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Biogenic amines in rainbow trout, tilapia, and cachama fish, available for consumption in Nariño, southern Colombia

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Abstract

Biogenic amines (BAs) are low molecular weight nitrogenous compounds, formed by the breakdown of proteins in highly perishable food products such as fish. BAs can affect human health and are associated with cases of food poisoning. The formation of Bas such as histamine, putrescine, and tyramine were determined, via Process Analytical Chemistry (PAC), in three species of freshwater fish available in markets of city of Pasto in southern Colombia: rainbow trout, tilapia, and cachama. We evaluated the formation of BAs during the fish conservation processes and considered a multifactorial design with two levels. The factors studied were: fish species, slaughter type, storage temperature, and time to purchase. Out of the three fish species studies, tilapia samples revealed the highest average content of putrescine and histamine, with values of 5.4 μg/g and 10.04 μg/g, respectively. Tyramine was not detected in any of the experiments performed. The observed values of BAs in the samples analyzed were below locally tolerated maximal values and the European standard (200 μg/g). However, their presence reveals that factors such as sample storage temperature and time to consumption triggered their formation.

Keywords: Biogenic amines; process analytical chemistry; freshwater fish; experimental design.

Introduction

Biogenic Amines (BAs) are low molecular weight nitrogenous compounds formed by decomposition of muscle tissue, especially in fish. BAs can affect human health and are associated with food poisoning cases, especially

after consuming large amounts of fish contaminated with these compounds (Hernández, 2001; Pons, 2005; Huss, 1995; Al-Bulushi *et al*., 2009; Biji *et al*., 2016).

BAs are toxic to humans; some act on the neurotransmitters of the central nervous system, and others have vasoactive properties, thus acting on the vascular system (Zarei *et al*., 2011; Veciana *et al*., 1995; Bito *et al*., 1983). BAs such as histamine, putrescine, cadaverine, tyramine, tryptamine, phenylethylamine, spermine, and spermidine have been identified as the cause of numerous food-poisoning episodes after consuming fish, ripened cheeses, wines, and meat products, such as dried sausages and unprocessed meats (Lopez *et al*., 1996, Bilgin & Gençcelep, 2015).

Nariño is a region in southern Colombia, where fresh-water fishes of the Salmonidae, Cichlidae, and Serrasalmidae families are farmed, marketed, and consumed. The most frequent species are rainbow trout, tilapia and cachama (Plan Nacional de Desarrollo de la Acuicultura Sostenible en Colombia. 2014, Gobernación de Nariño-Colombia, 2019). Studies by Hernandez (2001) have identified that the sanitary management of these fishes in local markets is poor and compromises food quality, thus being a possible cause of the poisonings associated with these fish. In 2019, 7.7 % of all food poisonings reported in Colombia occurred in the region of Nariño, and 8.6 % were associated with fish consumption (Instituto Nacional de Salud de Colombia, 2019). Food safety checks are necessary, along the entire production and market chain of these fish, to minimize the presence of agents, such as bacteria, that accelerate postmortem decomposition and increase the risk of BA driven food-poisoning in the consumer (Ministerio de Salud y Protección Social de Colombia, 2010). Currently, several analytical methods are available to keep track of BA formation in fish.

Process analytical chemistry (PAC) is one of the applications of analytical chemistry with specialized techniques, algorithms, and sampling equipment that addresses issues related to chemical processes. PAC is akin to process analytical technology (PAT), used in the pharmaceutical industry. Process analysis Chemistry has become in an important tool for quality control technology in research setups and analysis of physical and chemical composition of the desired products in several industries, particularly the food industry. (Workman-Jr. J *et al*., 2009).

In this work, we studied, via HPLC quantification, a PAC method, the main factors leading to the formation of BAs of the type putrescine, histamine, and tyramine, in the storage and conservation of rainbow trout, tilapia, and cachama in Nariño, Colombia.

Materials and Methods

Fish samples

Fish of the three species of interest, namely rainbow trout, tilapia, and cachama were obtained from a fish farm in Nariño - Colombia. A number of 24 fish of each species were captured at random, after an overall fasting period of 18 hours. The protein content of these fish was analyzed to define species-related protein levels, namely protein content in fish muscle per species studied (the fish species with the lowest and the highest protein content).

The fish were sacrificed by cold shock with ice water (0 ± 2 °C) and asphyxiation (as described in **Table 1**). Dead fish were cut along their ventral line, from the anus to the operculum, and their viscera and gills were removed. The fish were deboned to get their fillets. Prepared fish samples were packed and transported in isothermal boxes with ice (4 ± 2 °C) in a fish: ice ratio of 2:1 and stored at controlled temperatures of -18 °C, 4 °C, and 25 °C, for 5 and 10 days of storage following the experimental design.

To evaluate the quality of fish already in the market, the number of fish outlets in the city of Pasto (largest urban area in Nariño) was identified and three of them were selected. From each fish outlet, nine fish specimens of the three species were obtained (three from each site). This procedure was performed on market days. Samples from the different sampling points were packed in polystyrene bags, transported in isothermal boxes with ice (4 ± 2 °C) at a fish: ice ratio of 2:1, and filleted as previously mentioned. The samples were analyzed for BAs on the same day of acquisition.

Identification of factors determining BA levels in fish

To identify the relevant factors affecting BA levels in farmed fish, a complete factorial experimental design was followed. Factors such as fish species, type of fish slaughter, and storage time were considered with two levels, and fish storage temperature was a factor with three levels. A total of 24 experiments made up the entire experimental design was completely random, with the BAs concentration in µg/g being the response variable, the factors were selected following the conservation processes applied in the Nariño region. Table 1 shows the experimental factors and levels and **Table 2** shows the experiments of factorial design.

Table 1. Characteristics of the factors and levels full factorial design for analysis of biogenic amines. *Out of the three fish species studied, the one with the highest and the one with lowest protein content levels were considered in the experimental design. **Fish storage temperature was considered as a factor with three levels (-18 °C, 4 °C, and 25 °C).

Factors	lower	higher	Description
Species* (protein level)	0	1	(0) lower protein level (1) higher protein level: Proteins are susceptible to denaturation of the precursor amino acids of biogenic amines.
Sacrifice type	-1	1	(-1) Immersion in ice water (1) suffocation in the air: It produces a lot of stress being the cause of soft meat and poor quality.
Storage Time(days)	5	10	The most relevant situation for fishermen, traders and consumers is how long the fish is kept on ice.
Storage Temperature (°C)**	-18 4	25	Several BAs formed by loss of cold chain.

Determination of protein, water, and total fat in fish muscle

To relate BA content with the components that can affect the formation of BA in each sample, protein, water, and total fat content were determined in muscle samples (100 g); the corresponding determination methods are mentioned in **Table 3**. Since BAs are formed from proteins, protein content was the only variable considered in downstream analyses.

Extraction of biogenic amines from fish samples

Fish fillets were homogenized with a home mixer; 5 g of sample were weighed and 20 mL of 6 % TCA were added. The mixture was centrifuged at 11 200 RCF for 10 minutes at 4 °C in a HERMLE Z326K centrifuge (Germany)

Table 2. Experiments within the factorial experimental design.

Experiments	Species	Sacrifice	Time (days)	Temperature (°C)
1	1	1	10	-18
2	1	-1	5	25
3	1	-1	10	25
4	0	-1	5	4
5	0	-1	5	25
6	1	1	5	4
7	1	1	10	4
8	0	-1	10	-18
9	1	1	5	25
10	0	1	10	-18
11	1	-1	5	-18
12	1	-1	10	-18
13	0	-1	10	4
14	0	1	5	-18
15	0	1	5	4
16	0	1	10	25
17	0	1	5	25
18	1	-1	10	4
19	1	-1	5	4
20	0	1	10	4
21	1	1	5	-18
22	0	-1	10	25
23	1	1	10	25
24	0	-1	5	-18

and filtered with Whatman No. 2 paper. The extracts were brought to a final volume of 50 mL with 6 % TCA, and a total of 2.0 mL of the final extract was taken for derivatization.

Table 3. Methods of protein, water, and total fat analyses of the fish species studied.

Parameter	Method	Technique
Total Protein	Kjeldahl AOAC 981.10	Oxidation-Reduction
Water Content	Oven drying AOAC 930.15	Gravimetry
Total fat	Soxhlet extraction AOAC 960.39	Gravimetry

HPLC Analyses

Method Verification

We followed the method verification protocol by Argotty-Salazar & Lozada-Castro (2017). Linearity, accuracy, recovery percentage, detection limits, and quantification limits were assessed accordingly. Stock solutions of the standards (99.9 % histamine, tyramine, and putrescine; Sigma-Aldrich, USA). were prepared at a concentration of 100 mg/L in 0.1 M HCl, from these solutions seven mixtures of the standards were obtained at concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 25.0, and 100 mg/L.

We assessed linearity by constructing a graph with seven calibration points. For each level three replicas were performed to assess reproducibility. Within- and between-day precision were evaluated at a concentration of 10 mg/L of the standard mixture for five consecutive days and fifteen injections per day. Recovery percentages were evaluated in triplicate in tilapia muscle samples enriched with 2.0 mL of a mixture of 10 mg/L and 50 mg/L standards.

Derivatization and HPLC quantification of BAs

Each of the reserved 2.0 mL of the final extracts received 1.0 mL of 2.0 M NaOH and 15 μL of benzoyl chloride (99.9 %, Carlo Erba, Italy); the mixture was vortexed for 3 minutes and allowed to react at room temperature for 40 minutes. Subsequently, 2.0 mL of 5.0 M NaCl were added, and L-L

extraction was performed with 3.0 mL of diethyl ether (99.9 % AR-ACS, Burdick Jackson, USA). The upper organic layer was separated by decantation transferring it to a test tube, the solvent was evaporated to dryness with a stream of nitrogen (5.0 grade, Linde, Colombia), the residue was dissolved in 500 μL of methanol (HPLC grade, Fisher Scientific, USA) for analysis by HPLC with Photodiode array detector.

Derivatized extracts were injected in a HPLC Waters Breeze chromatography system (Maryland, USA). The system was equipped with a Waters 1525 binary pump, a Rheodyne 7525l injector, a 20-μL loop, and a Waters PDA 2998 photodiode array detector. A Kinetex C18 column (250 × 4.6 mm × 5.0 μm) (Phenomenex, USA) at 30 °C was used. The run included an isocratic separation mode, with a mobile phase consisting of Methanol: Water, in an 85:15 ratio and a flow of 0.8 mL/min. The photodiode array detector was set at 254 nm. The analysis of the chromatographic data was performed with the Empower II software of (Waters Corporation, USA).

Biogenic amines index (BAI)

Given that histamine is the only BA with legislated limits, a quality index for all BAs, namely the biogenic amines index (BAI = histamine + putrescine + tyramine) was taken into account. This BAI was proposed by Mieltz & Karmas (1977) and Veciana-Nogués *et al*. (1997) for salmon and tuna, respectively. To verify the usefulness of the BAI in our study, the following criteria were considered: the histamine limit proposed by the European Union (Commission Regulation (EC) No 2073/2005, Ministerio de Sanidad y Consumo de España, 1992) and the putrescine and cadaverine maximum levels proposed by Yamanaka *et al*. (1989) (Yamanaka *et al*. 1989, Al-Bulushi *et al*. 2009). Following these criteria, the concentrations of the total amines present in the fish samples were studied and the factors that lead to the presence of these biotoxins were determined. The BAI, as established by Veciana-Nogués *et al.* (1997), indicates that values below five are indicative of good quality; BAI values between five and twenty are acceptable but indicative of spoilage; and BAI values between twenty and fifty indicate poor quality of fish products. The values are expressed in parts per million (Francisco K.C.A *et al.* 2020).

Statistical analysis

The STAGRAPHICS centurion XVl software was applied at 95 % significance, to perform the analysis of variance.

Results and Discussion

Method Verification

Our analysis followed the method proposed by Argotty-Salazar & Lozada-Castro (2017). Shorter retention times were obtained and the peaks obtained were more acute and symmetrical. The retention factors were low and a good resolution was obtained. The calibration curves showed coefficients of determination between 0.9994-0.9997, recovery rates were in the range of 92 % to 101 %, and relative standard deviations were around 1.0 %. Limits of detection, limits of quantification, reproducibility, and recovery of the method are shown in **Table 4**.

Analysis of protein, water content, and total fat in fish muscle samples

Tilapia muscle samples revealed the highest average protein content (18.9 %), out of the three fish species tested. Cachama samples had the highest average water content (75.4 %), and trout had the highest average fat content (6.7 %), **Table 5** shows the average protein, water, and total fat content of the three species studied.

Putrescine, Histamine, and Tyramine in farmed fish

HPLC quantification of BAs in farmed fish samples revealed the presence of putrescine and histamine but no Tyramine. Putrescine was present in 50 % of the 24 samples analyzed; the highest concentrations of putrescine were observed in tilapia samples, the species with the highest protein content. The experiments with this fish species were 3, 7, and 23 (Suppl. 1 and Table 2; $P < 0.05$). The experimental conditions that most influenced the formation of putrescine were 10 days storage and storage at room temperature (25 °C). The lowest putrescine contents were observed in the samples with the least percentage of Nitrogen, corresponding to fish sacrificed with immersion in ice water, short storage time, and freezing storage temperatures conditions (> 0 °C). These experiments corresponded to numbers 5 and 13 in Suppl. 1 and table 2. These results were similar for the experiments 9, 17, and 20, in which the type of sacrifice was air asphyxiation. The difference in putrescine concentrations was due to the effect of factors such as time and storage temperature, which significantly affected the formation of BAs, accelerating the denaturation reactions of the precursor amino acids and decreasing the stability of the proteins (Rodríguez *et al*., 1994; Bover-Cid *et al*., 2009; Gardini *et al*, 2016).

Table 4. Analytical characteristics of the method for determining BAs *n=5, n=10, [a] average values mg/L. Intra-day and Inter-day accuracy.

BAs	LD mg/L	LQ mg/L	X[a]	RSD	X	Intra-day* RSD	Inter-day** 10 mg/L	Recovery % 25 mg/L
Putrescine	0.03	0.07	3.14	0.36	2.12	0.5	101.03	92.98
Histamine	0.09	0.29	7.68	0.17	0.08	0.6	98.79	96.88
Tyramine	19.48	21.18	3.13	0.12	1.60	0.2	93.46	90.01

Histamine showed a much lower increase throughout the storage of the samples. The fish samples with higher concentrations of this amine corresponded to experiments 16, 22, and 23; the latter sample presented the highest concentration of this toxin, due to the conditions to which it was exposed ($P < 0.05$). For instance, in experiment 23, two superimposed chromatograms were observed, in this sample the BA putrescine and histamine were identified (Suppl. 2).

Effect of species, sacrifice type, time, and storage temperature in BA formation

Total BAs contents in all samples analyzed and the treatments these samples were subjected to are shown in **Table 6**. These BAs range from 0.57 μg/g to 12.15 μg/g, with an average of 1.81 μg/g. The fish samples analyzed have an

Table 5. Relative content (g/100g) of water, total protein, and total fat in muscle samples of three fish species available in markets of the city of Pasto, Nariño-Colombia. [a] Average values, [b] relative standard deviation, (%) n=2

Species	Total Protein		Water (%)		Total Fat	
	Average[a]	RSD[b]	Average	RSD	Average	RSD
Tilapia	18.9	3.4	72.6	0.6	4.3	6.5
Trout	17.6	1.6	70.4	1.1	6.7	3.2
Cachama	16.8	2.5	75.4	1.0	4.6	4.6

Table 6. BAs content per fish sample, specifying fish species and treatments. BAI Index ranges from 0 to 5 μg/g of the BAs; *BAs minimum observed value (0.57 μg/g); **BAs maximum observed value (12.15 μg/g); BAs average of 1.81 μg/g; ND = not detected (see Suppl. 1 and Table 2).

Sample	Fish species	Type of sacrifice	Time after fish death (days)	Storage and market Temperature (°C)	Total BAs µg/g
1	1	1	10	-18	ND
2	1	-1	5	25	1.45
3	1	-1	10	25	5.33
4	0	-1	5	4	ND
5	0	-1	5	25	0.66
6	1	1	5	4	ND
7	1	1	10	4	5.44
8	0	-1	10	-18	ND
9	1	1	5	25	2.15
10	0	1	10	-18	ND
11	1	-1	5	-18	ND
12	1	-1	10	-18	ND
13	0	-1	10	4	0.57*
14	0	1	5	-18	ND
15	0	1	5	4	ND
16	0	1	10	25	5.80
17	0	1	5	25	3.06
18	1	-1	10	4	1.60
19	1	-1	5	4	ND
20	0	1	10	4	2.58
21	1	1	5	-18	ND
22	0	-1	10	25	2.72
23	1	1	10	25	12.15**
24	0	-1	5	-18	ND

acceptable content of BAs, according to their BAI values (BAI value below 5 indicate acceptable quality).

The statistical analysis on BAs contents per fish sample and the variables: fish species, type of fish sacrifice, time of storage, and storage temperature revealed that temperature and time of storage exerted a significant and positive influence on sample BAs content; whereas fish species and type of sacrifice had a negative, but not statistically significant, influence on sample BAs content (**Fig. 1**).

The interaction between storage time and temperature had a marked, positive influence on the formation and increase of BAs in the fish samples. Furthermore, fish species was a factor negatively related to BAs concentration via total protein content in the samples. In fish species with consistently low protein content, Nitrogen content was limited and so the formation of BAs (**Fig. 2**). Sample storage at -18 °C, overrode its concomitant factors by hindering the formation of biogenic amines. This is consistent with the observations of Ben-Gigirey *et al.* (1998) in that highly perishable products such as fish species can be stored for a period of 4 weeks at temperatures below -15 °C.

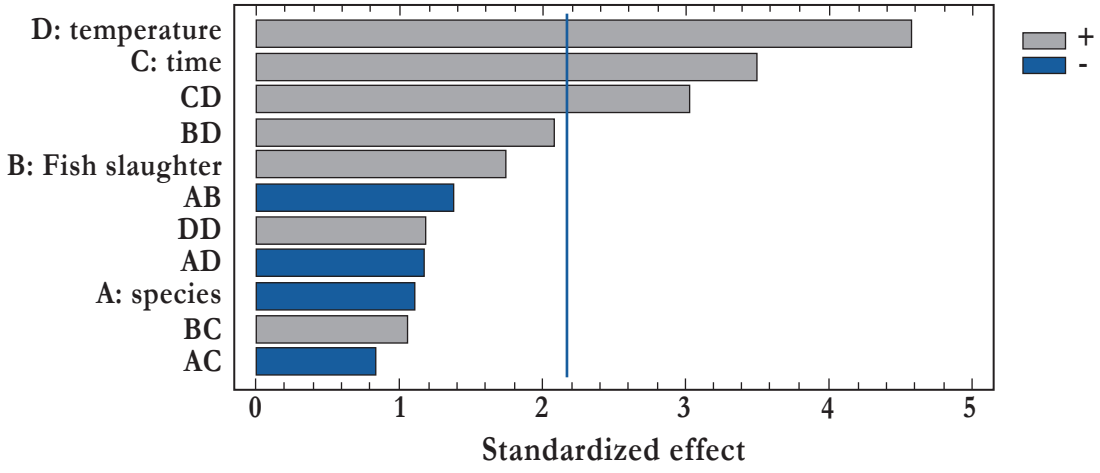

Figure 1. Pareto chart of size and direction of standardized treatment effects on biogenic amines formation. Keys, CD: time *temperature interaction; BD: sacrifice type*temperature interaction; AB: species*sacrifice type interaction; DD: quadratic effect of temperature; AD: species*temperature interaction; BC: sacrifice type*time interaction; AC: species*time interaction.

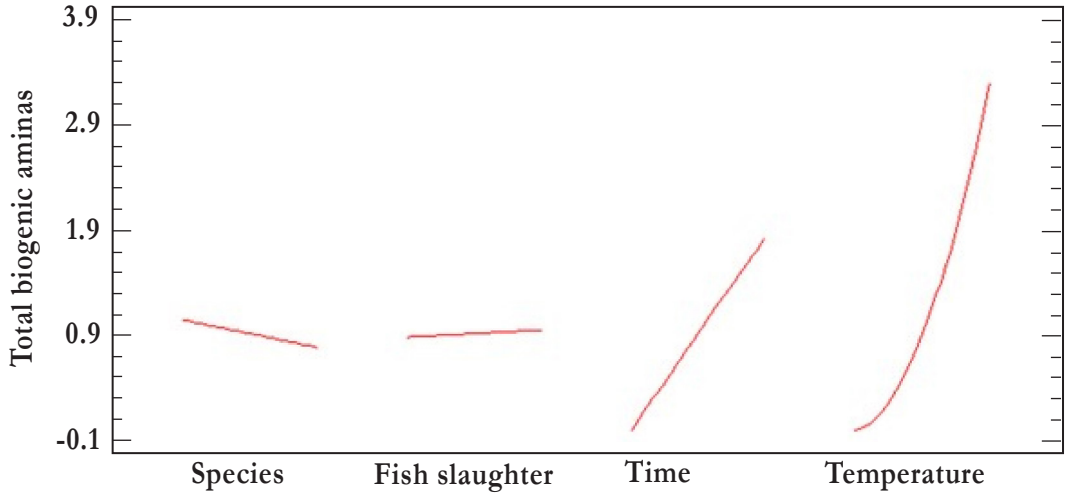

Figure 2. Main effects plot for BAs formation

BAs content in fish samples from markets

Out of the three biogenic amines investigated in samples of three fish species from outlets in the city of Pasto (Nariño), putrescine and histamine were detected. The concentration of these BAs was higher in trout, and the concentration of putrescine was overall higher than that of histamine (**Table 7**). Putrescine levels in the samples were, however, below the rejection limits for fish; indicating that these were suitable for human consumption.

Deficiencies in conservation and management were observed in some of the fish outlets included in this study; these deficiencies are reflected in the formation of BAs. A description of selected fish outlets in the city of Pasto is provided in the supplementary material (Suppl. 3). Our results are in accordance with the hypothesis that BA content depends on the amount of protein (Hernández 2001, Francisco K.C.A *et al*. 2020, Sentellas S *et al*. 2016), indicating that BAs and poisonous compounds in these fishes are produced due to cold chain loss during their conservation and storage. This factor leads to the growth of pathogenic microorganisms of the Enterobacteriaceae (that mediate the formation of these biotoxins). Cold chain loss and bacterial growth in fish are consequences of a lack of adequate infrastructure, the environmental conditions of fish markets, and non-compliance with sanitary and hygiene regulations by transporters and vendors.

Table 7. Identification and quantification of biogenic amines in samples from fish outlets in Pasto, Nariño Region, Colombia. ND: Not detected. Concentration in µg/g fresh weight. RSD: relative standard deviation (%)

Fish outlet	BA	Fish species					
		Tilapia		Cachama		Trout	
		µg/g	*RSD*	*µg/g*	*RSD*	*µg/g*	*RSD*
1	Putrescine	ND	ND	ND	ND	0.98	0.11
	Histamine	ND	ND	ND	ND	0.58	0.29
	Tyramine	ND	ND	ND	ND	ND	ND
2	Putrescine	1.24	0.20	ND	ND	0.55	0.12
	Histamine	ND	ND	ND	ND	ND	ND
	Tyramine	ND	ND	ND	ND	ND	ND
3	Putrescine	0.51	0.30	2.56	0.10	0.90	0.45
	Histamine	0.86	0.40	ND	ND	0.86	1.30
	Tyramine	ND	ND	ND	ND	ND	ND

Conclusions

The biogenic amines putrescine and histamine were present in samples from fish killed and stored under given conditions and from fish available in markets of the city of Pasto, Colombia. The concentrations of 10.04 μg/g and 5.44 μg/g for putrescine and histamine, respectively, indicate that in the fish samples have BAs contents below maximum established by the European Standard (EC) No. 2073/2005, of 200 mg/kg (200 μg/g) in fresh fish.

Through our experimental design we established the relationship, in real time between, the BA content of fish samples and four variables involved in the formation of these toxins along the fish marketing process. Fish storage temperature and time favor the production of BAs; whereas the content of free amino acids and type of sacrifice exert a negative effect on the formation of these compounds. The foregoing indicates that these compounds are largely associated with poor hygiene and handling practices by vendors, in addition to the loss of the cold chain in fish distribution.

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Conflict of Interests

The authors declare that no competing interests exist.

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Aminas biogénicas en trucha arcoíris, tilapia y cachama, disponibles para consumo en Nariño, sur de Colombia

Resumen: Las aminas biogénicas (BAs) son compuestos nitrogenados de bajo peso molecular, formados por la descomposición de proteínas en productos alimenticios altamente perecederos, como el pescado. Las BAs pueden afectar la salud humana y están asociadas con casos de envenenamiento por alimentos. Se determinó la formación de BAs como histamina, putrescina y tiramina por medio de Química Analítica de Procesos (PAC) en tres especies de pescado de agua dulce en mercados de la ciudad de Pasto, en el sur de Colombia: trucha arcoíris, tilapia y cachama. Se evaluó la formación de BAs durante el proceso de conservación y se consideró un diseño multifactorial con dos niveles. Los factores estudiados fueron: especie de pez, tipo de sacrificio, temperatura de almacenamiento y tiempo hasta la compra. De las tres especies estudiadas, las muestras de tilapia revelaron el contenido más alto de putrescina e histamina, con valores de 5.4 μg/g y 10.04 μg/g, respectivamente. No se detectó tiramina en ninguno de los experimentos que se llevaron a cabo. Los valores observados de BAs en las muestras analizadas estuvieron por debajo de los valores máximos tolerados localmente y del estándar europeo (200 μg/g). Sin embargo, su presencia revela que factores como la temperatura de almacenamiento de las muestras y el tiempo hasta el consumo desencadenan su formación.

Palabras clave: aminas biogénicas; química analítica de proceso; pescado de agua dulce: diseño experimental.

Aminas biogénicas em truta arco-íris, tilápia e cachama, disponíveis para consumo em Nariño, sul de Colômbia

Resumo: As aminas biogênicas (BAs) são compostos nitrogenados de baixo peso molecular, formados pela decomposição de proteínas em produtos alimentícios altamente perecíveis, como o p ixe. As BAs podem afetar a saúde humana e estão associadas com casos de intoxicação alimentar. A formação de BAs como histamina, putrecina e tiramina foi determinada por meio de Química Analítica de Processos (PAC) em três espécies de peixes de água doce em mercados da cidade de Pasto, ao sul da Colômbia: truta arco-íris, tilápia e cachama. Foi avaliada a formação de BAs durante o processo de conservação e considerou-se um desenho multifatorial com dois tempos. Os fatores estudados foram: espécie de peixe, tipo de sacrifício, temperatura de armazenamento e tempo até a compra. Das três espécies estudadas, as amostras de tilápia revelaram o teor mais alto de putrescina e histamina, com valores de 5,4 μg/g e 10,04 μg/g, respectivamente. Não se detectou tiramina em nenhum dos experimentos realizados. Os valores observados de BAs nas amostras analisadas estiveram abaixo dos valores máximos tolerados localmente e do padrão europeu (200 μg/g). Entretanto, sua presença revela que fatores como a temperatura de armazenamento das amostras e o tempo até o consumo desencadeiam sua formação.

Palavras-chave: Aminas biogênicas; química analítica de processos; peixe de água doce; desenho experimental.

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