MALDI-MS argininyl bufadienolide esters fingerprint from parotoid gland secretions of *Rhinella arenarum*: age, gender and seasonal variation

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Abstract

In many amphibians, the granular glands can be grouped in special regions forming macroglands. This is the case of toads, characterized by the presence of a pair of parotoid macroglands, strategically located to give protection by poison release in case of attacks. The product secreted consists of a wide variety of chemical compounds including proteins, peptides, biogenic amines, toxic steroidal bufadienolides, various alkaloids, depending on the species. In this work, using *Rhinella arenarum* we have performed, for the first time, the MALDI-MS and MS/MS characterization of the components of the secretion used as crude material, just suspended in MeOH (or MeCN). The crude sample as a whole (whole suspension), was spotted on the MALDI plate for analysis. ESI-Orbitrap was used for cross-checking experiments. The pattern of signals obtained at m/z ranges 600-800 and 1200-1600 could be assigned as the argininyl bufadienolide esters fingerprint characteristic of female and male. Variation patterns for gender (female, male), age (non-reproductive, reproductive) and season (non-reproductive, reproductive) are described.

Keywords: *Rhinella arenarum;* paratoid secretion; MALDI-MS, bufadienolides; fingerprint.

1. Introduction

Common toads are divided in genera *Rhinella* and *Rhaebo* in South America (used to be grouped in genus *Bufo*).[1] *Rhinella arenarum* is a native species from South America and is widely distributed in Argentina.[2]

Anurans have developed different resources to protect themselves against predators and microbial infection, which can include ecological, morphological, physiological or behavioural features.[3] Chemical defenses represent a common strategy in amphibians. The number and diversity of compounds produced by amphibians in their skin glands is surprisingly high. Those secretions contain biogenic amines (i.e., adrenaline and noradrenaline), steroids (bufogenines and bufotoxins), alkaloids (i.e., serotonin, batrachotoxin and tetrodotoxin) and peptides (including smaller oligopeptides, polypeptides and proteins).[4] The abundance and diversity of components of each of these family's compounds can vary according to the life history of the amphibian in question, gender and season.[5]

Chemical composition of secretion produced by *Rhinella arenarum* was studied for the first time by in 1933 by Chen et al. and described the presence of arenobufagin, arenobufotoxin and arenobufotenin in the alcoholic extract.[6] Later, Rees et al. (1959) characterized some bufogenins using IR spectroscopy in paratoid secretions.[7] More recently, Maciel et al. (2003) studied indolealkylamines by TLC and ESI-MS in extract from secretions.[8] Moreover, Mebs et al.(2007) using skin, instead of secretion, described the presence of bufogenine and bufalin by HPLC-ESI.[9]

Additionally, several studies identified compounds in secretion from other species such as *Rhinella* genus (*R. marina,*[10-15] *R. jimi,*[11] *R. crucifer,*[11] *R. granulosa,*[11] *R. major,*[11] *R. margaritifera,*[11] *R. icterica,*[11] *R. schneideri*[11,16,17]) and *Rhaebo* genus (R. *guttatus*)[10,11,13] found in Brazil. Ferreira et al. [10] showed significant differences in composition between *R. marina* and *R. guttatus* venoms but no relevant chemical difference were observed between male and

female secretions. Schmeda-Hirschmann et al. [16] isolated and identified by spectroscopic (RMN) and spectrometric methods (MS) 29 compounds (alkaloids and argininyl diacid derivatives of the bufadienolides bufalin, marinobufagin and telocinobufagin) from the *R. schneideri* secretion; Some of them were described for the first time. In order to characterize the peptides present in parotid secretion of *R. marina*, a combination of reversed phase HPLC and MALDI MS/MS was used.[12] The authors found that peptides are present in very low abundance being the lower molecular mass components (<900 Da) highly predominant.[12] Proteomic analysis by nano UPLC-ESI was performed recently in secretion of *R. schneideri*.[17] Sciani et al., [11] performed a characterization of the components of the poison of eight Rhinella species and one of Rhaebo in order to verify whether phylogenetic and biological differences were related with poison composition. In the secretions under studied they detected several bufadienolides (i.e., dehydrobufotenine, hellebrigenin, telocinobufagin, serotonin, N´-methyl-serotonin, resinobufagin, hellebrigenol-3-O-sulfate, bufalin, N´-N´-dimethyl-serotonin (bufotenin), desacetylcinobufagin and marinobufagin). Although some components were common among the secretions, they identified some molecules as exclusive to some species. They concluded that biological features, and not only evolution, seem to directly influence the skin secretion composition.

In the present work we studied the application of matrix assisted-ultraviolet laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) for the analysis of secretion from *R. arenarum*. Secretions were analyzed as got from the glands (crude samples). Thus, for the first time both MS techniques were used on this crude material to identify distinctive bufandienolide composition profiles (fingerprints), for male and female. Samples were collected from juvenile and adult specimens (non-reproductive the former and reproductive the latter, respectively) at two different times in the year, July (not reproductive season) and December (reproductive

season).

2. Experimental

2.1. Secretion samples.

Specimens of *Rhinella arenarum* were collected in the neighbouring areas of Buenos Aires city (Argentina) as follows: July 2014 (non-reproductive period), adults (female and male), three specimens of each gender; December 2014, (reproductive period), adults (female and male) and juvenile (male), six specimens of each; December 2016, (reproductive period), adult (female and male) five specimen of each gender. Paratoid gland secretions were collected separately in Eppendorf tubes by gentle pressing the parotoid glands. Secretions were kept at low temperature (-18 ^{o}C). For MALDI-MS analysis samples were prepared as suspension of the raw secretion in methanol (MeOH) or in acetonitrile (MeCN): TFA 0.1% 30:70 (v/v). Each spectrum shown corresponds to the secretion of one animal.

All of the experiments with toads were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales (UBA Res CD: 140/00) and the United States National Institutes of Health (Publication 8523, revised 1985).

2.2. MALDI-MS analysis.

Ultraviolet matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) was performed on the Bruker Daltonics Ultraflex II TOF/TOF mass spectrometer. Mass spectra were acquired in linear and reflectron positive modes and with the LIFT device in the MS/MS mode. The parameters used for MS/MS spectra adquisition were: Ion source voltage 1= 8 kV, Ion source voltage 2= 7.2 kV, lens voltage 3.6 kV, LIFT voltage 1=19 kV and LIFT

voltage 2=3.4 kV. External mass calibration was made using commercial peptides (bradykinin (1-7) (m.w. 757.39916), angiotensin II (m.w. 1046.54180), angiotensin I (m.w. 1296.68400) and insulin (m.w. 5734.52000)) and α-ciano-4-hydroxycinnamic acid (CHCA) as MALDI matrix, in positive ion mode. This matrix was selected as the best after matrix screening with: 2,5-dihydroxybenzoic acid (DHBA), 9H-pyrido[3,4-]indole (norharmane, nHo) and E-3,5-dimethoxy-4-hydroxycinnamic acid (E-sinapinic acid, SA). For MALDI-MS matrix solutions were prepared as nHo (1 mg/ml), E-SA (2 mg/ml) and CHCA (1mg/ml) in MeCN: TFA 0.1% 30:70 (v/v). Analyte and matrix solutions were spotted on a MTP 384 target plate polished steel from Bruker Daltonics (Germany). For MALDI-MS experiments samples were prepared by sandwich method loading successively 0.5 µl of matrix solution, analyte solution and matrix solution (x2) after drying each layer at normal atmosphere and room temperature.[18] The final matrix to analyte ratio was 3:1 (v/v) and the matrix and analyte solution loading sequence was: i) matrix, ii) analyte, iii) matrix, iv) matrix. Besides, on selected samples one additional layer (0.5 µl) of NaCl solution (0.1 to 1 mg/ml) was loaded in order to evaluate sodium effect on male and female secretion.

MALDI experiments were conducted as follows: each sample loaded on two spots (duplicate) with sample prepared with individual secretion. In order to check reproducibility of data, sample preparation and measurements were conducted independently, for each secretion sample, at least in three different days. Thus, our conclusions come from observation of results obtained (spectra) for each sample (each specimen), at least 6 times in 3 independent experiments**.**

Desorption/ionization was obtained by using the frequency-tripled Nd:YAG laser (355-nm). Experiments were performed using firstly the full range setting for laser firing position in order to select the optimal position for data collection, and secondly fixing the laser firing position in the sample sweet spots. The laser power was adjusted to obtain high signal-to-

noise ratio (S/N) while ensuring minimal fragmentation of the parent ions and each mass spectrum was generated by averaging 100 lasers pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively.

nHo, DHBA, CHCA, E-SA, bradykinin (1-7), insulin, angiotensin I and II were purchased from Sigma-Aldrich Chemical Co. TFA was purchased from Merck. MeCN HPLC grade was used. Water of very low conductivity (Milli Q grade) was used.

2.3. HRESI–MS analysis.

High resolution electrospray ionization (HRESI) mass spectrometry (MS) analysis was performed in positive ion mode using the mass spectrometer Q Exactive from Thermo Scientific (USA). Acquisition parameters: capillary temperature, 275 °C; spray voltage 3 kV, S lens voltage 50 V, auxiliar gas temperature 30ºC.

Fresh paratoid gland secretions suspended in MeOH or in MeCN: TFA 0.1% 30:70 (v/v) were used for the direct infusion and analysis.

2.4. Principal component analysis.

Statistical analysis was performed by principal component analysis (PCA) (SPSS Statistic 17.0, IBM software, USA). The data set consists of m/z values from 1300 to 1500 and the corresponding signal intensities of the spectra to be compared. Baseline correction was applied. Six different experiments were used for each condition.

3. Results and discussion

3.1 MALDI-MS

The chemical composition of the secretion of *Rhinella arenarum* was investigated. Secretions were collected by gentle pressing the parotid glands and were suspended/ dissolved in MeOH or in MeCN: TFA 0.1% 30:70 (v/v). The results obtained are summarized in Table 1. The structures of argininyl diacid derivatives from bufadienolides detected are shown in Scheme 1. As is shown in Table 1, the signals are distributed in four m/z regions (m/z < 450, 600 < m/z < 800, 1300 < m/z < 1500 and m/z > 6000).

3.2 MALDI-MS: analysis range m/z 600 -800

Figs. 1(a) and 1(b), (positive ion mode, matrix: CHCA, m/z region 600 to 800), show the MALDI mass spectra of secretions collected in December from adult specimens (Fig. 1(a) female and Fig. 1(b) male). Argininyl derivates from bufalin, marinobufagin, telocinobufagin, bufotalinin, resibufogenin, bufotalin and bufarenogin (or arenobufagin or hellebrigenin) were detected as protonated ($[M+H]^+$) and/or sodiated ($[M+Na]^+$) adduct in female and in male secretion. Some signals are observed in both spectra (m/z 669, 671, 685, 699, 701, 713, 715 and 729) but the patterns got from female and from male adults (specimen in reproductive stage) are quite different. In female secretion, the intensity is higher for sodiated adducts (for instance, m/z 724 and m/z 738 compared with the corresponding protonated signals at m/z 701 and 715, respectively). On the contrary, in adult male secretion, the intensity of the signals assigned to $[M+H]^+$ species (m/z 685 and m/z 699) are higher than $[M+Na]^+$ species (m/z 707 and 721, respectively). In order to compare chemical composition of the secretion from juvenile and adult specimens (non-reproductive and reproductive stages), MALDI-MS experiments were run in the same experimental conditions. Fig. S1 in supplementary material, shows the MALDI mass spectrum of secretion obtained from juvenile male specimen. Most of signals detected correspond to $[M+H]^+$ species (m/z 657, 669, 671, 685, 699, 701, 713 and 729). However, few signals with low intensity were assigned to $[M+Na]^+$ species (m/z 691, 707

and 721). No significant differences in chemical components (expressed as m/z values) were detected between juvenile and adult male secretion collected in the same season (December) although the relative intensities of the signals of the former and the latter are in general different (Fig 1(b), Fig S1 and Table 1). *i.e.,* in juvenile's secretion the signal at m/z 669 is in general the highest while in adult's secretion spectrum those at m/z 669 and 685 are the highest peaks. Results obtained using female and male secretions collected in July are shown in supplementary material (Figs. S2(a) and S2(b) and Table 1). In the region 600 < m/z < 800, protonated adducts were detected in both samples (m/z 657, 669, 671, 683, 685, 699, 701, 713, 715 and 729). Unlike results described before for samples collected in December, few sodiated adducts were detected in July samples. Here, chemical components and the relative intensities and as consequence the fingerprint of the adult specimens of female and male, collected in July are different.

The effect of NaCl addition was studied. Spectra of secretion from adult male and female obtained in December and July were recorded using CHCA as matrix, with and without addition of NaCl. No significant differences were observed.

In order to confirm the hypothesis than sodium concentration in secretion obtained from *Rhinella arenarum* in female is higher than male, atomic absorption spectroscopy measurements were conducted on samples collected in December. Values of 4.3 mg/L for male and 151.0 mg/L for female were obtained. This is the first time that sodium concentration in secretion was measured. However, others studies dealing with concentration of sodium in plasma from amphibians also obtained higher concentration in female respect to male. For instance, higher plasma level was obtained in American bullfrogs.[19]

For structural characterization of bufadienolides, MALDI-TOF MS/MS (fragmentation/decomposition induced by laser method: LID) was performed (Fig. 2, Figs. S3-S7, Tables 1-2, Table S1 and Schemes 1-2). As an example, in Fig. 2(a) is shown the MS/MS spectrum of the $[M+H]^+$ ion detected at m/z=685.009 in adult male secretion

collected in December (Fig. 1). Its fragmentation yielded ions at m/z=666.998 ($[M-18+H]^+$, lost of one H_2O moiety), at m/z=651.085 ($[M-18-18+H]^+$, lost of two H_2O moieties), and at m/z=556.043 ($[M-18-18-96+H]^+$, lost of two H_2O and the α-pyrone moieties). Argininyl diacids were previously detected by MS in positive ion mode. [16] Then, taking into account these previous results, the peak at m/z= 302.991 could be assigned as the $[M+H]^+$ species for adipoyl arginine and the peak at m/z=317.015 to the corresponding $[M+H]^+$ species for pimeloyl arginine. The simultaneous presence of those signals at m/z=317.015 ($[M-368+H]^+$ and m/z=302.991 ($[M-382+H]^+$ indicates the existence in the analyzed secretion of isomers containing the moieties bufalin and marinofufagin as bufadienolides, respectively (Fig. 2, Table 2, Table S1, entries 4 and 5 and Tables 1, entry 8). Furthermore, characteristic fragment from argininyl diacids were also detected at m/z=249.987, m/z=175.018 and m/z=158.017 (see Scheme 2). The two isomeric compounds present in the sample were tentative identified as 3-(N-pimeloyl argininyl)-bufalin and 3-(N-adipoyl argininyl)-marinobufagin (Table 1, entry 8 and Table S1, entries 4 and 5). Presence of 3-(N-adipoyl argininyl)-marinobufagin was previously detected in *R. scheneideri* using ESI-QTOF-MSMS.[16] No significant differences were observed in the fragmentation profile got for the selected precursor ion at m/z=685.012 from adult female secretion collected in July (Fig 2 (b)) or juvenile male secretion collected in December (Fig S3).

The MS/MS spectrum of the $[M + H]^+$ ion detected at m/z= 669.047 in adult male secretion was obtained (Figs. 1 and S4(a)). Its fragmentation yielded ions at m/z=651.014 ($[M-18+H]^+$, lost of one H_2O moiety), at m/z= 303.051 ($[M+H]^+$ for adipoyl arginine moiety), at m/z=250, at m/z=175 and at m/z=158. This compound was tentatively identified as 3-(N-adipoyl argininyl)-resibugenin (Table 1, entry 5 and Table S1, entry 1). No significant differences were observed in the fragmentation profile for the precursor ion at m/z=669 present in

juvenile male secretion (Fig S4 (b)). These spectra matches with the description provided for 3-(N-adipoyl argininyl)-resibugenin by Schmeda-Hirschmann et al. [15]

Interpretation of the MS/MS spectra obtained from other ions shown in Fig. 1, could be done in similar way. Thus for the $[M+H]^+$ precursor ion at m/z=698.925 (699) in adult male secretion collected in December a more complex signal pattern in the m/z region of argininyl diacids was observed (Fig. S5(a)). The fragments at m/z= 303.021 (adipoyl arginine), at m/z=317.055 (pimeloyl arginine) and at m/z=331.097 (suberoyl arginine) (Table 2), as it was discussed before [m/z= 303.021 ($[M-396+H]^+$, m/z=317.055 ($[M-382+H]^+$ and m/z=331.097 ($[M-368+H]^+$ indicate the presence of isomeric bufadienolides containing bufotalinin, marinobufagin and bufalin moietyes, respectivelys (Table 2; Table S1,entries 6-8, Table 1, entry 12). Characteristic fragment from argininyl diacids were also detected. Two of these isomers (3-(N- suberoyl argininyl)-bufalin and 3-(N- pimeloyl argininyl)-marinobufagin) where previously detected in R. *marina*[15] and in *R. scheneideri.* [16] The same fragments were detected in female secretion collected in July for this precursor ion (Fig S5(b)). However, the intensity of the signal assigned as suberoyl arginine was higher respect to adipoyl arginine for female secretion. On the contrary, adipoyl arginine was the highest signal detected when adult male secretion was studied.

The MS/MS spectrum of the $[M+H]^+$ ion detected at m/z=714.912 (715) in male secretion showed signals corresponding to two argininyl diacids detected as $[M+H]^+$ at m/z=317.667 (pimeloyl arginine) and at m/z=331.744 (suberoyl arginine) (Table 2 and Fig. S6(a)). Again, signals at m/z=317.667 ($[M-398+H]^+$ and m/z=331.097 ($[M-384+H]^+$ indicate the presence of isomers of bufadienolides (bufarenogin or arenobufagin or hellebrigenin and telocinobufagin, respectively) (Table S1, entries 14 and 15). 3-(N- suberoyl argininyl)-telocinobufagin was previously found in R. *marina*[15] and in R. *Schneideri.*[16]

The lost of two H_2O molecules was detected at m/z=678.929 ($[M-18-18+H]^+$ in female secretion (Fig. S6(b)). Additionally, the relative intensity of signals is quite different in the spectra obtained from male and female secretion. Then some difference in the chemical composition of both secretions (different mixture of isomers) can be concluded.

Similarly The MS/MS spectrum of the $[M+H]^+$ ion detected at m/z=728.912 (729) in adult male secretion collected in December (Fig. 1, Fig. S7) showed in the m/z region of the argininyl diacids a peak at m/z= 331.678 corresponding to an argininyl diacid (suberoyl arginine, Scheme 2) and a peak at m/z=303.682 corresponding to adipoyl arginine (Scheme 2). As several bufadienolides have the same molecular mass (bufadienolide isomers; bufarenogin, arenobufagin and hellebrigenin, Scheme 1 and Table 2) the MS information was not enough for full characterization of the peak. Then, the tentative precursor ion structure was assigned as 3-(N- adipoyl argininyl)-bufotalin and/or 3-(N- suberoyl argininyl)-bufarenogin and/or 3-(N- suberoyl argininyl)-arenobufagin and/or 3-(N- suberoyl argininyl)-hellebrigenin (Table S1, entries 16 and 17, Table 1, entry 21). 3-(N- suberoyl argininyl)-hellebrigenin was previously detected in R. *marina*[15] and in R. *scheneideri*[16] by ESI-QTOF-MSMS.

When fragmentation spectra was not available (very low intensity precursor ion), molecular mass and bibliography were used for tentative assignment of the compounds structure as is detailed in Tables 1. As a summary, at the m/z region < 800 were detected: (i) bufalin esters of glutaryl (entry 4), adipoyl (entries 6 and 10), pimeloyl (entries 8 and 14), suberoyl (entries 12 and 18), azelayl (entries 16 and 22) and sebacyl (entry 20) argininyl diacid derivatives; (ii) resibufogenin esters of adipoyl (entries 5 and 9) and pimeloyl (entry 7) argininyl diacid derivatives; (iii) marinobufagin esters of adipoyl (entries 8 and 14), pimeloyl (entries 12 and 18), suberoyl (entries 16 and 22) and azelayl (entry 20) argininyl diacid derivatives; (iv) For telocinobufagin, glutaryl (entry 11), adipoyl (entry 15), pimeloyl (entries 13 and 19), suberoyl

(entries 17 and 23) and undecadienoic (entry 26) acid argininyl diacid derivatives; (v) for bufotalinin, adipoyl (entries 12 and 18), pimeloyl (entries 16 and 22) and suberoyl (entry 20) argininyl diacid esters; (vi) adipoyl (entries 13 and 19), pimeloyl (entries 17 and 23), suberoyl (entries 21 and 25) and sebacyl (entry 26) argininyl diacid from hellebrigenin (bufarenogin/arenobufagin); and finally (vii) adipoyl (entry 21) and suberoyl (entry 26) argininyl diacid from bufotalin.

3.3. MALDI-MS: analysis range m/z 1200-1600

Going on with the analysis of the results obtained, in Figs 3 (a) and (b) are shown the unexpected groups of signals detected by MALDI in the m/z range 1200 to 1500 (specially 1300 to 1500), for the secretions collected from adult specimens, in December. Peaks at m/z=1339, m/z=1354, m/z=1369, m/z=1385, m/z=1397 and m/z=1414 were observed in female (Fig. 3(a)) and male (Fig. 3(b)) secretions. Additionally in the former, quite intense peaks at m/z=1429, m/z=1442, m/z=1458 and m/z=1472 were also detected. Similar fingerprint for female and male (always more complex the former than the latter) were obtained for secretions collected in July (Fig. S8). According to a recent survey of the literature, any information about compounds with these m.w. in secretions, was found. In order to shed some light to these results additionally experiments using direct sample infusion ESI-MS (ESI-Orbitrap) were conducted (cross check analysis), in order to show the presence of these compound in crude secretions. As can be seen in Fig. 4 similar pattern of signals (fingerprint) for the female secretion was obtained by ESI and MALDI (Figs. 3(a) and 4). Dilution of the secretion solution/suspensions (1/10. 1/100) yielded the same ESI pattern (results not shown). The results obtained with MALDI and ESI support the idea that these high m.w. new metabolites are in the secretions and that they are not just clusters (molecule aggregates) formed during MALDI experiments. They are in fact, argininyl bufadienolide

ester dimers. These dimers can be simetric (A and B argininyl bufadienolide ester. A-A; B-B;) or asimetric (A-B) species. The MS/MS analysis conducted from the precursor protonated ions, $[M+H]^+$, allowed to reach this conclusion.

The corresponding MS/MS spectra of precursor ions m/z= 1339, m/z= 1354, m/z=1369, m/z= 1385, m/z= 1397, m/z=1414, m/z=1429 and m/z=1442 (Fig. 3 and Table 1) are included in supplementary material as figures (Figs. S9 to S16), as a text (section: MALDI-MS/MS: analysis range m/z 1200-1500) and detailed in the Table S1 too. From the analysis of these spectra was clearly concluded that the butadienolide argininyl diacid moieties were present in these compounds (Table 2, bufadienolides ester moiety: i.e., m/z=669, m/z=671, m/z/=685, m/z=699, m/z=701, m/z=715, m/z=713 and m/z=729; argininyl diacids moiety, i.e., adipoyl arginine at m/z=303, pimeloyl arginine at m/z=317 and suberoyl arginine at m/z=331). Besides, the characteristic fragments from argininyl diacids were also detected at m/z=175 and m/z=158 as well as the peak at $[M+H-157]^+$ (Scheme 2) in agreement with the presence of argininyl diacid moieties in the molecules. The presence of more than one argininyl bufadienolide ester moiety as fragment in the m/z region 600-800 (Figs. S9-S16; signals at m/z 600-800) indicates the existence in the secretion of several dimeric isomers. of argininyl bufadienolide esters.

Another signals observed in the range 1210 <m/z <1270 (Figs. 3 and. S8) could be explained by the lost of an arginyl diacid moiety (m/z=157) from different dimers: m/z= 1214, m/z= 1229 and m/z=1244 from m/z=1369, m/z=1385 and m/z=1400 respectively (Table 1).

Similar results were obtained in mass spectra in the range 1300 < m/z < 1500 for juvenile compare with male adult specimens. Furthermore, no significant differences were observed when samples collected in July or December were studied (Figs. 3 vs S8). Thus, the dimeric butadienolide argininyl diacid are always present in the secretions of *Rhinella arenarum*. in spite of the fingerprint for male and female are quite different

3.4. MALDI-MS: analysis range m/z < 600

As it is indicated in Table 1, dehydrobufotenin was detected in all samples studied (Fig S17 ESI-Orbitrap, m/z range 0-300). Fragmentation of precursor ion with m/z=203 yields the expected fragments at m/z=188 $[M\text{-}CH_3]^+$, m/z=173 $[M\text{-}2(CH_3)]^+$ and m/z=145 $[N(CH_3)_3)]^+$ (Fig. S18).

The ESI-Orbitrap analysis in the m/z range 260-460 showed the protonated ions [M+H]+ corresponding to the following species: glutaryl arginine at m/z=289, adipoyl arginine at m/z=303, pimeloyl arginine at m/z=317, suberoyl arginine at m/z=331, azelayl arginine at m/z=345, (Fig S19, Table 2). Besides, scillaridin A at m/z=369, resibufogenin at m/z=385 $[M+H]^+$ and at m/z=407 $[M+Na]^+$, marinobufagin at m/z=401 $[M+H]^+$ and at m/z=423 $[M+Na]^+$, telocinobufagin at m/z=403 $[M+H]^+$ and at m/z=425 $[M+Na]^+$, bufotalin at m/z=415 $[M+H]^+$ and at m/z=437 $[M+Na]^+$, bufarenogin (arenofufagin or hellebrigenin) at m/z=417 $[M+H]^+$ and at m/z=439 $[M+Na]^+$ were also observed (Fig S20 and Tables 2).

3.5 MALDI-MS: analysis range m/z > 6000

Figs S21 (a) and (b) show signals in the region with m/z > 6000, detected in MALDI mass spectra of secretion collected from adult specimens, in July. Similar results were obtained from female and male secretions but higher intensity signals were always observed in male samples. Studies trying to characterize this components are under progress in our laboratory. MS/MS experiments are essential to be able to get conclusions about possible chemical structure of theses ions.

3.6 Principal component analysis. The MALDI spectra of male and female secretions were compared by principal component analysis (PCA) (Fig. S22). The data set consists of *m/z*

values and the corresponding signal intensities for several compounds. As is displayed in Fig S22 PCA shows that the variations in the intensity distribution contain enough information to obtain a clear differentiation between male and female adult specimens. Using PCA no significant differences were obtained between mass spectra of male juvenile or adult specimens. Furthermore, mass spectra from secretions collected in reproductive period (December) or non-reproductive period (July) did not present any significant difference.

4. Conclusions

The venom of amphibians is a fascinating source of chemical substances. Particularly, *Rhinella arenarum* paratoid glands were chosen for the present study. Among the different families of chemical compounds described in the gland's content, here the argininyl bufadienolide esters family has been easily studied by applying MALDI-MS, and complementary MALDI-MS/MS, straight on crude paratoid gland secretions. The analysis were performed using the whole suspensions (solid + supernatant) obtained just suspending crude secretions in MeOH (or MeCN TFA 0.1% 30:70 (v/v)). Female and male specimens showed characteristic fingerprints in two m/z regions: 600-800 and 1200-1600. In the former female and male secretions showed some differences in its fingerprints with additional variation depending on the season that secretions were obtained (July, non- reproductive; December, reproductive, in Argentina). In the latter, female showed in general a more complicated pattern of signals, with higher number of chemical species with m/z > 1400. Pour findings showed that MALDI-MS fingerprints from crude (raw) secretions can be a tool of choice for characterization of amphibian's secretions and for finding out new biomolecules naturally present in the material but may be lost during chemical manipulation (work-up) of the secretions.

Conflict of Interests

The authors state that they do not have any direct financial relationship with the commercial identities mentioned in this paper that might lead to conflict of interest for any of the authors. The authors declare that there are no conflicts of interest and affirm that this paper consists of original and unpublished work.

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Table 1. Bufalin argininyl diacids in parotoid gland secretions of *Rhinella arenarum* detected by MALDI-MS. Analysis of crude secretions for from male and female, juvenile and adult (not-reproductive and reproductive) specimens, collected in July and December (not reproductive and reproductive season). Experimental m/z values and probable structure for ion peaks observed.

	m/z	Female		Male			adduct	Tentative structure assigned [c]
		Adult [a]		Young [a]	Adult [a]			
		Jul[b]	Dec[b]	Dec[b]	Jul[b]	Dec[b]		
1	203 [d]	+	+	+	+	+	$[M+H]^+$	Dehydrobufotenin MS/MS: Table S1 and Fig. S18
2	633 [d]	-	-	-	-	+		Not assigned
3	645 [d]	-	-	+	-	+		Not assigned
4	657 [d]	+	-	+	+	+	$[M+H]^+$	3-(N- glutaryl argininyl)-bufalin
5	669 [d,e]	+	+	+	+	+	$[M+H]^+$	3-(N- adipoyl argininyl)-resibufogenin MS/MS: Table S1 and Fig. S4
6	671 [d]	+	+	+	+	+	$[M+H]^+$	3-(N- adipoyl argininyl)-bufalin
7	683 [d]	+	-	+	+	+	$[M+H]^+$	3-(N- pimeloyl argininyl)-resibufogenin
8	685 [d,f]	+	+	+	+	+	$[M+H]^+$	3-(N- pimeloyl argininyl)-bufalin/ 3-(N- adipoyl argininyl)-marinobufagin MS/MS: Table S1, Fig. 2 and Fig. S3
9	691 [d]	+	-	+	-	+	$[M+Na]^+$	3-(N- adipoyl argininyl)-resibufogenin
10	693 [d]	-	-	-	+	-	$[M+Na]^+$	3-(N- adipoyl argininyl)-bufalin
11	696 [d]	-	+	-	-	-	$[M+Na]^+$	3-(N-glutaryl argininyl)-telocinobufagin
12	699 [d-f]	+	+	+	+	+	$[M+H]^+$	3-(N-suberoyl argininyl)-bufalin (bufalitoxin)/ 3-(N- pimeloyl argininyl)-marinobufagin/)/ 3-(N- adipoyl)-bufotalinin MS/MS: Table S1 and Fig. S5
13	701 [d]	+	+	+	+	+	$[M+H]^+$	3-(N-pimeloyl argininyl)-telocinobufagin, 3-(N- adipoyl argininyl)-bufarenogin or arenobufagin or hellebrigenin
14	707 [d]	+	-	+	-	+	$[M+Na]^+$	3-(N- pimeloyl argininyl)-bufalin/ 3-(N- adipoyl argininyl)-marinobufagin
15	710 [d]	-	+	-	-	-	$[M+Na]^+$	3-(N-adipoyl argininyl)-telocinobufagin
16	713 [d]	+	+	+	+	+	$[M+H]^+$	3-(N-azelayl argininyl)-bufalin/3-(N- suberoyl argininyl)-marinobufagin (marinobufotoxin)/ 3-(N-pimeloyl argininyl)-bufotalinin
17	715 [d-fc]	+	+	-	+	+	$[M+H]^+$	3-(N-suberoyl argininyl)-telocinobufagin (telocinobufatoxin), 3-(N-pimeloyl argininyl)-bufarenogin or arenobufagin or hellebrigenin MS/MS: Table S1 and Fig. S6
18	721 [d]	+	-	+	+	+	$[M+Na]^+$	3-(N-suberoyl argininyl)-bufalin (bufalitoxin)/ 3-(N- pimeloyl argininyl)-marinobufagin/)/ 3-(N- adipoyl)-bufotalinin

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19	724 [d]	-	+	-	-	-	$[M+Na]^+$	3-(N-pimeloyl argininyl)-telocinobufagin, 3-(N- adipoyl argininyl)-bufarenogin or arenobufagin or hellebrigenin
20	727 [d]	+	-	-	-	-	$[M+H]^+$	3-(N-sebacyl argininyl)-bufalin/3-(N- azelayl argininyl)-marinobufagin/3-(N-suberoyl argininyl)-bufotalinin
21	729 [d–f]	+	+	+	+	+	$[M+H]^+$	3-(N- adipoyl argininyl)-bufotalin/ 3-(N- suberoyl argininyl)-bufarenogin or arenobufagin or hellebrigenin MS/MS: Table S1 and Fig. S7
22	736 [d]	-	+	-	-	-	$[M+Na]^+$	3-(N-azelayl argininyl)-bufalin/3-(N- suberoyl argininyl)-marinobufagin (marinobufotoxin)/ 3-(N-pimeloyl argininyl)-bufotalinin
23	738 [d]	-	+	-	-	-	$[M+Na]^+$	3-(N-suberoyl argininyl)-telocinobufagin (telocinobufatoxin), 3-(N-pimeloyl argininyl)-bufarenogin or arenobufagin or hellebrigenin
24	745 [d]	+	-	-	-	+		Not assigned
25	752 [d]	-	+	-	-	-	$[M+Na]^+$	3-(N- suberoyl argininyl)-bufarenogin or arenobufagin or hellebrigenin
26	757 [d]	+	-	-	-	+	$[M+H]^+$	3-(N- suberoyl argininyl)-bufotalin (bufotoxin) /3-(N-undecadienoyl argininyl)-telocinobufagin/3-(N-sebacyl argininyl)-bufarenogin or arenobufagin or hellebrigenin
27	760 [d]	-	+	-	-	-		Not assigned
28	1214 [g]	+	-	-	+	+	$[M+H]^+$	1369-157=1212 (Dimeric structure without one arginyl moiety)
29	1229 [g]	+	-	-	+	+	$[M+H]^+$	1385-157=1228 (Dimeric structure without one arginyl moiety)
30	1244 [g]	+	+	+	+	+	$[M+H]^+$	1400-157=1243 (Dimeric structure without one arginyl moiety)
31	1339 [g]	+	+	+	+	+	$[M+H]^+$	MS/MS: Table S1 and Fig. S9
32	1354 [g]	+	+	+	+	+	$[M+H]^+$	MS/MS: Table S1 and Fig. S10
33	1369 [g]	+	+	+	+	+	$[M+H]^+$	MS/MS: Table S1 and Fig. S11
34	1385 [g]	+	+	+	+	+	$[M+H]^+$	MS/MS: Table S1 and Fig. S12
35	1397 [g]	+	+	+	+	+	$[M+H]^+$	MS/MS: Table S1 and Fig. S13
36	1414 [g]	+	+	+	+	+	$[M+H]^+$	MS/MS: Table S1 and Fig. S14
37	1429 [g]	+	+	-	-	-	$[M+H]^+$	MS/MS: Table S1 and Fig. S15
38	1442 [g]	+	+	-	-	-	$[M+H]^+$	MS/MS: Table S1 and Fig. S16
39	1458 [g]	+	+	-	-	-		Not assigned
40	1472 [g]	+	+	-	-	-		Not assigned

41	6012 [h]	+	-		+	-		Not assigned
42	6028 [h]	+	+		+	+		Not assigned
43	6044 [h]	+	-		+	-		Not assigned
44	6068 [h]	-	-		+	-		Not assigned

[a] Adult: specimen in reproductive stage; Young: specimen in non-reproductive stage.
[b] July: non-reproductive period of the year in Argentina; December: reproductive period of the year in Argentina.
[c] As explained in the main text, several isomeric bufalin argininyl diacids as monomer species (m/z range 600-800) or dimeric species (m/z range 1200-1600) are suggested as the most probable structures.
[d] Fig. 1.
[e] G. Schmeda-Hirschmann, C. Quispe, C. Theoduloz, P. Teixeira de Sousa, C. Parizotto. Antiproliferative activity and new argininyl bufadienolide esters from the "Cururú" toad *Rhinella (Bufo) schneideri*. *J. Ethnopharmacol.* **2014**, *155*, 1076. Ref. [16].
[f] G. Schmeda-Hirschmann, C. Quispe, G.V. Arana, C. Theoduloz, F.A. Urra, C. Cárdenas. Antiproliferative activity and chemical composition of the venom from the Amazonian toad *Rhinella marina* (Anura: Bufonidae). *Toxicon.* **2016**, *121*, 119. Ref. [15].
[g] Fig. 3.
[h] Fig. S21 (supplementary information).

Table 2: Formula, molecular weight of protonated and sodiated adducts and principal fragments from argininyl bufadienolide esters.

					Argininyl bufadienolide esters $[M+H]^+$								
Bufadienolides	**formula**	**$[M+H]^+$**	**$[M+Na]^+$**	**MS MS**	**n**	3	4	5	6	7	8	9	10
					Argininyl diacids ($[M+H]^+$)	Glutaric (289)	Adipic ($303^{a,b}$)	Pimelic ($317^{a,b}$)	Suberic ($331^{a,b}$)	Azelaic ($345^{a,b,c}$)	Sebacic ($359^{a,b}$)	Undeca dienoic (373^{b})	Dodeca dienoc (387)
Resibufogenin	$C_{24}H_{32}O_4$	385^{d}	407	366		655	669^{e}	683	697	711	725	739	753
Bufalin	$C_{24}H_{34}O_4$	$387^{a,c,d}$	409	368		657	671	$685^{b,e}$	$699^{a,b,e}$	$713^{a,b}$	$727^{a,b}$	741	755
Marinobufagin	$C_{24}H_{32}O_5$	401^{a-d}	423	382		$671^{a,b}$	$685^{a,b,e}$	$699^{a,b,e}$	$713^{a,b,c}$	$727^{a,b}$	$741^{a,b}$	755^{a}	769^{a}
Telocinobufagin	$C_{24}H_{34}O_5$	$403^{a,c,d}$	425	384		673	687^{b}	$701^{a,b}$	$715^{a,b,e}$	729	743^{a}	757	771
Bufotalinin	$C_{24}H_{30}O_6$	415^{c}	437	396		685	699^{e}	713	727	741	755	769	783
Ψ Bufarenogin/ Bufarenogin	$C_{24}H_{32}O_6$	417^{c}	439	398		687	701	715^{e}	729^{e}	743	757	771	785
Arenobufagin	$C_{24}H_{32}O_6$	417^{c}	439	398		687	701	715^{e}	729^{e}	743	757	771	785
Hellebrigenin	$C_{24}H_{32}O_6$	417	439	398		687	701	715^{e}	$729^{a,b,e}$	743	757	771	785
Bufotalin	$C_{26}H_{36}O_6$	445	467	426		715	729^{e}	743	757	771	785	799	813

[a-d] Data from bibliography: [a] Schmeda-Hirshmann et al., 2014 (*Rhinella schneideri*), Ref. [16]; [b] Schmeda-Hirshmann et al., 2016 (*Rhinella marina*), Ref. [15]; [c] W.H.P. Córdova, S.G. Leitão, G. Cunha-Filho, R.A. Bosch, I.P. Alonso, R. Pereda-Miranda, R. Gervou, N.A.Touza, L.E.M. Quintas, F. Noël. Bufadienolides from parotoid gland secretions of Cuban toad *Peltophryne fustiger* (Bufonidae): Inhibition of human kidney Na+/K+-ATPase activity. *Toxicon* **2016**, *110*, 27; [d] Ferreira et al., 2013 (*Rhinella marina*), Ref. [10]; [e] tentative structure by MS/MS this work (*Rhinella arenarum*).

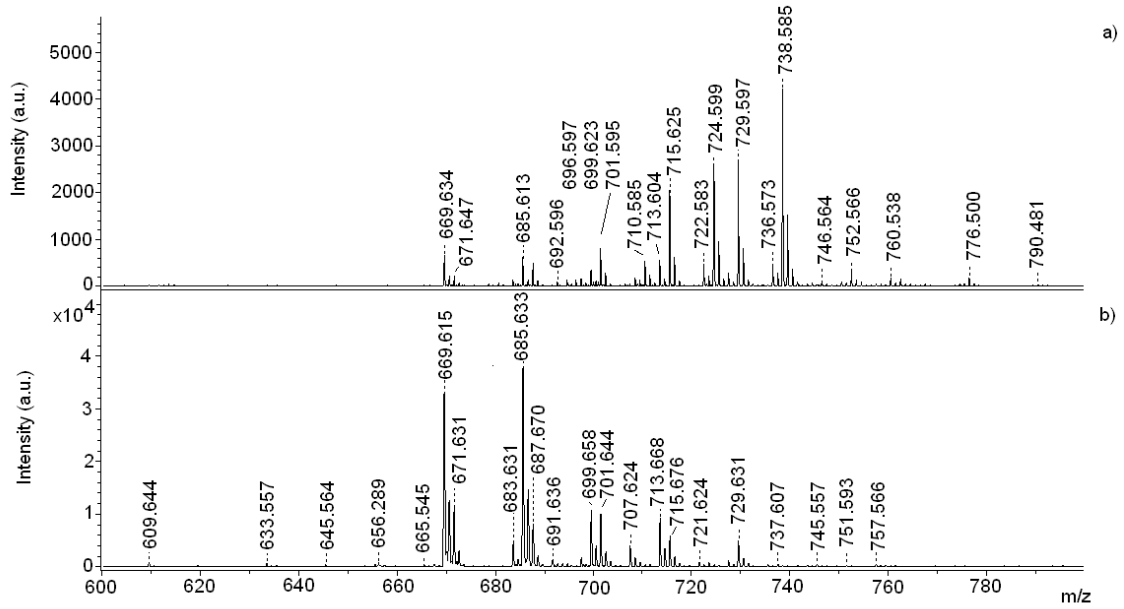

Figure 1. Positive ion MALDI mass spectra of secretion collected from adult specimens, in December: a) female, b) male. Solvent: MeOH. MALDI matrix: CHCA.

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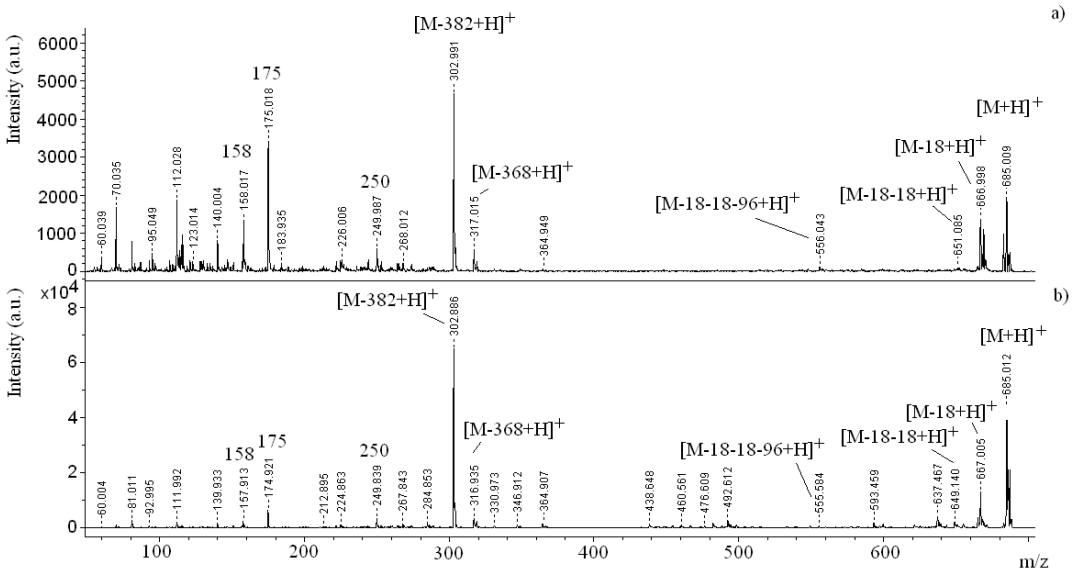


Figure 2. Positive ion MALDI MS/MS spectra of precursor ion m/z = 685 from a) adult male secretion collected in December, b) adult female secretion collected in July. MALDI matrix: CHCA.

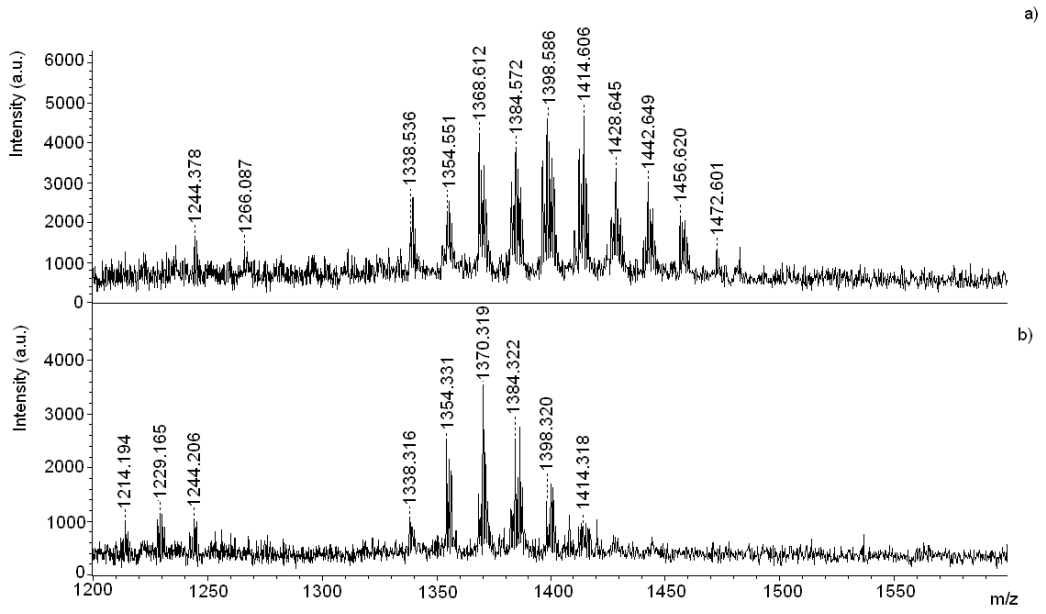


Figure 3. Positive ion MALDI mass spectra of secretion collected from adult specimens, in December: a) female, b) male. Solvent: MeOH. MALDI matrix: CHCA.

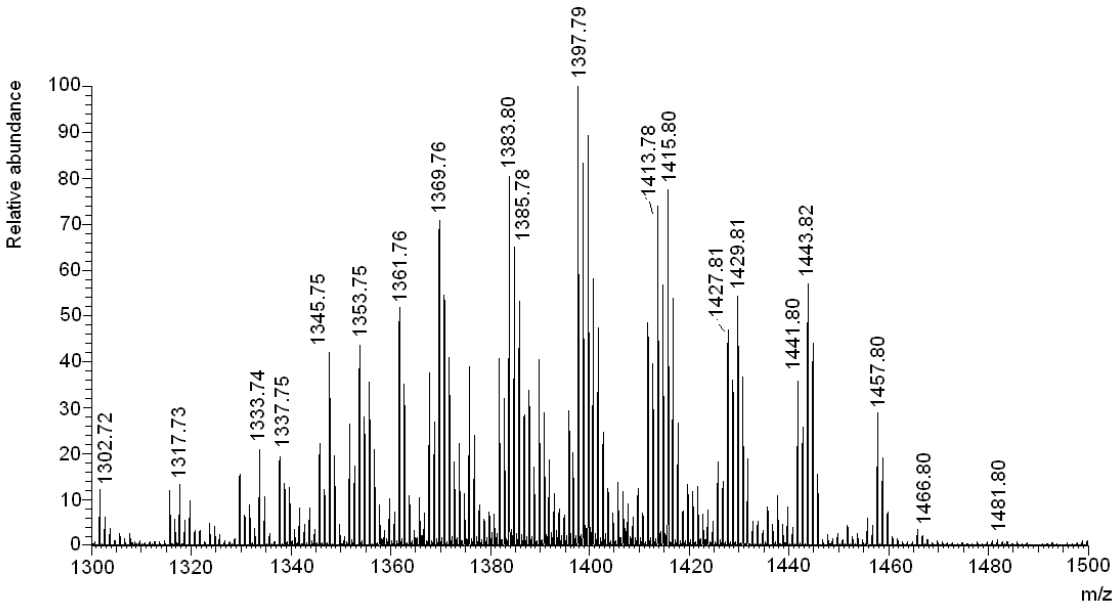
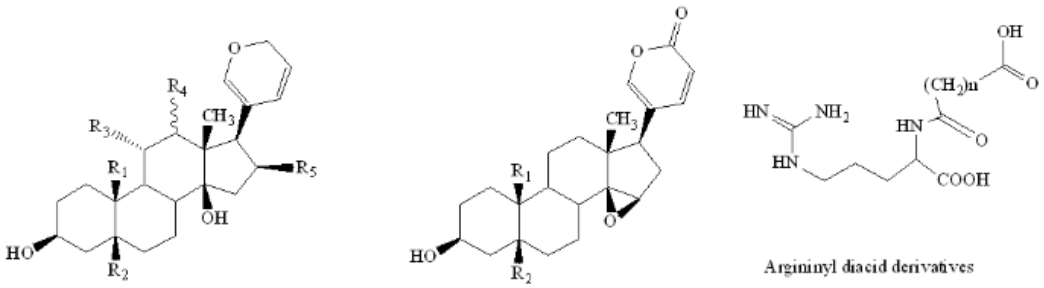


Figure 4. Positive ion ESI mass spectrum of secretion collected from adult female, in December. Solvent: MeOH.



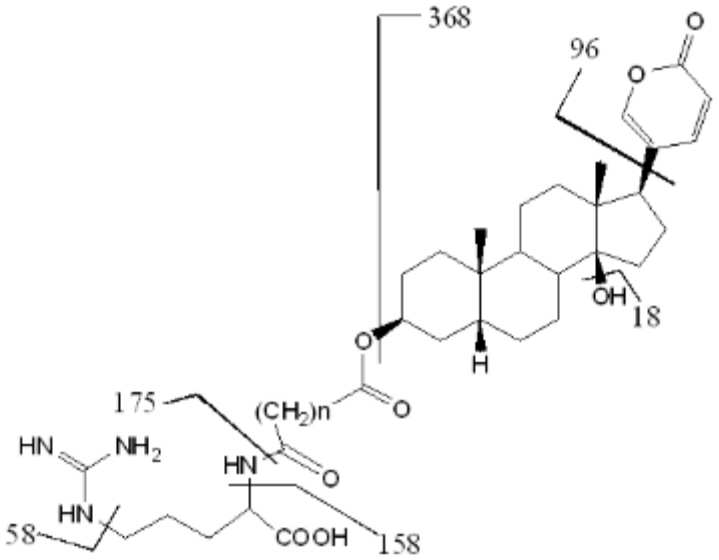
Trivial name	R_1	R_2	R_3	R_4	R_5
bufalin	CH_3	H	H	H	H
telocinobufagin	CH_3	OH	H	H	H
arenobufagin	CH_3	H	OH	=O	H
ψ-bufarenogin	CH_3	H	=O	α-OH	H
bufarenogin	CH_3	H	=O	β-OH	H
hellebrigenin	CHO	OH	H	H	H
bufotalin	CH_3	H	H	H	OAc

Trivial name	R_1	R_2
resibufogenin	CH_3	H
marinobufagin	CH_3	OH
bufotalinin	CHO	OH

Argininyl diacid derivatives

n	Diacid
3	glutaric acid
4	adipic acid
5	pimelic acid
6	suberic acid
7	azelaic acid
8	sebacic acid
9	undecadienoic acid
10	dodecadienoic acid

Scheme 1. Chemical structure of the steroids present in *Rhinella sp.* parotide secretion.



Scheme 2. Proposed fragmentation profile of bufalin argininyl diacid derivatives (n=3 to n=10) from *Rhinella sp.*; by MS/MS these compounds produce a fragment with m/z M-368. Similarly, resinobufogenin, marinobufagin, bufotalinin, telocinobufagin, Ψ-bufarenogin, bufarenogin, arenobufagin, hellegrigenin and bufotalin derivatives produce by MS/MS a fragment with with m/z M-366, M-382, M-396, M-384, M-398, M-398, M-398, M-398 and M-426 respectively.