

Chemotaxonomy of the Amazonian *Unonopsis* Species Based on Leaf Alkaloid Fingerprint Direct Infusion ESI-MS and Chemometric Analysis

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Unonopsis (Annonaceae) is a neotropical genus constituted by nearly fifty species, with fifteen described in Brazil. In the state of Amazonas seven species are found, including *U. guatterioides*, which displays problems from the botanical viewpoint. Previous studies showed this genus as a promising source of aporphinoid alkaloids. In order to investigate the potential of the leaf alkaloid fingerprint for chemotaxonomic approaches, twelve *Unonopsis* specimens, representing five species commonly found in the state of Amazonas were subjected to acid-base partitioning to yield the respective alkaloidal fractions. These fractions were analysed by direct infusion electrospray ionization multiple stage mass spectrometry (ESI-MSⁿ). The obtained data were treated through chemometric tools [principal components analysis (PCA) and hierarchical cluster analysis (HCA)]. Multivariate analysis pointed to aporphine, proaporphine and tetrahydroprotoberberine alkaloids as the responsible compounds for segregation of the investigated species, being these alkaloids tentatively identified by multiple stage mass spectrometry. The alkaloid fingerprint along with multivariate analysis provided a simple and effective approach to differentiate *Unonopsis* species commonly found in the state of Amazonas.

Keywords: alkaloid fingerprint, aporphinoid alkaloids, chemotaxonomy, *Unonopsis*

Introduction

Unonopsis (Annonaceae) is a Neotropical genus (Central and tropical South American regions) constituted by approximately fifty species, being twenty five relatively new.¹ This genus presents taxonomic problems clearly observed through the numerous attempts to position it within the Annonaceae family, as well as the difficulty of classify some species such as *U. guatterioides*, which recently had 13 species incorporated as synonyms.¹

In Brazil, fifteen *Unonopsis* species are described, some of them presenting restricted geographical distribution such as *U. duckei*. In the state of Amazonas besides *U. duckei*, the species *U. floribunda*, *U. guatterioides*, *U. rufescens*, *U. stipitata*, *U. spectabilis* and *U. veneficiorum* are also cataloged.¹ The leaves of some species are used for medicinal purposes, as example, leaves of *U. stipitata* and

U. veneficiorum are added in the food of indigenous people presenting brain disorders.²

Chemical studies point the *Unonopsis* genus as a promising source of aporphinoid alkaloids, being aporphine *sensu stricto*, oxoaporphines, azafluerenones, phenanthrenes and proaporphines recurrent in the literature.^{3,4} In addition, the triterpene polycarpol, considered a chemotaxonomic marker in Annonaceae family, has been identified in all *Unonopsis* species subjected to phytochemical approaches.⁵

Chemosystematics, the science by which chemical characters are applied in the formal systematics of plants, fungi, etc., is considered a useful tool, along with the morphology, anatomy and cytogenetics, in the systematic organization.⁶ However, it is observed that the success of this approach depends on the maximum accumulation of information about the distribution of secondary metabolites in a particular taxon.⁷ Considering the significant advancement of analytical instrumentation, mainly in the fields of mass spectrometry (MS) and chromatography,⁸ chemical analysis have become fast, and

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consequently a large amount of chemical data is obtained, making necessary the use of statistical tools for maximum exploitation of the information.^{9,10}

Electrospray ionization mass spectrometry (ESI-MS) fingerprinting has been demonstrated to be a useful technique for the screening of organic compounds of plant origin.¹¹ For example, the technique has been applied to the screening of alkaloids in *Unonopsis*⁴ and *Bocageopsis* species.¹² This technique provides immediate compositional information about ESI ionizable compounds through direct infusion, such as: products of acid-base, complexation and redox reactions,^{11,13} allowing the identification of characteristic compounds in extracts.^{14,15} Although direct infusion ESI-MS analyses present problems related to matrix effects, which results in reduced sensitivity and capabilities for metabolite identification,¹⁶ it has been successfully applied as a fast fingerprinting method.^{4,11,12,14} Some applications presented classification and prediction results comparable to LC-MS analysis.¹⁶

In this work, the alkaloid fractions from the leaves of the twelve *Unonopsis* specimens, representing five species commonly found in the state of Amazonas, were analyzed using direct infusion ESI-MS and multiple stage mass spectrometry (MSⁿ). The data were treated through chemometric tools to investigate the potential of the leaf alkaloid fingerprint for chemotaxonomic approaches.

Experimental

Plant material

For this study, twelve *Unonopsis* specimens were used. The leaves from the cited species were collected from the three main sites in the state of Amazonas: Distrito Agropecuário da SUFRAMA (DAS), Reserva Florestal Adolpho Ducke (RFAD) and Universidade Federal do Amazonas (UFAM) campus. From DAS, 4 specimens of *U. duckei*, 2 specimens of *U. floribunda*, and 1 specimen of *U. rufescens* were collected. From RFAD, 2 specimens of *U. duckei* were collected. From UFAM, 2 specimens of *U. stipitata* and 1 specimen of *U. guatterioides* were collected. All the species were sampled in September 2012. A voucher specimen of each individual is deposited according to Table 1.

Alkaloid extraction

The alkaloid fractions were obtained according to a previously reported method.¹² The leaves were immediately dried over ambient temperature (ca. 20 °C) during 20 days, then powdered. The pulverized (500 mg each, except

Table 1. *Unonopsis* collected in Amazonas state, Brazil

Plant	Code	Collection site	Voucher No.	Alkaloid fraction / mg
<i>U. duckei</i>	DRA	RFAD ^a	2627 ^d	1.2 (0.24%)
<i>U. duckei</i>	DRB	RFAD	3289 ^d	1.1 (0.22%)
<i>U. duckei</i>	DDA	DAS ^b	3478 ^e	1.1 (0.22%)
<i>U. duckei</i>	DDB	DAS	3504 ^e	1.2 (0.24%)
<i>U. floribunda</i>	FDA	DAS	6701 ^e	1.1 (0.11%)
<i>U. floribunda</i>	FDB	DAS	7394 ^e	1.0 (0.10%)
<i>U. rufescens</i>	RD	DAS	3767 ^e	1.1 (0.11%)
<i>U. duckei</i>	DDC	DAS	80 ^e	1.1 (0.22%)
<i>U. duckei</i>	DDD	DAS	610 ^e	1.2 (0.24%)
<i>U. stipitata</i>	SUA	UFAM ^c	8164 ^f	1.0 (0.20%)
<i>U. stipitata</i>	SUB	UFAM	8250 ^f	1.0 (0.20%)
<i>U. guatterioides</i>	GU	UFAM	8249 ^f	1.1 (0.11%)

^aReserva Florestal Adolpho Ducke; ^bDistrito Agropecuário da SUFRAMA; ^cUniversidade Federal do Amazonas; ^dherbarium of Instituto Nacional de Pesquisas da Amazônia (INPA); ^ebotany collection of Projeto Dinâmica Biológica de Fragmentos Florestais (PDBFF)/INPA; ^fherbarium of UFAM.

FDA, FDB, RD and GU, which were used 1,000 mg each) was vigorously stirred using a vortex mixer for 1 min with a mixture of 10% ammonium hydroxide (NH₄OH) aqueous solution (pH 11) (5 mL) and dichloromethane (CH₂Cl₂) (5 mL) in a glass container. The organic phase was transferred to another glass container and vigorously stirred (1 min) with a 10% acetic acid (CH₃CO₂H) aqueous solution (pH 0.5) (5 mL). The aqueous phase was removed, treated with NH₄OH to achieve pH 10 and extracted under vigorous stirring (1 min) with CH₂Cl₂ (5 mL). The organic phase was dried over anhydrous sodium sulfate, transferred to a new container and the solvent evaporated to dryness under a nitrogen gas stream to yield the crude alkaloid fraction. The obtained masses for the alkaloidal fractions are presented in Table 1.

Mass spectrometry analysis

Stock solutions (1 mg mL⁻¹) of the leaves alkaloidal fractions, were prepared with methanol. Aliquots (5 µL) of the stock solutions were further diluted to 5 µg mL⁻¹ and analyzed by direct infusion into the mass spectrometer.

All mass spectra were acquired in a continuous monitoring mode (Thermo LCQ Fleet Tune application) using a LCQ Fleet ion-trap mass spectrometer (Thermo LCQ Fleet, San Jose, CA, USA) with an electrospray ionization (ESI) interface and running in the positive ion mode to perform ESI-MS and ESI-MSⁿ analyses. Spectra were obtained from the mean of at least 10 scans *per*

spectrum. Samples were directly infused into the ion source through the instrument syringe pump ($10 \mu\text{L min}^{-1}$). The MS analytical conditions were: spray voltage, 5 kV; sheath gas, 10 arb; auxiliary gas, 5 arb; sweep gas, 0 arb; capillary temperature, $200 \text{ }^\circ\text{C}$; capillary voltage, 40 V; tube lens, 115 V; mass range, m/z 200 to 400. Helium was used as collision gas, and the ESI-MSⁿ spectra were obtained using collision energies ranging from 20 to 30%.

Chemometric analysis

The multivariate analysis was performed through the software Chemoface,¹⁷ version 1.5. Ions with intensity below 5% relative to the most abundant ion were neglected during data analysis.¹¹ Principal components analysis (PCA) was calculated through the variation of 54 variables, corresponding to the 54 registered ions. Hierarchical cluster analysis (HCA) was calculated through the Euclidian distances and average linkage of the first four principal components, whose cumulative variance represents 99.18%.

Results and Discussion

The fingerprint direct infusion of the leaves alkaloidal fractions from m/z 200 to 400 revealed several ions with even m/z values (Figures S1 to S12 in the Supplementary Information (SI) section), suggesting the potential presence of alkaloids. This proposal was based on previous studies with different isoquinoline-derived alkaloids with an odd number of nitrogens such as: aporphines,

tetrahydroprotoberberines, benzyloquinolines and proaporphines, where protonation process occurs in higher proportions when compared to other reactions.^{3,4,12} The base peaks corresponding to ions at m/z 268, 298 and 342 $[\text{M} + \text{H}]^+$ were observed in *U. guatteroides*, *U. duckei* and *U. stipitata* mass spectra, respectively, while the base peak at m/z 328 $[\text{M} + \text{H}]^+$ was observed in *U. floribunda* and *U. rufescens* mass spectra (Figure 1). Several others ions with even m/z values were observed pointing to complex samples with high diversity of alkaloids.

Analysis of the MS spectra revealed that 54 ions remained after elimination of the 5% least intense ions (Table S1 in the SI section). These 54 ions and their relative intensities were then subjected to the PCA and HCA analysis. In the PCA score plot (Figure 2a) were observed four main groups, being the specimens of *U. guatteroides* (group I), *U. stipitata* (group II) and *U. duckei* (group III) clearly separated. Group IV was constituted by *U. rufescens* and *U. floribunda* specimens, which is not surprising since these botanically close¹ species were recently suggested to be chemically close.¹⁸

According to the PCA biplot (Figures 2b and 2c), the ions at m/z 268, 298, 328 and 342 $[\text{M} + \text{H}]^+$ were the main responsible for the segregation of the groups I-IV. Through the MSⁿ spectra of these ions (Figures S13 to S17 in the SI section) were observed key fragmentations previously described for aporphine, proaporphine and tetrahydroprotoberberine alkaloids.^{3,4,12,19}

The MSⁿ spectra of the ions at m/z 268 and 298 $[\text{M} + \text{H}]^+$ presented strong evidence of the aporphine skeleton, being observed initial losses of 17 ($-\text{NH}_3$) (m/z 268 \rightarrow 251) and

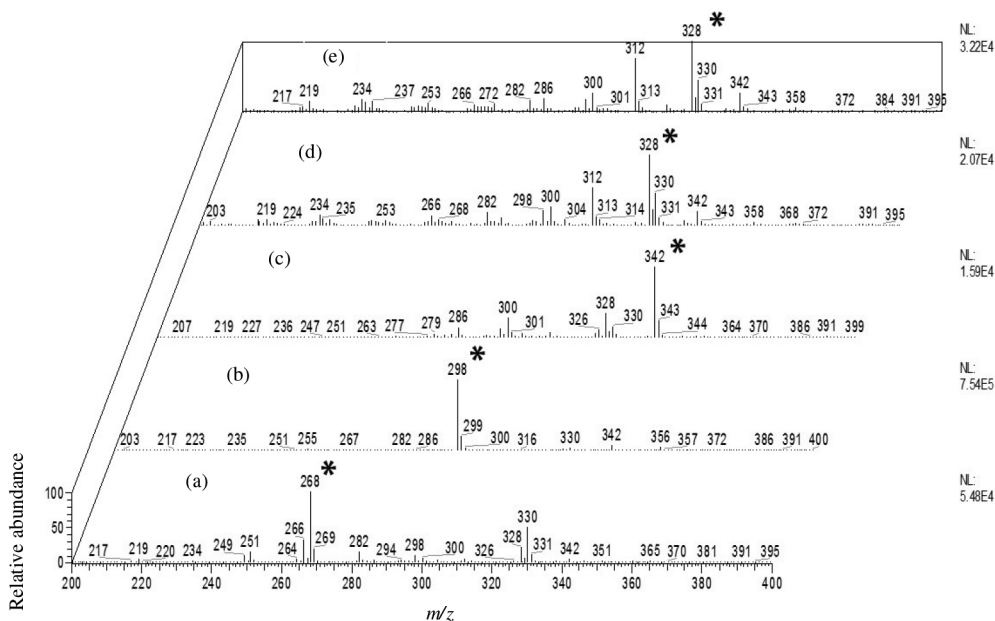


Figure 1. Total ion spectra of the alkaloidal fractions from leaves of *U. guatteroides* (a); *U. duckei* (b); *U. stipitata* (c); *U. floribunda* (d) and *U. rufescens* (e). The * denotes the base peaks for each sample.

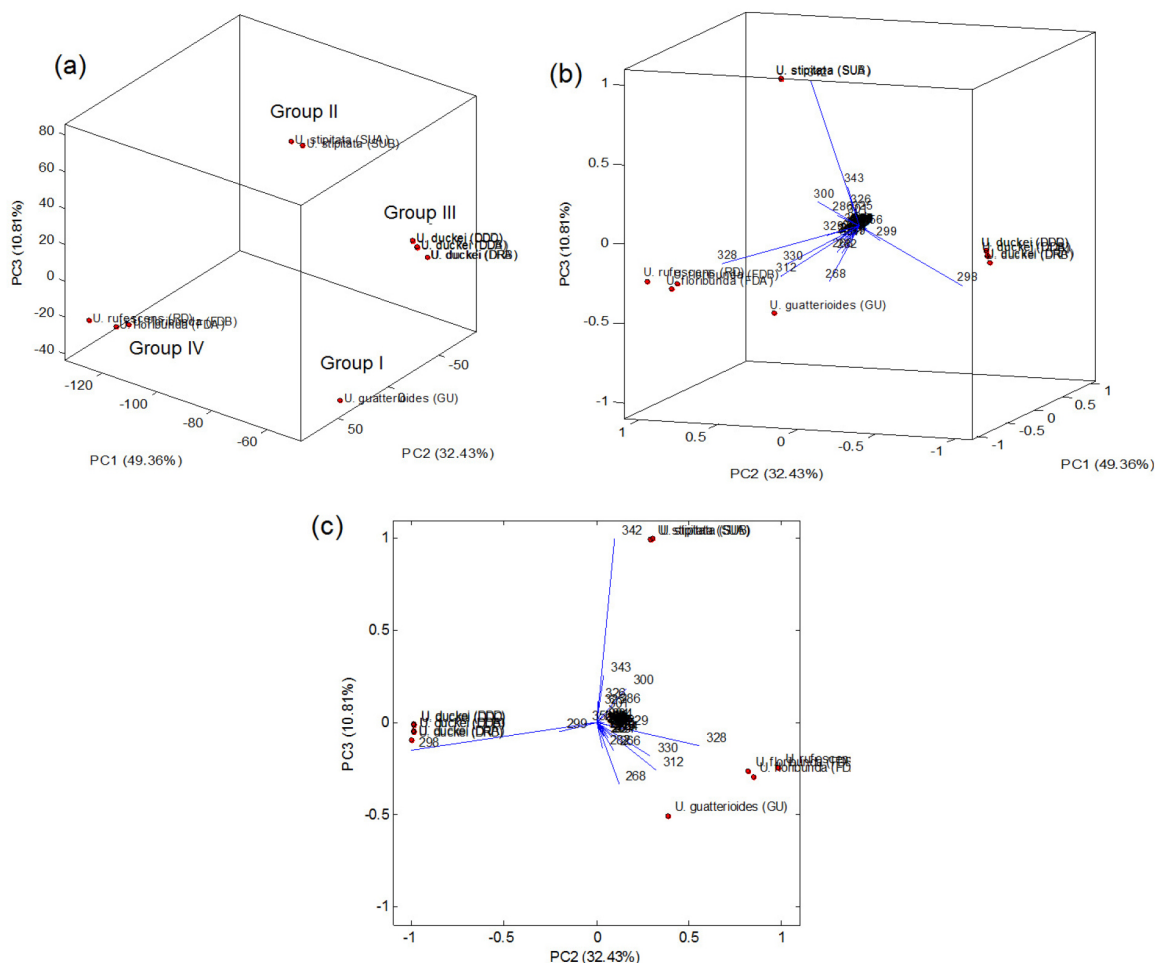


Figure 2. PCA score plot (a) and PCA biplots (b and c).

31 u ($-\text{NH}_2\text{CH}_3$) (m/z 298 \rightarrow 267), which is in accordance with the absence and presence, respectively, of a methyl at the heterocyclic nitrogen.¹⁹ In addition, subsequent losses of 32 u ($-\text{CH}_3\text{OH}$) (m/z 251 \rightarrow 219 and m/z 267 \rightarrow 235) and 28 u ($-\text{CO}$) (m/z 219 \rightarrow 191 and m/z 235 \rightarrow 207) were observed. These last fragmentations point to the presence of vicinal hydroxyl and methoxyl groups on aporphine skeletons.¹⁹ Fragmentation pathway observed to the ion at m/z 268 is consistent with the structure of the aporphine alkaloid asimilobine (**1**),^{3,4,12} previously reported in leaves of *U. guatterioides*,³ while the fragmentation observed to the ion at m/z 298 is in accordance with the structure of the proaporphine alkaloid glaziiovine (**2**), previously reported in leaves of *U. duckei*.⁴ In the MS^2 spectra of the ion at m/z 328 were observed several fragment ions highlighting an intense product ion at m/z 178 and a minor ion at m/z 151. High mass losses are unusual in aporphine skeleton but are commonly observed in benzyloquinolines and tetrahydroprotoberberines alkaloids.^{12,20,21} The major ion at m/z 178 and the minor at m/z 151 were previously described as key fragments for tetrahydroprotoberberine

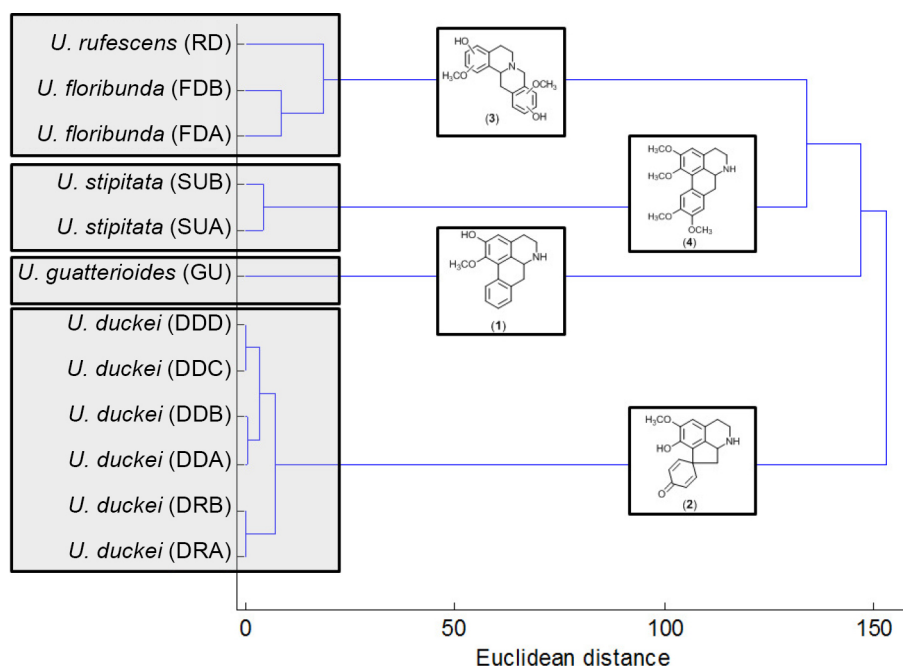
compounds containing methoxyl and hydroxyl groups at the ring A.¹² Although it is possible to predict the substitution patterns of this compound (scoulerine type) (**3**), it is not possible to guarantee the exact substitution positions once for this skeleton different positions with the same substituents will provide the same fragment ion.¹² In the MS^n spectra of the ions at m/z 342, besides the initial loss of 17 u ($-\text{NH}_3$) (m/z 342 \rightarrow 325), MS^3 losses of 15 u ($-\text{CH}_3$) (m/z 325 \rightarrow 310) and 31 u ($-\text{OCH}_3$) (m/z 325 \rightarrow 294), and subsequent MS^4 loss of 15 u ($-\text{CH}_3$) (m/z 310 \rightarrow 295) were observed. These key fragmentations are in accordance with the presence of aporphines skeleton containing a methyl group at the heterocyclic nitrogen and adjacent methoxyl groups.¹⁹ The fragmentation pathway observed to the ion at m/z 342 is consistent with the structure of the aporphine alkaloid norglaucine (**4**) previously reported in leaves of *U. duckei*.⁴ All the observed fragmentations were summarized in the Table 2.

Despite the high amount of alkaloids in the analyzed samples, PCA analysis showed that the tentatively identified asimilobine (aporphine), glaziiovine (proaporphine)

Table 2. Precursor ions and key fragments observed by ESI-MS^a experiments

Plant	[M + H] ⁺	MS ²	MS ³	MS ⁴	Alkaloid type
<i>U. guatterioides</i>	268 ^a	251 ^a (-17 u) ^b	219 ^a (-32 u)	191 (-28 u)	aporphine
<i>U. duckei</i>	298 ^a	267 ^a (-17 u)	235 ^a (-32 u)	207 (-28 u)	proaporphine
<i>U. stipitata</i>	342 ^a	325 ^a (-17 u)	310 ^a (-15 u) 294 (-31 u)	295 (-15 u)	aporphine
<i>U. floribunda</i>	328 ^a	178 (-150 u) 151 (-177 u)	–	–	tetrahydroprotoberberine
<i>U. rufescens</i>	328 ^a	178 (-150 u) 151 (-177 u)	–	–	tetrahydroprotoberberine

^aPrecursor ion; ^bneutral loss observed.

**Figure 3.** Dendrogram of HCA and the tentatively identified substances through fragmentation analysis.

and norglaucine (aporphine) play a fundamental role in the segregation of *U. guatterioides*, *U. duckei* and *U. stipitata* species, respectively. The unknown tetrahydroprotoberberine alkaloid was responsible for the formation of group IV, constituted by *U. floribunda* and *U. rufescens* species.

In the HCA dendrogram (Figure 3) were observed two subgroups in the *U. duckei* group, corresponding to different collection sites (RFAD and DAS). The chemical variability observed among these specimens was close to the observed between *U. stipitata* specimens (group II), while for the *U. floribunda* and *U. rufescens* (group IV) highest chemical variability was observed. The highest chemical similarity was observed between group II and IV, being this major group chemically close to the *U. guatterioides* (group I). Group III presented lower chemical similarity than the other groups.

In recent published work, the leaves essential oils of the same Amazonian *Unonopsis* species were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and the data treated by chemometric tools (PCA and HCA).¹⁸ Despite multivariate analysis showed significant differences between the species and their collection sites, the chemical variability among studied species was larger than presented in this work, suggesting that the non-volatile constituents (alkaloids) present better potential for chemotaxonomic purposes than volatile constituents (terpenoids). Another point that supports the use of the alkaloid fingerprint in chemotaxonomic approaches is the recurrent presence of these substances in *Unonopsis*. For example, glaziovine, norglaucine and glaucine were reported in the leaves of *U. duckei*,⁴ while asimilobine, anonaine, normuciferine, lisicamine and liriodenine were reported in the leaves of *U. guatterioides*.² The presence of tetrahydroprotoberberine

alkaloids in *U. rufescens* and *U. floribunda* species is an important fact, once this type of alkaloid was not reported in the *Unonopsis* genus. This observation along with previous chemical evidences¹⁸ could support a future approximation of these species. Morphologically, the difference between *U. floribunda* and *U. rufescens* is the glaucous monocarp and the thicker monocarp wall.¹

Conclusions

Multivariate analysis pointed to aporphine, proaporphine and tetrahydroprotoberberine alkaloids as responsables for segregation of five Amazonian *Unonopsis* species, being these compounds tentatively identified. New chemical evidences that support the botanical approximation between the close-related species *U. rufescens* and *U. floribunda* were found. The ESI-MS and ESI-MSⁿ data along with multivariate analysis provided a simple and effective approach to differentiate *Unonopsis* species commonly found in the state of Amazonas.

Supplementary Information

Supplementary information is available free of charge at <http://jbcs.sbc.org.br> as PDF file.

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