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Isotopic analysis method ($\delta^{13}C$) in apple-flavoured soft drinks

Método de análise isotópica ($\delta^{13}C$) em refrigerante de maçã

Autores | Authors

✉ Ricardo FIGUEIRA
Universidade Estadual Paulista (UNESP)
Faculdade de Ciências Agronômicas (FCA)
Campus de Botucatu
Caixa Postal: 237
CEP: 18603-970
Botucatu/SP - Brasil
e-mail: ricardofigueira@hotmail.com

Carlos DUCATTI
Universidade Estadual Paulista (UNESP)
Instituto de Biociências (IB)
e-mail: ducatti@ibb.unesp.br

Waldemar Gastoni VENTURINI FILHO
Universidade Estadual Paulista (UNESP)
Faculdade de Ciências Agronômicas (FCA)
e-mail: venturini@fca.unesp.br

✉ *Autor Correspondente | Corresponding Author*

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Summary

The objective was to develop an isotopic analysis method to quantify carbon from the C_3 photosynthesis cycle in apple-flavoured soft drinks. The beverage was produced in the laboratory according to Brazilian law. Adulterated soft drinks were also produced containing quantities of clarified juice below the legal limit. The isotopic analyses measured the relative isotopic enrichment of the apple soft drinks and their purified sugar fractions. From these results the quantity of source C_3 was estimated by means of the isotopic dilution equation. To determine the existence of adulteration in commercial soft drinks, it was necessary to create a legal limit according to Brazilian law. Three commercial brands of soft drinks were analyzed and one was classified as adulterated. The method developed was proved to be efficient to measure the quantity of carbon from a C_3 source in commercial apple-flavoured soft drink. The legal limit was an important methodological innovation which enabled the identification of beverages in conformity with Brazilian law. By following the steps of the present work, this methodology can be applied to other clarified fruit beverages as a means to verify their legality.

Key words: *Legislation; Adulteration; Fruit; Quality; Carbon-13; IRMS.*

Resumo

O objetivo deste trabalho foi desenvolver um método de análise isotópica para a quantificação de carbono do ciclo fotossintético C_3 em refrigerante de maçã. Esta bebida foi produzida em laboratório, conforme a legislação brasileira. Também foram produzidos refrigerantes adulterados com quantidade de suco clarificado de maçã abaixo do permitido. Nas análises isotópicas, foi mensurado o enriquecimento isotópico relativo dos refrigerantes e de sua fração açúcar purificado. Com estes resultados, foi estimada a quantidade de fonte C_3 por meio da equação da diluição isotópica. Para determinar a existência de adulteração nos refrigerantes comerciais, foi necessária a criação do limite de legalidade de acordo com a legislação brasileira. Três marcas de refrigerante comerciais foram analisadas. Uma foi classificada como adulterada. O método desenvolvido provou ser eficiente para mensurar a quantidade de carbono de origem C_3 em refrigerantes de maçã comerciais. O limite de legalidade foi uma importante inovação metodológica que permitiu identificar as bebidas que estão em conformidade com a legislação brasileira. Seguindo as etapas deste trabalho, esta metodologia pode ser aplicada para outras bebidas clarificadas de frutas como um meio para verificar sua legalidade.

Palavras-chave: *Legislação; Adulteração; Fruta; Qualidade; Carbono-13, IRMS.*

1 Introduction

Adulteration in food and beverages is a great challenge for the world market (ANTOLOVICH e ROBARDS, 2001). In the fruit beverage industries, the addition of fruit juice in quantities below the limits established by the legislation is a known practice.

Conventional physical-chemical analyses do not measure the quantity of apple juice (C_3 source) added to soft drink formulations. Consequently, product monitoring to verify whether this aspect satisfies the standards demanded by the law continues to be prejudiced. To overcome this problem, the isotopic procedure is widely published as the only way of measuring the quantity of fruit juice used in beverage manufacture (KELLY, 2003).

In Brazil, the Ministry of Agriculture, Livestock and Food Supply (MAPA) defines apple-flavoured soft drink as a gasified beverage obtained from the dilution of juice from its original source in potable water, with added sugar. The apple soft drink must contain at least five percent of apple juice (volume per volume) with a minimum concentration of 10 °Brix (BRASIL, 1998).

The objectives of the present work were to develop an isotopic analysis method to quantify the carbon from the C_3 photosynthesis cycle in commercial apple-flavoured soft drinks, and to determine the legal limit based on Brazilian legislation to identify the beverages in conformity with MAPA standards.

2 Material and methods

2.1 Raw material

The raw materials were supplied by Brazilian apple beverage companies. Two samples of concentrated clarified apple juice (soft drink standard) and twenty samples of sugarcane were provided.

2.2 Methods

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

$$\delta^{13}C\ (Sample, PDB) = (R_{sample} - R_{standard} / R_{standard}) * 10^3 \quad (1)$$

As there are only two isotopically different sources (clarified apple juice - source C_3 and sugarcane - source C_4), 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 from equations 2 and 3, whose value in relative isotopic enrichment of the product reflects the proportion of carbon-13 from each source. The symbols used in equations 2 and 3 are: δa and δb = relative isotopic enrichment of C_3 and C_4 carbon sources, respectively (adimensional); C_3 and C_4 = relative proportion of C_3 and C_4 sources in the product, respectively (adimensional); δproduct = relative isotopic enrichment of the product (adimensional).

$$\delta a * C_3 + \delta b * C_4 = \delta product \quad (2)$$

$$C_3 + C_4 = 1 \quad (3)$$

2.2.1 Laboratory production of apple-flavoured soft drinks

Starting from the raw materials (concentrated clarified apple juice, sugarcane and water), apple-flavoured soft drinks were produced in the laboratory according to Brazilian law. In addition, adulterated soft drinks were produced with a quantity of clarified juice below the limit permitted by MAPA. Thus the apple soft drinks were produced with soluble solids concentrations of 10.4 °Brix (smallest value found in commercial soft drinks). The soft drinks were produced with the addition of increasing quantities of clarified juice at 10 °Brix as follows: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% (volume per volume). The theoretical proportion of C_3 source in the soft drinks was calculated on a mass balance for soluble solids (Equation 4).

$$°Brix = \frac{\text{Mass of soluble solids}}{\text{Mass of solution}} \times 100 \quad (4)$$

2.2.2 Isotopic analysis of concentrated clarified apple juice - δa

For the isotopic analysis of concentrated juice, 0.35 microlitre (μL) of each juice, in duplicate, was added to a tin capsule, sealed and placed in an Elemental Analyzer (EA 1108 – CHN – Fisons Elemental Analyzer) for burning at 1020 °C to release CO_2. This gas was compared with standard CO_2 (PDB) to determine the relative isotopic enrichment by IRMS.

2.2.2.1 Isotopic analysis of the purified sugar extracted from concentrated clarified apple juices - δa

The method proposed by Koziet et al. (1993) was used to purify the sugar extracted from the concentrated clarified apple juices,. A solution of purified sugar (C_3 sugar + C_4 sugar) obtained at the end of the procedure was added to a tin capsule and placed in the Elemental Analyzer.

2.2.3 Isotopic analysis of sugarcane - δb

The liquid sugarcane samples were diluted with distilled water to a concentration of 10 °Brix, and then prepared and placed in the Elemental Analyzer.

The solid sugar samples were ground with liquid nitrogen in a cryogenic grinder (Spex CertiPrep 6750 Freezer/Mill) for three minutes at a temperature of –196 °C, to obtain a fine, homogeneous texture (≤65 µm). Each sample (0.03 mg) was placed in a tin capsule and inserted into the Elemental Analyzer.

2.2.4 Isotopic analysis of commercial and laboratory-manufactured apple-fllavoured soft drinks - δp

The methodology used was described in item 2.2.2.

2.2.4.1 Isotopic analysis of the purified sugar extracted from commercial and laboratory-manufactured apple-flavoured soft drinks - δp

The first step of this process followed the methodology described in item 2.2.2.1, and the samples were then prepared according to item 2.2.2.

2.2.5 Definition of the parameters δa, δb and δp in the isotopic analysis of apple-flavoured soft drinks manufactured in the laboratory

To manufacture the soft drinks in the laboratory, concentrated clarified juice (C_3) and sugarcane (C_4) were used as the raw materials. For the concentrated clarified juice, the isotopic analysis was applied to the concentrated juice itself (δa) and its purified sugar fraction (δa). In the laboratory-manufactured soft drink, the isotopic analysis was applied to the soft drink itself (δp) and to its purified sugar fraction (δp). The isotopic value for sugarcane (δb) was obtained from the databank. Since two isotopic values were obtained for δa and two more generated for δp, four C_3 percentages could be calculated (Equation 2). These practical results (IRMS) were compared with the theoretical C_3 source calculated ffrom the mass balance for the soluble solids (item 2.2.1.). The δa (concentrated juice or its purified sugar) and the δp (laboratory-manufactured soft drink or its purified sugar) that supplied the practical results closest to the theoretical results were chosen to quantify the carbon concentration originating from C_3.

2.2.6 Legal limit for commercial apple-flavoured soft drinks

To determine whether the commercial soft drinks were adulterated or not, it was necessary to create a legal limit to ascertain the conformity or nonconformity of the beverages with the Brazilian norms. The legal limit specifies the minimum concentration of C_3 source that the commercial soft drink must contain to be considered legal according to Brazilian law.

The legal limit was obtained from the theoretical measurement of the minimum quantity of C_3 source, calculated from the mass balance for the soluble solids (°Brix), in soft drinks with soluble solids concentrations of 10, 11, 12, 13 and 14 °Brix with the addition of 5% (volume/volume) of clarified apple juice (smallest value permitted by Brazilian law) at 10 °Brix. The minimum quantities of C_3 source were related to their respective concentrations of soluble solids (10, 11, 12, 13 and 14 °Brix). The resulting curve gave origin to the legal limit.

2.2.7 Concentration of C_3 source and determination of the legality of commercial apple-flavoured soft drinks

To determine the legality of commercial soft drinks, a range of C_3 source concentrations was calculated for each commercial product. For δa, the lightest and heaviest isotopic values of the concentrated clarified juice or its purified sugar fraction (item 2.2.5), were used. For δb the lightest and heaviest isotopic sugarcane values were employed, whereas δp used the isotopic value of the commercial soft drink or its purified sugar fraction (item 2.2.5). In equations 2 and 3, the heaviest and lightest isotopic values for δa were grouped with their respective counterparts in δb, jointly with the isotopic value for δp to obtain the maximum and minimum levels of C_3 source. In this manner a C_3 source concentration range was established for each commercial product.

The concentration range values for the C_3 source were plotted on a dispersion graph and related to the °Brix of the commercial soft drinks, and the legal limit values were inserted onto the same graph. When the 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, the product was classified as legally uncertain.

3 Results and discussion

3.1 Isotopic analysis of the raw materials

The relative isotopic enrichment of concentrated clarified juices was –27.33 ± 0.02‰ for sample 1, and –27.39 ± 0.04‰ for sample 2. In the isotopic analysis of the purified sugar, the relative isotopic enrichment was –27.41 ± 0.01‰ for sample 1 and –27.76 ± 0.01‰ for sample 2. Comparing the isotopic enrichment of the concentrated juice and the purified sugar, distinct values were found in samples 1 and 2. Such an observation was reported by Parker (1982), who, when analyzing concentrated juices, also found variation between the isotopic values of the juice and those of the purified sugar.

In Table 1, the mean isotopic value for the sugars used by apple beverage manufacturers was –12.72 ±

0.16‰. The lightest and heaviest isotopic values were, respectively, –13.06‰ (sample 16) and –12.51‰ (sample 17).

3.2 Isotopic analysis of laboratory-manufactured apple-flavoured soft drinks

The isotopic value for the soft drinks manufactured in the laboratory varied from –14.37 to –12.64‰. For the purified sugar the variation was from –14.18 to –12.88‰. From these values it can be concluded that the increasing addition of clarified apple juice impoverished the beverage in terms of carbon-13, thus making the isotopic value lighter (Table 2).

3.3 Definition of the parameters δa, δb and δp in the isotopic analysis of laboratory-manufactured apple-flavoured soft drinks

Using the isotopic values obtained for the concentrated clarified juice or that obtained for its purified sugar (item 3.1) in δa, for the sugar crystal (sample 3 – Table 1) in δb, for the soft drink manufactured in the laboratory or for its purified sugar (Table 2) in δp, the percentages for the practical quantities of C_3 source were obtained (Table 3).

Comparing the theoretical value (balance of soluble solids) with the practical C_3 value (IRMS) obtained, it was observed that when using the isotopic value of the purified sugar extracted from the concentrated juice (δa) *versus* that of the purified sugar extracted from the soft drink (δp), the most precise results were obtained from samples 29, 30, 31, 32, 33, 35, 36, 37 and 38. When employing the isotopic value of the purified sugar extracted from the concentrated juice (δa) *versus* that of the soft drink (δp), the greatest precision was obtained from samples 28 and 34. In the combination of the isotopic value of the concentrated juice (δa) *versus* the value of the soft drink (δp), only sample 28 provided a precise result (Table 3).

Comparing the sum of errors, the best fit was verified using the isotopic value for the purified sugar extracted from the concentrated juice (δa) *versus* that of the purified sugar extracted from the soft drink (δp) (Table 3).

Table 1. Relative isotopic enrichment of carbon-13 in sugarcane samples

N°	Sugars	Mean (δ‰)	Average deviation
3	Crystal	–12.63	0.01
4	Crystal	–12.92	0.07
5	Crystal	–12.71	0.04
6	Crystal	–12.69	0.08
7	Crystal	–12.89	0.08
8	Crystal	–12.56	0.04
9	Refined	–12.62	0.02
10	Refined	–12.98	0.13
11	Refined	–12.78	0.01
12	Liquid	–12.69	0.03
13	Liquid	–12.56	0.01
14	Liquid	–12.88	0.02
15	Liquid	–12.54	0.15
16	Liquid	–13.06	0.11
17	Inverted	–12.51	0.07
18	Inverted	–12.69	0.01
19	Inverted	–12.79	0.01
20	Caramel Syrup	–12.51	0.06
21	Glazed	–12.84	0.05
22	Demerara	–12.56	0.04
	Mean	–12.72	
	Standard deviation	0.16	

3.4 Legal limit for apple-flavoured soft drink

To determine the legality of commercial soft drinks, it was necessary to create a legal limit. This calculation was employed in apple-flavoured soft drinks with soluble solids concentrations of 10, 11, 12, 13 and 14 °Brix with the addition of 5% in volume of clarified apple juice at 10 °Brix (minimum juice quantity permitted in accordance with Brazil, 2000). Using Equation 4 it was possible to calculate the minimum C_3 source percentage in a commercial soft drink produced with 5% of clarified juice (volume/volume) at 10 °Brix (Table 4).

3.5 Isotopic analysis of commercial apple-flavoured soft drinks

The three brands of apple soft drink found on the Brazilian market were identified by the sample numbers 56, 57 and 58. In these beverages the isotopic analysis was applied to the purified sugar extracted from the

Table 2. Relative isotopic enrichment in carbon-13 in laboratory-manufactured soft drinks and in their purified sugar fractions.

N°	Juice (%)[1]	Soft drink (δ‰)	Average deviation	Purified sugar (δ‰)	Average deviation
28	0.00	–12.64	0.01	–12.82	0.12
29	1.00	–12.99	0.01	–12.91	0.04
30	2.00	–13.19	0.02	–13.05	0.11
31	3.00	–13.37	0.11	–13.14	0.04
32	4.00	–13.46	0.11	–13.24	0.16
33	5.00	–13.51	0.01	–13.43	0.04
34	6.00	–13.56	0.03	–13.72	0.01
35	7.00	–13.80	0.13	–13.76	0.09
36	8.00	–13.98	0.08	–13.81	0.05
37	9.00	–13.99	0.07	–13.98	0.02
38	10.00	–14.37	0.08	–14.18	0.02

[1]Percentage of clarified concentrated juice (sample 2) added, at 10 °Brix (volume per volume).

Table 3. Comparison between the theoretical and practical values for C_3 source and the estimated error in combinations of δa and δp for the laboratory-manufactured apple-flavoured soft drink.

N°	%C_3 theoretical[1]	CJ *versus* S	Error (%)[2]	PCJ *versus* S	Error (%)	CJ *versus* PS	Error (%)	PCJ *versus* PS	Error (%)
28	0.00	0.07	0.07	0.07	0.07	1.29	1.29	1.26	1.26
29	0.96	2.41	1.45	2.35	1.39	1.90	0.94	1.85	0.89
30	1.92	3.79	1.87	3.70	1.78	2.85	0.93	2.78	0.86
31	2.88	5.01	2.13	4.89	2.01	3.46	0.58	3.37	0.49
32	3.84	5.62	1.78	5.49	1.65	4.13	0.29	4.03	0.19
33	4.80	5.93	1.13	5.78	0.98	5.42	0.62	5.29	0.49
34	5.76	6.27	0.51	6.11	0.35	7.35	1.59	7.17	1.41
35	6.72	7.93	1.21	7.73	1.01	7.62	0.90	7.44	0.72
36	7.68	9.11	1.43	8.89	1.21	7.99	0.31	7.80	0.12
37	8.64	9.18	0.54	8.96	0.32	9.15	0.51	8.92	0.28
38	9.60	11.75	2.15	11.47	1.87	10.50	0.90	10.24	0.64
Sum of errors (%)			14.27		12.64		8.86		7.35

[1]Percentage of theoretical C_3 source obtained from the mass balance of soluble solids (item 2.2.1.); [2]percentage of theoretical C_3 source – percentage of practical C_3 source; CJ *versus* S - concentrated clarified juice (δa) *versus* soft drink manufactured in the laboratory (δp); PCJ *versus* S - purified sugar extracted from concentrated clarified juice (δa) *versus* laboratory-manufactured soft drink (δp); CJ *versus* PS - concentrated clarified juice (δa) *versus* purified sugar extracted from laboratory-manufactured soft drink (δp); PCJ *versus* PS - purified sugar extracted from concentrated clarified juice (δa) *versus* purified sugar extracted from laboratory-manufactured soft drink (δp).

Table 4. Mass balance for apple-flavoured soft drinks produced with 5% of clarified juice.

N°	Juice (%)[1]	Juice (mL)	Sugar (°Brix)	Sugar (g)	Water (mL)	Soft drink (°Brix)	Soft drink (mL)	Legal limit (%Source C_3)
51	5.00	12.50	100.00	24.70	222.31	10.00	250.00	5.00
52	5.00	12.50	100.00	27.41	220.63	11.00	250.00	4.53
53	5.00	12.50	100.00	30.15	218.94	12.00	250.00	4.13
54	5.00	12.50	100.00	32.91	217.24	13.00	250.00	3.80
55	5.00	12.50	100.00	35.69	215.52	14.00	250.00	3.51

[1]Percent of clarified apple juice (volume per volume) at 10°Brix.

commercial soft drink (item 3.3.). The relative isotopic enrichments of samples 56, 57 and 58 were, respectively, –14.24 ± 0.01, –13.41 ± 0.05 and –13.18 ± 0.05‰.

3.6 Concentration of C_3 source and determination of the legality of commercial apple-flavoured soft drinks

To calculate the C_3 source concentration range in commercial apple-flavoured soft drinks, in δa, the lightest (–27.76‰ - sample 2) and the heaviest (–27.41‰ - sample 1) isotopic values for the concentrated clarified apple juice were used (item 3.1). In δb, the lightest (–13.06‰ - sample 16) and heaviest (–12.51‰ - sample 17) isotopic values for sugarcane were employed (Table 1). In δp the isotopic value for the purified sugar extracted from commercial apple-flavoured soft drinks was used (item 3.5). The results are shown in Figure 1.

Taking the C_3 source concentration range and the °Brix of the commercial soft drinks (Figure 1) together with the legal limit (Table 4), it was concluded that sample 56 conformed with the Brazilian law. Sample 57 could be classified as legal or adulterated according to the relative isotopic enrichment of the raw materials. Therefore this soft drink was classified as legally uncertain. For sample 58 all the quantifications of C_3 source were below the legal limit. So, this beverage was classified as adulterated (Figure 1).

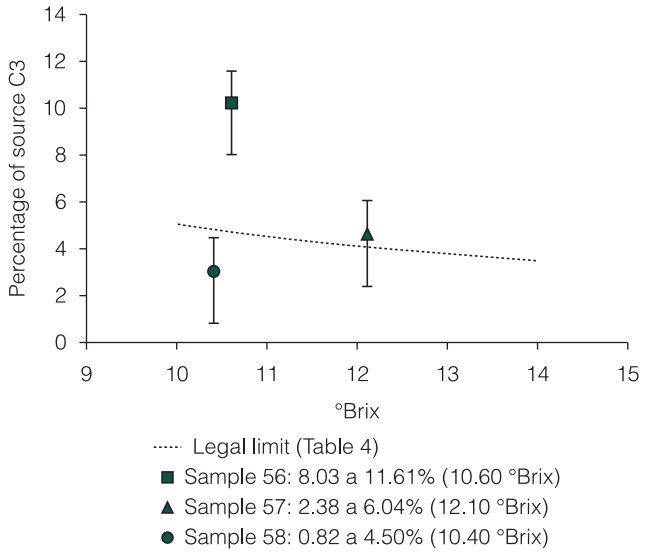

Figure 1. Concentration of carbon from C_3 origin in commercial apple-flavoured soft drinks.

4 Conclusions

The method developed was proved to be efficient for measuring the quantity of carbon from C_3 source in commercial apple-flavoured soft drinks.

The legal limit was an important methodological innovation which enabled the identification of beverages in conformity with the Brazilian law.

By following the steps in the present work, this methodology can be applied to other clarified fruit beverages as a means of verifying their legality.

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