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Technological research article

## Development and validation of a new rapid reversed-phase HPLC method for the simultaneous quantification of melittin and apamin in apitoxin

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### SUMMARY

**Context:** High performance liquid chromatography (HPLC) is a high sensitivity and precision to apitoxin samples analysis. However, it has the disadvantage of incurring high financial, environmental and time costs. **Aims:** The aim of this study is to develop a shorter HPLC method for the quantification of apitoxin samples. **Methods:** The method was performed and validated on an RP-C18 column, mobile phases A (water and trifluoroacetic acid (TFA) 0.1%) and B (acetonitrile and TFA 0.1 %), gradient mode, flow rate 1.5 mL/min and 220 nm absorbance. **Key results:** The gradient elution 5% - 70% phase B over 20 minutes, after a 4-minute isocratic elution with 5% B at 25°C. Results showed a linear in the range between 0.084 and 0.84 mg/mL ( $R^2=0.995$ ) for melittin and between 0.02 and 0.20 mg/mL ( $R^2=0.995$ ) for apamin, accurate method (recovery rate 101.11  $\pm$ 9.47 % and 113.57  $\pm$ 15.06 % respectively). Precision: 10.37 and 9.33 for melittin and apamin respectively, and result of a selective method in its usual matrix. The LOD was 0.00178 and 0.00117 mg/mL, and LOQ was 0.00541 and 0.00353 mg/mL, for melittin and apamin, respectively.

**Keywords:** apamin; *Apis mellifera*; bee venom; HPLC analysis; melittin.

### RESUMEN

#### Desarrollo y validación de un nuevo método rápido de HPLC en fase reversa para la cuantificación simultánea de melitina y apamina en apitoxina

**Contexto:** La cromatografía líquida de alta resolución (HPLC) es un método de alta sensibilidad y precisión para el análisis de muestras de apitoxina. Sin embargo, tiene la desventaja de generar altos costos financieros, ambientales y de tiempo. **Objetivos:** El objetivo de este estudio es desarrollar un método de HPLC más corto para la cuantificación de muestras de apitoxina. **Métodos:** El método se realizó y validó en una columna RP-C18, fases móviles A (agua y ácido trifluoroacético (TFA) 0,1%) y B (acetonitrilo y TFA 0,1%), modo gradiente, velocidad de flujo 1,5 mL/min y absorbancia A 220 nm. **Resultados clave:** La elución en gradiente 5% - 70% de la fase B durante 20 minutos, después de una elución isocrática de 4 minutos con 5% de B a 25 °C. Los resultados mostraron una linealidad en el rango entre 0,084

y 0,84 mg/mL ( $R^2=0,995$ ) para melitina y entre 0,02 y 0,20 mg/mL ( $R^2=0,995$ ) para apamina, método preciso (tasa de recuperación  $101,11 \pm 9,47\%$  y  $113,57 \pm 15,06\%$  respectivamente). Precisión: 10,37 y 9,33 para melitina y apamina respectivamente, y resultado de un método selectivo en su matriz habitual. El LOD fue 0,00178 y 0,00117 mg/mL, mientras que el LOQ fue 0,00541 y 0,00353 mg/mL, para melitina y apamina, respectivamente.

**Palabras clave:** apamina; *Apis mellifera*; veneno de abeja; análisis HPLC; melitina.

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## RESUMO

### Desenvolvimento e validação de um novo método rápido de HPLC de fase reversa para quantificação simultânea de melitina e apamina em apitoxina

**Contexto:** A cromatografia líquida de alta eficiência (HPLC) é uma análise de amostras de apitoxina de alta sensibilidade e precisão. No entanto, tem a desvantagem de incorrer em altos custos financeiros, ambientais e de tempo. **Objetivos:** O objetivo deste estudo é desenvolver um método de HPLC mais curto para quantificação de amostras de apitoxina. **Métodos:** O método foi realizado e validado em uma coluna RP-C18, fases móveis A (água e ácido trifluoroacético (TFA) 0,1%) e B (acetonitrila e TFA 0,1%), modo gradiente, vazão de 1,5 mL/min e absorvância de 220 nm. **Principais resultados:** A eluição do gradiente de 5% - 70% da fase B ao longo de 20 minutos, após uma eluição isocrática de 4 minutos com 5% de B a 25 °C. Os resultados mostraram uma linearidade no intervalo entre 0,084 e 0,84 mg/mL ( $R^2=0,995$ ) para melitina e entre 0,02 e 0,20 mg/mL ( $R^2=0,995$ ) para apamina, método preciso (taxa de recuperação de  $101,11 \pm 9,47\%$  e  $113,57 \pm 15,06\%$  respectivamente). Precisão: 10,37 e 9,33 para melitina e apamina respectivamente, e resultado de um método seletivo em sua matriz usual. O LOD foi de 0,00178 e 0,00117 mg/mL, e o LOQ foi de 0,00541 e 0,00353 mg/mL, para melitina e apamina, respectivamente.

**Palavras-chave:** apamina; *Apis mellifera*; veneno de abelha; análise por HPLC; melitina.

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## 1. INTRODUCTION

Apitoxin is the solid residue that results from the evaporation of bee venom (*Apis mellifera*) [1]. It is usually a complex mixture of nitrogenous compounds in which peptides predominate. It also contains enzymes, biogenic amines, carbohydrates and amino acids [2]. In medicine, it can be used for the treatment of various diseases [3-5]. It is presented in different ways, sometimes with separate components, or it has been shown that the synergy between some components can make a better contribution to the treatment of certain diseases [6-8]. These characteristics make it a product with high potential for use in the pharmaceutical industry [9].

Apitoxin has the particularity of being a natural base, so its quantitative composition can vary depending on different factors [10]. The genetics of the bee, the environment in which it develops (climate, availability and abundance of natural food), as well as the health and age of the bee are factors that have been shown to influence the production and properties of apitoxin [1, 11, 12]. It has been reported that environmental and biological factors can determine the quantitative differences in apitoxin, while human factors, such as the method and timing of venom extraction, including processing and storage conditions, should also be considered [11, 13, 14]. Therefore, due to the large number of factors that conditions the composition of apitoxin, its qualitative-quantitative analysis is necessary to control and ensure that this product meets the requirements requested for its commercialization and application in medicine.

High performance liquid chromatography (HPLC) was chosen for the analysis of apitoxin, because it offers advantages in terms of cost-benefit ratio and shorter analysis times compared to other methods such as size exclusion chromatography or capillary electrophoresis (Pacfikovfi *et al.*, 1995) [15]. Melittin, apamin and phospholipase A2 are the main components of

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apitoxin and are therefore analyzed using HPLC methods. In addition, the cost of using standard methods would make these analyses prohibitively expensive. Reports on the analytical methods achieved with this technology include those by Rybak-Chmielewska and Szczêsna (2004) [16] and Ferreira Junior *et al.* (2010) [11]. However, a disadvantage of methods described is the long analysis time of around 35 to 50 minutes per sample, which means a high workload for carrying out these studies with a large number of samples. For this reason, the aim of this study was to develop and validate an HPLC analysis method with a shorter analysis time that still preserves the resolution between the peaks of the main components of apitoxin like melittin and apamin, as the main active ingredients sought in the venom. In addition, the applicability of the developed method in the qualitative-quantitative determination of different samples of apitoxin was investigated.

## 2. METHODOLOGY

The study consisted of two parts. Firstly, the development of a short analytical method using HPLC for the qualitative-quantitative analysis of apitoxin, while maintaining a good resolution of the peaks corresponding to the components of apitoxin. Once the optimized method was obtained, its validation was performed with standards for two of its main components: melittin and apamin. The HPLC system used was an HPLC-UV Dionex, Ultimate 3000, with standard LPG 3400 pump, WPS 3000 PL autosampler, detector (DAD) 3000, 50  $\mu$ L and 250  $\mu$ L loops, (from Thermo Fisher Scientific, Massachusetts, United States) and a C18 reverse phase chromatographic column (Hibar® 5  $\mu$ m, 150  $\times$  4.6 mm, Purospher STAR, from Merck, Darmstadt, Germany). The reagents and solvents used were Carlo Erba ppa., suitable for HPLC. The standards used were bee melittin with a purity of  $\geq$  85 % and a bee apamin standard with a purity of  $\geq$  95 %, acquired through Sigma-Aldrich. The apitoxin used was obtained commercially from Uruguayan producers, it was in its dry form and stored in amber bottles in a freezer at  $-4$  °C, until the time of the analysis.

To prepare the samples, 21 mg of dry apitoxin solubilized in 25 mL of 0.1 % formic acid water solution were used. Once the sample was homogenized, it was filtered through a syringe filter with a 0.45  $\mu$ m membrane and 1 - 1.5 mL was loaded into vials.

### 2.1. Method optimization

They were then placed in HPLC for analysis using the chromatographic specifications described by Ferreira Junior *et al.* (2010) [11]. They were placed in a two-solvent system, where phase A was 0.1% trifluoroacetic acid (TFA) in water ( $H_2O$ ) and phase B was 0.1% TFA in acetonitrile (MeCN). The sample was eluted at a flow rate of 1 mL/min with a gradient elution of 10 to 100 % phase B for 31 minutes, after a 5 minutes isocratic elution with 10 % B at a temperature of 25 °C. The diode array detector was set to a fixed wavelength of 220 and 280 nanometers (nm).

In order to optimize the starting method, 11 different methods were tested, whereby the flow rate and the composition of the mobile phase were varied by the elution gradient. The changes were made starting from the conditions of the starting method, which were monitored by the successive chromatograms, until the selected method was reached. The other conditions remained unchanged

## 2.2. Validation of the analytical method

Validation of the method was performed with melittin and apamin standards to determine linearity, limit of quantification, limit of detection, accuracy, precision and selectivity at two wavelengths ( $\lambda = 220$  nm and 280 nm).

The linearity test consisted of chromatographic analysis of three copies of the same standard concentration used to calculate the calibration curve. The concentrations distributed over the entire range were 0.84, 0.168 and 0.084 mg/mL for melittin and 0.2, 0.04 and 0.02 mg/mL for apamin. Linearity was verified by statistical processing of the data using the correlation coefficient ( $R^2$ ) of the calibration curve, analyzed at the two wavelengths studied.

Detection limit of an assay is the lowest concentration that can be detected but not necessarily quantified and the quantification limit is the lowest concentration that can be quantified with acceptable precision. The limits can be determined by three different methodologies according to the ICH guidelines: visual determination, signal-to-noise determination, and standard deviation and slope method [17, 18]. In this work, we were calculated using the results for linearity and the calibration curve. The following equations were applied:

- Detection limit:  $= \frac{3.3 \times \sigma}{S}$
- Quantification limit:  $= \frac{10 \times \sigma}{S}$

Were  $\sigma$  is the standard deviation of the peak areas for each lowest studied standard concentration level and  $S$  is the slope of the calibration curve.

To check the accuracy, solutions with a concentration of 60, 80, 100, 120 and 140 % of the theoretical concentration were prepared and analyzed three times each. They were then compared with the accepted reference values.

Precision was determined by repeatability and reproducibility. Repeatability is the precision under conditions where independent analytical results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short time intervals. And reproducibility is the precision when the analysis results are obtained with the same method in identical analysis items under different conditions, whether in the laboratory, different operators, using different equipment, among others [17, 18]. For repeatability, the analysis of variance was performed for three replicates were prepared at a concentration of 0.1 mg/mL and each was analyzed six times. Intermediate precision was also measured to determine the test using the repeatability results and comparing them to the results of the same test performed the following day. For intra-laboratory reproducibility (i.e., precision within a laboratory), sample determination was performed in triplicate on 3 different days, and for interlaboratory reproducibility sample determination was performed ten times in the same day. The analysis of variance was performed for both trials. The coefficient of variation (CV%) was calculated as:  $\%CV = (S/X) \times 100$ , where  $X$  is the average of the concentrations.

The selectivity was determined under acidic, alkaline, oxidizing, reducing, thermal and photolytic degradation conditions using a reference solution of 0.1 mg/mL. A 1 M solution of hydrochloric acid (HCl) was added for acidic degradation and 1 M solution of sodium hydroxide (NaOH) was added for alkaline degradation. Both determinations were allowed to act for 1 hour and then analyzed by HPLC. For oxidation degradation, a 10 % hydrogen peroxide ( $H_2O_2$ ) solution was added and left for 10 days, then the corresponding chromatogram was prepared. For the photolysis test, the sample was exposed to sunlight for 10 days and for the thermolysis test, the sample was left at a temperature of 100 °C for 10 days. The samples were then analyzed using HPLC.

### 3. RESULTS AND DISCUSSION

The results obtained by the different modifications compared to the initial method are shown in Table 1. Given the increase in flow rate, the resulting chromatogram showed a decrease in the resolution of the peaks and a reduction in the elution time of the last component. The same happened when the elution gradient was increased. Subsequently, the gradient of the mobile phase was reduced, which led to a decrease in the elution time of the components and the resolution of the peaks. Based on these results, method 11 was selected for validation, with a final analysis time was 24 minutes. The required flow rate for the selected method was 1.5 mL/min, with an elution gradient of 5 to 70 % of phase B from analysis time 0 to 15, followed by an isocratic elution of 5 minutes with 70 % of phase B. Finally, the system stabilizes from 20.01 to 24 minutes with an isocratic flow of 5 % of phase B at a temperature of 25 °C. The retention time for melittin was 10.4 min, for apamin 5.0 min and for phospholipase A2 8.0 min.

**Table 1.** Variations made to optimize the starting method<sup>a</sup>.

| Method | Final time (min) | Changes <sup>b</sup>                                   | Observations on the chromatograms                    |
|--------|------------------|--|--|
| 1      | 38               | Increase flow rate                                     | Increase time and inadequate peak resolution         |
| 2      | 38               | Change gradient elution (5% - 100% phase B)            | Increase time and inadequate peak resolution         |
| 3      | 35               | Change gradient elution (5% - 80%/ 80% - 100% phase B) | Inadequate peak resolution                           |
| 4      | 35               | Change gradient elution (10% - 95% phase B)            | Inadequate peak resolution                           |
| 5      | 30               | Change gradient elution (10% - 95% phase B)            | 2 peaks are observed, and the resolution is low (<2) |
| 6      | 25               | Change gradient elution (5% - 80% phase B)             | 4 peaks are observed, but the resolution is low (<2) |
| 7      | 20               | Change gradient elution (5% - 70% phase B)             | 4 peaks are observed, but the resolution is low (<2) |
| 8      | 20               | Change gradient elution (5% - 60% phase B)             | 4 peaks are observed, but the resolution is low (<2) |
| 9      | 20               | Change gradient elution (2.5% - 70% phase B)           | 4 peaks are observed, but the resolution is low (<2) |
| 10     | 20               | Change gradient elution (10% - 70% phase B)            | 4 peaks are observed, but the resolution is low (<2) |
| 11     | 24               | Change gradient elution (5% - 70% phase B)             | 7 peaks are observed, with an adequate               |

<sup>a</sup> The conditions of the starting method were: flow rate 1.0 mL/min, two-solvent system where phase A was 0.1% TFA in H<sub>2</sub>O and phase B was 0.1% TFA in MeCN, with a gradient elution of 10 to 100 % phase B for 31 minutes, and 5 minutes more with 10 % B, 25 °C, wavelength of 220 and 280 nm. <sup>b</sup> All tests were performed at a flow rate of 1.5 mL/min.

Thus, the results for the various validation parameters linearity, limits of detection and quantification, accuracy, selectivity, precision and selectivity, were as follows:

### 3.1. Linearity, detection limits and quantification limits

Table 2 shows the equations of the corresponding calibration curves for melittin and apamin, which were examined at a wavelength of 220 and 280 nm, respectively. In addition, the linearity result is included; in each case examined the value obtained by the  $R^2$  exceeds 0.995. The values of the limits of quantification and detection are also included.

**Table 2.** Results for melittin and apamin at a wavelength of 220 and 280 nm, from the calibration curve equation, correlation coefficient, quantification limit and detection limit.

| $\lambda^a$ (nm) | Components | Calibration curve      | $R^2$ <sup>b</sup> | Quantification limit (mg/mL) | Detection limit (mg/mL) |
|------------------|------------|------------------------|--------------------|------------------------------|-------------------------|
| 220              | Melittin   | $y = 412159x - 6891.2$ | 0.999              | 0.00541                      | 0.00178                 |
|                  | Apamin     | $y = 727866x - 2444.8$ | 1.000              | 0.00353                      | 0.00117                 |
| 280              | Melittin   | $y = 56307x - 2043.8$  | 0.999              | 0.00310                      | 0.00101                 |
|                  | Apamin     | $y = 10817x - 26.272$  | 0.999              | 0.00571                      | 0.00189                 |

<sup>a</sup>  $\lambda$  (Wavelength). <sup>b</sup>  $R^2$  (correlation coefficient).

### 3.2. Accuracy

Next, the precision is determined (Table 3). For apamin, none of the values obtained at wavelength of 280 nm were within the acceptable range, so they were not considered. For melittin, the results were acceptable at concentrations greater than 0.08. As for the results at 220 nm, with the exception of apamin at a concentration of 0.06 and 0.14, all values examined were acceptable.

**Table 3.** Results of the accuracy study for melittin at 220 and 280 nm and for apamin at 220 nm.

| $\lambda^a$ (nm)      |                         | 220                   |                     | 280                   |
|-----------------------|-------------------------|-----------------------|---------------------|-----------------------|
| Concentration (mg/mL) | Acceptance requirements | Melittin recovery (%) | Apamin recovery (%) | Melittin recovery (%) |
| 0.06                  | 60-115                  | 113.90                | 141.29              | 129.49                |
|                       |                         | Accepted              | Not accepted        | Not accepted          |
| 0.08                  | 60-115                  | 88.09                 | 105.47              | 100.89                |
|                       |                         | Accepted              | Accepted            | Accepted              |
| 0.100                 | 80-110                  | 99.78                 | 105.67              | 112.36                |
|                       |                         | Accepted              | Accepted            | Not accepted          |
| 0.120                 | 80-110                  | 94.39                 | 98.51               | 108.46                |
|                       |                         | Accepted              | Accepted            | Accepted              |
| 0.140                 | 80-110                  | 109.40                | 116.89              | 134.080               |
|                       |                         | Accepted              | Not accepted        | Not accepted          |

<sup>a</sup>  $\lambda$ : Wavelength.

### 3.3. Precision

In the case of precision, measured by analysis of variance of repeatability (Table 4). Acceptance values were achieved in all tests except test 1 for apamin at 280 nm. In the case of mean precision (Table 5), all values fulfil the acceptance criterion.

**Table 4.** Repeatability data obtained for melittin and apamin, corresponding to the two wavelengths studied.

| Component | $\lambda^a$<br>(nm) | Experiment 1    |                  |                 | Experiment 2    |                  |                 | Experiment 3    |                  |                 |
|-----------|---------------------|-----------------|------------------|-----------------|-----------------|------------------|-----------------|-----------------|------------------|-----------------|
|           |                     | CV <sup>b</sup> | CVh <sup>c</sup> | CA <sup>d</sup> | CV <sup>b</sup> | CVh <sup>c</sup> | CA <sup>d</sup> | CV <sup>b</sup> | CVh <sup>c</sup> | CA <sup>d</sup> |
| Melittin  | 220                 | 10.37           | 0.085            | Accepted        | 1.03            | 0.10             | Accepted        | 13.40           | 0.078            | Accepted        |
|           | 280                 | 11.62           | 0.080            | Accepted        | 1.67            | 0.098            | Accepted        | 13.81           | 0.074            | Accepted        |
| Apamine   | 220                 | 9.33            | 0.020            | Accepted        | 1.16            | 0.020            | Accepted        | 10.38           | 0.020            | Accepted        |
|           | 280                 | 1.78            | 0.020            | Not accepted    | 2.42            | 0.020            | Accepted        | 13.50           | 0.020            | Accepted        |

<sup>a</sup>  $\lambda$ : Wavelength. <sup>b</sup> Coefficient of variation (CV) (%) calculated. <sup>c</sup> Horwitz coefficient of variation (CVh) % Horwitz. <sup>d</sup> Acceptance requirements: CV% > CVh %.

**Table 5.** Data obtained from the intermediate precision for melittin and apamin, corresponding to the two wavelengths studied.

| Component                            | Melittin         |          | Apamin           |          |
|--------------------------------------|------------------|----------|------------------|----------|
|                                      | $\lambda^a$ (nm) |          | $\lambda^a$ (nm) |          |
|                                      | 220              | 280      | 220              | 280      |
| CVRi% calculado <sup>b</sup>         | 13.61            | 14.52    | 12.19            | 14.39    |
| CVhRi % Horwitz <sup>c</sup>         | 0.086            | 0.080    | 0.020            | 0.020    |
| Acceptance requirements, CV% > CVh % | Accepted         | Accepted | Accepted         | Accepted |

<sup>a</sup>  $\lambda$ : Wavelength. <sup>b</sup> CVRi (Internal Repeatability Coefficient of Variation) % calculated. <sup>c</sup> CVhRi % Horwitz (Horwitz coefficient of variation of internal repeatability).

The precision measured by analysis of variance of intra-laboratory and interlaboratory reproducibility showed in the table 6. Acceptance values were achieved in all tests except test 1 for apamin at 280 nm. Referring to the interlaboratory assay, only the quantification of melittin was carried out, apamin was not analyzed. Prior to this test, linearity was determined, resulting in non-linearity at the wavelength of 280 nm for that equipment. For this reason, reproducibility was not determined at that wavelength.

**Table 6.** Intra-laboratory and interlaboratory reproducibility data obtained for melittin and apamin, corresponding to the two wavelengths studied.

| Component | $\lambda^a$<br>(nm) | Intra-laboratory |                  |                 | Interlaboratory |                  |                 |
|-----------|---------------------|------------------|------------------|-----------------|-----------------|------------------|-----------------|
|           |                     | CV <sup>b</sup>  | CVh <sup>c</sup> | CA <sup>d</sup> | CV <sup>b</sup> | CVh <sup>c</sup> | CA <sup>d</sup> |
| Melittin  | 220                 | 6.34             | 20.68            | Accepted        | 2.26            | 17.61            | Accepted        |
|           | 280                 | 8.52             | 21.51            | Accepted        | NA              | NA               | NA              |
| Apamine   | 220                 | 7.88             | 34.92            | Accepted        | NA              | NA               | NA              |
|           | 280                 | NA               | NA               | NA              | NA              | NA               | NA              |

<sup>a</sup>  $\lambda$ : Wavelength. <sup>b</sup> Coefficient of variation (CV) (%) calculated. <sup>c</sup> Horwitz coefficient of variation (CVh) % Horwitz. <sup>d</sup> Acceptance requirements: CVRintra% < (2CVh%/3). <sup>e</sup> Acceptance requirements: CVRinterlab% < CVh%, NA.: not applicable.

### 3.4. Selectivity

The results of the selectivity study (Table 7) show that while all values are acceptable melittin at 220 nm, it does not achieve the expected results for acid degradation and the photolysis test at 280 nm. Since apamin was not quantifiable under the working conditions at 280 nm, the corresponding measurements were not performed. At 220 nm, although the method has shown that resolutions greater than 2 are not achieved, it would not be possible to quantify the product, therefore the method is not selective in this case, but it is possible to identify the compound in each of the tests to which it was subjected.

**Table 7.** Selectivity data obtained for melittin corresponding to the two working wavelengths and for apamin at 220 nm.

| Component         |                         | Solution not subject to degradation | Acid degradation   | Basic degradation  | Oxidation degradation | Photolysis        | Thermolysis        |
|-------------------|-------------------------|-------------------------------------|--------------------|--------------------|-----------------------|-------------------|--------------------|
| Melittin (220 nm) | RP <sup>a</sup> (%)     | 100.00                              | 37.83              | 29.80              | 5.70                  | 65.34             | 96.08              |
|                   | Resolution <sup>b</sup> | -                                   | 13.14 <sup>c</sup> | 11.95 <sup>c</sup> | 19.23 <sup>c</sup>    | 3.64 <sup>c</sup> | 13.02 <sup>c</sup> |
| Melittin (280 nm) | RP <sup>a</sup> (%)     | 100.00                              | 10.12              | 11.64              | 12.03                 | 13.30             | 9.69               |
|                   | Resolution <sup>b</sup> |                                     | 9.03 <sup>c</sup>  | 1.62 <sup>d</sup>  | 3.07 <sup>c</sup>     | 0.78 <sup>d</sup> | 2.54 <sup>c</sup>  |
| Apamin (220 nm)   | RP <sup>a</sup> (%)     | 100.00                              | 123.16             | 117.40             | 86.83                 | 105.59            | 105.22             |
|                   | Resolution <sup>b</sup> |                                     | 0.68 <sup>d</sup>  | 1.12 <sup>d</sup>  | 0.96 <sup>d</sup>     | 1.20 <sup>d</sup> | 0.40 <sup>d</sup>  |

<sup>a</sup> RP: Recovery percentage. <sup>b</sup> Acceptance criteria:  $R > 2$  for adjacent peaks (R: resolution). <sup>c</sup> Fulfills the acceptance criteria. <sup>d</sup> Doesn't fulfill the acceptance criteria.

Among the methods studied, there were some that had a shorter analysis time than the one chosen as a starting point, but with unacceptable resolutions between peaks ( $R \geq 2$ ), this parameter being defined as a numerical measure that indicates the quality of a separation between two compounds [11, 18]. Low resolution results leads to insufficient analysis to identify and quantify the main constituents and thus do not fulfil the stated objectives.

When selecting the variables for method optimization, it was assumed that each one of them exhibited compromised results in the chromatographic test. These compromised results were considered for achieving a method that not only reduces analysis time but is also effective in quali-quantify assessing its main components.

The variables chosen for the method optimization included the flux and composition of the mobile phase determined by gradient variation. Among these, increasing the flux in a chromatographic analysis causes the main components of the injected mixture to elute more quickly, thereby shortening the run time. However, this may compromise the resolution of the peaks. Hence, adjustments to the variation of mobile phase gradients need to be complemented.

On the other hand, a higher concentration of the mobile phase organic modifier used (MeCN) resulted in a quicker elution of the mixture's components, leading to a decreased resolution between the peaks. Consequently, the elution gradient was adjusted by varying the proportion of acetonitrile, aiming to reduce retention times while attempting to approximate isocratic compositions of the mobile phase.

It was possible to establish a gradient that facilitates the elution of all components within the mixture in a 20-minute timeframe, with an additional 4 minutes required for final stabilization before commencing the next run.



To assess optimized method, linearity is determined within a range of useful concentrations, with the acceptance criterion set at  $R^2 \geq 0.995$  for linear regression. All the obtained results surpassed this threshold, indicating that the method meets the linearity parameter within the study range.

While most accuracy and precision parameters for melittin complied within acceptance criteria at the two working wavelengths, certain parameters for apamin at 280 nm, such as accuracy and some precision tests, did not meet the established limits. This discrepancy is attributed to the lower absorptivity of apamin at 280 nm in the absorption spectrum, making it less visible to the detector and compromising its quantification. Despite this, due to the considerably higher concentration of melittin compared to apamin, quantification of melittin is still possible at that wavelength.

Attempts to increasing the concentration of apamin in apitoxin for analysis were hindered by the apitoxin maximum solubility. Consequently, it was determined that at 280 nm, a qualitative-quantitative analysis of the apitoxin is not feasible.

After validating the method for the two major components of the apitoxin, future researchers are encouraged to explore the possibility of validating the method for phospholipase A2, thereby enriching the analytical approach.

This work has some limitations that should be considered while interpreting the data. First, it was not possible to quantify apamin at the wavelength of 280 nm, so the use of the method is restricted to 220 nm. This is due to the low concentrations of apamin and that it has only 1 heterocycle in its structure that can be detected at 280 nm. Second, this method has been optimized for the quantification of melittin and apamin at 220, but the quantification of other main components of apitoxin will be included later. Third, it was not possible to determine the interlaboratory reproducibility for apamin since there were no standards to perform this assay, and it was also not possible to determine for melittin at 280 nm as wavelength since the response of the detector was not linear with the melittin concentrations used.

In conclusion, an HPLC analytical method was developed and validated to determine the qualitative-quantitative composition of the main components of apitoxin at 220 and 280 nm. The developed method presents a reduction in analysis times per sample of 12 minutes compared to the initial method, allowing a greater number of samples to be processed in less time. Regarding the validation carried out, it was found that the validated method is linear in the range between 0.084 and 0.84 mg/mL ( $R^2=0.995$ ) for melittin and from 0.02 to 0.20 mg/mL ( $R^2=0.995$ ) for apamin, accurate (recovery rate  $101.11 \pm 9.47\%$  and  $113.57 \pm 15.06\%$  respectively), and selective in its usual matrix for the quantification of melittin and apamin at 220 nm. This method was also precise, showing compliance with the acceptance criteria for intra and interlaboratory reproducibility and repeatability tests. The LOD was 0.00178 and 0.00117 mg/mL, and LOQ was 0.00541 and 0.00353 mg/mL for melittin and apamin, respectively. According to the results obtained, the appropriate wavelength for the qualitative-quantitative analysis of apitoxin is 220 nm.

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## CONFLICT OF INTEREST

All authors report that they do not have any conflicts of interest.

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