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Full Paper

Free Amino Acids Analysis in the Venom of the Social Wasp *Polistes lanio* Under Different Forms of Preservation

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Abstract*:* Venoms of social wasps have in their composition proteins, peptides, among others and several of these compounds were evaluated for their function in the venom, however, there is still no study of the quantification of free amino acids of venom of social wasps. The peptides and proteins of the venom can originate in the medium amino acids or other molecules by the rupture of the peptide bonds. Therefore, the objective was evaluated the composition of the free amino acid in venom of the social wasp *Polistes lanio* and validated a method for free amino acids analysis using different forms of preservation of this venom. For this, the venom of *P. lanio* was analyzed direct and partitioned (liquid-liquid with organic solvents) by High Performance Liquid Chromatography. For validation of the amino acids, parameters as linearity, stability and recovery of the assay method were evaluated by the analyzing it fresh or frozen, and before or after extraction with organic solvents. The free amino acids were recovered from these samples at rates ranging from 93.2-99.0%. The data obtained indicate that the freezing, storage and extraction with organic solvents does not lead to an increase in the amino acids content of proteins and peptides.

Keywords: DAD; HPLC; threonine; tryptophan

1. INTRODUCTION

Social wasps, together with some species of bees and all ants and termites constitute the social insects, which present reproductive division of labor with presence of castes (queen and workers), cooperative brood care and overlap of adult generations [1]. The evolutionary success of these insects is directly related to the efficiency in communication, exchange of information and maintenance of cohesion in the tasks of their colonies [2]. This efficient mechanism of communication is carried out by acoustic, visual, tactile, magnetic and mainly chemical signals [3].

In addition to efficiency in communication, social wasps are highly effective in catching prey and defending their colonies [1], and for this purpose they have a specific apparatus known as the sting apparatus [4], capable of injecting toxic substances (venom) into prey and natural enemies. This apparatus is formed by venom glands responsible for its production, a reservoir for storage, and the sting, which is the injecting structure of this venom [5, 6].

The primary function of this sting apparatus is related to prey capture; however, it has become an important defense weapon against natural enemies [7]. In vertebrates, the venom injected during a sting causes severe pain with local edema and erythema, due to the increased permeability of the blood vessels, which can cause tissue damage at the site and occasionally the death of large vertebrates, including humans [8].

A complex mixture of biologically active molecules composes this venom, ranging from low molecular mass compounds to peptides and allergenic proteins [9, 10]. Furthermore, volatile compounds have also been found such as esters, alkanes, alkenes, spiroacetates and acetanamides [11-16], which are related to alarm responses and recruitment to attack when colonies are disturbed [17].

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The biological activities already described for venom of social wasps as desnatured venom of *Polybia paulista* show anticonvulsant effects in freely rats, and did not show toxic effects on motor performance [18], anti-inflammatory activity in the venom of *Polybia paulista* [19], anticancer properties in the venom in *P. paulista*, which increases the permeability of the membrane, facilitating the formation of larger transmembrane pores in cancer cells [20], besides others. However, despite the potential activities found in the venom of these social wasps, there are still few studies on chemical composition, especially in species of the genus *Polistes* and even less on the free amino acids present in the venom [21, 22]. In studies with snakes Bieber [23] argues that free amino acids in the venom can be related to increased toxic action or activation of other venom proteins.

In this study a composition of the free amino acid in venom of the social wasp *Polistes lanio* evaluated in fresh and frozen, and before or after extraction with organic solvents.

2. MATERIAL AND METHODS

2.1 Reagents

Spectroscopic grade acetonitrile, ethyl acetate and hexane were purchased from Merck (Germany), DL-2-aminobutyric acid. (98 %, Sigma Aldrich, USA), diethyl ethoxymethyl-enemalonate (DEEM) (99%, Sigma Aldrich, USA), boric acid (99,8%, Dinâmica, Brazil), sodium borate (99 %, Sigma Aldrich, USA), sodium azide (99%, Sigma Aldrich, USA), ethyl ether (Dinâmica, Brazil) and amino acids (alanine, arginine, cysteine, isoleucine, metionine, proline, serine, threonine, tryptophan and valine (98-99 %, Sigma-Aldrich, USA). Stock mixture of amino acids was constituted from individual solution, dissolved in water and then used as external standard. All aqueous solutions and Chromatography analyses were performed with purified ultrapure water (18.2 MΩ), which was obtained from a Milli-Q (Songpa-gu, South Korea).

2.2 Obtaining venom and sample preparation for HPLC analysis

Three hundred females of *P. lanio* were collected in September 08, 2015 from innumerable colonies in the municipality of Castilho, São Paulo State, Brazil (20º57'13.4" S; 51º38'13" W). *P. lanio* females (Fig. 1A) were captured with entomological net or plastic containers containing cotton moistened with ethyl ether, which was then removed to avoid degradation/decomposition of the chemical compounds. For venom extraction the females were anesthetized by freezing and the sting was pulled with tweezers to expose the venom reservoir (Fig. 1B) under stereomicroscope.

The 300 reservoirs were placed in 600 µL of ultrapure water, macerated to expel the venom and centrifuged, and then the total venom was divided into four groups, as shown in Figure 2. For each group, triplicates were performed in order to develop the method for free amino acid analysis.

Among the twelve samples obtained from the venom of *P. lanio* (Figure 2), three with pure venom (P1) were immediately submitted to the derivatization process for sample preparation and analysis by High Performance Liquid Chromatography (HPLC) with Diode Array Detector (DAD). Three others pure venom samples were frozen at -4 ºC for 24 hours (P2) and then subjected to the derivatization process and further HPLC analysis.

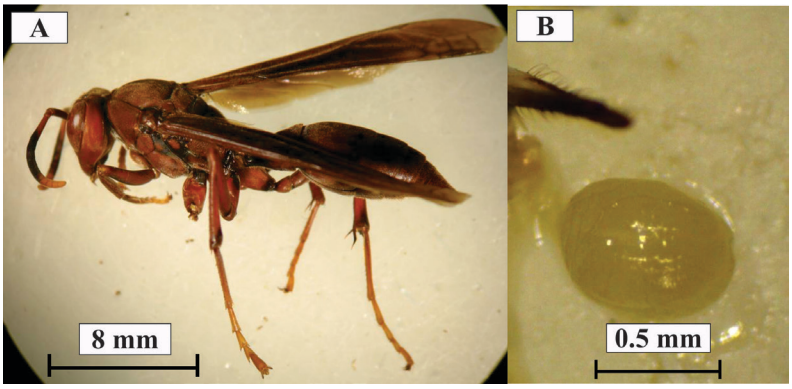

Figure 1. Female of *Polistes lanio* (A); and their venom reservoir.

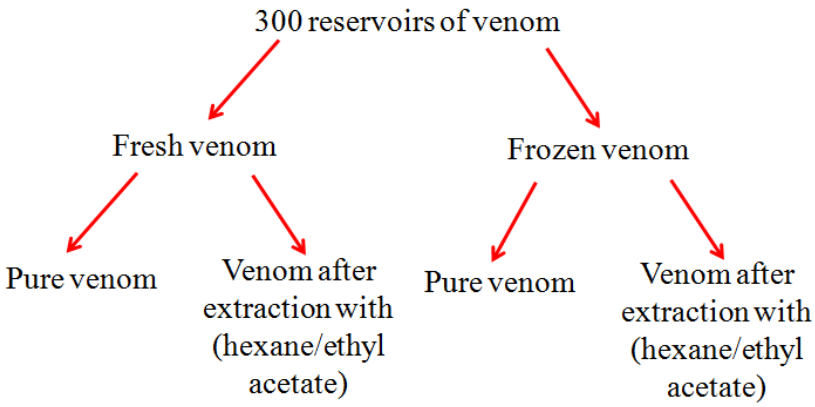

Figure 2. Four groups of venom of *P. lanio* used for free amino acids analyses.

In six samples, the venom obtained was extracted with 200 µL of hexane during 2 min. The hexane phase was removed and then 200 µL of ethyl acetate was added remaining in contact for 12 hours. The ethyl acetate phase was removed and combined with the hexane phase for analysis in another study by GC-MS. From these six samples three were submitted to the derivatization process after extraction with the organic solvents (hexane and ethyl acetate) (P3) and the other three were frozen (-4 ºC) for 24 hours (P4) also undergoing the derivatization process and HPLC analysis.

To prepare the samples for amino acids, the method described by Megías et al. [24] in *Vicia disperma* (Fabaceae) seeds with adaptations was used. Twenty mL of the extracted venom fraction, 3 mL of borate buffer (pH = 9), 0.1 mol L^{-1}, 24 µL of DL-2-aminobutyric acid (0.200 g L^{-1}) and 2 µL of diethyl ethoxymethyl-enemalonate (DEEM). The solution was stirred and incubated at 50 °C for 50 minutes. Subsequently, it was filtered with 0.20 µm ultrafiltration for HPLC analysis (method 1). Method 2 was similar to method 1, but in the latter samples were not subjected to heating, instead they were only maintained at room temperature 25 °C for 50 minutes.

A sample of each amino acid was dissolved in a volumetric flask of 5 mL with ultrapure water, filtered through a 0.45 µm Millex filter resulting in the stock solution. The stock solution was dissolved in ultrapure water in order to obtain solutions for analysis by HPLC.

2.3 Free amino acids analysis

The extraction efficiency (recovery) was determined by analyzing aliquots of each sample spiked with standards corresponding to 9 µg L^{-1} concentration. The venoms were prepared as described above, spiked during a period of 24 hours, and then the samples were derivatized and analyzed by HPLC.

The detection limit was determined by injecting (n= 5) solutions of amino acids of known concentration (20 µL each) and then decreasing the concentrations of the samples until detection of a peak with a signal/noise ratio of 3. The corresponding concentration was considered the minimal detectable concentration. The quantification limit was determined by performing the same methodology and, thus, the quantification limit was defined as the chromatographic peak having a signal/noise ratio of 10.

The content estimation of the amino acids in the samples was performed by adding a standard to the sample. Aliquots of 20 µL of the dilutions were analyzed by HPLC with each determination being carried out five times. A linear least-square regression of the peak areas as a function of the concentrations was performed to determine the correlation coefficients. The equation parameters (slope and intercept) of the standard curve were used to obtain the concentration values for the samples. Samples whose concentration exceeded the analytical curve were re-assayed upon appropriate dilution of the samples.

Identification of the free amino acids was performed by comparing retention times and spectra of the amino acid patterns: alanine, arginine, cysteine, isoleucine, methionine, proline, serine, threonine, tryptophan and valine, in the region of 200 to 800 nm, with the peaks obtained in real samples.

2.4 High Performance Liquid Chromatography

The aqueous extracts of venom obtained from the samples were analyzed in an analytical HPLC (LC-6AD, Shimadzu, Kyoto, Japan) system with a binary

solvent a diode array detector (DAD) monitored at λ = 200-800 nm. The HPLC column was a C-18 (15 cm × 4.6 mm; particle size, 5 µm; Luna, Thermo Electron Corporation, Torrance, CA, USA). In each analysis, the flow rate and the injected volume were set as 0.9 mL min^{-1} and 20 µL, respectively. All chromatographic analyses were performed at 18 ºC. Elution was carried out using the following solvent-gradient programs: Mobile phase A consisting of a 25 mM solution of acetic acid and 0.02% sodium azide in ultrapure water, adjusted for pH 6 and mobile phase B was acetonitrile (M1). The mobile phase A consisted of a 25 mM solution of acetic acid in ultrapure water, adjusted to pH 6 and mobile phase B was acetonitrile (M2). The gradient programming is described in Table 1.

Table 1. Gradient elution for analysis of amino acids by HPLC in M1 and M2.

Retention time (min)	Eluent A (%)	Eluent B (%)
0	96	4
3	88	12
13	88	12
30	69	31
35	69	31
40	96	4

3. RESULTS AND DISCUSSION

This study developed a fast, reproducible and sensitive analytical method for quantitative analysis of these compounds (Figure 3). Amino acids found in extracts of venom were identified by performing co-injection experiments in which aliquots of the venom and standards were mixed and diluted to a known volume and analyzed by HPLC. Identification of the amino acids with the aid of a DAD detector scanning in the spectral range of 200-800 nm did not reveal interferences in retention time of the venom of *P. lanio* in HPLC by the developed elution method (Figure 4). The relative standard deviation for the retention times of replicated injections (n= 5) were less than 2%, thereby demonstrating good repeatability.

The linearity was determined by linear regression using HPLC in 5 concentration ranges (Tables 2 and 3). The average standard errors for peak areas of replicated injections (n= 5) were less than 1%, thus showing good repeatability of the calibration curve.

Analyzing the data obtained with the two mobile phases (Tables 2 and 3) used in this study and the similarity between them, M2 mobile phase was chosen for the recovery studies and limits of detection and quantification.

The analytical procedure showed linearity of the assayed method (Tables 2 and 3). The efficiency of each analytical procedure was evaluated by calculating the recovery values. Recovery was evaluated by analyzing each sample spiked with a known amount of the analyte at a concentration of 9 µg L^{-1}, which showed that the response was proportional to the concentration of the samples. Recovery results were between 93.2-99.0% in four methods evaluated showing that the procedures employed for extraction of amino acids from the venom were efficient with a relative standard deviation lower than ± 5% (Table 4), thus ensuring good recovery of these amino acids in the four methods employed.

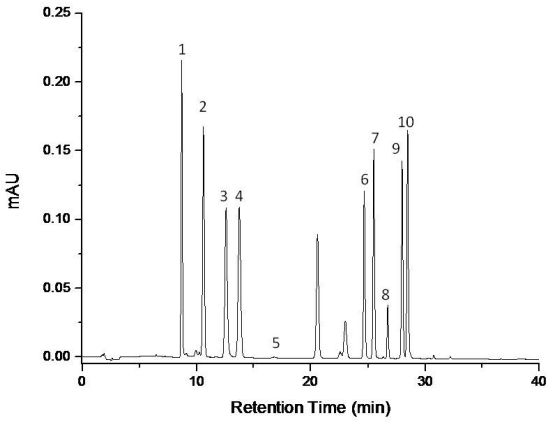

Figure 3. Chromatogram pattern of amino acids obtained by HPLC. (1. Serine; 2. Treonine; 3. Arginine; 4. Alanine; 5. Proline; 6. Valine; 7. Methionine; 8. Cysteine; 9. Isoleucine; 10. Tryptophan).

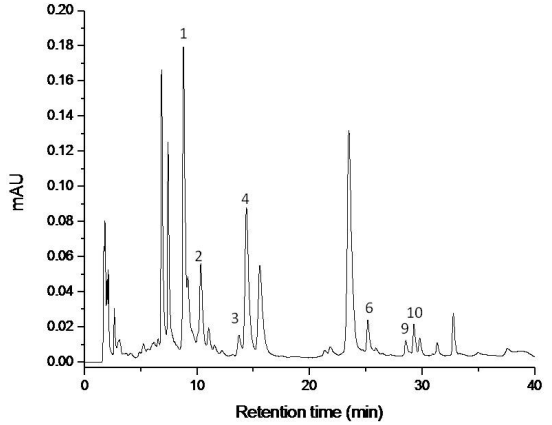

Figure 4. Chromatogram of amino acids obtained of the venom of *P. lanio* employing HPLC (1. Serine; 2. Treonine; 3. Arginine; 4. Alanine; 6. Valine; 9. Isoleucine; 10. Tryptophan).

Table 2. Analysis of amino acids employing HPLC (M1).

Peak	Retention time (min)	Amino acids	Linear range (µg/L)	A	b(10^{10})	R
1	8.87	Serine	8.45-101.46	77158	2.70	0.99698
2	10.98	Treonine	8.51-102.06	10511	2.91	0.99970
3	13.49	Arginine	8.36 100.28	49917	2.61	0.99794
4	14.26	Alanine	8.76-105.14	42139	2.47	0.97011
5	16.66	Proline	8.67-104.00	12380	2.41	0.95816
6	24.99	Valine	8.36-100.37	29532	1.92	0.98508
7	25.87	Methionine	8.35-100.19	6363	2.40	0.99971
8	26.95	Cysteine	8.27- 99.30	8664	50.01	0.99928
9	28.29	Isoleucine	8.29 -99.52	47234	2.01	0.98364
10	28.88	Tryptophan	8.30-99.54	10714	2.80	0.99751

Formula: y = a + bx, where y = ratio of peak areas; x = concentration (µg L^{-1}); a = intercept and b = slope.

Table 3. Analysis of amino acids employing HPLC (M2).

Peak	Retention time (min)	Amino acids	Linear range (µg/L)	A	b(10^{10})	R
1	8.86	Serine	8.45-101.46	77177	2.78	0.99688
2	10.92	Treonine	8.51-102.06	10517	2.87	0.99972
3	13.48	Arginine	8.36 100.28	49924	2.65	0.99792
4	14.24	Alanine	8.76-105.14	42147	2.49	0.97123
5	16.64	Proline	8.67-104.00	12389	2.47	0.96678
6	24.96	Valine	8.36-100.37	29544	2.05	0.98512
7	25.85	Methionine	8.35– 100.19	6369	2.47	0.99979
8	26.92	Cysteine	8.27- 99.30	8656	48.12	0.99945
9	28.28	Isoleucine	8.29 -99.52	47249	2.12	0.98456
10	28.86	Tryptophan	8.30-99.54	10723	2.89	0.99766

Formula: y = a + bx, where y = ratio of peak areas; x = concentration (µg L^{-1}); a = intercept and b = slope.

The detection limit was determined for amino acids based on the signal-to-noise ratio (S\N) of 3 (Table 4). The quantification limit was defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than 10 (Table 4).

Table 4. Data of the recovery study and limits of detection and quantification of the amino acids in venom of *Polistes lanio.*

Amino acids	P1 Recovery (%) Mean±SD	P2 Recovery (%) Mean±SD	P3 Recovery (%) Mean±SD	P4 Recovery (%) Mean±SD	Detection limit (pg)	Quantification limit Q (pg)
Serine	93.2±1.2	94.3±1.9	93.9±1.4	94.1±1.0	1.6	5.3
Treonine	95.6±1.1	94.9±2.3	94.9±1.5	94.7±0.9	1.5	5.0
Arginine	93.0±1.9	93.6±1.7	93.3±1.6	94.0±1.2	2.9	9.7
Alanine	99.0±2.2	98.7±0.9	99.0±1.3	98.4±0.7	2.5	8.3
Proline	93.5±1.0	93.9±1.3	93.2±1.1	93.4±1.1	4.5	15.0
Valine	95.7±1.5	96.1±1.1	95.9±1.4	96.0±0.8	1.4	4.7
Methionine	94.8±1.7	95.2±1.5	94.9±1.1	95.0±1.0	1.9	6.3
Cysteine	98.4±2.6	98.7±2.0	98.1±1.9	98.4±2.1	2.2	7.2
Isoleucine	97.3±1.2	97.6±2.1	97.8±1.5	97.9±0.9	1.9	6.3
Tryptophan	96.2±1.3	96.7±1.6	96.4±1.0	96.4±1.7	1.6	5.3

SD= Standard deviation**. P1=** pure venom immediately submitted to the derivatization process, P2= pure venom submitted frozen at-4 ºC for 24 hours before the of derivatization process. **P3=** pure venom submitted organic extraction to the derivatization process, P4= pure venom submitted organic extraction and stored in frozen at -4 ºC for 24 hours antes the of derivatization process.

Alterations were not detected in working solutions of amino acids prepared after 24 hours at 22 ºC, two months at 4 °C and six months of storage at -4 °C. Therefore, this validated method for determination

of amino acids can be considered adequate to indicate the stability of the solutions.

The experiment employing different samples (Table 5) to measure the contents of amino acids showed a coefficient of variation lower than 5%, indicating that the venom did not present considerable variation in relation to the previously frozen samples and also in relation to the samples that were subjected to extraction with organic solvents. In presence of organic solvents, the peptides and proteins of the venom can originate in the medium amino acids or other molecules by the rupture of the peptide bonds.

Table 5. Concentration values in μg L^{-1} of the amino acids in venom samples from social wasp *Polistes lanio.*

Amino Acids	P1 Mean ± SD	P2 Mean ± SD	P3 Mean ± SD	P4 Mean ± SD
Serine	38.35 ± 0.55	38.37 ± 0.92	38.30 ± 0.66	38.43 ± 0.87
Treonine	40.20 ± 1.00	39.99 ± 1.01	40.50 ± 0.84	39.93± 0.77
Arginine	64.45 ± 2.05	63.87 ± 2.49	65.77 ± 2.15	64.37 ± 2.12
Alanine	61.05 ± 0.45	61.43 ± 0.90	61.13 ± 0.87	61.50 ± 0.45
Proline	-	-	-	-
Valine	18.70 ±0.70	17.93 ± 0.66	18.43 ± 0.62	17.97 ± 0.78
Methionine	11.71± 0.24	11.67 ± 0.11	11.59 ± 0.22	11.64 ± 0.18
Cysteine	-	-	-	-
Isoleucine	11.56 ± 0.43	11.63 ± 0.34	11.47 ± 0.41	11.71 ± 0.46
Tryptophan	17.60 ± 1.00	17.77 ± 1.07	17.73 ± 1.07	17.87 ± 1.02

SD= Standard deviation**. P1=** pure venom immediately submitted to the derivatization process, P2= pure venom submitted frozen at -4 ºC for 24 hours before the of derivatization process. **P3=** pure venom submitted organic extraction to the derivatization process, P4= pure venom submitted organic extraction and stored in frozen at -4 ºC for 24 hours antes the of derivatization process.

The contents (Table 5) obtained from the venom sample subjected to freezing for 24 hours were similar to the data obtained with the non-frozen venom. The sample submitted to extraction with organic solvent in relation to the directly analyzed venom sample presented similar results. Based on the results of table 5 it can be inferred that freezing or extraction with hexane and ethyl acetate did not affect the amounts of amino acids, indicating that the samples can be subjected to these processes prior to the analysis of free amino acids.

In the free amino acid analysis, 8 of the 10 analyzed were identified in *P. lanio* venom (Table 5), with arginine and alanine having relatively higher concentration, in which there was no qualitative variation and little quantitative variation among the samples evaluated which demonstrates the high diversity of these compounds. This result demonstrates that the presence of free amino acids as constituents of the venom seems to be common, as has been described in studies of the venom of other animals [25, 26]. According to Ikan and Ishay [22] alanine had higher concentration in venom of *Vespa orientalis,* followed by proline that was not found in *P. lanio* venom. Among the amino acids detected in the gland and in the venom bag of *Apis florea* are serine and valine [27], also found in *P. lanio* venom. This method of analysis also identified these 10 free amino acids used as standards in the venom of *Polistes erythrocephalus* (unpublished data), in which alanine and proline showed higher concentrations. In ants, free amino acids were identified in the venom of *Pseudomyrmex triplarinus*, with aspartic acid, glutamic acid and proline constituting more than 72% of the total mass of the free amino acids of the venom.

As amino acids are essential for the construction of peptides, proteins, phospholipids, neurotransmitters and biogenic amines, changes in the availability of these molecules affect the regulation of cell signaling, gene expression and the transport of amino acids themselves [26]. In this sense, studies on free amino acids are still scarce and little is known about their function, especially in venom and in social insects. According to Bieber [23] in studies with snake, it is argued that free amino acids in the venom may be related to increased toxic action or activation of other venom proteins.

Studies with social wasps demonstrate the

presence of neuroactive amino acids acting as inhibitory neurotransmitters, including alanine [28, 29]. Therefore, a high concentration of these neurotransmitter amino acids can cause paralysis in the prey, explaining its presence in the venom [28].

In addition, amino acids can generate numerous catabolites, including ammonia, carbon dioxide, fatty acids, glucose, hydrogen sulfide, ketone bodies, nitric oxide, urea, uric acid, polyamines and other nitrogenous substances of enormous biological importance. Amino acids can lead to the formation of important biogenic amines, common to venom, used for both defensive and offensive purposes [29].

These formed biogenic amines can act by accentuating pain, in edematous reactions of the skin or even increase the permeability of venom, consequently acting as toxins, along with proteins and peptides that are also commonly found in venom [29, 30]. Among the biogenic amines already found in Hymenoptera species are: histamine, serotonin, dopamine and epinephrine (adrenaline), which are respectively metabolites of the amino acids histidine, tryptophan, and the last two of the amino acid tyrosine [29-31]

Among the identified functions of these biogenic amines, serotonin, which is a metabolite of the amino acid tryptophan, also identified in *P. lanio* venom acts causing pain and potentiating the algogenic effect of other venom compounds [32, 33]. In addition, it may facilitate the penetration of venom into the cell, as it causes increased cell permeability [34]. Epinephrine also acts to prolong pain, since it obstructs capillaries and venules [35], whereas histamine, in addition to contributing to pain, aids in vasodilation, leading to vascular collapse when present at high concentrations [30, 36]. Finally, dopamine acts by increasing the insect's heart rate, and consequently leads to faster venom circulation in the body [37].

4. CONCLUSION

The results show a high diversity of the free amino acids in the social wasp venom *P. lanio*, which do not change in samples fresh or frozen, and before or after extraction with organic solvents, wich demonstrates that there is stability in the free amino acid of the venom. Besides the method for analysis of the free amino acids in venom of the social wasp *P. lanio* was validated with sufficient sensitivity, linearity, stability and good recovery (93.2-99.0%) by High Performance Liquid Chromatography. The coefficients of variation in the quantitative analysis of the free amino acids were under 5% in fresh or frozen venom or when previously submitted to the extraction process with organic solvents, therefore, the method was considered sensitive for quantification of free amino acids in venom. The data obtained indicate that the freezing, storage and extraction with organic solvents does not lead to an increase in the amino acids content of proteins and peptides.

5. ACKNOWLEDGMENTS

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