factors (photosynthetic capacity, genetic variation, competition, etc.) have the potential to influence the carbon isotope composition in C_3 and C_4 plants (BOUTTON, 1996).

No statistically significant difference (Tukey test, $\alpha = 0.05$) (Table 1) was detected in the comparison the isotope value of the four kinds of sugars tested.

With regard to the relative isotope enrichment of the additives utilized in the production of pulpy apple juices, xanthan gum (-26.07 ± 0.03‰) presented an isotope value characteristic of C_3source. However, ascorbic acid (-13.91 ± 0.17‰) and citric acid (-10.99 ± 0.04‰) presented isotope values characteristic of C_4source. The isotope values of ascorbic and citric acid corroborated with the results obtained by Nogueira (2008).

3.2 Isotope analysis and definition of the parameter δp of laboratory-manufactured pulpy apple juices

The isotope analyses of the laboratory-manufactured pulpy juices, their pulp fractions, and purified sugar are displayed in Table 2. Analyzing the isotope values of the pulpy juices and their purified sugar fraction, it can be concluded that the increasing addition of sugarcane enriched the samples of carbon-13 (Table 2). With regard to the pulp fraction, according to the internal standard, this variation in relative isotope enrichment did not occur. This finding was reported by Jamin et al. (1998) and Kelly (2003), who conducted studies on the internal standard of juices without quantifying adulterations with sugars.

By utilizing the isotope value of the pulp extracted from juices manufactured in the laboratory (Table 2) in δa, crystal sugar (sample 1 – Table 1) in δb, and juice or its purified sugar (Table 2) in δp, the practical quantifications of C_3source percentage (Table 3) were obtained.

In the covariance analysis, the *F* test values were statistically significant ($p < 0.05$). The Tukey test indicated the existence of statistical difference between the combinations. Since combination 2 had the lowest mean *Errors*, it was chosen to measure the C_3 source concentration in pulpy apple juices (Table 3).

Table 2. Relative isotope enrichment of carbon-13 in laboratory-manufactured juices and their pulpy and purified sugar fractions.

Nº	Sugar (%)[1]	δa (δ‰)		δp (δ‰)		δp (δ‰)	
		Pulp	Average deviation	Juice	Average deviation	Purified sugar	Average deviation
24	0.00	-27.48	0.07	-27.62	0.01	-27.53	0.08
25	2.50	-27.35	0.06	-24.53	0.05	-24.54	0.05
26	5.00	-27.31	0.08	-22.70	0.02	-22.59	0.08
27	7.50	-27.45	0.06	-21.49	0.07	-21.30	0.07
28	10.00	-27.46	0.06	-20.35	0.02	-20.31	0.00
29	12.50	-27.36	0.04	-19.36	0.07	-19.33	0.06
30	15.00	-27.31	0.03	-18.80	0.07	-18.72	0.06
31	17.50	-27.31	0.08	-18.22	0.05	-18.17	0.03
32	20.00	-27.47	0.04	-17.87	0.03	-17.72	0.14

[1]percentage of sugar (sample 1) added (mass/mass).

Queiroz et al. (2007) verified the conformity of orange juices 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 the purified sugar fraction extracted from the juices (δp).

3.3 Comparison between pulpy apple juices manufactured with and without additives

Statistical comparison of pulpy juices manufactured with and without additives revealed no differences (Table 4). This finding was also reported by Figueira et al. (2010), who performed the same comparative study with grape juice produced with and without additives. It is likely that the small amount of additives added in apple juice was not representative

Table 3. Comparison between the theoretical and practical values of C_3 source and error estimate in combinations of δa and δp in pulpy apple juices manufactured in the laboratory.

Nº	Sugar (%)[1]	%C_3 Theoretical[2]	1		2	
			P vs. J	Error (%)[3]	P vs. SJ	Error (%)
24	0.00	100.00	100.94	0.94	100.34	0.34
25	2.50	80.77	80.87	0.10	80.90	0.13
26	5.00	67.74	68.60	0.86	67.81	0.07
27	7.50	58.33	59.78	1.45	58.50	0.17
28	10.00	51.22	52.04	0.82	51.80	0.58
29	12.50	45.65	45.70	0.05	45.50	0.15
30	15.00	41.18	42.00	0.82	41.49	0.31
31	17.50	37.50	38.04	0.54	37.74	0.24
32	20.00	34.43	35.31	0.88	34.30	0.13
Mean			-	0.72[a]	-	0.24[b]
Standard deviation			-	0.43	-	0.16

[1]% sugar (sample 1) (mass per mass); [2]%theoretical source C_3 obtained by balance of soluble solids (item 2.1.); [3]% theoretical source C_3 –% practical source C_3; P vs. J - pulp extracted from juice manufactured in the laboratory (δa) vs. pulpy juice manufactured in the laboratory (δp); P vs. SJ - pulp extracted from juice manufactured in the laboratory (δa) vs. purified sugar extracted from pulpy juices manufactured in the laboratory (δp).

Table 4. Comparison between pulpy juices manufactured with and without additives.

Nº	Sugar (%)[1]	%C_3 Theoretical[2]	Additive	%C_3 Practical[3]	Error (%)[4]
33	0.00	100.00	Absent	101.06[a]	0.94
34	0.00	100.00	Present	100.12[a]	
35	5.00	67.74	Absent	67.45[b]	0.46
36	5.00	67.74	Present	66.99[b]	
37	10.00	51.22	Absent	51.20[d]	0.69
38	10.00	51.22	Present	51.89[d]	
39	15.00	41.18	Absent	41.07[e]	0.42
40	15.00	41.18	Present	41.49[e]	
41	20.00	34.43	Absent	34.76[f]	0.19
42	20.00	34.43	Present	34.57[f]	

[1]% sugar (sample 1) added (mass per mass); [2]% theoretical source C_3 obtained by balance of soluble solids (item 2.1.); [3]same letters do not differ statistically (*t*-test for paired samples, $\alpha < 0.05$); [4]% source C_3 without additive –% source C_3 with additive.

Table 5. Isotope analysis ($\delta^{13}C$) and C_3 source concentration values of whole commercial pulpy apple juices.

Nº	°Brix	δa (δ‰)		δp (δ‰)		Legal limit (%)	δb = -13,06‰	δb = -12,51‰
		Pulp	Average deviation	Purified sugar	Average deviation		Maximum C_3 source (%)	Minimum C_3 source (%)
43	11.9	-27.01	0.04	-27.29	0.01	100	102,01	101,93
44	11.2	-27.60	0.02	-27.62	0.01		100,14	100,13
45	10.5	-30.71	0.01	-28.06	0.03		84,99	85,44

in relation to other raw materials (apple juice concentrate and sugarcane).

3.4 Legal limit for whole pulpy apple juices

By definition, whole apple juices must not contain sugarcane (BRASIL, 2009). Therefore, the percentage of C_3 source should be 100%, independently of the ºBrix of the sample (legal limit = 100% of carbon originating from C_3source).

3.5 Isotope analysis, concentration of C_3source, and determination of legality in commercial pulpy apple juices

Three samples of whole pulpy apple juice were found in the Brazilian market. Both the pulp and the purified sugar of those three beverages were analyzed isotopically. The variation in relative isotope enrichment ranged from -30.71 to -27.01‰ for pulp and from -28.06 to -27.29‰ for purified sugar (Table 5). Jamin et al. (1997) obtained isotope values for apple juices manufactured in Europe from -26.00 to -25.10‰. These values were lighter than the ones shown in Table 5.

To calculate the concentration range of C_3source in whole pulpy apple juices, the isotope value of pulp was utilized as the standard for δa (Table 5), two isotope values of commercial sugars for δb, and the isotope value of purified sugar for δp (Table 5). For δb, the isotope value of -13.06‰ was used as the lightest and -12.51‰ as the heaviest standard isotope value of sugarcane samples (Table 1). The results of the C_3 source concentration range are displayed in Table 5.

Comparing the C_3source concentration range (Table 5) with the legal limit (item 3.4), it was possible to conclude that samples 43 and 44 are in accordance with the Brazilian law. However, sample 45 certainly contains sugarcane in its composition and was thus classified as adulterated (Table 5).

With regard to the soluble solids concentrations, all whole apple juices were in accordance with the MAPA, which established the minimum concentration value as 10.5 °Brix (BRASIL, 2000) (Table 5). However, as seen in the isotope analysis, the sample 45 was not in conformity with the Brazilian law.

4 Conclusions

Analyzing only the chemical patterns established by the MAPA were not sufficient to determine the legality of the apple juices produced in Brazil.

The carbon isotope analysis methodology ($^{13}C/^{12}C$) based on the C_3 and C_4 photosynthetic metabolisms determined efficiently the C_3 source concentration of commercial clarified apple juices allowing an accurate and fair identification of the legality of those beverages.

The legal limit enabled to clearly identify the juice that was not in conformity with the Brazilian law.

By analyzing only three brands of commercial whole apple juice, it was possible to identify the sample with addition of sugarcane. This finding reinforces the need for inspection by responsible corporations.

Following the steps presented in the present study, this methodology can be applied to pulpy beverages derived from other fruits as a tool to check conformity of those products.

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