VALIDATION STUDY OF A HPLC METHOD ABLE TO MEASURE BIOGENIC AMINES IN CHICKEN MEAT

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Abstract
The work describes an internal study for validating the measuring method of the biogenic amines in refrigerated chicken meat by means of the high performance liquid chromatography (HPLC). The evaluated features for validating the measuring method by means of the high performance liquid chromatography are as follows: linearity, precision, accuracy (repeatability and reproductibility), selectivity, sensitivity (detection limit, quantification limit), robustness. The analysed biogenic amines are: tryptamine, phenyl-ethylamine, putrescin (tetramethylene dimine), cadaverine, histamine, serotonin, tyramine, spermidine and spermine. The calibration curves for the biogenic amines are linear and the values of the linearity coefficients (r^2) are greater than 0.996. The average recovery in the concentration levels 0.5 – 2 µg/ml for the chicken samples recorded the following values: tryptamine 83-85%; phenylethylamine 85-90%; putrescin 94-97%; cadaverine 95-103%; histamine 93-99%; serotonin 88-91%; tyramine 92-93%; spermidine 95-98%; spermine 99-103%. The standard deviation value for inter-laboratory reproducibility evaluation and the precision regarding the chicken meat samples which were artificially contaminated with biogenic amines solutions having a concentration of 1 µg/ml and 2 µg/ml are less than 1.

Keywords: validation, HPLC, biogenic amines, chicken meat.

1. Introduction

Validation, as per SR EN ISO 9000:2001, consists in confirming, by supplying objective proves that the requests for a certain purpose or application were fulfilled (SR EN ISO 17025/2005).

The validation objective is the one of demonstrating that a defined analytic system leads to obtaining some precise and reproducible results, for a given feature. Therefore, it is necessary to investigate different validation parameters depending on the analysis type: qualitative and/or semi-quantitative, screening or quantification.

The validation can represent an internal validation study (intra-laboratory) or large-scale validation, through inter-laboratories studies.

The production and implicitly the chicken meat consumption have increased very much in Romania recently. The chicken products became popular due to their specific sensorial properties and the consumers increasing tendency to consider the chicken meat safer than the beef, pork, etc. The chicken meat spoilage is an economical burden; in some cases it could be a danger to the consumers' health because meat can contain pathogen and spoilage microorganisms (Georgnaras *et al.*, 1995).

2. Materials and methods

The refrigerated carcases of entire chicken weighing between 1.1…1.5 kg were purchased from Agricola International Bacau, transported to the HORTING Bucharest research laboratory and stored at +4°C in

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the refrigerator. The sampling process was done according to actual laws (***, 2005).

The biogenic amines were determined using the method recommended by the Food Research Institute from Helsinki, Finland and adapted by the team members from the Institute for Research-Development of the Horticultural Products Marketing and Industrialization-Horting (Eerola *et. al*, 2001, Rokka *et. al*, 2004, Alberto *et. al*, 2004, Lorentz, 2004, Jercan, 1982, Liteanu *et. al*, 1976, Phyllis, 1973).

All the reagents had analytical purity for HPLC grade. The reagents were purchased from the Merck and Sigma-Aldricht Company. We used biogenic amine standard solutions: hydrochloric tryptamine ($C_{10}H_{12}N_2 \cdot HCl$), hydrochloric phenylethylamine ($C_8H_{11}N \cdot HCl$), bi-hydrochloric putrescin ($C_4H_{12}N_2 \cdot 2HCl$), bi-hydrochloric cadaverine ($C_5H_{14}N_2 \cdot 2HCl$), bi-hydrochloric histamine ($C_5H_9N_3 \cdot 2HCl$), hydrochloric serotonin ($C_{10}H_{12}N_2O \cdot HCl$), hydrochloric tyramine ($C_8H_{11}NO \cdot HCl$), spermidine phosphate hexahydrate ($C_{14}H_{47}N_6O_{12}P_3 \cdot 6H_2O$), spermina bi-phosphate ($C_{10}H_{26}N_4 \cdot 2H_3PO_4$), shown in which were purchased from Sigma-Aldrich Company. Working solutions were prepared with a concentration of 100 µg/ml and 10µg/ml. The internal standard solution, 1,7-diaminoheptan ($C_7H_{18}N_2$) was also purchased from Sigma-Aldrich Company. The concentration stock solution was prepared at 1mg/ml concentration and the working solution at 100µg/ml.

Installations and equipment: homogenisation type blender, Kern analytical balance, Silent CrusherM, centrifuge EBA 21, filter paper of ∅=55 mm, syringe filters having porosity of 0.45µm, agitator REAX control, ultrasonic water tank Aquawave TM, incubator BMT INCUCELL 55, water cleaning system EASY pure RoDi (18.2 MΩcm and total organic carbon <5ppb), filtering system with vacuum pump.

The HPLC analysis system consists in: pump, column thermostat, UV-VIS detector with diode array, computer system, and printer. Chromatography column are BDS Hypersil C18 250 x 4.6 mm, having the particles size of 5 µm and Hypersil Gold precolumns 10 x 2.1 mm.

In order to make different biogenic amine concentrations (from 0.1 up to 7 µg/ml), we prepared the standard working solutions of 100µg/ml and 10µg/ml concentrations as well as the known internal standard working solution. Then we added different volumes of perchloric acid in order to obtain a final volume of 0.5 ml.

Quantitative measurements were performed depending on the internal standard, using the chromatography peaks obtained for each biogenic amine. The absorbance of derivatised biogenic amines was measured at 254 nm and the peaks were integrated with CromQuest software. Each biogenic amine concentration was expressed in µg/ml.

The results obtained are of 10 determinations; the mean values were calculated with Microsoft Excel software from Microsoft Office suite.

3. Results and Discussion

3.1. Linearity

In order to determine the biogenic amines from the chicken meat by means of the high performance chromatography liquid, a calibration curve was made with standard concentrations of 8 biogenic amines (with three replicates), in the concentration ranging from 0.1 µg/ml to 7 µg/ml, as shown in table 1.

For making the calibration curve, in order to underline linearity, the biogenic amine concentrations were done with three repetitions.

Table 1. Operating conditions

Time, min	Gradient		Flow, ml/min	Wave length, nm	Column pressure, bar	Column temperature, ºC	Sample room temperature, ºC	Injected sample volume, µl
	Ammonia acetate, %	Nitrile acetate, %						
0.01	40	60	1.00	254	min. 70	40	7	20
15	40	60						
20	30	70						
25	5	95						
30	40	60						

The calibration curves for the biogenic amines are linear having r^2 as follows: tryptamine $r^2 = 0.9953$; β-phenylethylamine $r^2 = 0.9983$; putrescin $r^2 = 0.9985$; cadaverine $r^2 = 0.9985$; histamine $r^2 = 0.9981$; serotonin $r^2 = 0.9966$; tyramine $r^2 = 0.9986$; spermidine $r^2 = 0.9986$; spermine $r^2 = 0.9982$.

The values of r^2 are a fraction between 0.0 and 1.0 and have no units. An r^2 value of 0.0 means that knowing X does not help predict Y, so there is no linear relationships between X and Y, and best fit line is an horizontal line going through the mean of all Y values.

When r^2 is 1.0, all points lie exactly on a straight line with no scatter. Knowing X lets you predict Y perfectly. So, the values of r^2 for each biogenic amine are as a linear relationship between area ratio (Y) and amount ratio (X) which are the coordinates that characterise the two axes of the graph. As it can be seen, the values of r^2 for every biogenic amine are close to 1.0.

3.2. Precision

The precision reflects the ability to perform an analysis with small differences between the real and experimental values. In case of the biogenic amine method on animal origin samples by means of the HPLC, the precision is expressed by recovery.

In order to establish the recovery, in case of this method, the chicken samples were analysed as they are (blank), then standard solutions biogenic amine were added in different concentrations. Thus, working solutions of biogenic amine were added in order to obtain injection concentrations of: 0.5 µg/ml, 1 µg/ml and 2 µg/ml for each amine. Ten measurements were performed for each added concentration of biogenic amine. The same analyst did the preparation. The liquid chromatography measurement was done three times for each sample. The mean values were calculated with Microsoft Excel software from Microsoft Office suite.

As presented in Table 2 data, the best recovery is for cadaverine and spermine while tryptamine has the lowest recovery. Recoveries exceeding 100% are normal but they do not have to exceed 105%, because in this case there is a problem of equipment (column and detector).

It can be noticed in Table 3 that histamine and spermine have the best recovery and tryptamine has the lowest recovery.

Table 2. The recovery values for each studied amine having a 0.5 µg/ml concentration added

Mean recovery (%) for the 0.5 µg/ml concentration	
Tryptamine	83.26
Phenylethylamine	85.67
Putrescin	97.13
Cadaverine	103.00
Histamine	93.06
Serotonin	88.55
Tyramine	93.91
Spermidine	95.48
Spermine	104.43

Table 3. The recovery values for each studied amine having a 1 µg/ml concentration added

Mean recovery (%) for the 1 µg/ml concentration	
Tryptamine	84.05
Phenylethylamine	90.55
Putrescin	94.49
Cadaverine	95.81
Histamine	99.69
Serotonin	91.59
Tyramine	92.61
Spermidine	98.52
Spermine	99.65

In Table 4 the best recovery is for histamine and spermine. Tryptamine and serotonin had the lowest recovery.

Table 4. The recovery values for each studied amine having a 2 µg/ml concentration added

Mean recovery (%) for the 2 µg/mL concentration	
Tryptamine	85.77
Phenylethylamine	86.96
Putrescin	97.71
Cadaverine	96.54
Histamine	97.97
Serotonin	89.28
Tyramine	93.75
Spermidine	98.06
Spermine	103.94

For the small concentrations of the analysed biogenic amines (0.5%…2%), cadaverine, histamine and spermine had the best recovery: > 95%. Tryptamine has the lowest recovery: 83…85%.

Per global, the recoveries of nine biogenic amines at little concentrations added is good, tryptamine, phenylethylamine and serotonin having the lowest recovery for small concentration added.

3.3. Accuracy

The accuracy represents "the matching level among the independent analytical results obtained under the specified conditions" (SR EN ISO 17025/2005, 2005).

The accuracy is an indicator of the result dispersion and is determined under repeatability and reproducibility conditions. Repeatability (r) – the value of the absolute difference among the independent results, obtained on the same sample, the same device, by the same performer and after a short period, is in range of the specific probability limits (usually 95 %).

Reproducibility (R) – the value of the absolute difference among the results of a single test, obtained for an identical material, in two or many laboratories by different performers on different machines, is in range of the specific probability limits (usually 95 %). The obtained values are presented in Table 5.

Intra-laboratory reproducibility assessment was done by two analysts working on 10 samples (analyst A – 6 samples, analyst B – 4 samples) that were "artificially" contaminated with solution of biogenic amines solution having a 1µg/ml concentration and were calculated:

- Standard deviation value for retention time, peak range and determined the concentration of biogenic amines;
- Limit reproducibility.

The obtained values are shown in Table 6.

3.4. Selectivity

It reflects the ability to find and measure a specified compound when are present other interested compounds. It means that, for the chromatography methods, the analysed substance can be separated with enough resolution by all the neighbouring peaks and that it can be detected with a proper instrument.

The peaks of biogenic amines are separated very well by the base line and by the peaks of other compounds. The achieved resolution is higher than 1 when using a BDS Hypersil C 18, 250 x 4.6 mm chromatography column with 5 µm particle dimension.

The proposed method for the measurement of biogenic amines by means of the high performance liquid chromatography is selective.

The corresponding peaks of biogenic amines are separated from the base line and the peaks of other composites. The achieved resolution is higher than 1, which means that the two adjacently peaks are separated very well (as it can be seen in the figures). In figure 1, 2 and 3, the biogenic amines with known concentration of the standard solution chromatograms are presented, as well as the chromatogram of biogenic amines of a chicken meat sample.

3.5. The detector sensitivity

Reflects the ability of the detector to detect samples with a lower content of the specified compound.

The detection limit (LOD) is defined as the concentration that will give an absorbance signal three times higher than background noise. The detection limit is the value that establishes the lower limit of the working field.

The quantification limit (LOQ) is defined as being the lowest analysed substance concentration that can be determined with an acceptable precision under the analysis method conditions. As a value, the quantification limit is the detection limit doubled.

Table 5. Repeatability values and standard deviation centralized for each studied amine

Biogenic amines	Standard deviation (SD)			Repeatability		
	Retention time	Peak area	Concentration	Retention time	Peak area	Concentration
Tryptamine	0.026	19213.155	0.020	0.072	53796.834	0.056
Phenylethylamine	0.026	18559.942	0.018	0.072	51967.836	0.050
Putrescin	0.032	70679.688	0.021	0.089	197903.125	0.058
Cadaverine	0.035	92569.639	0.037	0.098	259194.990	0.105
Histamine	0.036	46433.270	0.018	0.100	130013.155	0.051
Serotonin	0.043	18302.081	0.009	0.120	51245.828	0.025
Tyramine	0.015	71264.565	0.039	0.042	199540.781	0.110
Spermidine	0.009	32193.968	0.016	0.025	90143.111	0.044
Spermine	0.011	30775.764	0.047	0.032	86172.139	0.131

Table 6. Reproducibility values and standard deviation centralizer for each studied amine

Biogenic amines	Standard deviation			Reproducibility		
	Retention time	Peak area	Concentration	Retention time	Peak area	Concentration
Tryptamine	0.027	10424.664	0.016	0.074	29189.058	0.044
Phenylethylamine	0.028	18353.585	0.013	0.078	51390.038	0.036
Putrescin	0.034	46577.329	0.014	0.094	130416.521	0.038
Cadaverine	0.038	35994.537	0.014	0.108	100784.705	0.039
Histamine	0.039	35796.861	0.015	0.109	100231.211	0.043
Serotonin	0.049	14904.839	0.011	0.136	41733.548	0.030
Tyramine	0.032	29937.416	0.015	0.089	83824.765	0.043
Spermidine	0.018	53437.604	0.023	0.049	149625.292	0.064
Spermine	0.012	77512.750	0.045	0.033	217035.700	0.127

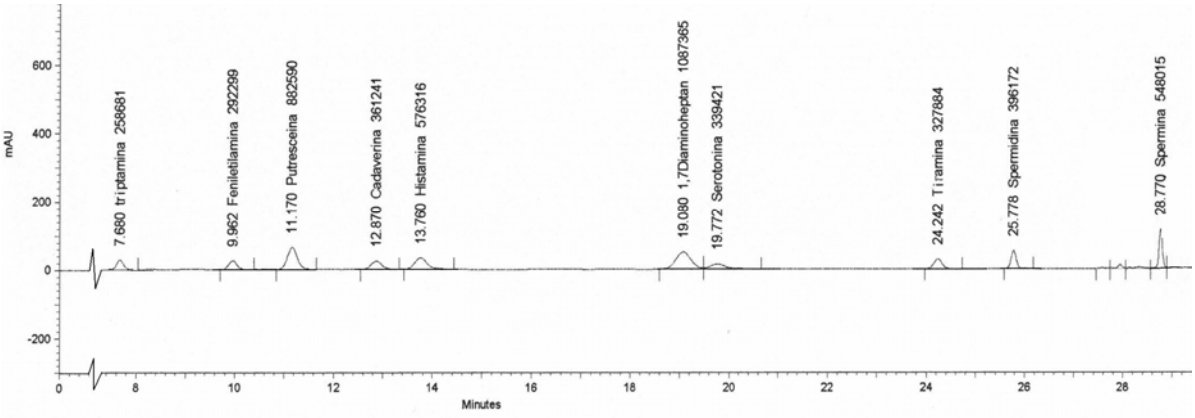

Figure 1. Chromatogram of standard solutions of the biogenic amines for a 0.5µg/ml concentration

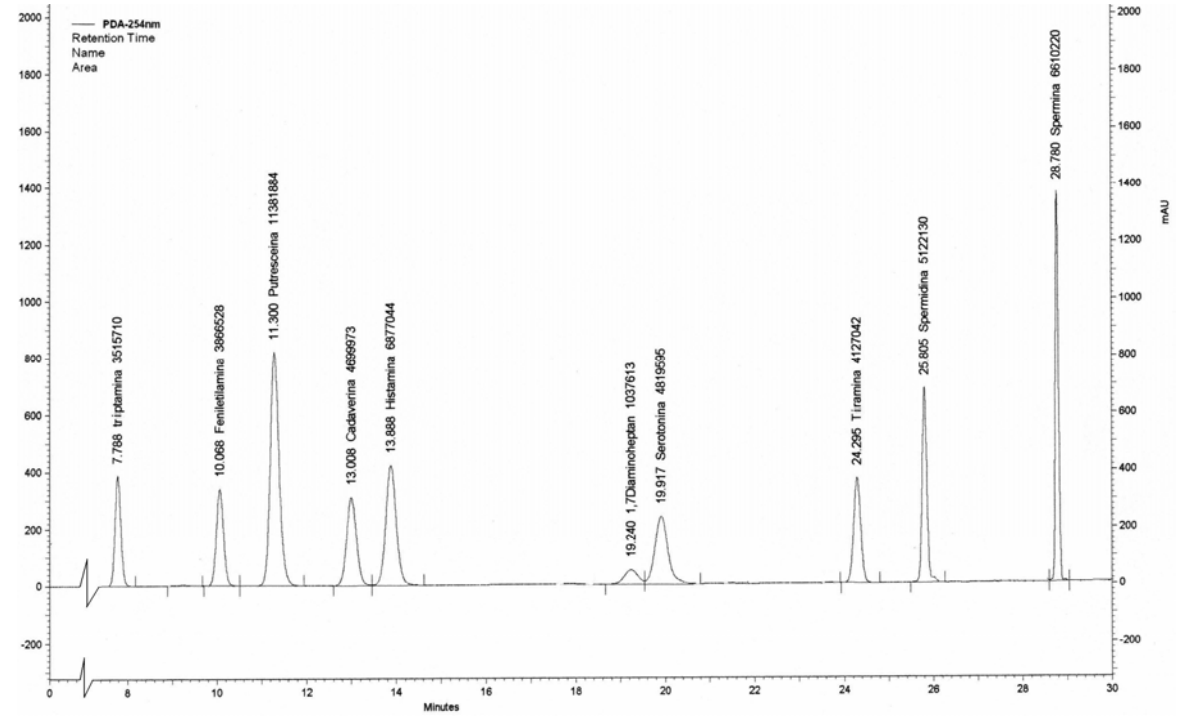

Figure 2. Chromatogram of standard solutions of the biogenic amines for a 7µg/ml concentration

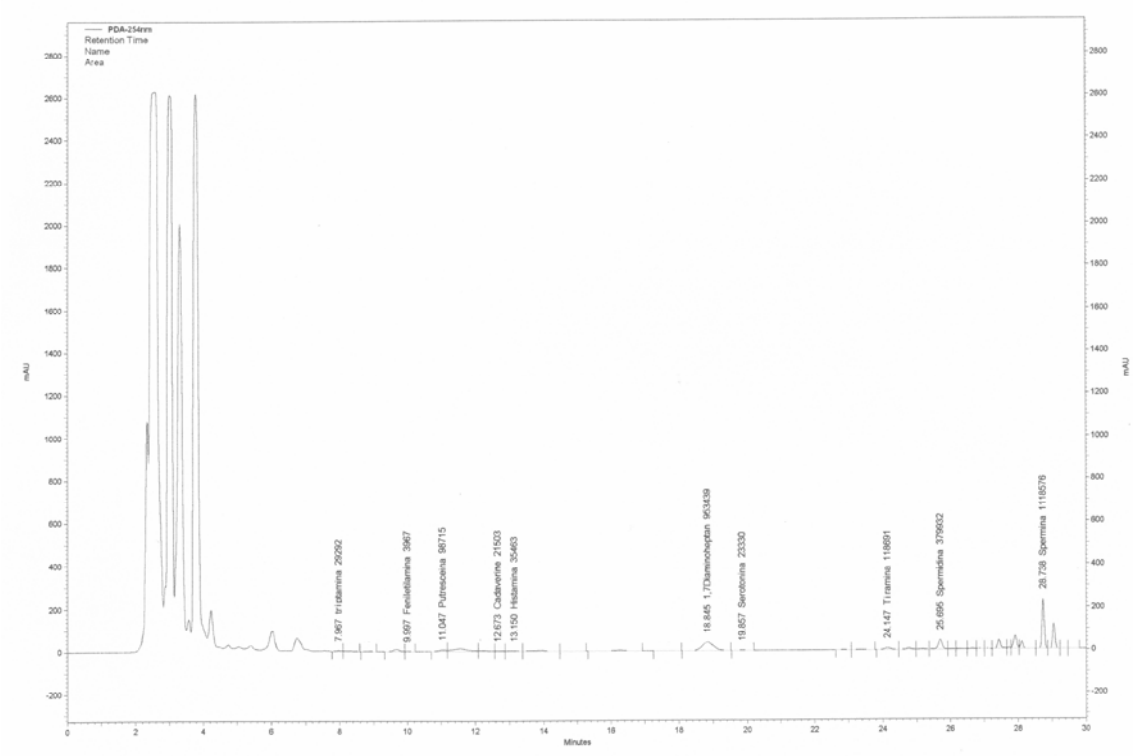

Figure 3. Chromatogram of chicken meat sample (mAU = mili Absorbance Unit)

Using the ratio signal/noise recorded by the high performance liquid chromatograph for the biogenic amine measurement in case of the standard solutions of biogenic amines, the detection limit and the quantification limit were calculated.

LOD and LOQ measurement was performed on the standard solutions of biogenic amines with 0.1, 0.5, 1, 1.5, 2, 3, 5 and 7 concentrations. The obtained results are shown in Table 7.

Table 7. LOD and LOQ values for each studied amine

Biogenic amines	Mean detection limit (µg/ml)	Mean quantification limit (µg/ml)
Tryptamine	0.006	0.012
Phenylethylamine	0.050	0.100
Putrescin	0.022	0.044
Cadaverine	0.030	0.060
Histamine	0.035	0.070
Serotonin	0.015	0.030
Tyramine	0.006	0.012
Spermidine	0.005	0.010
Spermine	0.009	0.018

3.6. The robustness

It reflects the method sensibility to operate condition changes. In order to asses the robustness of the proposed method of measuring the biogenic amines by means of the high performance liquid chromatography, the following parameters varied: wavelength and injection volume.

3.6.1. Wavelength variation

Chicken meat samples spiked with different volumes of 2 µg/ml biogenic amine solution were tested at λ = 249 nm and λ = 259 nm. The obtained results are shown in Table 8.
From the obtained data, it can be noticed that the recovery is good for cadaverine and phenylethylamine. Serotonin has the lowest recovery for both wavelengths.

3.6.2. Injection volume variation

Chicken meat samples spiked with different volumes of 2 µg/ml biogenic amine solution were tested at λ = 254 nm and injection volume of 18 µl, λ = 254 nm and injection volume of 22 µl. The obtained results are shown in Table 9. From the obtained data, it can

be noticed that the best recovery was for cadaverine and then phenylethylamine. Serotonin had the lowest recovery for both injection volume variations.

Table 8. The mean recovery values for each studied biogenic amine

Biogenic amines	Mean recovery (%)	
	249 nm	259 nm
Tryptamine	80.28	90.65
Phenylethylamine	91.02	91.62
Putrescin	86.22	86.75
Cadaverine	101.75	103.58
Histamine	89.25	79.85
Serotonin	67.48	51.98
Tyramine	85.25	84.97
Spermidine	84.22	80.07
Spermine	72.62	79.07

The results obtained in tables 9 and 10 underline the fact that the method is sensitive to wavelength and injection volume changes.

Table 9. The mean recovery values for each studied biogenic amine

Biogenic amines	Mean recovery (%)	
	18 µl	22 µl
Tryptamine	82.98	83.50
Phenylethylamine	91.82	91.52
Putrescin	86.80	86.23
Cadaverine	103.11	102.63
Histamine	83.82	83.53
Serotonin	68.77	68.98
Tyramine	89.70	89.13
Spermidine	87.60	87.03
Spermine	85.27	85.00

Table 10. The mean recovery values for each studied biogenic amine

Biogenic amines	Mean recovery (%)	
	18 µl	22 µl
Tryptamine	82.98	83.50
Phenylethylamine	91.82	91.52
Putrescin	86.80	86.23
Cadaverine	103.11	102.63
Histamine	83.82	83.53
Serotonin	68.77	68.98
Tyramine	89.70	89.13
Spermidine	87.60	87.03
Spermine	85.27	85.00

4. Conclusions

The results confirm the validity of the biogenic amine measurement in the chicken meat by means of the high performance liquid chromatography. Biogenic amine lower detection limit is 0.1 µg/ml.

This method is complex, precise, sensible, selective, reproducible, to quantify the nine biogenic amines. The method can be used to determine the biogenic amines in the chicken meat.

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