

Boron Isotope Dilution in Cellular Fractions of Coffee Leaves Evaluated by Inductively Coupled Plasma Mass Spectrometry with Direct Injection Nebulization (DIN-ICP-MS)

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A diluição isotópica do B enriquecido em 94,14 átomos % em ^{10}B fornecido durante o cultivo de mudas de cafeeiro foi avaliada na parede celular, núcleo e cloroplastos de células foliares. Para isto foi necessária a determinação de $^{11}\text{B}/^{10}\text{B}$ em pequenos volumes de amostras conseguidas pelo processo de fracionamento por centrifugação diferenciada. As determinações isotópicas foram efetuadas por espectrometria de massas com fonte de plasma acoplado indutivamente (ICP-MS) utilizando-se nebulizador de injeção direta (DIN). Razões isotópicas de B entre 1,002 e 1,326 foram determinadas em $50\ \mu\text{L}$ das amostras enriquecidas com precisão caracterizada por $\text{RSD} < 1,5\%$. As concentrações de B encontradas nas frações celulares variaram entre 3,3 e $10,8\ \mu\text{g g}^{-1}$, e em média os átomos % de ^{10}B foram 46,7; 44,5 e 48,8 na parede celular, núcleo e cloroplasto, respectivamente. O limite de detecção foi estimado em $0,5\ \text{ng B mL}^{-1}$.

Enriched ^{10}B (94.14 atom %) was supplied to coffee plantlets for three months. Then boron isotope ratios were determined in the leaf cell compartments, cell wall, nuclei and chloroplast, after a sub-cellular fractionation procedure. The isotopic measurements were performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) provided with a direct injection nebulizer (DIN), introducing a sample volume of $50\ \mu\text{L}$. Isotopic ratios from 1.002 to 1.326 were determined with precision characterized by RSD lower than 1.5% for the enriched cell fractions with B concentrations ranging from 3.3 to $10.8\ \mu\text{g g}^{-1}$. The detection limit (3σ) was $0.5\ \text{ng B mL}^{-1}$. The average enrichments in ^{10}B atom % found in the cell walls, nuclei and chloroplasts were 46.7, 44.5 and 48.8, respectively.

Keywords: isotope dilution, inductively coupled plasma mass spectrometry, biological cell fractionation, boron isotope ratios, plant analysis

Introduction

The interest in determining traces of boron has increased in recent years, since boron is an essential element for plants and may also be essential for animals and humans.¹ Besides, the application of ^{10}B in neutron capture therapy (BNCT) is increasing as well as its isotopic determination in biological tissues.²

Thermal ionization mass spectrometry (TI-MS) may be used for boron isotope ratio measurements with precisions ranging from 0.2 and 0.3%.³ The main disadvantage of this method is the time-consuming sample preparation. With inductively coupled plasma mass spectrometry (ICP-MS) boron concentrations and isotope ratios are determined with minimal requirements in sample preparation.⁴

The principal concerns when using ICP-MS for B isotopic determinations concern memory effects and mass discrimination. Memory effects are mainly due to interactions of B with the heated quartz torch. Some authors use washout times of several minutes to reduce boron memory.⁵⁻⁶ Others use a sodium fluoride solution,⁷ Triton X-100 surfactant,⁸ mannitol⁹ or ammonia¹⁰ as washing agents. These methods slowly remove the memory effect when solutions with more than $0.5\ \text{mg L}^{-1}$ of boron are analyzed. No memory effect occurs using direct injection nebulization (DIN). This device promotes liquid sample transport inside a capillary by pumping at high pressure, introducing its aerosol into the plasma, avoiding contact with the torch and spray chambers.¹¹⁻¹² Mass discrimination between ^{11}B and ^{10}B is accentuated due to the 10% isotopic mass difference and arises during ion transport caused by instrumental adjustments or by the sample matrix.¹³ The instrumental effect is corrected by analyzing a reference standard with a certified $^{11}\text{B}/^{10}\text{B}$ ratio (NIST SRM 951).¹⁴

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The sample matrix effect on mass discrimination can be corrected using internal standards.^{12, 15-16}

Also, for low-resolution quadrupole-based mass spectrometers, spectral overlap of ¹²C on ¹¹B could occur, which is relevant for B isotope measurements in biological digests. Evans and Krahenbuhl⁷ noted a significant interference of ¹²C on B when analyzing microwave-digested samples using ICP-MS in the normal resolution mode (0.8 u). In the high-resolution mode (0.6 u) Gregoire¹⁴ reported good results for geological solutions, but not for biological digests.¹⁷

In the present paper, intrinsic ¹⁰B labeled coffee leaves were submitted to a sub-cellular fractionation procedure. Inductively coupled plasma mass spectrometry with a direct injection nebulizer was used to determine boron isotope ratios from the separated cell walls, nuclei and chloroplasts.

Experimental

Apparatus

A VG plasma Quad PQII ICP-MS, (VG Elemental, Winsford, Cheshire, UK) was used for isotopic measurements. A computer controlled direct injection nebulizer system, DIN, model Microneb 2000 (CETAC Technologies, Omaha, NE, USA) provided with two six-port solenoid valves, a gas displacement pump (GDP), and a quartz torch formed by four concentric tubes was used. A fused-silica capillary is inside the middle torch tube, and with the other tubes the nebulization, the auxiliary and the plasma gases are introduced. The ICP-MS and DIN operating conditions are shown in Table 1. An inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 3000 DV (Perkin Elmer, Norwalk, USA) was

Table 1. DIN-ICP-MS operating conditions

Plasma	Forward power, W	1350
	Reflected power, W	<5
Argon flow rate, L min ⁻¹	Plasma	12.0
	Auxiliary	0.7
	Nebulizer	0.2
DIN	GDP pressure, kPa	827
	Nebulizer pressure, kPa	550
	Capillary, i.d.(mm)	0.075
	Carrier	2% (v/v) HNO ₃
Data acquisition	Detection Mode	Scan (m/z)10-11
	Channels/amu	19
	Dwell Time, μs	320
	Total acquisition time, s	30

employed for B and C determination at 249.773 nm and 193.3 nm, respectively. For sample preparation a Sorvall Super T21 refrigerated centrifuge (Du Pont, Wilmington, USA), a 801851 optical microscope (Nikon, Tokio, Japan) and silicone-treated paper (CalBiochem, San Diego, USA) were utilized for cell fractionation. A NT 245 water bath (Nova Técnica, Piracicaba, Brazil) and a 259E mechanical shaker (Fanem, São Paulo, Brazil) were used for sample preparation.

Reagents and solutions

Concentrated nitric and hydrochloric acids, pro-analyze quality (Merck, Darmstadt, Germany), purified by sub-boiling distillation, and water deionized to 18.2 MΩ cm in a Milli-Q system (Millipore, Bedford, MA, USA) were used throughout.

A stock solution of C (10000 mg L⁻¹) was prepared from (COOH)₂·2H₂O (Merck, Darmstadt, Germany). Stock solutions of B (1000 mg L⁻¹) were prepared from H₃BO₃ isotopic certified reference material, from National Institute of Standard and Technology (NIST SRM-951) and boric acid enriched in ¹⁰B (50 atom %), produced by the Instituto de Engenharia Nuclear (IEN, Rio de Janeiro, Brazil).

Working solutions of B (25 μg L⁻¹) and B + C (25 μg L⁻¹ + 2000 mg L⁻¹) were prepared by appropriate dilutions in 1 mol L⁻¹ HCl from the certified B stock solutions and C stock solution. Solutions of 2% v/v HNO₃, 0.05 mol L⁻¹ Tris-HCl (tris-[hydroxymethyl] aminomethane, Sigma, St. Louis, USA) and 0.3 mol L⁻¹ sucrose (Merck), adjusted to pH 7, were also prepared.

Samples

Coffee plantlets were cultivated in nutrient solutions for 3 months. Nine plantlets received H₃BO₃ enriched with 94.14 atom % ¹⁰B (enriched sample), and four plantlets received H₃BO₃ in natural isotope composition (control sample). At the end of the treatment plants were collected and separated into leaves, stems and roots.

Sample preparation

Samples of leaves, stems and roots from six plants were rinsed in deionized water and dried at 50 °C in a drying oven. The dried samples were ground until passing through a 20-mesh screen. After that, the extraction of B was carried out by using 100 μg of sample mixed with 5 μL of 1 mol L⁻¹ HCl and heating in a water bath at 80 °C for 15 min. The cold solutions were shaken for 1 h at 250 rpm and filtered (Whatman-40).

The leaves of seven plants were destined for cell fractionation and isotopic analysis. The cell fractionation steps are shown in Figure 1.

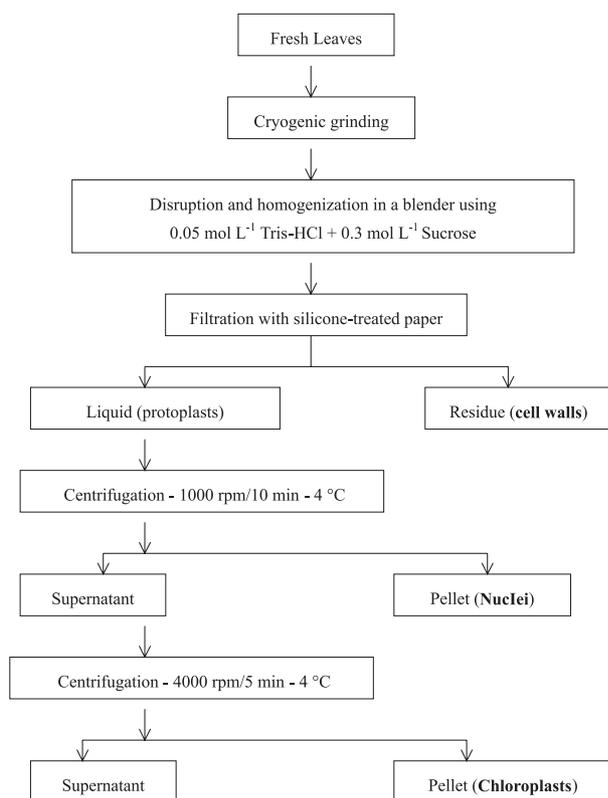


Figure 1. Scheme of the cell fractionation process used in this research to separate cell walls, nuclei and chloroplasts of coffee leaves.

The fractionation procedure was performed at cold-room temperature (10 °C) using N₂ cryogenic grinding and a refrigerated centrifuge in order to minimize possible enzymatic changes in the sub-cellular components. The rupturing of the cell wall was performed in a blender using Tris-HCl and sucrose. This medium preserved the structure of the cell components since it constituted of an isotonic solution that guarantees the osmotic activity while the buffering characteristics of Tris-HCl maintained the pH during the release of organic acid from the ruptured vacuoles. Confirmation of each separated component was corroborated by optical microscopy. Then, the B present in the separated fractions (cell walls, nuclei and chloroplasts) were extracted with 1 mol L⁻¹ HCl. A blank solution of the extractor was used to determine the limit of detection.

Results and Discussion

Total B concentrations were determined in leaves,

stems and roots from three replicates of two controls and four plants that received the enriched ¹⁰B. No difference in total B concentrations was noticed between samples with and without enriched B. The B concentrations were 78.3 ± 2.6 μg g⁻¹ in leaves, 55.6 ± 2.6 μg g⁻¹ in stems and 69.1 ± 2.4 μg g⁻¹ in roots.

From 2 g of fresh leaves and after the fractionation steps, around 800 μg of cell walls, 200 μg of nuclei and 300 μg chloroplasts were separated. Sub-samples of 100 μg were extracted, producing around 4 mL of solution for determining total B concentration and the isotope ratios. Results presented in Table 2 correspond to total B determined by ICP-OES in each cellular fraction of the different samples. The low variance of results in each fraction reflects the high reproducibility of the process. The average content of B in the fractions was around 20 μg per g of fresh leaves with 70% of humidity, that means that 83% of the total B is in the analyzed cell compartments.

Table 2. Total boron concentration (μg g⁻¹) in the cell fractions (Mean values, n=6)

Coffee leaves	Mean ± SD		
	Cell walls	Nuclei	Chloroplasts
Control - 01	10.8 ± 0.3	5.2 ± 0.1	3.3 ± 0.1
Control - 02	9.9 ± 0.4	5.9 ± 0.2	3.1 ± 0.1
Enriched - 01	11.6 ± 0.2	6.1 ± 0.1	3.3 ± 0.1
Enriched - 02	10.4 ± 0.3	5.6 ± 0.1	3.2 ± 0.1
Enriched - 03	9.3 ± 0.1	5.2 ± 0.1	3.2 ± 0.1
Enriched - 04	10.6 ± 0.1	6.3 ± 0.1	3.2 ± 0.1
Enriched - 05	11.4 ± 0.3	5.4 ± 0.1	3.0 ± 0.1

The small sample volumes obtained after B extraction were used to perform several analyses using the DIN system. Injected volumes of 20, 50 and 100 μL of a 25 μg L⁻¹ B standard solution were transported through the capillary to the plasma by the 2% v/v HNO₃ carrier solution pressurized by gas at 827 kPa. Under these conditions the transient signals presented in Figure 2 were attained monitoring *m/z* 11. A rapid increase after sample injection, a steady state dependent on the injected volume and a long slowdown characterize the peaks. Returning the injection valve to the original position the baseline is attained faster. The B isotope ratios were measured during 30 s, thus, a 50 μL sample volume was chosen. The ICP-MS data acquisition was synchronized with the DIN injection, programming a delay of 5 s to start acquisition after sample injection to ensure that data were collected at the peak maximum.

Results of boron isotope measurements by ICP-MS were affected by mass discrimination due to the gas pressure

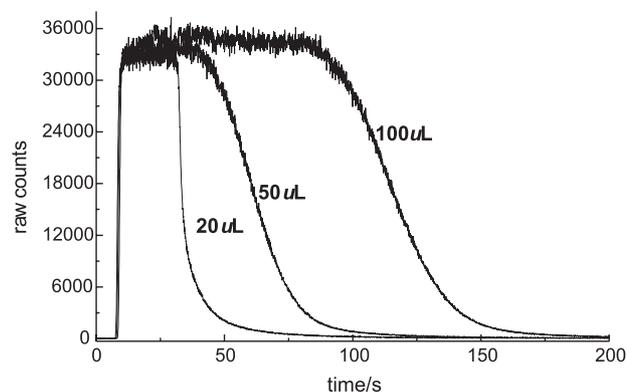


Figure 2. Transient signals at m/z 11 obtained with ICP-MS by injection using DIN of discrete volumes (25, 50 and 100 μL) of a 25 $\mu\text{g L}^{-1}$ solution of B.

forming the aerosol by DIN, as shown in Figure 3. The nebulization conditions affect ^{10}B and ^{11}B measurements, consequently, the $^{11}\text{B}/^{10}\text{B}$ ratio. The closest isotope ratio for the certified standard solution NIST 951 (4.044) was

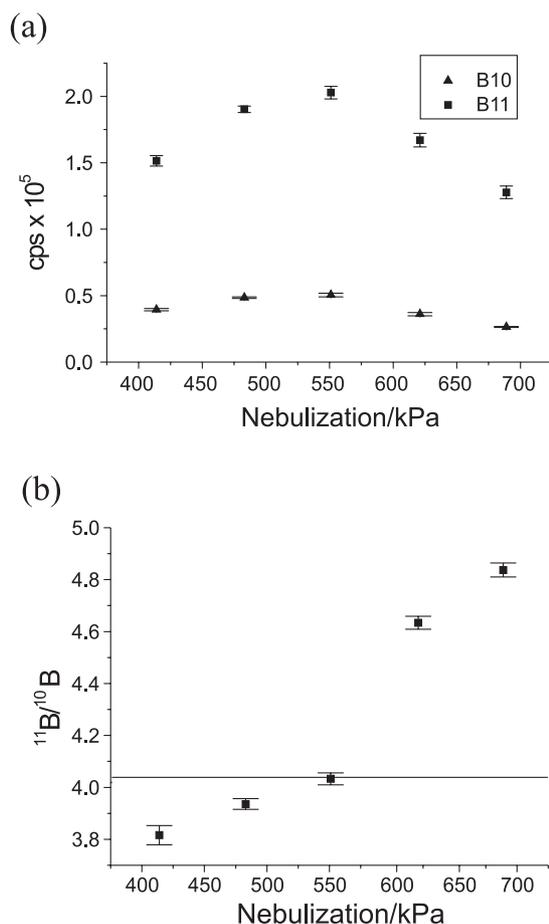


Figure 3. Effect of DIN gas nebulization pressure on B isotopes signals (a) Sensitivity in counts per seconds (cps) for ^{11}B and ^{10}B (b) $^{11}\text{B}/^{10}\text{B}$ measurements.

obtained at 550 kPa. The total mass bias on B isotope ratio determination was calculated by comparing the reference and measured values of a 25 $\mu\text{g L}^{-1}$ of NIST 951. The mass bias was less than 3%, which was lower than reported early values.¹⁸⁻¹⁹ The detection limit of 0.5 ng B mL^{-1} was calculated from data (3σ) obtained by 10 sequential measurements at m/z 10 of the blank solution.

The residual carbon contents in the sub-cellular solutions determined by ICP-OES were lower than 2000 mg L^{-1} . Adjusting the resolution to 0.6 u using the criteria of 10% peak height, the overlap of ^{12}C on the ^{11}B signals in the ICP-MS was minimized. This parameter was defined by analyzing a solution containing 25 $\mu\text{g L}^{-1}$ of the IEN standard ($^{11}\text{B}/^{10}\text{B} = 1$) alone and with 2000 mg L^{-1} of C.

Results presented in Table 3 correspond to B isotope ratios corrected for instrumental mass bias, following the recommendations of Gregoire.¹⁴ The atom % of ^{10}B were calculated from the relation: atom % $^{10}\text{B} = 100 / R + 1$, with R being the isotope ratio. The precision of the $^{11}\text{B}/^{10}\text{B}$ measurements was characterized by RSD lower than 1.5%. The control samples presented isotope ratios from 3.976 to 4.062, which are close to those reported in other agricultural studies.⁴ Isotope ratios ranges from 1.111 to 1.171 in the cell walls, from 1.195 to 1.340 in the nuclei and from 0.9920 to 1.074 in the chloroplast samples were

Table 3. Boron isotope ratios in the cell fractions of the samples

Sample		$^{11}\text{B}/^{10}\text{B}$	Atom % ^{10}B
Control - 01	Cell walls	4.053 \pm 0.054	19.7
	Nuclei	4.013 \pm 0.054	19.9
	Chloroplasts	4.021 \pm 0.035	19.9
Control - 02	Cell walls	4.059 \pm 0.016	19.7
	Nuclei	4.062 \pm 0.049	19.8
	Chloroplasts	3.976 \pm 0.073	20.1
Enriched - 01	Cell walls	1.123 \pm 0.012	47.1
	Nuclei	1.326 \pm 0.014	43.0
	Chloroplasts	1.058 \pm 0.012	48.6
Enriched - 02	Cell walls	1.146 \pm 0.016	46.6
	Nuclei	1.209 \pm 0.014	45.3
	Chloroplasts	1.058 \pm 0.013	48.6
Enriched - 03	Cell walls	1.156 \pm 0.015	46.4
	Nuclei	1.212 \pm 0.017	45.2
	Chloroplasts	1.002 \pm 0.010	49.9
Enriched - 04	Cell walls	1.124 \pm 0.012	47.1
	Nuclei	1.211 \pm 0.023	45.2
	Chloroplasts	1.058 \pm 0.016	48.6
Enriched - 05	Cell walls	1.140 \pm 0.016	46.7
	Nuclei	1.253 \pm 0.012	44.4
	Chloroplasts	1.065 \pm 0.009	48.4

found. These ratios reflect a slight discrimination between fractions. In terms of atom % ^{10}B , the chloroplasts present values on average 2 atom % higher than the cell wall and 4 atom % more than the nuclei. The ^{10}B atom % found in the samples was two times higher than that of the controls, as a consequence of the addition of 3 mg (94.14 atom %) of ^{10}B per plant.

Conclusions

The feasibility of evaluation of the isotope dilution of ^{10}B in compartments produced by cell fractionation of leaves was demonstrated by obtaining reliable experimental data using ICP-MS and DIN.

In terms of comparison with the control sample, the ^{10}B enrichment was approximately 29, 27 and 25 atom % in the chloroplast, cell wall and nucleus fractions, respectively, which were well discriminated by the proposed methodology.

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