# VALIDATION STUDY OF A HPLC METHOD FOR BIOGENIC AMINES QUANTIFICATION IN BANANAS

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*In this paper we describe an internal study for validating high performance liquid chromatography (HPLC) method for quantification of the biogenic amines in bananas. The evaluated features for validating the measuring method by means of the high performance liquid chromatography are as follows: linearity, precision, accuracy (repeatability and reproducibility), selectivity, sensitivity (detection limit, quantification limit), and robustness. The analyzed biogenic amines are: tryptamine, phenyl-ethylamine, putrescine, 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 as follow: 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 average recovery in the concentration levels 0.5 ... 2 µg/ml for the banana samples recorded the following values: tryptamine 63-93%; β-phenylethylamine 80-87%; putrescine 85-99%; cadaverine 96-110%; histamine 80-93%; serotonin 60-85%; tyramine 82-98%; spermidine 80-105% and spermine 90-110%. The proposed method for biogenic amines quantification in bananas by HPLC is selective. The peaks for every biogenic amine are separated by the baseline and by the vicinity peaks. The resolution that characterize the selectivity is greater than 1. LOD (limit of detection) for every amine are as follows: tryptamine 0.006 µg/ml, β-phenylethylamine 0.050 µg/ml, putrescin 0.022 µg/ml, cadaverine 0.030 µg/ml, histamine 0.035 µg/ml, serotonin 0.015 µg/ml, tyramine 0.006 µg/ml, spermidine 0.005 µg/ml, spermine 0.009 µg/ml. LOQ (limit of quantitation) are as follows: tryptamine 0.012 µg/ml, β-phenylethylamine 0.100 µg/ml, putrescin 0.044 µg/ml, cadaverine 0.060 µg/ml, histamine 0.07 µg/ml, serotonin 0.030 µg/ml, tyramine 0.012 µg/ml, spermidine 0.01 µg/ml, spermine 0.018 µg/ml.*

***Key words:*** *validation, HPLC, biogenic amines, banana pulp*

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 [10]. 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.

Romania is a country in witch bananas does not characterize its fruit grow. In Romanian markets banana are found everywhere, being consumed by all people categories. Therefore, it is very interesting to know witch biologically active amines are present and their extent.

## MATERIAL AND METHOD

The bananas at room temperature are purchased from the Plus retailer at 16°C, transported to the HORTING Bucharest research laboratory and maintained at laboratory room temperature (16…20°C). Here we determined biogenic amines content of bananas. The sampling process was done according to actual laws [1]. In our analysis we took only the edible part of banana: banana pulp. 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, Bucharest [2,3,4,5,6,7,8]. 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 purchased from Sigma-Aldrich Company. Working solutions were prepared with a concentration of 100µg/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 Φ=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, 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 100 µg/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, and the biogenic amines content were expressed in mg/kg. The results obtained are of 10 determinations; the mean values were calculated with Microsoft Excel software from Microsoft Office suite.

## RESULTS AND DISCUSSIONS

In order to determine the biogenic amines from the banana pulp by means of the high performance chromate graphy 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

**HPLC operating conditions**

| Time, min | Gradient | | Flow, ml/min | Wave lenght, nm | Column pres., bar | Column temp., °C | Sample room temp., °C | Injected sample volume, µl |
|---|---|---|---|---|---|---|---|---|
| | Ammonia acetate,% | Nitrile acetate,% | | | | | | |
| 0,01 | 40 | 60 | 1.00 | 254 | minim 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 characterize the two axes of the graph. As it can be seen from anterior results, the values of $r^2$ for every biogenic amine are close to 1.0.

**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 vegetal 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 banana samples were analyzed as they are (blank), then standard solutions biogenic amines were added in different concentrations (spiked). 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 serotonin and tryptamine has the lowest recovery. Recoveries exceeding 100% are normal but they do not have to exceed 110%, because in this case there is a problem of equipment (column and detector).

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 | 63.26 |
| β-phenylethylamine | 85.67 |
| Putrescin | 87.13 |
| Cadaverine | 110.00 |
| Histamine | 83.06 |
| Serotonin | 58.55 |
| Tyramine | 83.91 |
| Spermidine | 85.48 |
| Spermine | 109.43 |

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

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 | 64.05 |
| β-phenylethylamine | 80.55 |
| Putrescin | 74.49 |
| Cadaverine | 95.81 |
| Histamine | 79.69 |
| Serotonin | 71.59 |
| Tyramine | 82.61 |
| Spermidine | 78.52 |
| Spermine | 89.65 |

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 | 75.77 |
| β-phenylethylamine | 86.96 |
| Putrescin | 90.71 |
| Cadaverine | 96.54 |
| Histamine | 87.97 |
| Serotonin | 75.28 |
| Tyramine | 87.75 |
| Spermidine | 91.06 |
| Spermine | 103.94 |

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

For the small concentrations of the analyzed biogenic amines (0.5%…2%), only cadaverine had the best recovery: > 95%. Tryptamine and serotonin had the lowest recovery: <75%. Per global, the recoveries of nine biogenic amines at little concentrations added is good, tryptamine, β-phenylethylamine and serotonin having the lowest recovery for small concentration added.

**Accuracy**

The accuracy represents "the matching level among the independent analytical results obtained under the specified conditions" [10]. 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.*

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 | 0.027 | 21397.276 | 0.049 | 0.076 | 59912.374 | 0.137 |
| Phenylethylamine | 0.037 | 36211.485 | 0.063 | 0.103 | 101392.158 | 0.176 |
| Putrescin | 0.050 | 87102.100 | 0.058 | 0.139 | 243885.880 | 0.162 |
| Cadaverine | 0.060 | 77872.830 | 0.059 | 0.167 | 218043.923 | 0.164 |
| Histamine | 0.066 | 25716.808 | 0.027 | 0.185 | 72007.062 | 0.075 |
| Serotonin | 0.118 | 12632.689 | 0.011 | 0.330 | 35371.528 | 0.029 |
| Tyramine | 0.083 | 62645.843 | 0.057 | 0.232 | 175408.360 | 0.161 |
| Spermidine | 0.057 | 69127.529 | 0.055 | 0.159 | 193557.081 | 0.155 |
| Spermine | 0.040 | 48675.120 | 0.029 | 0.111 | 136290.335 | 0.081 |

Intra-laboratory reproducibility assessment was done by two analysts working on 10 samples (analyst A – 6 samples, analyst B – 4 samples) that were spiked with biogenic amines solution having a 2 µg/ml concentration. Then we determined the following:

- standard deviation value for retention time, peak range and biogenic amines concentration;
- limit reproducibility.

The obtained values are shown in *Table 6.*

**Selectivity**

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. After we

analyzed the peaks with the CromQuest software, we can say that the proposed method for the measurement of biogenic amines by means of the high performance liquid chromatography is selective.

Table 6

**Reproducibility values and standard deviation centralized for each studied amine**

| Biogenic amines | Standard deviation | | | Reproducibility | | |
|---|---|---|---|---|---|---|
| | Retention time | Peak area | Concentration | Retention time | Peak area | Concentration |
| Tryptamine | 0.074 | 63935.352 | 0.050 | 0.207 | 179018.987 | 0.141 |
| Phenylethylamine | 0.098 | 101263.190 | 0.069 | 0.275 | 283536.933 | 0.193 |
| Putrescin | 0.127 | 345756.836 | 0.059 | 0.354 | 968119.140 | 0.166 |
| Cadaverine | 0.149 | 206901.762 | 0.066 | 0.417 | 579324.934 | 0.184 |
| Histamine | 0.157 | 155249.088 | 0.031 | 0.439 | 434697.445 | 0.087 |
| Serotonin | 0.228 | 89811.898 | 0.028 | 0.637 | 251473.313 | 0.078 |
| Tyramine | 0.131 | 165930.721 | 0.058 | 0.367 | 464606.019 | 0.162 |
| Spermidine | 0.081 | 221700.353 | 0.057 | 0.227 | 620760.988 | 0.158 |
| Spermine | 0.044 | 120208.081 | 0.026 | 0.123 | 336582.626 | 0.073 |

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 banana pulp sample.

**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 analyzed 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. mAU is in fact 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*.

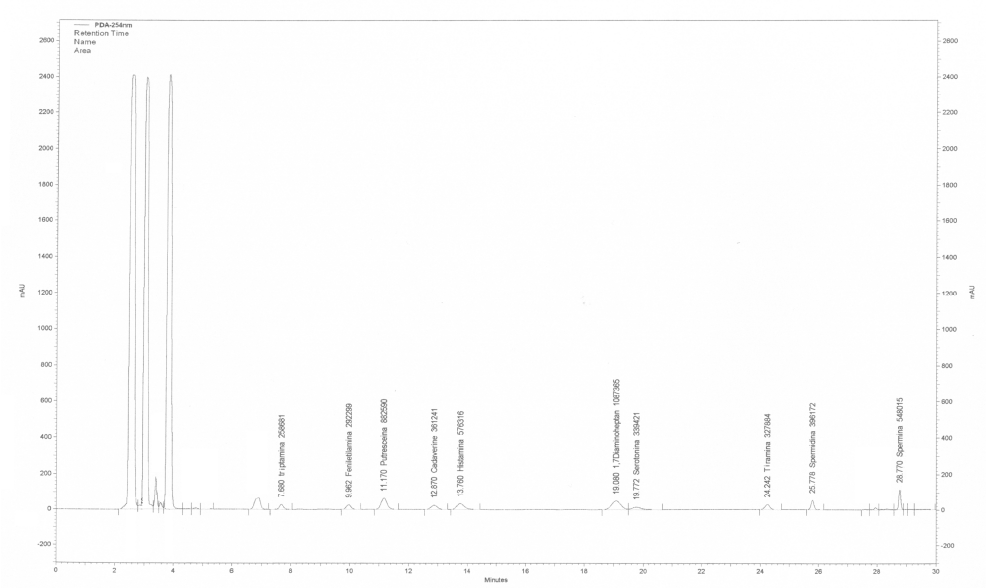


Figure 1 **Chromatogram of standard solutions of the biogenic amines for a 0.5 µg/ml concentration**

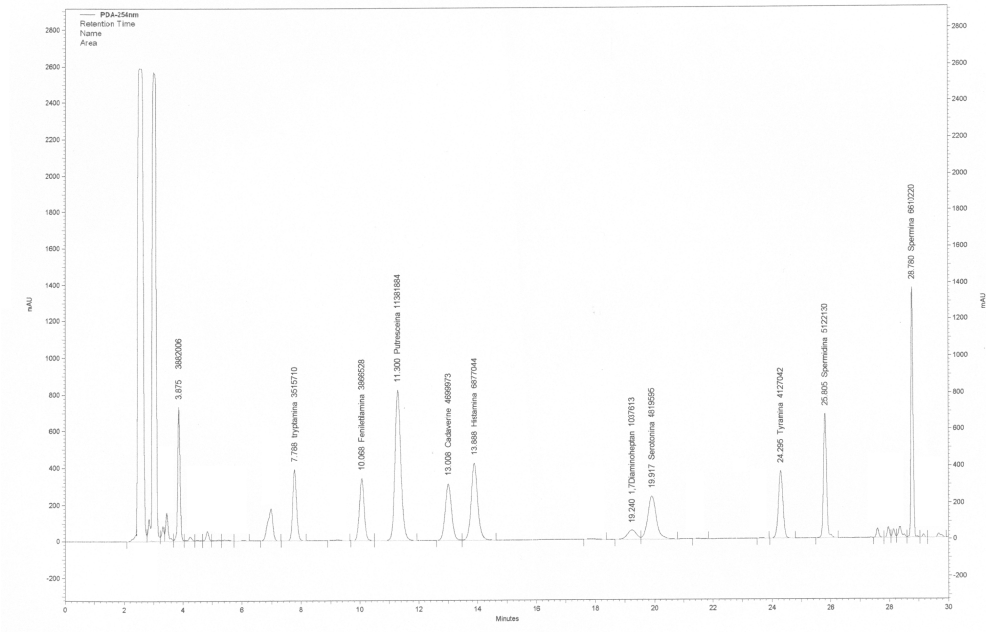


Figure 2 **Chromatogram of standard solutions of the biogenic amines for a 7 µg/ml concentration**

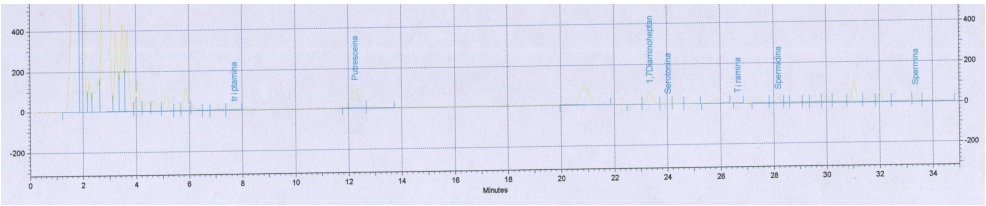


Figure 3 **Chromatogram of banana sample**

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 | 0.07 |
| Serotonin | 0.015 | 0.030 |
| Tyramine | 0.006 | 0.012 |
| Spermidine | 0.005 | 0.01 |
| Spermine | 0.009 | 0.018 |

**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.

**1. Wavelength variation**

Banana samples spiked with different volumes of 2 µg/ml biogenic amine solution were tested at $\lambda = 249$ nm and $\lambda = 259$ 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.

**2. Injection volume variation**

Banana samples spiked with different volumes of 2 µg/ml biogenic amine solution were tested at $\lambda = 254$ nm and injection volume of 18 µl, $\lambda = 254$ 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.

The results obtained in *tables 8* and *9* underline the fact that the method is sensitive to wavelength and injection volume changes.

Table 8

**The mean recovery values for each studied biogenic amine**

| Biogenic amines | Average recovery (%) | |
|---|---|---|
| | 249nm | 259nm |
| Tryptamine | 80.28 | 90.65 |
| Phenylethylamine | 91.02 | 91.62 |
| Putrescin | 76.22 | 76.75 |
| Cadaverine | 101.75 | 103.58 |
| Histamine | 69.25 | 79.85 |
| Serotonin | 37.48 | 41.98 |
| Tyramine | 75.25 | 84.97 |
| Spermidine | 74.22 | 80.07 |
| Spermine | 52.62 | 59.07 |

Table 9

**The mean recovery values for each studied biogenic amine**

| Biogenic amines | Average recovery (%) | |
|---|---|---|
| | 18 µl | 22 µl |
| Tryptamine | 82.98 | 83.50 |
| Phenylethylamine | 91.82 | 91.52 |
| Putrescin | 76.80 | 76.23 |
| Cadaverine | 103.11 | 102.63 |
| Histamine | 73.82 | 73.53 |
| Serotonin | 38.77 | 38.98 |
| Tyramine | 79.70 | 79.13 |
| Spermidine | 77.60 | 77.03 |
| Spermine | 55.27 | 55.00 |

## CONCLUSIONS

The results confirm the validity of the biogenic amine measurement in the banana pulp 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 banana pulp.

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