



## Detection of biogenic amines in mead of social bee

Irana Paim Silva<sup>a,\*</sup>, Luís Guimarães Dias<sup>b</sup>, Macela Oliveira da Silva<sup>a</sup>, Cerilene Santiago Machado<sup>a</sup>,  
Vanessa Marina Branco Paula<sup>b</sup>, Norma Suely Evangelista-Barreto<sup>a</sup>,  
Carlos Alfredo Lopes de Carvalho<sup>a</sup>, Letícia M. Estevinho<sup>b</sup>

<sup>a</sup> Centro de Ciências Agrárias, Ambientais e Biológicas, Universidade Federal Do Recôncavo da Bahia, Cruz Das Almas, Bahia, Brazil

<sup>b</sup> Centro de Investigação de Montanha - CIMO, Escola Superior Agrária, Instituto Politécnico de Bragança, Campus Santa Apolónia, 5301-855, Bragança, Portugal

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### ABSTRACT

Mead is an alcoholic beverage, resulting from the fermentation of honey and water, although it is an old drink, some fermentation products are still unknown. Biogenic amines have driven research on food and beverages due to their toxicity and capacity to cause damage to the human health. This study aimed to evaluate the presence of biogenic amines in mead from honey of two species of social bees, using different fining agents (bentonite, banana peel flour, and passion fruit peel flour) and storage in oak barrel for 120 days. We observed the presence of three biogenic amines for each type of mead. Putrescine was reported only in samples of mead of *Apis mellifera*. Tryptamine and histamine were not detected, while tyramine was observed in the mead samples of honey from *Melipona quadrifasciata anthidioides*. Samples clarified with fruit peels increased the number of amines. In the analysis of the histamine, used as standard, obtained limits of detection and quantification of 2.47 mg/L and of 7.48 mg/L, respectively. In this study, the amines observed remained below the limit of detection, and did not pose a risk to consumers. The finings used demonstrated a profile similar to the control mead, and its use was satisfactory.

### 1. Introduction

Biogenic amines are nitrogen compounds of low molecular weight (Ke, Weic, Bogdald, Göktaş, & Xiao, 2018), which play an important role as endogenous regulators of physiological processes in animals, plants, and microorganisms (García-Marino, Trigueros, & Escribano-Bailón, 2010; Schumacher, Gardin, Colimo, Bettoni, & Messerschmidt, 2012). Amines can be classified as biogenic, such as histamine, serotonin, tyramine, phenylethylamine, tryptamine, putrescine, cadaverine and agmatine, when formed by the decarboxylation of amino acids by microbial enzymes, and as natural, whose formation occurs *in situ* in cells, such as putrescine, agmatine, spermine, spermidine, and histamine (Gomes, Pires, Fracalanza, & Marin, 2014). Among the biogenic amines, histamine, tyramine, putrescine, cadaverine (OIV, 2015, p. 26), spermine, spermidine, phenylethylamine, and tryptamine (Ke et al., 2018; Romano et al., 2012) stand out as they are abundant in some fruits and vegetables (Romano et al., 2012) and mainly in fermented foods, seasoned or preserved (cheese, wine, beer, fish and chocolate) (Lorenzo et al., 2017).

The consumption of products containing high levels of amines can generate severe toxicological conditions, which become more severe in

consumers whose activity of mono and diamine oxidase is reduced (Gomes et al., 2014; Romano et al., 2012). Intoxication may be aggravated if there is concomitant use of alcohol and/or medication (García-Marino et al., 2010; Schumacher et al., 2012). Histamine is widely studied because of its ability to act as a neurotransmitter and vasodilator in the central and cardiovascular nervous systems (Gomes et al., 2014), causing migraines, edema and rashes, hypotension, vomiting, palpitations, diarrhea, and heart problems (Ke et al., 2018; Ordóñez, Troncoso, García-Parrilha, & Callejón, 2016). Other amines, such as tyramine and phenylethylamine, are associated with bouts of hypertension (Gomes et al., 2014; Ordóñez, Troncoso, García-Parrilha, & Callejón, 2016) and symptoms, such as vasoconstriction, cerebral hemorrhages, and migraines (García-Marino et al., 2010; Schumacher et al., 2012).

Putrescine and cadaverine, although not toxic *per se*, potentiate toxicity of other amines, such as histamine and tyramine, favoring their absorption (Hernández-Orte et al., 2008). In addition, they may cause unpleasant aromas in fermented beverages and foods (Ordóñez et al., 2016; Schumacher et al., 2012).

The complexity of amine interactions with other substances has created difficulty in establishing safe limits for these compounds in

\* Corresponding author.

E-mail address: [anaripaim@gmail.com](mailto:anaripaim@gmail.com) (I.P. Silva).

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foods and beverages (García-Marino et al., 2010). In general, in alcoholic beverages, toxic doses range from 8 to 20 mg/L for histamine, from 25 to 40 mg/L for tyramine, and for phenylethylamine, at 3 mg/L (Schumacher et al., 2012). Some countries have already established limits for histamine in wine, such as Germany (2 mg/L), Belgium (2 mg/L), France (3.5 mg/L), Australia (10 mg/L), and Switzerland (10 mg/L) (Restuccia, Loizzo, & Spizzirri, 2018).

In recent years the presence of biogenic amines in wines has been extensively studied (García-Marino et al., 2010; Ke et al., 2018; Lorenzo et al., 2017; Romano et al., 2012; Schumacher et al., 2012), because it is related to product quality and also indicative of hygienic conditions of product production (Ke et al., 2018). Therefore, identification of biogenic amines is crucial in fermented and alcoholic beverages. Nevertheless, this is a pioneering study have investigated biogenic amines in mead, particularly in mead aged in oak barrels; thus, this study aimed to detect the presence of biogenic amines in meads from honey of two species of social bees (*Apis mellifera* and *Melipona quadrifasciata anthidioides*), using different finings and storage systems in oak barrel.

## 2. Material and methods

### 2.1. Samples

We used 12 mead samples, six corresponding to mead from *Apis mellifera* honey (three samples at time 0 [T0] after being potted, three samples at 120 days [T120] of storage in oak barrel), and six samples corresponding to mead from *Melipona quadrifasciata anthidioides* honey (three at time 0 [T0] and three samples at time 120 days [T120]).

For the preparation of the mead samples, we used honey from the semiarid region of Bahia State (Brazil), acquired directly from beekeepers. The features and quality of honey were satisfactory, in compliance with the requirements established in the Brazilian and European Legislation (Brazil, 2000; Codex, 2001). Mead was elaborated with honey, mineral water, nutrients (Fermaid E/Proenol), and used in combination with yeasts of *Saccharomyces cerevisiae* (Ferm BDX grape) and *Torulasporea delbrueckii* (Biodiva Tm - TD 291) (Silva, Carvalho, Machado, & Estevinho, 2018). The mead was fined with bentonite (Bentogran Rapid-AEB), banana peel flour (*Apis mellifera* honey mead) and passion fruit peel meal (*M. q. anthidioides* honey mead) obtained manually, according to Storck, Nunes, Oliveira, and Basso (2013) and Oliveira, Gurak, Cladera-Oliveria, and Marczak (2016). Mead without fining was used as control.

### 2.2. Detection biogenic amines

For the detection of biogenic amines tryptamine, putrescine, histamine, tyramine, spermidine, and spermine, we used chromatography in thin layer (TLC, silica gel with fluorescence). Dansyl chloride (5-dimethylaminonaphthalene-1-sulfonylchloride) was used as derivatizing agent.

Standard solutions of biogenic amines were prepared at the concentration of 0.1g/100 mL using 5% of trichloroacetic acid (TCA) as solvent. In 25 mL volumetric flasks, six calibration solutions were prepared for histamine using different volumes of a concentrated solution (0.1 mL, 0.2 mL, 0.3 mL, 0.5 mL, 0.7 mL, and 1.0 mL). The flasks were calibrated with 5% TCA. To detect the other amines, the reference concentration of 0.7 mL of histamine was used, concentration best seen in TLC plates between the minimum and maximum reference level of "histamine".

#### 2.2.1. Derivatization process

We added 0.2 mL of sodium carbonate (17 g/50 mL), 1 mL of dansyl chloride (0.1 g/10 mL of acetone), and 1 mL of the mead in a test tube. The mixture was vortexed under ultra-sonic bath with light protection for 30 min at 40 °C. Afterward, 0.1 mL of ammonia hydroxide (25%) was added to the mixture and placed to rest in the dark for 30 min.

Subsequently, the mixture was centrifuged (3400 rpm/10 min) and the supernatant transferred to a new test tube.

The biogenic amines were extracted using three times the volume of 2 mL of diethyl ether (vortexing for 30 s after each addition). The amines were then placed into a glass container for solvent evaporation (hotte). The dried extract was re-dissolved in 1 mL of ethyl acetate and transferred to a vial for further analysis by TLC (Shalaby, 1999; Shruti, Hae-Kyong, Jong-Kyu, & Myunghee, 2010).

#### 2.2.2. Chromatographic process

We used commercial aluminum plates coated with silica gel added with a fluorescent additive (Silica gel 60 F254, Merck, 20 × 20 cm). A baseline (2.0 cm from the plate edge) was drawn on each plate and marked points 1 cm apart, where standard calibration solutions and samples were applied. To apply each derivatized solution to the TLC plate, we used 1 µL of solution measured with a microsyringe (Merck) positioned at each point marked on the baseline (Vogel, 1986).

#### 2.2.3. Plates preparation

After application of the samples and standards on the TLC plate, the plates were placed in a chromatographic chamber containing the solvent (Chloroform, 40 mL; diethyl ether 10 mL; triethylamine, 10 mL) about 1 cm high (Lapa-Guimarães & Pickova, 2004). The chromatographic chamber was placed on a flat horizontal surface to avoid undulations in the plate.

In order to homogenize the internal atmosphere of the chamber, embedded filter paper was immersed in the eluent and placed on one of the walls to be facing the plate side with the stationary phase (silica gel). The development proceeded until the solvent reached the desired distance (12 cm from the top). The plate was removed from the chamber immediately marking the position of the solvent front. Subsequently, the TLC plates were oven dried at 40 °C, visualized under a UV light (365 nm) placed inside a dark camera, and photographed.

### 2.3. Qualitative and quantitative analyses

The quantitative analysis was performed by treatment of the image obtained from the photograph of the TLC plate, using the program Image J (Version 1.51J8). A densitogram was obtained from these data. Subsequently, a calibration line was constructed using various concentrations of histamine. The equation of the line was determined using the linear regression model that relate histamine concentrations to areas of the peaks obtained from the densitogram of stains.

The qualitative analysis corresponding to the identification of the compounds was obtained using the retention factor (RF) (Vogel, 1986). Given by:

$$RF = \frac{\text{"Distance from stain to baseline" (D1)}}{\text{"Distance from the solvent front to the baseline" (D2)}}$$

The biogenic amine concentration was obtained by direct interpolation of the peak area of this amine in the corresponding linear calibration curve (peak versus concentration area). The limit of detection and quantification was calculated from the linear regression (Ribani, Bottoli, Collins, Jardim, & Melo, 2004).

The limit of detection (LOD) considered was the minimum concentration of the substance detected, expressed by:

$$LOD = \frac{3.3 \times \text{Standard deviation of the ordinate}}{\text{Slope}}$$

The limit of quantification (LOQ) for the substance concentration was obtained by:

$$LOQ = \frac{10 \times \text{Standard deviation of the ordinate}}{\text{Slope}}$$

**Table 1**

Average retention factor for biogenic amine patterns in 12 mead samples of two species of social bees (*Apis mellifera* and *Melipona quadrifasciata anthidioides*) with different finings and stored for 120 days in oak barrel.

Biogenic amines	<i>Apis mellifera</i>						<i>Melipona quadrifasciata anthidioides</i>						RF
	Control [T0]	Control [T120]	Bentonite[T0]	Bentonite [T120]	Banana[T0]	Banana [T120]	Control [T0]	Control [T120]	Bentonite[T0]	Bentonite [T120]	Passion fruit [T0]	Passion fruit [T120]	
Cu <sub>1</sub>	d	d	d	d	d	d	d	d	d	d	d	d	0.11
Putrescine	nd	d	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.25
Tryptamine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.31
Spermidine	d	d	d	d	d	d	d	d	d	d	d	d	0.40
Histamine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.52
Spermine	nd	d	nd	nd	d	nd	d	nd	nd	nd	nd	d	0.54
Tyramine	nd	nd	nd	nd	nd	nd	d	d	d	d	d	d	0.63
Cu <sub>2</sub>	d	d	d	d	d	d	d	d	d	d	d	d	0.75

[T0] – Time zero, mead collection; [T120] – Mead stored in oak barrel for 120 days; C<sub>u</sub>: compound unknown; nd: not detected; d: detected; RF: Retention Factor. The experiment was performed in triplicate.

### 3. Results and discussions

#### 3.1. Qualitative analysis

Considering the retention factors obtained from the stains on the TLC plate (Table 1), we observed six compounds in the mead samples of *Apis mellifera* and *Melipona quadrifasciata anthidioides*. Two compounds (Cu<sub>1</sub> and Cu<sub>2</sub>) were not identified and may be associated with the nature of the matrix used in the mead preparation or products formed during the fermentation process (Table 1). In general, fermented beverages have a complex composition that increases the difficulty of analysis and interferes in the results (Ordóñez et al., 2016).

Considering the retention factor (RF), the elution order of biogenic amines on the TLC plate was putrescine (RF = 0.25), which presented a lower value and remained closer to the baseline, followed by tryptamine (RF = 0.31), spermidine (RF = 0.40), histamine (RF = 0.52), spermine (RF = 0.54), and tyramine (RF = 0.63) (Table 1).

The method used showed good chromatographic separation, with no overlap of the standard compounds of the biogenic amines used. Latorre-Moratalla, Bover-Cidb, Vecina-Nogués, and Vidal-Caroua (2009) also used the detection of amines by TLC in wines and observed the same order of separation of the compounds with RF values also similar (putrescine (RF = 0.26), tryptamine (RF = 0.30), spermidine (RF = 0.44), histamine (RF = 0.57), spermine (RF = 0.62) and tyramine (RF = 0.76)).

In general, three biogenic amines were detected in the two types of mead. In mead of *A. mellifera* honey, we observed putrescine, spermidine, and spermine and in mead of *M. q. anthidioides* honey, we detected spermine and tyramine. Natural polyamines, such as putrescine, spermine, and spermidine, are present at low levels in microorganisms, plants, animals, and human cells, being involved in physiological functions (Restuccia et al., 2018). The presence of these amines in mead may be attributed to honey, which contains plant phytochemicals, bee salivary substances.

Similar behavior can be observed in wines, where biogenic amines levels are relatively low at the end of alcoholic fermentation, attributed to the grape used and fermentative processes. However, biogenic amines levels may increase during malolactic fermentation and aging, suggesting that lactic acid bacteria may be responsible for their production during this period (Gomes et al., 2014). In addition, biogenic amines may also be formed by proteolysis during yeast autolysis (Restuccia et al., 2018).

Putrescine was observed in *A. mellifera* mead samples stored for 120 days in oak barrel (Control/*Apis* and Banana/*Apis*) (Table 1). Putrescine is a catabolic product of the ornithine or arginine pathways, which can be converted to spermidine and form spermine, these three are interconvertible molecules (Russo et al., 2010).

Mota, Amorim, Fávero, Gloria, and Regina (2009) studied wines and reported high putrescine levels in the winter in two harvests. For the authors, one of the reasons for putrescine accumulation in the grape occurred because the grapes were cultivated during the dry season and underwent water stress or because there was potassium deficiency, evidencing that this amine may be related those factors other than the hygienic conditions.

Kelly, Blaise, and Larroque (2010) investigated 11 wines [Cabernet sauvignons (n = 4) and Merlots (n = 3) from Romania, and Merlots (n = 2) and Syrahs (n = 2) from France] and found that putrescine was the main biogenic amine (and the only one in the Merlots). In the other wines, there was also the presence of cadaverine in small amounts.

Spermidine was detected in all samples analyzed, whereas spermine was detected only in Control/*Apis* [T120], Banana/*Apis* [T0] and [T120], Control/*Melipona* [T120], Passionflower/*Melipona* [T120]. Spermidine and spermine are associated with plant morphogenesis, acting as growth regulators and as response to environmental conditions (Mota et al., 2009).

Tryptamine and histamine were absent in all samples analyzed, a positive factor for mead production and consumption. Absence of tryptamine is desirable, due to its toxic effects in humans and contribution to increase blood pressure, although there is no tolerance maximum threshold of consumption regarding the toxic effect (Gomes et al., 2014). The absence of tryptamine is consistent with the results obtained by Ke et al. (2018) for red and white wines (n = 456).

Among biogenic amines, histamine is the most investigated because of its toxicity, which can be potentiated in the presence of other amines, such as spermine, spermidine, putrescine, or agmatine (García-Marino et al., 2010) and also associated with alcohol and medications (García-Marino et al., 2010; Schumacher et al., 2012). Histamine acts as a neurotransmitter and a vasodilator in the central nervous system and cardiovascular system. It is considered responsible for episodes of food poisoning, which are presented by allergic reactions, characterized by respiratory bronchoconstrictor effect, pruritus, rash, vomiting, fever, and hypertension (Gomes et al., 2014).

Tyramine was detected in all mead samples of *M. q. anthidioides* honey and this amine may be related to fermentation of *Melipona* honey. This honey has peculiar characteristics because these are native bee species it is little known, more studies are needed to characterize this matrix. Landete, Ferrer, and Pardo (2007) demonstrated that lactic acid bacteria in wines produced histamine, tyramine, phenylethylamine, and putrescine.

Moreno-Arribas, Torlois, Joyeux, Bertrand, and Lonvaud-Funel (2000) detected tyramine in 11 of 14 wine samples analyzed. The authors isolated several strains producing tyramine from wines that had undergone malolactic fermentation. The authors reported that tyramine production could be related to the presence of the amino acid (tyrosine)

available.

When fruit peels (Banana/*Apis* and Passionfruit/*Melipona*) were used as finings, there was an increase in the number of amines from two ( $n = 2$ ) at [T0] to three ( $n = 3$ ) with aging (Table 1). These results indicate that lactic acid bacteria may have developed during aging or extracellular decarboxylases may have been occurred during this period (Hernández-Orte et al., 2008).

In all mead samples fined with bentonite, the number of amines during storage remained constant and was positive for mead production and consumption. Only spermidine was detected in Control/*Apis* at [T0] and in Bentonite/*Apis* meads at [T0] and [T120]. However, in Control/*Apis* at [T120] and in the mead fined with Banana/*Apis* at [T0] and [T120], three amines (spermidine, spermine, and putrescine) were present, which could have originated from the banana peel, since they are present in banana morphogenesis.

The mead obtained with honey from *M. q. anthidioides*, in Control/*Melipona* at [T0] and fined with bentonite at [T0] and [T120] presented the same number of amines ( $n = 2$ ), spermidine and tyramine. The presence of three amines (tyramine, spermidine, and spermine) was observed in Control/*Melipona* [T120]. However, spermine may be the result of chemical reactions from spermidine. Both spermine and spermidine are synthesized by sequential addition to putrescine of an aminopropyl moiety, provided by decarboxylated S-adenosylmethionine. The enzyme, S-adenosylmethionine decarboxylase, catalyzes the synthesis of decarboxylated S-adenosylmethionine from S-adenosylmethionine and is a key step in the regulation of polyamines (Srivastava et al., 2007). This effect may be reduced in the treatment with bentonite once bentonite is capable of reducing the volatile compounds in mead and wine, according to Pascoal et al. (2017) and Marchal and Jeandet (2009), chap. 5.

The mead of Passionfruit/*Melipona* at time [T0] and [T120] did not differ in relation to the presence of amines at Control/*Melipona*, demonstrating that the use of this fining is satisfactory, since it did not generate any new compounds.

The results obtained by García-Marino et al. (2010) confirm the observations of this study. These researchers also observed a progressive increase in the content of certain amines during aging, particularly histamine, tyramine, putrescine, and diaminobutane.

Restuccia et al. (2018) investigated wines and reported that conventional fining agents can influence biogenic amines concentration by increasing the concentration of precursor amino acids or favoring the development of biogenic amines producing microorganisms. In addition, these agents influence other parameters (fermentation duration in the presence of pulp and peel, alcohol content, sulfur dioxide concentration, added nutrients, pH, temperature, and quantity and type of fining). Therefore, the use of natural matrices for fining mead can be a viable and easily accessible alternative to honey producer.

### 3.2. Quantitative analysis

For the quantitative evaluation, histamine analysis was used, since histamine is generally the most analyzed amines, in addition to being considered the most toxic biogenic amine (García-Marino et al., 2010). Moreover, the combination of this amine with alcohol and other amines may increase its toxicity by inhibiting the action of enzymes, such as methyl transferase, diamine oxidase, and monoamine oxidase, which are involved in the detoxification process of the human body (Restuccia et al., 2018).

Fig. 1 shows a densitogram and a photograph of a TLC plate used for the calibration of histamine. For that purpose, different amine concentrations were used. The figure shows an increase in the stain intensity, directly proportional to the height and area of peaks corresponding to the level and concentration of histamine.

From the densitogram of the histamine, the area of each peak was obtained, which allowed making the calibration line. There was a direct linear relationship between the areas obtained from the densitogram

peaks and the respective concentrations of each level of the standard solution.

Table 2 shows the linear regression parameters obtained for the histamine calibration (concentration range, correlation coefficient, calibration equation, limit of detection, and quantification). The relationship between concentrations and peak areas was linear along the time interval tested, with a correlation coefficient of 0.996, which shows that the linear model was obtained with points very close to the adjusted line. Pereira, Pontes, Câmara, and Marques (2008) reported similar results by analyzing amino acids and biogenic amines in honey and wines ( $R = 0.996$ , histamine).

The calibration of the histamine standard solution allowed to calculate the LOD (2.47 mg/L), which was below the minimum concentration used for the standards (4 mg/L) and the LOQ was established at 7.48 mg/L (Table 2).

Ke et al. (2018) reported significantly higher concentrations for the LOD of histamine in red wine (22 mg/L) and white wine (1.3 mg/L) using a derivatization method with dansyl chloride and HPLC.

Tuberoso, Congiu, Serreli, and Mameli (2015) reported LOD for nitrogen compounds ranging from 0.004 mg/L to 0.23 mg/L and the limit of quantification below 1 mg/L for all compounds. The authors used derivatization with dansyl chloride and HPLC with fluorescence detection as the analytical method.

This study is one of the few aimed at detecting the presence of biogenic amines in meads, especially mead from *M. q. anthidioides* honey. This information may contribute to aggregate information to the quality of this fermented beverage.

## 4. Conclusion

The amines detected had a content lower than the estimated limit of detection for histamine, presenting no risk to consumers. Bentonite is a good fining for mead, since the number of biogenic amines remained constant during aging. In the meads fined with the other agents (banana peel flour and passion fruit peel meal), we demonstrate to be a viable alternative to the producer, since it showed a profile similar to the control.

### Disclosure statement

No potential conflict of interest was reported by the authors.

### Author contributions section

I.P.S. performed the laboratory work, including the analysis and interpretation of data, discussion of results and wrote the manuscript; L.G.D. supervised the biogenic amine analyses, contributed to statistical analysis and interpretation of data, discussion of results and revision of the manuscript; M.O.S. performed the laboratory work; C.S.M. contributed on the discussion of results and revision of the manuscript; V.M.B.P. supervised the biogenic amine analyses; N.S.E.B. contributed to the discussion of results and revision of the manuscript; C.A.L.C. supervised the work, discussion of results and revision of the manuscript; L.M.E. conceived and designed the experiments and supervised the work, discussion of results and revision of the manuscript. All authors revised and approved the submitted version.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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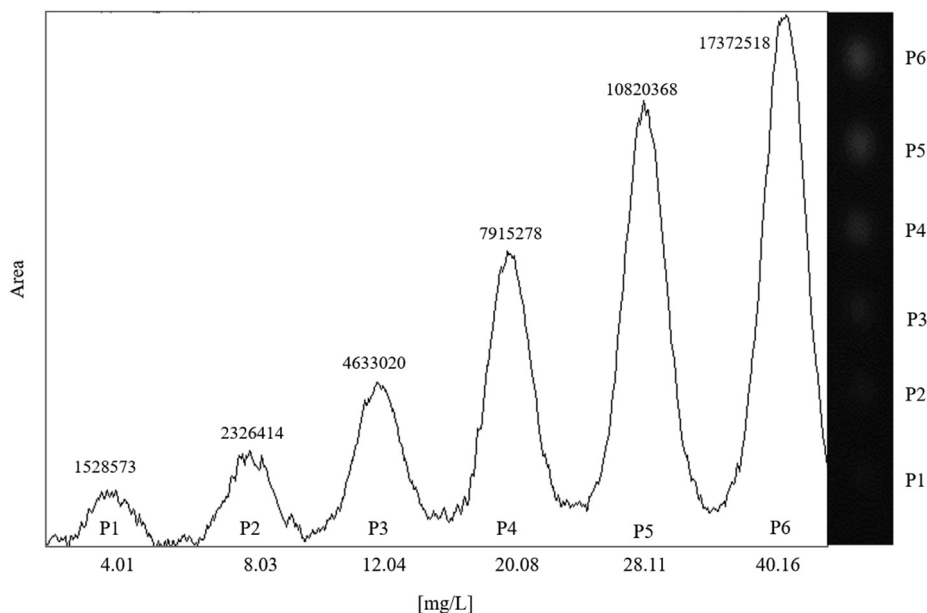


Fig. 1. Densitogram and TLC plate obtained from a grayscale photograph in the analysis of the six histamine calibration solutions.

Table 2

Calibration obtained for standard histamine solution.

Biogenic amine	CR	R	CE	LOD	LOQ
Histamine	4.01 to 40.16	0.996	$y = 448571x - 810439$	2.47	7.48

CR: Concentration range [mg/L]; R: correlation coefficients; CE: Calibration equation; LOD: Limit of Detection [mg/L]; LOQ: Limit of quantification [mg/L]. The experiment was performed in triplicate.

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