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Ecology

Free amino acids in nectar: its composition and variability among bat-pollinated plants

Aminoácidos libres en el néctar: composición y variabilidad en plantas polinizadas por murciélagos

Nelly Rodríguez-Peña [a], Kathryn E. Stoner [b], Jorge E. Schondube [c], Angel Durán-Díaz [d], Luis Barbo Hernández-Portilla [a, e], César Mateo Flores-Ortiz [a, e, *]

[a] *Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala, UBIPRO, Laboratorio de Fisiología Vegetal, Av. de los Barrios Núm. 1, Los Reyes Iztacala, 54090 Tlalnepantla, Estado de México, Mexico*

[b] *Colorado State University, Wildlife and Conservation Biology, Department of Fish, Fort Collins, 805223 Colorado, USA*

[c] *Universidad Nacional Autónoma de México, Centro de Investigaciones en Ecosistemas, Apartado postal 27-3 (Xangari), 58089 Morelia, Michoacán, Mexico*

[d] *Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala, Departamento de Bioestadística, Av. de los Barrios Núm. 1, Los Reyes Iztacala, 54090 Tlalnepantla, Estado de México, Mexico*

[e] *Universidad Nacional Autónoma de México, Facultad de Estudios Superiores Iztacala, Laboratorio Nacional en Salud, Av. de los Barrios Núm. 1, Los Reyes Iztacala, 54090 Tlalnepantla, Estado de México, Mexico*

*Corresponding author: cmflores@unam.mx (C.M. Flores-Ortiz)

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Abstract

Regardless of the low concentrations at which amino acids are present in floral nectar of bat-pollinated plants, their role as nectar flavor providers and their influence on bats' foraging decisions have been recognized. Nevertheless, variation in the free amino acids among bat-pollinated plant species has been less studied. The goal of this study was to analyze the nectar free amino acids and to determine its variability among 8 bat-pollinated plant species from 5 families in a Tropical Dry Forest in Mexico. Nectar collections were made opportunistically depending on plants flowering season. We quantified 17 amino acids by HPLC. All 17 free amino acids were present in nectar from the 8 analyzed plant species. The concentration of 12 amino acids is explained by plant species by 19-58%. Analyses showed that *Ceiba grandiflora* (Malvaceae) was significantly different in asparagine content when compared to *Bahuinia pauletia* (Fabaceae) and *Ceiba aesculifolia* (Malvaceae), and in glutamic acid when compared to *Ipomoea ampullacea* (Convolvulaceae). We discuss the importance of free amino acids in nectar among plant species and their influence on bat pollination ecology.

Keywords: Bat-pollinated plants; Free amino acid nectar types; Nectar chemistry; Nectar flavor; Pollination ecology

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Resumen

Independientemente de las bajas concentraciones a las que los aminoácidos están presentes en el néctar floral de las plantas polinizadas por murciélagos, se ha reconocido su papel como proveedores de sabor al néctar y su influencia en las decisiones de alimentación de los murciélagos. Sin embargo, la variación en los aminoácidos libres entre las especies de plantas polinizadas por murciélagos ha sido menos estudiada. El objetivo de este estudio fue analizar los aminoácidos libres en el néctar y determinar su variabilidad entre 8 especies de plantas polinizadas por murciélagos de 5 familias en un bosque tropical seco en México. La colecta de néctar se realizó de manera oportunista dependiendo de la temporada de floración de las plantas. Cuantificamos 17 aminoácidos por HPLC. Los 17 aminoácidos libres estuvieron presentes en el néctar de las 8 especies de plantas analizadas. La concentración de 12 aminoácidos se explicó por la especie de planta en 19-58%. Los análisis mostraron que *Ceiba grandiflora* (Malvaceae) fue significativamente diferente en el contenido de asparagina cuando se comparó con *Bahuinia pauletia* (Fabaceae) y *Ceiba aesculifolia* (Malvaceae), y en ácido glutámico en comparación con *Ipomoea ampullacea* (Convolvulaceae). Discutimos la importancia de los aminoácidos libres en el néctar entre las especies de plantas y su influencia en la ecología de la polinización de los murciélagos.

Palabras clave: Plantas polinizadas por murciélagos; Tipos de aminoácidos libres en el néctar; Química del néctar; Sabor del néctar; Ecología de la polinización

Introduction

In addition to sugars, proteins and free amino acids commonly occur in floral nectar of most plant species (Baker & Baker, 1975, 1977, 1982; Nicolson & Thornburg, 2007). Although free amino acids in nectar are known to influence the foraging behavior of pollinator guilds (Alm et al., 1990; Bluthgen & Fiedler, 2004; Hainsworth & Wolf, 1976; Ignell et al., 2010; Inouye & Waller, 1984; Rodríguez-Peña et al., 2013), it is unknown whether particular free amino acid types, constancy or variability in nectar confer advantages to the plant within the context of pollination ecology.

Several of the early studies within the field of nectar focused on describing sugars, amino acids, proteins, lipids and alkaloids among other metabolites, and their relation to plants' principal pollinator and plant taxonomy (Baker, 1978; Baker & Baker, 1973a, b, 1975, 1977, 1982, 1983, 1986). These studies show that nectar protein and amino acid concentration are related to the plant´s principal pollinator and usually differ among species. For example, they show that while the presence of single amino acids is constant within plant species, the nectar of bird and bat-pollinated plant species, compared to plants preferred by other pollinator types, is usually characterized as low in protein and amino acid total content as both bats and birds have alternative sources of these nutrients in their diet (i.e., pollen, fruit, insects). Recent studies within the context of pollination ecology have characterized the nectar protein and free amino acid total content of plant species from several regions and pollinator guilds (Galetto & Bernardello, 2004; Gottsberger et al., 1984; Herrera, 1989; Petanidou et al., 2006). A few other studies have focused on describing the free amino acid profile in nectar (Dress et al., 1997; Goldberg 2009; Nepi et al., 2012; Nicolson & Thornburg, 2007; Piechowski et al., 2010; Scogin, 1986; Tiedge & Lohaus, 2017).

The relative abundance of sugars and amino acid types is important for providing characteristic taste profiles (Baker, 1978; Gardener & Gillman, 2002; Nepi, 2014; Nicolson & Thornburg, 2007), and it is well known that particular amino acid flavors in nectar solution vary with its concentration (Birch & Kemp, 1989). The influence of amino acids on nectar taste is widely documented for insect-pollinated plants where particular mixes and types of free amino acids and non-proteic amino acids (Carter et al., 2006; Lanza & Krauss, 1984; Nepi et al., 2012; Petanidou, 2007; Petanidou et al., 2006, Potter & Bertin, 1988) have been characterized as phagostimulants (Gardener & Gillman, 2002; Hansen et al., 1998; Shiraishi & Kuwabara, 1970; Teulier et al., 2016). However, this is not well documented for plants pollinated by other guilds. In the case of bat-pollination interactions, nectarivore bats have the capacity to differentiate and to show preferences between the 3 sugars that commonly occur in nectar, regardless of their concentration (Ayala-Berdon et al., 2013); however, they do not exhibit preferences for sugars found in the nectar of flowers they usually consume (Herrera, 1999; Rodríguez-Peña et al., 2016). Further, when the role of nitrogen and the relative abundance of free amino acids were tested, bats showed a preference for the most concentrated sugar-only nectar but were indifferent when nectar contained nitrogen (Rodríguez-Peña et al., 2013). In fact, nectarivore bats were capable of discriminating the nectar flavor of a particular plant species provided by its free amino acid relative abundance, when compared with that found in the nectar of other species. This suggests that plants might manipulate the relative abundance of their free amino acids in nectar influencing the foraging decisions of their pollinators.

To date, only a few studies have analyzed the occurrence and relative abundance of some amino acids in nectar from a few bat-pollinated plant species (Goldberg, 2009; Gottlinger et al., 2019; Piechowski et al., 2010; Scogin, 1986). However, these studies mainly focused on identifying the occurrence of a few types of amino acids and documenting which of these have the highest concentration. The variation of amino acids in nectar from bat-pollinated species has yet to be determined. Identifying if particular amino acids vary among bat-pollinated plant species within a plant community will contribute to a better understanding of their significance in the context of pollination ecology. The overall goal of this study was to document the occurrence of 17 free amino acids in nectar and assess whether they differ among 8 bat-pollinated plant species from 5 families in a Tropical Dry Forest in Mexico. We focused on the following questions: *i)* do the same types of free amino acids occur in the nectar of bat-pollinated plant species? and *ii)* which free amino acid types differ in concentration among bat-pollinated plant species? Finally, we discuss the implications of our results in the context of bat pollination ecology.

Study site and plant species

Field collections were made at the Chamela-Cuixmala Biosphere Reserve on the central Pacific coast of Mexico (ca. 19º22’ - 19º35’ N, 104º56’ - 105º03’ W). The predominant vegetation type is tropical lowland deciduous forest with patches of riparian forest (Lott 1993).

Nectar collection

We sampled the 8 most common plant species in the study area and well documented for their importance to the diet of nectarivorous bats in this region: *Crescentia alata* (Bignoniaceae; n = 5); *Pachycereus pecten* (Cactaceae; n = 2); *Ipomoea ampullacea* (Convolvulaceae; n = 4); *Bahuinia pauletia* (Fabaceae; n = 5); *Ceiba aesculifolia* (n = 5), *Ceiba grandiflora* (n = 4), *Ceiba pentandra* (n = 5) and *Pseudobombax ellipticum* (Malvaceae; n = 5) (Herrera et al., 2001; Sánchez-Casas & Álvarez, 2000; Stoner et al., 2003). Sample collection was carried out opportunistically, following the flowering season of each plant species throughout a year period. In brief, one mature flower bud from different individuals of each species, were covered with mesh bags 1 h before sunset. Bags were removed 1 h after anthesis, and nectar was extracted with capillary glass tubes. In order to avoid possible nectar contamination with pollen grains amino acid content, particular care was taken to carefully remove stigma to one side of the flower, due to the capillary tube manipulation, until reaching the nectary. Nectar samples were immediately stored in eppendorf tubes at -80 ºC until analysis.

Nectar amino acid quantification

High Performance Liquid Chromatography (HPLC) was used to measure concentrations of 17 free amino acids: aspartic acid (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr) cysteine (Cys), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys) and proline (Pro). Analyses were performed in an Agilent 1100 Series (Hewlett Packard), following the Agilent 1090 series method, by using a two-step precolumn derivatization, with ortho-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for the secondary amines as previously published for *C. aesculifolia* (Malvaceae) and *Pachycereus pecten* (Cactaceae; Rodríguez-Peña et al., 2013). In brief, a 0.4 N borate buffer was used with pH 10.4. Separation was performed by using a Hypersil AA - ODS2.1 x 200 mm Agilent column. We used a solvent gradient system with 2 mobile phases. The first mobile phase (A) consisted of sodium acetate, trietilamine and tetrahydrofuran water mix, and the second mobile phase (B) consisted of sodium acetate, acetonitrile and methanol water mix buffers (pH 7.20). The gradient started with 100% A, at 17 minutes 60% B, at 18.1 minutes flow 0.45, at 18.5 minutes flow 0.8, at 23.9 minutes flow 0.8, at 24 minutes 100% B and flow 0.45, at 25 minutes 0% B. Detection was via a Perkin Elmer (LS50B) Luminescence Spectrometer (excitation at 340 nm and emission at 450 nm for primary amino acids and excitation at 266 nm and emission at 305 nm for secondary amino acids). Data collection was done by FL Win Lab Perkin Elmer software. Chromatograms were compared with authentic standards of individual amino acids with known concentrations for identification and quantification. Total concentration and percentage of each amino acid was calculated for each sample.

Data analysis

We used Kruskall-Wallis to analyze whether differences exist in the relative abundance of the 17 amino acids among the 8 plant species. We used eta-squared (η^2) as measure of effect size, in which, scales of magnitude indicate a small effect ($0.01 \leq 0.06$); moderate effect ($0.06 \leq 0.14$); and a large effect (0.14). Further, η^2 values multiplied by 100 indicate the percentage (%) of variance in the dependent variable (amino acid type) which is explained by the independent variable (plant species;

Snyder & Lawson, 1993), meaning that the larger the effect size is, the stronger the relationship between the 2 variables (Cohen, 1988; Lalongo, 2016; Tomczak & Tomczak, 2014). To find which pairs of groups differ significantly we used Dunn´s post-hoc tests. Analyses were performed in Statistica ® Version 7 and SPSS ® Version 20 (IBM, 2011).

Nectar free amino acids among bat-pollinated plant species

All 17 free amino acids were found in the nectar of the 8 plant species (Table 1). Kruskall-Wallis test showed significant differences in the concentration of Asp (H = 23.7; p = 0.001), Glu (H = 20.8; p = 0.004), Ser (H = 15.1; p = 0.035), Gly (H = 14.6; p = 0.041), and Cys (H = 19.9; p = 0.006), among all 8 of the bat-pollinated plants (Table 2).

When calculated η^2 values for Thr, Arg and Ile, a small size effect was found (0.01 ≤ 0.06); while for Asp, Glu, Ser, His, Gly, Tyr, Cys, Val, Met, Phe, Lys and Pro the obtained size effect was large (≥ 0.14); showing that in the case of these last twelve amino acids, plant species explained its concentration by up to 19 to 58% depending on the amino acid type. No effect size was detected for Ala and Leu (Table 3).

Finally, significant differences were found for Asp concentration between *Ceiba grandiflora* (Malvaceae) when compared with both, *Bahuinia pauletia* (Fabaceae; p = 0.005) and *Ceiba aesculifolia* (Malvaceae; p = 0.038); and for Glu concentration when compared to *Ipomoea ampullacea* (Convolvulaceae; p = 0.009). No significant differences were found among other plant species and amino acid types.

Free amino acid total concentration in nectar has been found to greatly vary among plant species (Baker & Baker, 1977; Galetto & Bernardello, 2004; Gardener & Gilman, 2001; Gottsberger et al., 1984; Scogin, 1986). However, due to the low concentration of free amino acids in the nectar of bat-pollinated flowers, it has been less studied in the past compared to flowers pollinated by other animals. This study provides the first attempt to identify differences in particular free amino acids in nectar among bat-pollinated plants collected in a uniform way within one study site. In this section, we discuss the ecological implications of our findings.

Table 1

Free nectar amino acids presence for 8 species and 5 families of bat-pollinated plants in a Tropical Dry Forest in Mexico.

Amino acid	Bignoniaceae	Cactaceae	Convolvulaceae	Fabaceae	Malvaceae			
	C. alata	*P. pecten*	*I. ampullacea*	*B. pauletia*	*C. aesculifolia*	*C. grandiflora*	*C. pentandra*	*P. ellipticum*
Asp	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Glu	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Ser	4/5	2/2	3/4	5/5	5/5	4/4	5/5	4/5
His	5/5	2/2	2/4	4/5	5/5	4/4	5/5	5/5
Gly	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Thr	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Ala	5/5	2/2	4/4	4/5	4/5	4/4	5/5	5/5
Arg	5/5	2/2	4/4	4/5	5/5	4/4	5/5	5/5
Tyr	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Cys	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Val	5/5	2/2	3/4	5/5	4/5	4/4	5/5	5/5
Met	5/5	2/2	4/4	5/5	4/5	4/4	5/5	5/5
Phe	5/5	2/2	4/4	5/5	4/5	3/4	5/5	5/5
Ile	4/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Leu	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Lys	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5
Pro	5/5	2/2	4/4	5/5	5/5	4/4	5/5	5/5

Numbers in parentheses indicate (particular amino acid occurrence / n = sampled flowers) for each plant species. For all plant species, n = 5, except *P. pecten* (n = 2), and *C. grandiflora* and *I. ampullaceae* (n = 4).

Table 2

Free nectar amino acid concentration (µg/ml) for 8 species and 5 families of bat-pollinated plants in a Tropical Dry Forest in Mexico.

Amino acid	Bignoniaceae	Cactaceae	Convolvulaceae	Fabaceae	Malvaceae			
	C. alata	*P. pecten*	*I. ampullacea*	*B. pauletia*	*C. aesculifolia*	*C. grandiflora*	*C. pentandra*	*P. ellipticum*
	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD	mean±SD
Asp	11.75±6.09	33.69±0.46	3.99±2.36	2.71±2.16	5.18±5.09	38.75±5.96	6.17±1.99	6.48±2.09
Glu	17.46±9.13	34.56±6.02	2.85±1.14	5.66±4.05	7.41±7.57	37.43±7.55	6.87±1.55	5.84±3.13
Ser	11.95±23.79	29.70±26.07	1.04±1.16	4.35±2.78	20.02±13.73	15.66±4.52	4.73±3.61	12.97±24.69
His	28.87±19.54	54.54±7.62	12.99±21.25	8.72±13.97	17.95±21.11	10.69±6.32	2.91±1.21	8.76±5.49
Gly	5.59±4.84	19.675.92	10.88±9.20	36.44±33.74	2.41±1.79	17.46±2.19	2.83±2.95	4.87±2.73
Thr	4.31±2.32	3.42±0.66	6.49±3.46	5.84±5.75	6.72±7.36	2.23±1.21	3.21±4.51	2.90±2.87
Ala	5.19±6.67	1.19±0.04	1.34±1.63	1.92±2.22	1.54±1.86	1.07±0.06	1.88±1.05	6.36±10.58
Arg	11.78±6.66	10.86±7.88	10.47±15.10	11.61±10.35	9.68±15.23	5.79±0.56	2.31±1.37	12.72±20.52
Tyr	2.03±1.75	15.29±14.47	4.68±3.92	1.65±1.49	0.40±0.26	0.23±0.10	1.62±1.73	1.44±0.59
Cys	3.23±2.03	1.32±0.82	5.96±4.20	4.79±4.90	0.36±0.35	0.83±0.07	0.33±0.25	1.09±0.69
Val	0.81±0.54	12.95±5.65	5.68±8.31	2.74±3.53	5.76±6.72	17.28±3.80	1.22±0.68	1.47±1.42
Met	1.11±1.28	5.53±3.39	0.75±0.32	5.17±5.42	1.82±1.71	1.95±0.26	2.90±2.95	0.83±0.62
Phe	6.20±6.39	0.89±0.46	0.78±0.52	2.461.79	1.04±0.75	0.16±0.11	1.20±1.24	0.78±0.17
Ile	7.95±4.77	12.59±5.69	9.05±2.40	8.65±3.81	10.63±4.91	9.54±0.33	12.55±5.65	5.23±4.69
Leu	14.44±17.10	23.44±1.39	51.35±52.88	20.32±24.07	18.10±8.91	3.21±2.79	9.64±5.13	22.35±24.24
Lys	14.65±5.42	32.46±1.25	35.29±22.78	30.63±11.11	27.52±21.79	24.05±4.71	23.33±3.36	170.05±208.67
Pro	7.44±2.70	32.08±29.14	8.69±9.28	8.66±3.25	23.68±32.76	2.34±1.28	18.40±16.27	2.52±1.48

For all plant species n = 5, except *P. pecten* (n = 2), and *C. grandiflora* and *I. ampullaceae* (n = 4).

Are the same types of free amino acids present in the nectar of all bat-pollinated plant species?

The presence of the 17 quantified free amino acid types in this study was found in all analyzed bat-pollinated plant species. This concurs with Baker and Baker (1977, 1986), who found that the occurrence of individual amino acids in floral nectar is generally constant within a species, and accordingly to Tiedge and Lohaus (2017). Our results clearly showed that most free amino acid types occur in floral nectars of bat-pollinated plants, although they occur in variable concentrations. In contrast with our findings, several other studies have reported fewer amino acids in bat-pollinated species. For example, Scogin (1986) only found 5 amino acids (Ala, Ser, His, Thr and Gly) in the nectar of *Cheirosemon platanoides* (Sterculiaceae) and *Kigelia pinnata* (Bignoniaceae). Similarly, Goldberg (2009) only found cysteine (Cys) in all nectar samples of *Helicteres baruensis* (Sterculiaceae) even though their study evaluated the same 17 free amino acids found in our study. In the bat-pollinated *Parkia pendula* (Fabaceae), Piechowski et al. (2010) documented 5 of 14 amino acids analyzed and only found proline (Pro) and one other unidentified amino acid in all 5 samples.

Results also showed that within the same species, not all amino acid types were present in all flowers. Nectar amino acids differences have been explained due that nectar composition reflects the amino acid composition of the phloem and nectaries owing to amino acids are leaching from nectaries (Nicolson & Thornburg, 2007). Also, carbon and nitrogen content in soil have been found to be related to nectar amino acids composition (Gijbels et al., 2014).

Which free amino acid types discriminate among bat-pollinated plant species?

Our results showed that particular amino acid concentrations were different among *Ceiba grandiflora* (Malvaceae) with *Bahuinia pauletia* and *Ceiba aesculifolia* and with *Ipomoea ampullacea* for Asp and Glu, respectively. It is well recognized that plants can regulate the feeding behavior of animals via manipulating nectar metabolites

Table 3

Nectar amino acids statistics by Kruskall-Wallis test and η^2 (%) for 8 species and 5 families of bat - pollinated plants in a Tropical Dry Forest in Mexico. Significant values ($p < 0.05$) are presented in bold.

Amino acid type	H	*p*-value	η^2 %
Asp	23.69	**0.0013**	58.1
Glu	20.82	**0.0041**	51.2
Ser	15.08	**0.0350**	29.9
His	12.86	0.0756	21.7
Gly	14.61	**0.0413**	28.2
Thr	8.21	0.3140	4.5
Ala	3.21	0.8651	0
Arg	7.91	0.341	3.4
Tyr	13.76	0.0555	25
Cys	19.91	**0.0058**	47.8
Val	12.39	0.0885	19.9
Met	12.82	0.0765	21.6
Phe	13.12	0.0691	22.7
Ile	7.57	0.3715	2.1
Leu	5.19	0.6363	0
Lys	12.79	0.0775	21.4
Pro	13.12	0.0691	22.9

Eta-squared (η^2) values are presented as percentages (%), indicating the variance in the dependent variable (amino acid type) explained by the independent variable (plant species).

such as sugars and free amino acids (Borghi & Ferni, 2017; Shoonhoven et al., 2005). While sugars constitute the energy content in nectar, free amino acids provide the discriminant taste and act as stimulators and contributors to the overall nectar palatability (Brito, 2011; Gardener & Gillman, 2002; Petanidou et al., 2006). Moreover, a positive relationship has been recognized between a particular amino acid taste in nectar and its concentration (Birch & Kemp, 1989). In the case of bats, only one study has evaluated the potential phagostimulant effect of amino acids and experimentally demonstrated that the flavor provided by the relative abundance of amino acids in nectar can be perceived and preferred by bats and influence their foraging decisions (Rodríguez-Peña et al., 2013).

Although free amino acid types were present in nectar from all bat-pollinated studied species, differences on amino acid concentrations were only found in 2 out of 17 amino acids types and among few plant species. Future studies should focus on the study of nectar amino acids ecology within a phylogenetical framework.

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