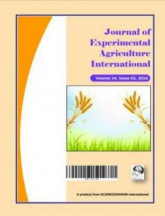

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Multiple Element Isotopic Analysis as a Tool to Discriminate the Geographical Origin of *Ipomoea violácea*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LMCP and MMPS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MCSM and ETS managed the analyses of the study. Author ARPS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed at testing the possibility of differentiating the geographical origin of *I. violacea* wild specimens by its carbon, nitrogen and oxygen stable isotopic compositions.
Study Design: Completely randomized design with 3 replications.
Place and Duration of Study: Sample: the laboratorial analyzes were carried out at the *Institute of Biosciences in Botucatu, Brazil* between January 2016 and December 2016.
Methodology: We collected the shoots of ten *I. violacea* wild specimens at each of the two selected regions in Brazil (Botucatu – SP and Três Lagoas – MS). Samples were dried in a forced-ventilation oven at 60ºC for 48 hours, and they were placed individually in plastic capped recipients containing

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lead spheres for the milling process. After that, the samples were weighed within individual capsules in a high precision balance and stored until analysis. For carbon and nitrogen analyses, tin capsules were used, and the average weight was 60 µg and 1100 µg, respectively. For oxygen analysis, the amount weighted was 170 µg within silver capsules. Isotopic analyses were performed by isotopic ratio mass spectrometry (IRMS) in triplicate. The isotope values were expressed in the standard delta notation (δ[‰]) with analytical errors of 0.2‰, 0.3‰ and 1.0‰ for carbon, nitrogen and oxygen, respectively. Results are presented as mean ± standard deviation. Principal component analysis was performed to define which isotope presents the greater variability.

Results: Results allowed to clearly differentiate *I. violacea* specimens collected from different regions. They provide the possibility of discriminating the geographical origin of *I. violacea* specimens by multiple element stable isotope analysis. Results also certified that multiple element isotope analysis can contribute to the building of a database containing the isotopic signature of *I. violacea* specimens with known origin.

Conclusion: The isotopic composition varied between plants of different regions. Thus, the technique is effective in *I. violacea*'s geographical origin discrimination.

Keywords: Stable isotopes; carbon-13; nitrogen-15; oxygen-18; LSA; morning glory.

1. INTRODUCTION

There are many psychoactive plants around the Earth, and they can be easily found due to its easy identification as well as already established geographical distribution. These plants do not share the same active principle, so the neurotoxic substances within them are different [1]. Historically, the use of a convolvulaceae with bell-shaped flowers named *Ipomoea violacea* was common in pre-Columbian rituals [2].

I. violacea has ergot-alkaloids of the LSD family such as ergine – or lysergic acid amide (LSA, from the German word lysergsäureamid) and its isomer, isoergine [3]. They are high activity L-tryptophan-derived natural products [4] which have affinity with the receptors of human serotonin and dopamine [5].

Recently, several countries (e.g. Brazil and U.S.) have listed LSA as a controlled substance, and thus the possession and/or distribution of LSA-containing materials within these countries are illegal. As a result, there have been interest in such materials particularly by law enforcement agencies.

The Brazilian Federal Police is seeking to find ways to map illicit plants in South America in order to create a database to compare the chemical signatures of seized plants with those of known origin plants [6]. Many studies utilized the isotopic signature, mainly from carbon and nitrogen, to identify traceability patterns of psychoactive substances or plants [7,8,9].

A previous study found significant differences in carbon and nitrogen isotopic composition between *I. violacea* specimens [10]. They analyzed leaves collected from both north and south of the Mobile-Bay Causeway in Mobile-Tensaw river delta, USA.

This study aims at testing the possibility of discriminating the geographical origin of *I. violacea* specimens collected at regions with distinct developing conditions. The experiment consisted of applying multiple element stable isotope analysis to determine carbon, nitrogen, and oxygen isotopic compositions of *I. violacea* specimens.

2. MATERIALS AND METHODS

We collected the shoots of ten *Ipomoea violacea* wild specimens in each of the two selected regions, Três Lagoas (TL), in Mato Grosso do Sul state, and Botucatu (BT), in São Paulo state. The relative location between the cities is shown in Fig. 1.

The TL region has coordinates 20º45'04" S and 51º40'42" W and is situated at 319 meters above sea level [11]. Its annual average temperature ranges from 24 to 26ºC, and the average annual precipitation varies between 1250 and 1450 mm (H_2O) yr-1 [12]. The characteristic biome within this city is the Brazilian cerrado and the soil is predominantly a Dark Red Latosol [13].

The BT region is situated in Midwest São Paulo state, has coordinates 22º53'09" S and 48º26'42" W, and is located at 804 meters above sea level [11]. Its annual average temperature ranges from 20 to 22ºC and the average annual precipitation ranges between 1250 and 1450 mm (H_2O) yr-1 [12]. The soil is mostly a Dystrophic Red Latosol

Fig. 1. Location of TL and BT regions of *I. violacea* on the map of Brazil (scale = 1:31,500,000 cm). A straight-line distance between the cities has approximately 409 km.

(27.71%) and an Orthic Quartzarenic Neosoil (24.47%) [14], and its observed biome is a transition between the Atlantic forest and the Brazilian cerrado [15].

Plant shoots were separated into leaves, flowers and seeds. Prior to isotopic analysis, samples were dried in a forced-ventilation oven (Marconi – MA 035) at 60ºC for 48 hours.

Then, the dry parts were placed individually in plastic capped recipients containing lead spheres for the milling process. Samples were subjected to low temperature (-196 ºC) for a few seconds by using liquid nitrogen and placed into a cryogenic mill (Spex 6700-230 freezer/mill – Spex Industries) at rotation frequency of 870 rpm for six minutes.

After that, the samples were weighed within individual capsules in a high precision balance scale (d = 1 µg; Excellence Plus Balance XP6 – Mettler Toledo International Inc.) and stored until analysis. For carbon and nitrogen analysis, tin capsules were used, and the average weight was 60 µg and 1100 µg, respectively. For oxygen analysis, the amount weighted was 170 µg within silver capsules.

Isotope analyses were performed by isotopic ratio mass spectrometry (IRMS) to determine the isotope ratio (r_{sample}) between the two most abundant isotopes, thus allowing the calculation of its isotopic composition (δX) using the following equation [10]:

$$\delta X = \left[\frac{r_{sample}}{r_{std}} - 1 \right]$$

where *X* is the heavier stable isotope (^{13}C, ^{15}N or ^{18}O), r_{sample} is the ratio of the heavier isotope to the light one (e. g. $^{13}C/^{12}C$), and r_{std} is based on an established reference material. The international reference standard for carbon, nitrogen, and oxygen isotopes are the Pee Dee Belemnite (PDB), the atmospheric air and the Vienna-Standard Mean Ocean Water (V-SMOW), respectively [7,10].

Carbon and nitrogen analyses were performed in an online system where the samples were

introduced individually in the elemental analyzer (EA 1108 – CHN – Fisons Instruments) by an autosampler to generate CO_2 and NOx. The latter was then reduced to N_2 by the presence of copper. The resulting gases were separated by gas chromatography and then analyzed in dual inlet IRMS system (Delta S – Finnigan MAT). All analyses were performed in triplicate. At the time between analyses, measurements of certified standard (IVA33802174 – Urea Isotopic Working Standard) were made.

The system used for oxygen analysis was automatic. Here, the elemental analyzer (Thermo Finnigan™ TC/EA) uses a pyrolysis system to convert organic matter into H_2 and CO gases that are separated by gas chromatography, and then, the formed CO is analyzed in dual inlet IRMS system (Delta V ADVANTAGE). This process was run in triplicate, and the reference standard was the aspartic acid (2-aminobutanedioic acid).

The isotope values were expressed in the standard delta notation (δ[‰]) with analytical errors of 0.2‰, 0.3‰ and 1.0‰ for carbon, nitrogen and oxygen, respectively. Results are presented as mean ± standard deviation.

Principal component analysis was performed to define which isotope had the greater variability (as described by [16]). The isotopic difference was assessed by discriminant analysis, allowing the distinction of the response groups of shoots' parts between regions. Mahalanobis distance was used to show the dissimilarity between parts of the shoots of different regions.

3. RESULTS

Plants grown in BT region were more enriched in carbon-13, nitrogen-15 and oxygen-18 (Table 1). By comparing $\delta^{13}C$ values among the parts of the shoots of different regions, the variation ranged from 0.04‰ to 3.14‰. Taking $\delta^{15}N$ values into account, the variations were between 2.11‰ and 2.84‰. And the variation observed in $\delta^{18}O$ values for shoots ranged from 0.87‰ to 6.68‰.

Principal component analysis showed the formation of distinct response groups for each part of the shoot, as well as the separation of whole plants within its corresponding region (Fig. 2). This was also showed by discriminant analysis ($p = 0.01$). The first component accounted for 55% of the total variability, and $\delta^{18}O$ was the most influential variable in the discrimination of this axis (Table 2). The second component accounted for 29% information, and it was more influenced by $\delta^{15}N$.

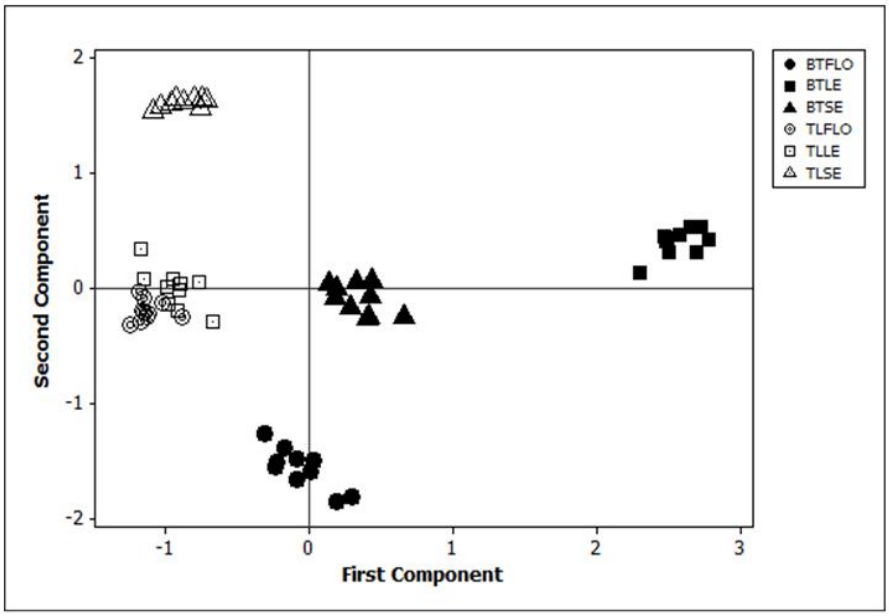

Fig. 2. Principal component analysis of parts of the shoot of *I. violacea* from different regions. BTFLO, BTLE and BTSE: Flowers, leaves and seeds from BT region, respectively; TLFLO, TLLE and TLSE: Flowers, leaves and seeds from TL region, respectively.

Table 1. Carbon, nitrogen, and oxygen isotopic compositions of *I. violacea*. means and standard deviations presented (n = 10). Flowers, leaves and seeds were from different regions of occurrence (BT and TL)

	Part of the shoot	BT	TL
$\delta^{13}C$ [‰]	Flower	-29.85 ± 0.14	-29.89 ± 0.08
	Leaf	-27.29 ± 0.12	-30.43 ± 0.12
	Seed	-28.23 ± 0.11	-29.53 ± 0.13
$\delta^{15}N$ [‰]	Flower	12.18 ± 0.34	9.85 ± 0.12
	Leaf	11.34 ± 0.15	9.23 ± 0.26
	Seed	11.06 ± 0.22	8.22± 0.08
$\delta^{18}O$ [‰]	Flower	26.11 ± 0.60	19.43 ± 0.40
	Leaf	19.29 ± 0.42	18.42 ± 0.35
	Seed	21.19 ± 0.36	19.23 ± 0.27

Table 2. Coefficients of principal components for delta variables of *I. violacea* samples

Variable	PC1	PC2	PC3
$\delta^{13}C$	0.582	0.564	-0.586
$\delta^{15}N$	0.466	-0.822	-0.327
$\delta^{18}O$	0.666	0.083	0.741
Variance [%]	0.553	0.291	0.157

Mahalanobis distance analysis showed that leaf had the greater dissimilarity between regions, with a squared distance of 910.67 (Table 3). For the flower and the seed, the squared distances were 122.36 and 183.19, respectively.

Table 3. Squared distances between parts of the shoots of *I. violacea* from different regions

	TLFLO	TLLE	TLSE
BTFLO	122.359		
BTLE		910.667	
BTSE			183.187

4. DISCUSSION

Multiple element stable isotope analysis is a useful tool for discriminating plants collected in distinct locations with varied developing conditions. This justifies its use in experiments aimed at gathering information about the geographical origin of *I. violacea*. Carbon, nitrogen and oxygen isotopic compositions of the plant vary as its developing condition varies. This allows the generation of an isotopic profile of the plant based on its region of occurrence.

Carbon isotopic composition is regulated by plants' photosynthetic pathway throughout the relationship between CO_2 concentrations inside the stomata (c_i) and in the atmospheric air (c_a) as described in the equation below [6]:

$$\delta^{13}C_{plant} = \delta^{13}C_{atm} - a - (b-a)\frac{c_i}{c_a}$$

where $\delta^{13}C_{atm}$ is the carbon isotopic composition of the atmospheric air, a is the CO_2 physical fractionation from the air to the stomata and b is the fractionation involved in the photosynthetic pathway [6].

The small variation found for carbon isotopic composition between regions was expected because the samples belong to the same species, and thus they displayed the same photosynthetic pathway. Such variation might be observable due to a difference in atmospheric CO_2 bioavailability within the regions. A previous study analyzed the carbon isotopic composition of *Ipomoea*'s leaves and found similar variations [10].

The photosynthetic mechanisms are Hatch-Slack-Kortschak (C_4), Benson-Calvin (C_3) and Crassulacean Acid Metabolism (CAM) [6]. A C_3-type plant has $\delta^{13}C$ values ranging from -35 to -24‰ [16]. Thus, by looking at the $\delta^{13}C$ results presented in this work, *I. violacea* is clearly a C_3-type plant.

The factors relative to nitrogen isotopic composition are dry and wet deposition, its fixation from the air by nitrogen-fixing bacteria and soil conditions [6]. Those factors may have different isotopic compositions that are transferred to the plant. Organic matter tends to have higher $\delta^{15}N$ values [7]. As a result, plants developed in nutrient-poor soils will display low $\delta^{15}N$ values. Samples from BT region showed

higher $\delta^{15}N$ values (Table 1). This could be due to region's nutrient-richer soil, as compared with the soil in TL region.

There were greater variations when compared with $\delta^{15}N$ values from a previous study [10]. This was due to the wider difference in plants' developing conditions between experiments. That wider difference was characterized by the distance between collection regions [17].

The most significant geographical indicator among elements of biological interest (e.g. hydrogen, carbon, nitrogen, oxygen and sulfur) is the oxygen isotopic composition [18]. It exhibits a systematic variation as a function of its developing region, i.e. it is extremely latitude-dependent. The $\delta^{18}O$ variation found between developing regions was expected because of their different latitudes. Besides, our results corroborated the significance of oxygen isotopic composition as an important geographical indicator (Table 2, Fig. 2).

Our results clearly differentiated *I. violacea* specimens collected within different regions (Fig. 2, Table 3). This proves the possibility of discriminating the geographical origin of *I. violacea* specimens by multiple element stable isotope analysis. Besides, finding greater isotope dissimilarity for leaves was beneficial, as *I. violacea* is a perennial rather than a deciduous plant. Results also certified that multiple element isotope analysis can contribute to the building of a database containing the isotopic signature of *I. violacea* specimens with known origin.

I. violacea plants differ in the total composition of their alkaloids as their native/geografic growth region varies [19]. Thus, the plant's psychoactive potentiality could be possibly estimated based on its region of occurrence. Besides, this multiple element stable isotopes approach, coupled with high performance chromatography methods, allows the verification, in further studies, of the relationship between the total composition of alkaloids and the region of occurrence of *I. violacea* samples.

5. CONCLUSION

The isotopic composition varied between plants of different regions, and thus the technique can be applied to discriminate the geographical region of *I. violacea*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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