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k_0-INAA as a contributor in nutrition and health: multielemental determination in *Stevia rebaudiana* Bertoni, leaves and stevioside product

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Abstract
Concerned about food safety and considering the advantages that bring the use of the powerful INAA technique based in k_0 method, we have decided to perform a complete multi element determination in the leaves and in the product stevioside, of *Stevia rebaudiana* Bertoni, which use as a sweetener has increased in the last decade. Using *Stevia* leaves from different origin, thirty seven elements were determined using k_0-INAA; copper was quantified using FAAS, while sixteen elements were determined in the stevioside product from different manufacturers. The results indicated that it is necessary to make a strict quality control on the content of elements in leaves, which are used both as a raw material of the stevioside product, as a sweetener.

Keywords Additives in nutrition · k_0-method · Neutron activation analysis · *Stevia rebaudiana* Bertoni

Introduction

Stevia rebaudiana Bertoni is the name of an herb of the Asteraceae family, native from the north east of Paraguay and extended to Brazil and Argentina in areas where a tropical and wet weather exist. Today its cultivation has spread to other regions of the world. At present it is known because of its sweetening properties due to the high content of sweet diterpene (4–20%) in dry leaf matter. Among the 230 species in the genus *Stevia*, only the species rebaudiana and phlebophylla produce steviol glycosides [1].

This last compound is the sweet principle which was isolated (1909) and the extract purified (1931) to produce stevioside, which is about 300 times sweeter than saccharose, allowing its use as a substitute of it. People diagnosed with diabetes, hypertension or obesity is the most in use. It is considered for the Codex Alimentarius as a food additive [2, 3].

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It is well known that the nutrients are the basic components of food and enable our body to grow and stay healthy. They are divided into six classes: carbohydrates, lipids, proteins, vitamins, minerals and water. The first three are macronutrients and the following two, micronutrients. Minerals are important to maintain specific concentrations of certain inorganic ions in intracellular and extracellular fluids. For a good health it is necessary some quantity of inorganic elements that the body did not synthesise [4]. One way to obtain them is through food, thereof the importance to know the multielemental composition of the sweetener *S. rebaudiana* Bertoni, leaves and stevioside.

Considering that one of the many applications of neutron activation analysis technique is for the analysis of biological samples for its excellent sensitivity, free of analytical blank and the easy sample preparation, it is a good contributor to research into the roles of inorganic elements in nutrition, physiology, pathology and toxicology [5]. The analysis of the stevioside it is not an easy task by other analytical techniques such as; ICP-OES, ICP-MS, or AAS where samples should be analysed in form of solution, the sample form a kind of emulsion with acids, the best way to digest it could be the calcination of sample, which implies a lot of manipulation.

Authors concerned about food safety, would like to investigate and provide the most complete information about the multielemental content in this novel additive food that allows characterize it, as well as, to enhance the use of neutron activation analysis technique in this type of applications.

Experimental

Samples and comparators preparation

For NAA, two samples of leaves, from different provenance place of the country (sample A and sample B, respectively), were collected from the market and carried to the laboratory to be milled using a cryogenic mill. Then a portion of 300 mg were weighed and prepared pellets of 13 mm diameter. Two sets of three replicates of each sample were prepared to be irradiated (one set for short lived radionuclides and the other for intermediate and long lived radionuclide determination). As well, two samples of processed *Stevia* were acquired from de market. One manufactured in Colombia (sample C) and the other one in Brazil (sample D). Two sets of three replicates of pellets of 300 mg were also prepared for irradiation.

A reference material IAEA-336, trace and minor elements in lichen was also prepared in the same way that sample for quality control.

A set of sodium comparators were prepare depositing 0.200 mL of a primary standard sodium solution of 9990 ± 50 mg L^{-1}, in a disk of filter paper of 5.9 cm diameter, then allowed to dry to prepare pellets of 2 mm height and 13 mm diameter, using a hydraulic press. Other set of zinc comparators were prepare in the same way that sodium but depositing 0.100 mL of zinc standard solution of 9990 ± 50 mg L^{-1}. The samples and comparators kept the same shape and size.

For atomic absorption spectrometry, four replicates of 0.50000 ± 0.00500 g of each sample leave were weighted in a vessel to be pre-digested for 12 h and then, digested with 10 mL of nitric acid suprapur quality, 2 mL of perchloric acid was added after total digestion of organic material and boiled until dryness and white fumes. Then, sample was quantitatively transferred to a volumetric flask of 25 mL, using ultra-pure water in nitric media and it was analyzed by flame method. A blank test of reagents was also considered. In this case the standard reference material was used as quality control: SRM NIST 1515, Apple Leaves.

Irradiation and measurement

For the quantification of short lived radionuclides; ^{28}Al, ^{38}Cl, ^{42}K, ^{24}Na, ^{27}Mg, ^{56}Mn, ^{51}Ti and ^{52}V, samples were packed in polyethylene vials with sodium comparators for irradiation during 600 s at 0.320 MW using the pneumatic transfer system at 10 MW reactors (RP-10). The thermal neutron flux at this facility was 1.0×10^{12} $cm^{-2}s^{-1}$ and the epithermal neutron flux was 2.3×10^{10} cm^{-2} s^{-1}. The *f* and α parameter at this irradiation position were 60.9 ± 1.0 and 0.033 ± 0.004, respectively.

After a decay time of 300 s samples were measured to a distance of 185 mm using a HPGe detector (Canberra GC7019; relative efficiency = 70%, FWHM = 1.9 keV at 1332.5 keV ^{60}Co).

For intermediate and long lived radionuclides, samples were packed in an aluminium can for irradiation during 9000 s in a well thermalized core position at 10 MW power reactors. The thermal neutron flux at this position was 1.8×10^{13} cm^{-2} s^{-1} and the epithermal neutron flux is 2.1×10^{11} cm^{-2} s^{-1}. The parameters at this irradiation position *f* and α were 49 ± 2 and 0.33 ± 0.01, respectively. Gamma ray attenuation factor and flux variation inside the irradiation capsule was fixed no more than 1%.

After a decay time of 5 days, a first counting of samples was performed by 5000–6000 s using the HPGe -mentioned above. The comparators were measured by 1000 s after 6 decay days. The measurements were done to a distance of 12 mm from the detector cover. The radionuclides ^{76}As, ^{82}Br, ^{42}K, ^{140}La, ^{177}Lu, ^{99}Mo, ^{24}Na, ^{239}Np, ^{122}Sb, ^{153}Sm, ^{189}W and ^{175}Yb were determined. A second counting was performed after a decay time of 15–17 days by 10,000 s using the same detector and distance. The nuclides determined were ^{131}Ba, ^{141}Ce, ^{60}Co, ^{51}Cr, ^{134}Cs, ^{152}Eu, ^{59}Fe, ^{181}Hf, ^{147}Nd, ^{86}Rb, ^{46}Sc, ^{75}Se, ^{85}Sr, ^{182}Ta, ^{160}Tb, ^{233}Pa, ^{65}Zn and ^{95}Zr.

Gamma spectrum analysis was done using the Canberra software Genie 2000 (v 2.1) and for concentration calculations, an in house developed software application was used, based on an excel spreadsheet and macros written in the Visual Basic for Applications (VBA) tool from Microsoft [6].

A Perkin Elmer AAnalyst 800 atomic absorption spectrometer was used to measure copper in leave samples.

Results and discussion

Tables 1 and 2 shown the mass fraction obtained in the analysis of *Stevia* leaves and the stevioside, respectively, and the detection and quantification limits, calculated according to [7]. Results expressed with the symbol less

Table 1 Results of mass fraction in *Stevia rebaudiana* Bertoni expressed in mg kg^{-1}, dry weight. LOD and LQD calculated using sample B

Leaves ($n = 3$)				
Ele	Sample A	Sample B	Detection limit (LOD)	Quantification limit (LQD)
Al	249 ± 15	1058 ± 65	2.20	8.0
As	0.052 ± 0.003	0.635 ± 0.032	0.011	0.035
Ba	ND	18.0 ± 1.6	4.0	12
Br	12.50 ± 0.60	1.50 ± 0.070	0.17	0.55
Ce	0.700 ± 0.040	1.50 ± 0.10	0.17	0.50
Cl	531 ± 31	1470 ± 85	30	110
Co	0.095 ± 0.006	0.400 ± 0.024	0.004	0.010
Cr	6.00 ± 0.30	5.50 ± 0.30	0.08	0.24
Cs	0.022 ± 0.002	0.245 ± 0.020	0.010	0.034
Cu[a]	8.00 ± 0.20	15.0 ± 0.30	0.001	0.006
Eu	< 0.04	0.024 ± 0.004	0.013	0.04
Fe	198 ± 10	780 ± 39	5.0	15
Hf	0.13 ± 0.010	0.100 ± 0.010	0.010	0.020
K	29670 ± 1400	26590 ± 1280	70	220
La	0.260 ± 0.013	0.650 ± 0.030	0.0040	0.010
Lu	< 0.006	0.0090 ± 0.0010	0.002	0.006
Mg	1930 ± 150	2880 ± 220	255	850
Mn	51.0 ± 3.0	63.0 ± 4.0	1.0	4.0
Mo	< 6.0	10.0 ± 1.0	1.8	6
Na	22.0 ± 1.0	194.0 ± 9.0	0.43	1.0
Nd	< 1.0	< 1.0	0.40	1.0
Rb	6.00 ± 0.30	15.0 ± 1.0	0.25	0.78
Sb	< 0.010	0.030 ± 0.002	0.004	0.010
Sc	0.054 ± 0.003	0.260 ± 0.012	0.0025	0.01
Se	ND	0.200 ± 0.020	0.070	0.220
Sm	0.030 ± 0.001	0.140 ± 0.007	0.0010	0.0030
Sr	ND	23.5 ± 1.7	3.0	10
Ta	0.013 ± 0.001	0.020 ± 0.002	0.0035	0.010
Tb	ND	< 0.020	0.010	0.020
Ti	< 200	< 200	70	200
Th	0.130 ± 0.010	0.200 ± 0.010	0.010	0.020
U	< 0.070	< 0.070	0.020	0.070
V	< 1.0	1.80 ± 0.20	0.30	1.0
W	< 0.10	< 0.10	0.04	0.10
Yb	0.032 ± 0.003	0.062 ± 0.006	0.020	0.070
Zn	80.0 ± 4.0	82.0 ± 4.0	0.20	0.60
Zr	< 16	< 16	5.0	16

ND not detected

[a]AAS

than, means that, they were detected but the counting statistic was not good enough to be quantified. So the quantification limit is expressed.

The determination of the short-lived radionuclide Mg were corrected by the contribution of aluminum interference of the threshold reaction ^{27}Al (n,p) ^{27}Mg, being the correction factor 12.5. Similarly, to avoid interference in the determination of Mn by 843.7 keV photopeak the 1810.7 keV peak was evaluated instead of 846.7 keV photopeak. Due to the presence of ^{239}Np, the 103.18 keV photopeak of ^{153}Sm were corrected (1.89 correction factor). The interfering ^{27}Al nuclide in the determination of ^{24}Na due to the threshold reaction ^{27}Al (n,α) ^{24}Na, was not

Table 2 Results of mass fraction in stevioside expressed in mg kg^{-1}, dry weight. LOD and LQD calculated using sample C for As, Eu and sample D for other elements

Stevioside ($n = 3$)				
Ele	Sample C	Sample D	Detection limit (LOD)	Quantitation limit (LQD
Al	21.0 ± 1.0	34.0 ± 2.0	0.70	2.0
As	0.014 ± 0.0013	ND	0.0030	0.010
Ba	ND	< 1.0	0.30	1.0
Br	ND	< 0.050	0.020	0.050
Ce	0.051 ± 0.004	< 0.050	0.020	0.050
Cl	46.0 ± 3.0	814 ± 45	4.0	13
Eu	< 1.5	ND	0.37	1.5
Fe	0.430 ± 0.040	0.700 ± 0.060	0.090	0.30
K	4.4 ± 1.0	140 ± 40	108	340
La	0.022 ± 0.003	0.0100 ± 0.0010	0.0010	0.0020
Mg	< 170	< 170	53	170
Mn	< 0.25	0.500 ± 0.035	0.070	0.250
Na	95.0 ± 5.0	630 ± 30	0.320	1.0
Rb	ND	0.150 ± 0.020	0.030	0.100
Sb	< 0.010	< 0.010	0.0040	0.010
Zn	< 6.0	< 6.0	1.5	6.0

ND not detected

taken into account. Upcoming experiments will be carried out to evaluate this contribution.

The determination of copper by neutron activation analysis was complicated in leaves samples. In sample A it was not detected and in sample B it was detected but not quantified because of the poor counting statistics. The detection limit was 15 mg kg^{-1} and the quantification limit 50 mg kg^{-1}. The high content of manganese and sodium interfere. However, copper is among those elements determined frequently by atomic absorption spectrometry. It can be atomized relatively easily and exhibits practically no interferences in the air-acetylene flame, is virtually independent of the stoichiometry of the flame and the lamp current, and is therefore frequently used as a standard to test an instrument or procedure [8].

Sample B of *S. rebaudiana* leaves has more quantified elements that sample A. Some elements such as chlorine, cobalt, copper, chromium, iron, potassium, magnesium, manganese, molybdenum, sodium, selenium and zinc have known nutritional function and are significantly contained in *Stevia* leaves. Sodium, potassium and chlorine maintain adequate concentrations of salts in body fluids, cobalt is part of the B_{12} vitamin; copper is part of oxidative enzymes, interact with iron and help in the formation of hemoglobin; chromium act in the efficient use of insulin; manganese in mucopolysaccharide metabolism and superoxide dismutase enzyme; iron transports oxygen and electrons to the body, molybdenum is important in the metabolism and intestinal absorption of iron, selenium is a mineral that protect the body of the oxidative processes and it is part of the peroxidase enzyme and zinc is part of many enzymes related with the energetic metabolism. It is needed for the immune system [4]. Other group of quantified elements in Stevia leaves has unknown functions such as barium, bromide, rubidium, hafnium, antimony, titanium, vanadium. It is known that arsenic is highly toxic in its inorganic form. The Brazilian legislation through the Agência Nacional de Vigilância Sanitária (ANVISA), as well as, the international legislation referred in [9], establishes a maximum values to additives and inorganic contaminants in sugar. The international legislation recommends a maximum arsenic concentration of 0.5 mg kg^{-1} and the Brazilian one a value of 1.0 mg kg^{-1}. The concentration of this element found in leaves samples and in the stevioside, does not exceeds the value proposed; however arsenic content must be determined and assessed due to its cumulative effect in the body. Long-term exposure to arsenic from food can cause cancer and skin lesions. It has also been associated with development effects, cardiovascular disease, neurotoxicity and diabetes [10].

High mass fraction is shown in aluminum: 249 ± 15 and 1058 ± 65 mg kg in sample A and B, respectively. This element in leaves can derive from that which is present naturally, considering that is the third most abundant element (8%) of the earth's crust. Aluminum has been evaluated extensively in food additives. It can cause neurotoxicity and play a role in Alzheimer's disease, when the brain concentration exceeds 10–20 times the normal value [11]. A group of rare earth elements (REEs) cerium, lanthanum, lutetium, scandium, samarium, thorium and

ytterbium were also found in *Stevia* leaves. Vegetables can significantly transfer REEs from soil to edible part and can accumulate them [12]. There is no information available about the potential health effects of exposure to REEs.

Sixteen elements were detected in the stevioside product, only aluminum, chlorine, iron, potassium and sodium has significant concentrations. The last four elements are nutrients. Aluminum was evaluated by WHO and declared acceptable for daily intake [11]. Its presence could be due to either its use in the manufacturing process of the stevioside or because it was originally present in the raw material.

Table 3 shows the results in units of mass fraction (mg kg^{-1}) of the reference material IAEA-336 used as quality control. Although the reference material does not report the expanded uncertainty, the confidence interval 95% considered in the certificate is used to assess the E_n-number. The $|E_n| \leq 1$ obtained showed a satisfactory results for the elements analyzed by k_0-INAA technique. The NIST SRM-1515 Apple Leaves was used as quality control for copper determination by FAAS technique. It was obtained 5.76 ± 0.30 mg kg^{-1} being the certified value 5.64 ± 0.24 mg kg^{-1}. The En- number was 0.31. This is, as well, a satisfactory result.

Table 3 "E_n number" obtained in the performance evaluation of laboratory results

Element	$X_{Lab} \pm U$ ($k = 2$)	$X_{ref} \pm IC$	E_n-number
Al[a]	693 ± 43	680 ± 110	0.11
As	0.720 ± 0.040	0.630 ± 0.080	0.98
Ba	6.70 ± 0.60	6.40 ± 1.10	0.26
Br	12.20 ± 0.60	12.90 ± 1.70	0.39
Ce	1.310 ± 0.080	1.28 ± 0.17	0.18
Cl[a]	1992 ± 116	1900 ± 300	0.29
Co	0.240 ± 0.014	0.29 ± 0.05	0.96
Cr[a]	0.900 ± 0.047	1.06 ± 0.17	0.92
Cs	0.1160 ± 0.0010	0.110 ± 0.013	0.40
Eu[a]	0.0225 ± 0.0036	0.023 ± 0.004	0.08
Fe	462 ± 23	430 ± 50	0.58
K	1824 ± 88	1840 ± 200	0.07
La	0.570 ± 0.029	0.66 ± 0.10	0.85
Lu[a]	0.00540 ± 0.00040	0.0066 ± 0.0026	0.46
Mn	64.4 ± 4.0	63 ± 7	0.18
Na	312 ± 15	320 ± 40	0.20
Nd[a]	0.59 ± 0.11	0.60 ± 0.18	0.05
Th	0.150 ± 0.010	0.140 ± 0.020	0.29
Sb	0.068 ± 0.010	0.073 ± 0.010	0.44
Sc[a]	0.193 ± 0.010	0.170 ± 0.020	1.0
Se	0.255 ± 0.012	0.220 ± 0.040	0.84
Sm	0.118 ± 0.010	0.106 ± 0.014	0.80
Sr	9.9 ± 1.0	9.3 ± 1.1	0.42
Tb[a]	0.0130 ± 0.0014	0.0140 ± 0.0020	0.45
V[a]	1.40 ± 0.14	1.47 ± 0.22	0.28
Yb	0.0400 ± 0.0040	0.037 ± 0.012	0.21
Zn[a]	33.3 ± 1.6	30.4 ± 3.4	0.76

[a]RM information values

Conclusions

Neutron activation analysis has proven to be a powerful technique to characterize the food additive S*tevia rebaudiana* Bertoni and provide information to nutrition science.

Further studies must be continued to evaluate the presence of arsenic in leaves of *Stevia rebaudiana* Bertoni from different provenance.

Nutritionists could use the information provided by this paper to evaluate the acceptable daily intake (ADI) for food additives, which is an estimate of the amount of a food additive in food or beverages expressed on a body weight (bw) basis that can be ingested daily over a lifetime without appreciable health risk to the consumer. The acceptable value is 0–4 mg kg^{-1} bw [13].

The stevioside product of *S. rebaudiana* Bertoni, is safe enough to be considered a food additive in terms of mineral content. However, aluminum must be monitored considering it could cause Alzheimer's disease.

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