ISOTOPE ANALYSIS METHOD ($\delta^{13}C$) IN CLARIFIED APPLE JUICE

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The aims of this study were to develop the method of isotope analysis to quantify the carbon of the C_3 photosynthetic cycle in commercial clarified apple juices and to measure the legal limit based on Brazilian legislation in order to identify the beverages that do not conform to the guidelines of the Ministry of Agriculture, Livestock and Food Supply (MAPA). This beverage was produced in a laboratory, according to Brazilian legislation. Adulterated juices with a sugarcane quantity above the legal limit were also produced. Isotope analyses measured the relative isotope enrichment of clarified apple juices and their purified sugar fraction. From these results the C_3 source concentration 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 Brazilian legislation. Two commercial brands of clarified juice were analyzed. Taking the C_3 source concentration and the ºBrix of commercial clarified juices, together with the legal limit, it was possible to verify that one sample certainly contained more sugarcane than the quantity established by the MAPA. The development of a legal limit was an important methodological innovation that made it possible to identify the beverages that did not conform to Brazilian legislation. The methodology developed proved efficient for quantifying the carbon of C_3 origin in commercial clarified apple juices.

KEY-WORDS: ADULTERATION; CARBON-13; IRMS; Malus domestica; SUGARCANE.

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1 INTRODUCTION

Adulteration in beverages is a great challenge for the world markets (ANTOLOVICH, LI & ROBARDS, 2001). In the fruit juice industry, one known practice is the addition of sugar originating from cane sugar (*Saccharum officinarum*). When sugar is added to apple juice above the quantities permitted by Brazilian legislation there is a reduction in costs which causes economic disadvantages for honest producers (ROSSMANN, 2001). To identify this adulteration, isotope analysis is the most sophisticated and specific technique widely used in the areas of food and beverages (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; JAMIN *et al.*, 2005).

The methodology of the carbon isotope ratio ($^{13}C/^{12}C$) is based on the mixture of compounds produced from plants of the C_3 photosynthetic cycle (apple, grape, orange, etc.) and C_4 (cane sugar, corn, etc.). The C_3 vegetables have relative isotope enrichment ($\delta^{13}C$) between -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 and can determine with accuracy the botanical source of carbon (ROSSMANN, 2001).

Most isotope studies (NOGUEIRA *et al.*, 2011; QUEIROZ *et al.*, 2009, FIGUEIRA *et al.*, 2011) use an internal standard (insoluble solids) to estimate the composition and to check the amount of sugar added in non-alcoholic beverages. The internal component, used as an isotope reference, decreases errors caused by the isotope variability regarding raw materials (KELLY, 2003). When the beverage is clarified, the insoluble solids are removed by technological processes. In that case, the isotope technique requires the use of a database coming from the isotope in relation to the values of raw materials (fruit juice and sugar) as a reference for comparison, to estimate the composition of the commercial products to be analyzed (KELLY, 2003). Clarified concentrated juices and sugarcane are usually used as isotope references for C_3 and C_4 sources, respectively (DONER, 1995).

The quality control of non-alcoholic beverages manufactured in Brazil is performed by the Ministry of Agriculture, Livestock and Food Supply (MAPA). The MAPA defines apple juice as a non-fermented non-diluted beverage obtained from the edible part of the apple *(Malus domestica*) through an adequate technological process (BRAZIL, 2000). For this beverage, the maximum quantity of sugar that may be added is 10 % (m/m) (BRAZIL, 2009). The proportion of soluble solids cannot be less than 10.5 °Brix (BRAZIL, 2000).

Even with the standards currently codified in Brazilian legislation, conventional chemical analyses do not measure the quantity of sugarcane (source C_4) added into clarified apple juices (source C_3). Consequently, the product enforcement, which must verify whether these beverages conform to the standards demanded by law, is prejudiced.

The aims of this study were to develop the method of isotope analysis to quantify the carbon of C_3 the photosynthetic cycle in commercial clarified apple juices and to measure the legal limit, based on Brazilian legislation, in order to identify the beverages that do not conform to the MAPA's guidelines.

2 MATERIAL AND METHODS

The value of the relative isotope enrichment of carbon ($\delta^{13}C$) was obtained by Isotope Ratio Mass Spectrometry (IRMS) (Delta S Finnigan Mat). The $^{13}C/^{12}C$ ratio in relation to the international Pee Dee Belemnite (PDB) standard was calculated by Equation 1, where: $\delta^{13}C$ = relative isotope enrichment of the sample in relation to PDB (adimensional); R = isotope ratio $^{13}C/^{12}C$ of the sample and of the standard (adimensional). The negative sign of $\delta^{13}C$ means that the sample contains less carbon-13 than standard (PDB) (ROSSMANN, 2001):

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

As there were two different isotope sources (clarified apple juice - source C_3; sugarcane - source C_4), stable isotopes of the chemical element carbon were utilized (^{13}C and ^{12}C) to quantify the participation of the C_3 and C_4 sources. This measurement was obtained by Equation 2, whose value of relative isotope enrichment of the product reflects the proportion of carbon-13 from each source. The symbols are: δa, δb and $\delta product$ = relative isotope enrichment of C_3, C_4 carbon source and of product, respectively (adimensional); *A* and *B* = relative proportion of C_3 and C_4 source in product, respectively, where $A+B = 1$ (adimensional) (DUCATTI, 2005; ROSSMANN, 2001):

$$\delta a * A + \delta b * B = \delta product \quad (2)$$

The raw materials were supplied by Brazilian apple beverage companies. Three samples of clarified concentrated apple juice and seventeen samples of sugarcane were provided. Two brands of clarified apple juice were purchased, in duplicate, in supermarkets in the city of Botucatu, SP (BRAZIL).

2.1 LABORATORY PRODUCTION OF CLARIFIED APPLE JUICES

Starting with the raw materials, the clarified apple juices were produced in the laboratory according to Brazilian legislation. In addition, adulterated juices were produced with a sugarcane quantity above the limit permitted by the MAPA. The apple juices were produced with a concentration of soluble solids of 10.5 °Brix (BRAZIL, 2000) and then increasing quantities of sugarcane of 0, 2.5 to 20.0 % (m/m) were added. The clarified juice was produced used the mass balance for soluble solids (Equation 3), derived from °Brix = (mass of soluble solids / mass of solution) * 100 (FIGUEIRA & VENTURINI FILHO, 2009). These beverages were used to calculate the theoretical C_3 source concentration (*% C_3*) (Equation 4). The symbols are: °Brix = proportion of soluble solids of the reconstituted juice, sugarcane (100 °Brix) and the clarified juice, *M* = mass of reconstituted juice, sugar and sweetened clarified juice:

$$^{\circ}Brix_{Juice} * M_{Juice} + {}^{\circ}Brix_{Sugar} * M_{Sugar} = {}^{\circ}Brix_{Clarified} * M_{Clarified} \quad (3)$$

$$\%C_3 = \frac{{}^{\circ}Brix_{Juice} * M_{Juice}}{{}^{\circ}Brix_{Juice} * M_{Juice} + {}^{\circ}Brix_{Sugar} * M_{Sugar}} * 100 \quad (4)$$

2.2 ISOTOPE ANALYSIS OF CLARIFIED CONCENTRATED JUICE (δa), LABORATORY-FABRICATED JUICES (δp) AND COMMERCIAL APPLE JUICE (δp)

For the isotope analysis, 0.35 microliter (*µ*L) of each apple products, in duplicate, was conditioned in a tin capsule, packed and placed in an Elemental Analyzer (EA 1108 Fisons Elemental Analyzer) for burning at 1020 *°C* to release CO_2. This gas was compared with standard CO_2 (PDB) to determine the $\delta^{13}C$ by IRMS (Delta S Finnigan Mat) (FIGUEIRA, DUCATTI & VENTURINI FILHO, 2010a).

2.3 ISOTOPE ANALYSIS OF PURIFIED SUGAR EXTRACTED FROM CONCENTRATED JUICE (δa), LABORATORY-FABRICATED JUICES (δp) AND COMMERCIAL APPLE JUICE (δp)

For the purification of the sugar fraction extracted from the apple products, the method proposed by Koziet *et al*. (1995) was utilized. The purified sugar fraction was inserted into a tin capsule and placed in the Elemental Analyzer (EA).

2.4 ISOTOPE ANALYSIS OF SUGARCANE (δb)

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

2.5 DEFINITION OF PARAMETERS δa AND δp IN ISOTOPE ANALYSIS OF LABORATORY-FABRICATED CLARIFIED APPLE JUICE

The clarified concentrated juice (C_3), sugarcane (C_4) and laboratory-fabricated clarified apple juice were utilized as raw materials. In the clarified concentrated juice, the isotope analysis was accomplished in the concentrated juice itself (δa) and in its purified sugar fraction (δa). In the laboratory-fabricated clarified juice, the isotope analysis was performed on the juice itself (δp) and on its purified sugar fraction (δp). The isotope value of sugarcane (δb) was obtained from the databank. As two isotope values were obtained in δa and two more values for δp, four combinations were obtained to calculate the practical C_3 source concentration (Equation 2).

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) using the “SAS” program, according to Equation 5, 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 *eij* = random error (ZAR, 1999):

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

As the value of the *F* test was meaningful ($p < 0.05$), the errors of each combination were compared using Tukey‘s Test (α = 0.05) (ZAR, 1999; VIEIRA, 2006). Moreover, the mean and standard deviation of errors were measured. Based on statistical analysis and the mean of errors, it was checked which combination had the practical result closest to the theoretical results. The chosen combination was used to quantify the carbon concentration from the C_3 source in the next stages of method development.

2.6 DEFINITION OF THE ISOTOPE VALUE FOR δa AND δb, METHOD ACCURACY AND ERROR ESTIMATE

To verify the accuracy of the method, the laboratory-fabricated clarified juices were analyzed as if they were commercial beverages. Thus, the isotope values of δa and δb were obtained from the databases of concentrate juice and sugarcane, respectively.

To estimate the accuracy of the method, three combinations with the values of δa and δb were made. In δa, the lightest, medium and heaviest isotope value of the concentrated juice, or of its purified sugar fraction, were used (item 2.5). In δb, the lightest, medium and heaviest isotope value of the sugarcane were used. In δp, the isotope value of the laboratory-fabricated juices or of its purified sugar fraction were used (item 2.5). By using Equation 2, the heaviest, medium and lightest isotope values of δa were grouped respectively with the heaviest, medium and lightest δb isotope values, together with the isotope value of δp, to measure the C_3 source concentrations.

The values of these three measurements were subtracted from the theoretical C_3 source concentration (item 2.1) to estimate the error and compared following the statistical treatments of item 2.5. Furthermore, the total error of the method (mean of errors + standard deviation) for each combination was calculated. Having defined the best combination, the isotope values of δa and δb

were used to quantify the C_3 source concentration in the commercial juices. Using these values the total error of the method were added and subtracted.

2.7 LEGAL LIMIT FOR COMMERCIAL CLARIFIED APPLE JUICES

To determine whether the commercial clarified juices were adulterated or unadulterated, it was necessary to create a legal limit to ascertain the conformity or nonconformity of the beverages with Brazilian norms. The legal limit specifies the minimum C_3 source concentration that commercial clarified apple juice must present to be considered legal under Brazilian legislation.

To obtain the legal limit the mass balance for soluble solids was measured (Equation 3) in clarified juices with concentrations of soluble solids from 10.5, 11.0 to 14.0 °Brix with 10 % (m/m) added sugarcane. The minimum C_3 source concentrations were calculated using Equation 4 and related to their respective concentrations of soluble solids. The resultant curve originated the legal limit.

2.8 ISOTOPE ANALYSIS AND LEGALITY OF COMMERCIAL CLARIFIED APPLE JUICES

To calculate the C_3 source concentration in the commercial clarified apple juices, the isotope values of δa and δb were used according to item 2.6. For δp the isotope value of the commercial juice or of its purified sugar fraction was used (item 2.5). For each C_3 source concentration, the total error of the method was inserted (item 2.6). These values were related to the ºBrix of the commercial clarified juices. Onto this same graph the legal limit values were inserted (item 2.7). When the C_3 source concentration surpassed or matched the legal limit values the juice was considered legal. When the concentration was below those limits the juice was defined as adulterated.

3 RESULTS AND DISCUSSION

3.1 ISOTOPE ANALYSIS OF RAW MATERIALS

The relative isotope enrichment of sample 3 was -20.07 ‰. That sample probably contained sugarcane in its composition. When isotope analysis was performed on the purified sugar fraction, the value was -20.01 ‰. That datum confirms the presence of sugarcane in this product (Table 1).

Comparing the isotope values of the concentrated juice and of the purified sugar fraction, there was no statistical difference (*t* Test, α = 0.05). This observation was also reported by Parker (1982). In analyzing the concentrated juices no statistical difference was found between the isotope values of the juice and of the purified sugar fraction.

Jamin *et al*. (1997) measured the isotope ratio of apple juices and concentrates produced in Europe, the United States of America (USA) and South Africa. In that study, the authors reported the isotope value of -27.50 ‰ as the lightest isotope reference measured for malic acid (source C_3). This result corroborates with the isotope values of samples 1 and 2 in the present study (Table 1).

Table 2 shows that the mean isotope value for the sugars utilized by apple juice manufacturers was -12.72±0.16 ‰. Studies of grape juices (FIGUEIRA *et al.*, 2010b) and mango and guava non-alcoholic beverages (NOGUEIRA, 2012) reported mean isotope values of sugarcane of -13.10 ‰. Other researches with cashew juices and pulps (FIGUEIRA *et al*., 2011), peach nectars (NOGUEIRA *et al*., 2011) and orange non-alcoholic beverages (QUEIROZ *et al*., 2009), indicated mean isotope values of -12.83 ‰, which were equal to thouse found in the present study. These variations in the isotope values may be related to different conditions of soil, climate and sugarcane variety. Environmental factors (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).

Comparing the isotope values of the four kinds of sugars, there was no statistical difference (Tukey's Test, α = 0.05) (Table 2). This observation confirms the results obtained by Nogueira (2012) and Figueira *et al*. (2011).

TABLE 1 - ISOTOPE ANALYSIS ($\delta^{13}C$) OF CLARIFIED CONCENTRATED APPLE JUICES AND IN ITS PURIFIED SUGAR FRACTION DATABASE FOR δa

Nº	Juice (δ ‰)	A.D.[1]	Purified sugar (δ ‰)	A.D.
1	-27.66	0.01	-27.72	0.01
2	-27.24	0.02	-27.24	0.02
3	-20.07	0.04	-20.01	0.01
Mean	-24.99a[2]		-24.99a	
S.D.[3]	4.27		4.32	

[1]Average deviation; [2]*t* Test (α = 0.05); [3]Standard deviation.

TABLE 2 - ISOTOPE ANALYSIS ($\delta^{13}C$) IN SUGARCANES DATABASE FOR δb

Sugar	n	Mean ± S.D.[1] (lightest / heaviest) (δ ‰)
Crystal	6	-12.73a[2] ± 0.14 (-12.92 / -12.56)
Refined	3	-12.73a ± 0.10 (-12.80 / -12.62)
Liquid	5	-12.75a ± 0.22 (-13.06 / -12.54)
Inverted	3	-12.66a ± 0.14 (-12.79 / -12.69)
General mean ± S.D.		**-12.72 ± 0.16**

[1]Standard deviation; [2]*t* Test (α = 0.05).

The isotope values of apple juice concentrate (Table 1) and sugarcane (Table 2) were used in items 3.3 and 3.4.

3.2 ISOTOPE ANALYSIS OF LABORATORY-FABRICATED CLARIFIED APPLE JUICES

The isotope value for laboratory-fabricated clarified juice varied from 27.78 to -17.81 ‰. For the purified sugar fraction, the variation was from -27.78 to -18.07 ‰. From these values it can be verified that the increased addition of sugarcane enriches the beverage in carbon-13, thus making the isotope value heavier (Table 3).

TABLE 3 - ISOTOPE ANALYSIS ($\delta^{13}C$) OF LABORATORY-FABRICATED CLARIFIED JUICE AND THEIR PURIFIED SUGAR FRACTION

Nº	Sugar (%)[1]	δp (δ ‰)			
		Clarified juice	Average deviation	Purified sugar	Average deviation
24	0.0	-27.78	0.01	-27.78	0.01
25	2.5	-24.79	0.01	-24.75	0.02
26	5.0	-22.72	0.02	-22.49	0.01
27	7.5	-21.43	0.01	-21.23	0.01
28	10.0	-20.32	0.01	-20.30	0.01
29	12.5	-19.53	0.03	-19.46	0.01
30	15.0	-18.82	0.01	-18.78	0.03
31	17.5	-18.25	0.05	-18.60	0.04
32	20.0	-17.81	0.01	-18.07	0.01

[1]sugar (m/m) (-12.64 ‰).

The isotope values of laboratory-fabricated apple juices and their purified sugar fraction (Table 3) were used in items 3.3 and 3.4.

3.3 DEFINITION OF PARAMETERS δa AND δp IN ISOTOPE ANALYSIS OF LABORATORY-FABRICATED CLARIFIED APPLE JUICES

Utilizing the isotope values of concentrated apple juices or their purified sugar fractions (sample 1 - Table 1) in δa, crystal sugar (-12.63 ‰) in δb and laboratory-fabricated juice or its purified sugar fraction (Table 3) in δp, the percentages of the practical C_3 source concentration were obtained (Table 4).

In the covariance analysis, the *F* Test values were meaningful ($p < 0.05$). Tukey's Test indicated the existence of statistical difference between combinations 1 and 3 and combinations 2 and 4. Because combination 1 had the lowest mean errors, it was indicated to measure the C_3 source concentration in the clarified apple juice. However, as evidenced by the statistical analysis, combination 3 could also be used for this purpose (Table 4).

Figueira, Ducatti & Venturini Filho (2010) verified the legality of soft drink apple sold in the Brazilian market through carbon isotope analysis. In their study, the best result for the C_3 source quantification was obtained using the isotope values of the purified sugar fraction extracted from concentrated juice (δa) and the purified sugar fraction extracted from laboratory-fabricated soft drink (δp). This observation indicates the necessity for the individual development of the isotope method, even among beverages made with the same raw material (apple).

TABLE 4 - COMPARISON BETWEEN THE THEORETICAL AND PRACTICAL VALUES OF SOURCE C_3 AND THE ERROR ESTIMATE IN COMBINATIONS OF δA AND δP IN LABORATORY-FABRICATED CLARIFIED APPLE JUICE

Nº	Sugar (%)[1]	% C_3 Theorical[2]	1		2		3		4	
			C *vs.* J	Error (%)[3]	C *vs.* SJ	Error (%)	SC *vs.* J	Error (%)	SC *vs.* SJ	Error (%)
50	0.00	100.00	100.80	0.80	100.80	0.80	100.40	0.40	100.40	0.40
51	2.50	80.77	80.90	0.13	80.63	0.14	80.91	0.14	80.32	0.45
52	5.00	67.74	67.13	0.61	65.60	2.14	66.87	0.87	65.34	2.40
53	7.50	58.33	58.55	0.22	57.19	1.14	58.32	0.01	56.96	1.37
54	10.00	51.22	51.16	0.06	51.03	0.19	50.96	0.26	50.83	0.39
55	12.50	45.65	45.87	0.22	45.41	0.24	45.69	0.04	45.23	0.42
56	15.00	41.18	41.18	0.00	40.92	0.26	41.02	0.16	40.76	0.42
57	17.50	37.50	37.39	0.11	39.69	2.19	37.24	0.26	39.53	2.03
58	20.00	34.43	34.46	0.03	36.19	1.76	34.33	0.10	36.05	1.62
Mean				0.24b[4]		0.98a		0.25b		1.06a
Standard deviation				0.28		0.86		0.26		0.81

[1]sugar (m/m) (-12.63 ‰); [2]% theoretical C_3 source concentration (item 2.1.); [3]|% theoretical C_3 source concentration – %practical C_3 source concentration|; [4]Tukey's Test (α = 0.05); C *vs.* J - clarified concentrated juice (δa) *vs.* laboratory-fabricated clarified juice (δp); C *vs.* SJ - clarified concentrated juice (δa) *vs.* purified sugar extracted from laboratory-fabricated juice (δp); SC *vs.* J - purified sugar extracted from clarified concentrated juice (δa) *vs.* laboratory-fabricated clarified juice (δp); SC *vs.* SJ - purified sugar extracted from clarified concentrated juice (δa) *vs.* purified sugar extracted from laboratory-fabricated juice (δp).

Combination 1 (clarified concentrated juice - δa *vs.* laboratory fabricated clarified juice - δp) (Table 4) was used in items 3.4 and 3.6.

3.4 DEFINITION OF THE ISOTOPE VALUES FOR δa AND δb, METHOD ACCURACY AND ERROR ESTIMATE

To calculate the C_3 source concentrations in laboratory-fabricated juices, in δa (concentrated juice - Table 1) the isotope values of -27.66 ‰ (sample 1), -27.45 ‰ (mean value between samples 1 and 2) and -27.24 ‰ (sample 2) were used. For δb (sugarcane - Table 2), the values -13.06 ‰ (sample 17), -12.72 ‰ (mean) and -12.51 ‰ (sample 18) were used. For δp, the isotope values of laboratory-fabricated juices (Table 3) were used according to the result item 3.3.

In the covariance analysis, the *F* Test values were meaningful ($p < 0.05$). The Tukey's Test showed statistical difference between combinations 2 and 3. However, the combination 1 did not differ from the others. As the patterns of δa (-27.45 ‰) and δb (-12.72 ‰) from combination 2 provided the lowest mean errors, these patterns, along with the total error (±1.34 %) (Table 5), were used to measure the C_3 source concentration in the commercial clarified apple juices.

TABLE 5 - DEFINITION OF THE BEST ISOTOPE VALUES OF δa AND δb TO QUANTIFY THE C_3 SOURCE CONCENTRATION IN COMMERCIAL CLARIFIED APPLE JUICES

N°	Sugar (%)[1]	% C_3 Theorical[2]	1		2		3	
			-27.66 ‰ (δa); -13.06 ‰ (δb)	***Erro* (%)[3]**	**-27.45 ‰ (δa); -12.72 ‰ (δb)**	***Erro* (%)**	**-27.24 ‰ (δa); -12.51 ‰ (δb)**	***Erro* (%)**
50	0	100.00	100.+82	0.82	102.24	2.24	103.67	3.67
51	2.5	80.77	80.34	0.43	81.94	1.17	83.37	2.60
52	5	67.74	66.16	1.58	67.89	0.15	69.31	1.57
53	7.5	58.33	57.33	1.00	59.13	0.80	60.56	2.23
54	10	51.22	49.73	1.49	51.60	0.38	53.02	1.80
55	12.5	45.65	44.32	1.33	46.23	0.58	47.66	2.01
56	15	41.18	39.45	1.73	41.41	0.23	42.84	1.66
57	17.5	37.50	35.55	1.95	37.54	0.04	38.97	1.47
58	20	34.43	32.53	1.90	34.56	0.13	35.98	1.55
Mean				1.36ab[4]		0.64b		2.06a
Standard Deviation				0.52		0.70		0.70
Total Error (±)[5]				1.87		1.34		2.76

[1]sugar (m/m) (-12.63 ‰); [2]% theoretical C_3 source concentration (item 2.1.); [3]|% theoretical C_3 source concentration – % practical C_3 source concentration|; [4]Tukey's test (α = 0.05); [5]Total error = mean of the error + standard deviation.

The isotope values of δa (-27.45 ‰) and δb (-12.72 ‰) from combination 2, along with the total error (±1.34%), were used in item 3.6.

3.5 LEGAL LIMIT FOR CLARIFIED APPLE JUICES

To determine the legality of commercial clarified sweetened juice, it was necessary to create a legal limit. This calculation was performed in relation to the clarified apple juices with concentrations of soluble solids of 10.5, 11.0 to 14.0 °Brix with the addition of 10 % sugar (BRAZIL, 2009). Utilizing Equation 4 it was possible to calculate the minimum C_3 source concentrations of commercial clarified juices with 10 % sugarcane (m/m) (Table 6).

TABLE 6 - MASS BALANCE FOR CLARIFIED APPLE JUICES PRODUCED WITH 10 % SUGARCANE (m/m)

Nº	Juice (ºBrix)	Juice (g)	Sugar (ºBrix)	Sugar (%)	Sugar (g)	Sweetened juice (g)	Sweetened juice (ºBrix)	Legal limit (%Source C_3)
33	10.5	250	100	10	25	275	18.64	51.22
34	11.0	250	100	10	25	275	19.09	52.38
35	11.5	250	100	10	25	275	19.55	53.49
36	12.0	250	100	10	25	275	20.00	54.55
37	12.5	250	100	10	25	275	20.45	55.56
38	13.0	250	100	10	25	275	20.91	56.52
39	13.5	250	100	10	25	275	21.36	57.45
40	14.0	250	100	10	25	275	21.82	58.33

The legal limit (Table 6) was used in item 3.6 for separating the adulterated juices from the unadulterate juices.

3.6 ISOTOPE ANALYSIS AND LEGALITY OF COMMERCIAL CLARIFIED APPLE JUICES

The two brands of sweetened clarified apple juice found in the Brazilian market were identified by the sample numbers 41 and 42. The relative isotope enrichment was -20.90±0.02 ‰ and -15.90±0.01 ‰, respectively. Jamin *et al*. (1997) obtained isotope values for apple juice manufactured in Europe ranging from -26.00 to -25.10 ‰. These values were lighter than the ones found in samples 41 and 42. This observation does not mean that the European apple juices contained less sugar than the juices manufactured in Brazil. In Europe, the sugar is produced from beet sugar (Source C_3). Therefore, even with added sugar, Europeans juices have isotope values that are characteristic of those from source C_3. In Brazil, beet sugar is not added in fruit juices due to the high cost compared to sugarcane and fruit juices.

To calculate the C_3 source concentration in commercial clarified apple juices, the isotope values of -27.45 ‰ (δa) and -12.72 ‰ (δb) were used (item 3.4). In δp, the isotope values of commercial clarified juice were used (item 3.3). For each quantification of C_3 source, the total error of ±1.34 % was inserted (item 3.4).

Taking the C_3 source concentration and the ºBrix of commercial clarified juices (Figure 1), together with the legal limit (Table 6), it was possible to verify that sample 41 was in accordance

with Brazilian legislation. However, sample 42 certainly contained more sugarcane than the quantity established by the MAPA, and therefore classified as adulterated (Figure 1).

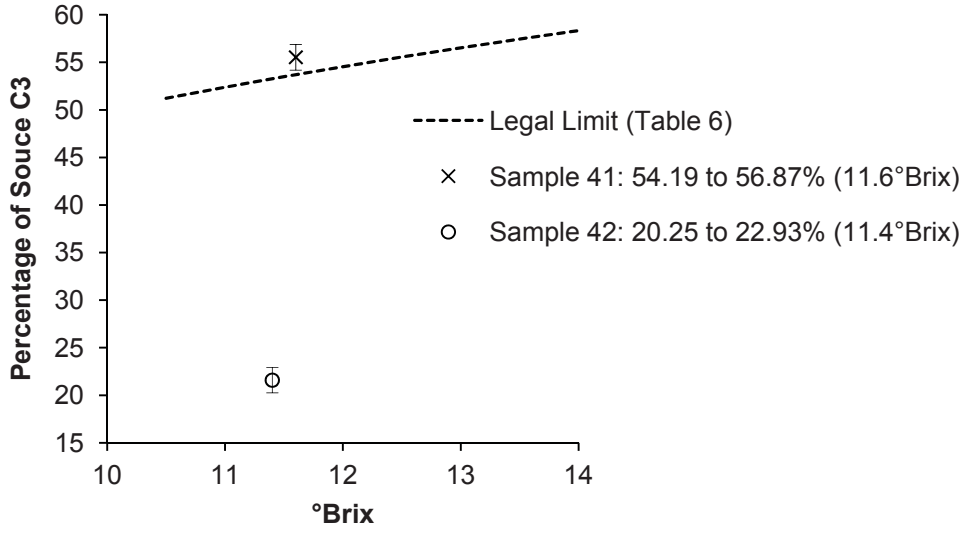

FIGURE 1 - CARBON CONCENTRATION FROM C_3 SOURCE IN COMMERCIAL CLARIFIED APPLE JUICES

4 CONCLUSION

The creation of a legal limit was an important methodological innovation that made it possible to identify beverages with a quantity of sugarcane above that allowed by Brazilian legislation. However, the legal limit can only be calculated when the MAPA determines the minimum proportion of soluble solids (°Brix) of the raw material that originates from the fruit.

The small number of clarified apple juice samples meant that it was not possible to conclude that the adulteration of this product is a common practice in non-alcoholic apple beverages.

The carbon isotope analysis methodology ($^{13}C/^{12}C$) based on the C_3 and C_4 photosynthetic metabolisms made it possible to identify the legality of the clarified apple juices.

Even working with database for isotope values of raw materials (concentrated juice and sugarcane), the proposed method enabled the efficient measurment of the C_3 source concentration in these beverages. By following the steps set out in this study, this methodology can be applied to other clarified fruit beverages as a means to verify their legality.

RESUMO

MÉTODO DE ANÁLISE ISOTÓPICA ($\delta^{13}C$) EM SUCO CLARIFICADO DE MAÇÃ

Os objetivos deste trabalho foram desenvolver método de análise isotópica para quantificar o carbono do ciclo fotossintético C_3 em sucos clarificados de maçã comerciais 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). Produziu-se a bebida em laboratório conforme a legislação brasileira. Também foram produzidos sucos adulterados com quantidade de açúcar de cana acima do permitido. Nas análises isotópicas mensurou-se o enriquecimento isotópico relativo dos sucos clarificados de maçã e de sua fração açúcar purificado. Com esses resultados estimou-se a quantidade de fonte C_3 por meio da 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. Duas marcas de suco clarificado de maçã foram analisadas. Relacionando a concentração de fonte C_3 e o °Brix dos sucos clarificados comerciais, juntamente com o limite de legalidade, foi possível verificar que uma amostra certamente contém mais açúcar que a quantidade estabelecida pelo MAPA. O limite de legalidade constituiu importante inovação metodológica que possibilitou identificar as bebidas que estavam em inconformidade com a legislação brasileira. A metodologia

desenvolvida provou ser eficiente para quantificar o carbono de origem C_3 em sucos clarificados comerciais de maçã.

PALAVRAS-CHAVE: ADULTERAÇÃO; CARBONO-13; IRMS; Malus domestica; AÇÚCAR DE CANA.

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