

Original Research Article

Multiple element isotopic analysis as a tool to discriminate the geographical origin of *Ipomoea violácea*

ABSTRACT

Aims: The importance of this study is to test 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.

Place and Duration of Study: Sample: the laboratorial analyzes we were on *Institute of Biosciences, Botucatu, Brazil* between January 2016 and December 2016.

Methodology: We collected the shoots of ten *I. violacea* wild specimens in each of the two selected regions (Botucatu - SP, Três Lagoas – MS, Brazil). The samples we submitted at dry (60 °C for 48 hours in a forced ventilation oven), of dry parts were placed individually in plastic capped recipients containing 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 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. Isotopic analyzes were performed by isotopic ratio mass spectrometry (IRMS). Analyses were also made in triplicate. The isotope values were expressed in the standard delta notation ($\delta[\text{‰}]$) with analytical errors of 0.2‰, 0.3‰ and 1.0‰ for carbon, nitrogen and oxygen, respectively. Work results are presented as mean $\pm$ standard deviation. Principal component analysis was performed to define which isotope presents the greater variability.

Results: Our results clearly differentiated *I. violacea* specimens collected within different regions (Fig. 2; Table 3). This certifies 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 its already established geographical distribution. These plants don't 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, many countries (e.g. Brazil and USA) placed LSA under special control. This makes illegal to possess or to distribute LSA-containing materials within these countries and increases the interest in such materials, mainly by law enforcement agencies.

The Brazilian Federal Police is trying 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* [10]. They analyzed leaves collected both north and south of the Mobile-Bay Causeway in Mobile-Tensaw river delta, USA.

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

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

We collected the shoots of ten *I. violacea* wild specimens in each of the two selected regions. These regions are respectively comprised of the Brazilian cities of 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.



Fig. 1. Relative location between the regions on the map of Brazil (scale = 1:31,500,000 cm). A straight-line distance between the cities has approximately 409 km.

The TL region has coordinates 20°45'04"S and 51°40'42"W, and is located 319 meters above sea level [11]. Its annual average temperature ranges from 24 to 26 °C and the precipitation level varies between 1250 and 1450 mm(H₂O) yr⁻¹ [12]. The characteristic biome within this city is the Brazilian cerrado and the soil is predominantly a Dark Red Latosol [14].

The BT region is situated in Midwest São Paulo state and has coordinates 22°53'09"S and 48°26'42"W. The average height above sea level is 804 meters [11]. Its annual average temperature ranges from 20 to 22 °C and the precipitation level ranges between 1250 and 1450 mm(H₂O) yr⁻¹ [12]. The soil is mostly a Dystrophic Red Latosol (27.71%) and an Orthic Quartzarenic Neosol (24.47%) [13], and its observed biome is a transition between the Atlantic forest and the Brazilian cerrado [15].

We separated the plants' shoots in leaves, flowers and seeds. Prior to isotopic analysis, all samples were submitted to drying at 60 °C for 48 hours in a forced ventilation oven (Marconi – MA 035).

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

After that, the samples were weighed within individual capsules in a high precision balance (*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.

Isotopic analyzes were performed by isotopic ratio mass spectrometry (IRMS), these can determine the sample's isotope ratio (*r*_{sample}) between the two most abundant isotopes, thus allowing the calculation of its isotopic composition (δ*X*) throughout the following equation [10]:

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

Where *X* is the element's heavier stable isotope (¹³C, ¹⁵N or ¹⁸O) and *r*_{sample} is the ratio of the heavy isotope to the light one, e. g. ¹³C/¹²C. The *r*_{std} utilized for carbon, nitrogen and oxygen are the international standards values of PeeDee belemnite (PDB), atmospheric air and 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 CO₂ and NO_x. The last one was then reduced to N₂ 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.

For oxygen analyses, the system in use was also online. Here, the elemental analyzer (Thermo Finnigan™ TC/EA) uses a pyrolysis system to convert organic matter into H₂ and CO gases that are separated by gas chromatography, and then, the formed CO is analyzed in dual inlet IRMS system (Delta V ADVANTAGE). Analyses were also made in triplicate. In this case, the certified standard is 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. Work results are presented as mean ± standard deviation.

Principal component analysis was performed to define which isotope presents 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. And the Mahalanobis distance analysis was used to show the dissimilarity between parts of the shoots of different regions.

3. RESULTS

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

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
$\delta^{13}\text{C}$ [‰]	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
$\delta^{15}\text{N}$ [‰]	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
$\delta^{18}\text{O}$ [‰]	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

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 proved by discriminant analysis ($p = 0.01$). The first component represents 55% information, and $\delta^{18}\text{O}$ is the most influential variable in the discrimination of this axis (Table 2). The second component represents 29% information, and it is more influenced by $\delta^{15}\text{N}$.

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

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

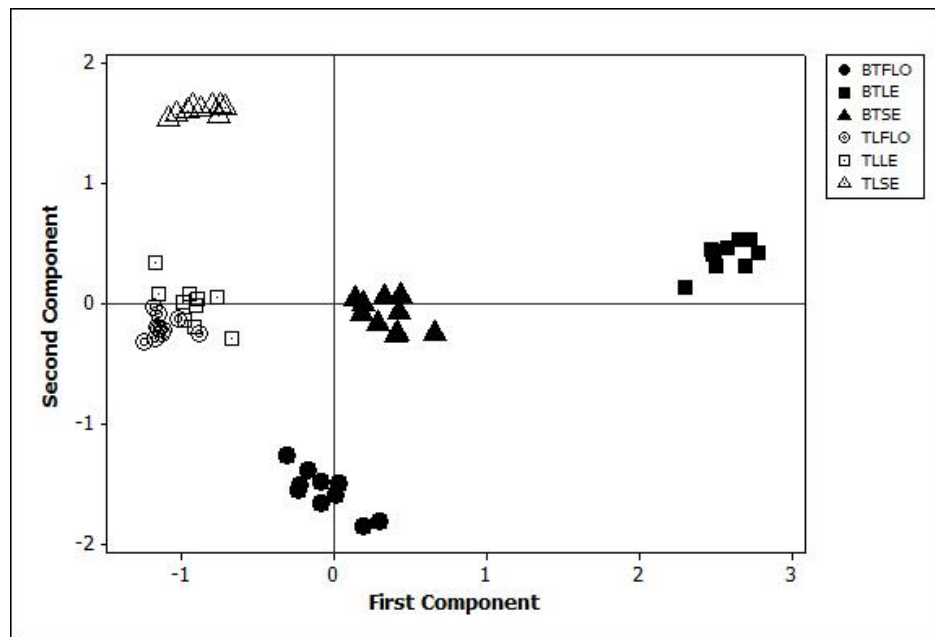


Fig. 2. Principal component analysis of parts of the shoot from different regions. BTFLO, BTLE and BTSE: flowers, leaves and seeds from BT region, respectively; TLFLO, TLLE and TLSE: flowers, leaves and seeds from TL region, respectively.

Mahalanobis distance analysis showed that leaf is the part of the shoot with greater dissimilarity between regions, with a squared distance of 910.67 (Table 3). For the flower and the seed, the squared distances are 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 in 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 CO₂ 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; hence they have 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 also 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 present low $\delta^{15}N$ values. Samples from BT region presented higher $\delta^{15}N$ values (Table 1). This could be due to region's nutrient-rich soil, as compared with the soil in TL region.

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

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 certifies 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 growth region varies [19]. Thus, the plant's psychoactive potentiality could be possibly estimated with a basis on its region of occurrence. Besides, this multiple element stable isotopes approach, combined 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. Thus, the technique is effective in *I. violacea*'s geographical origin discrimination.

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