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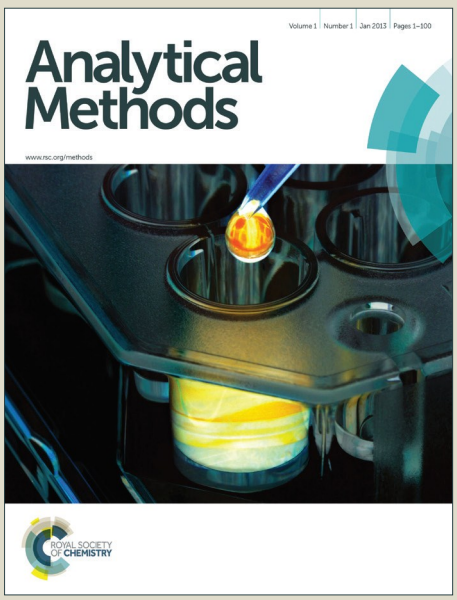

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Simultaneous Detection of Fifteen Biogenic Amines in Animal Derived Products by HPLC-FLD with Solid-Phase Extraction after Derivatization with Dansyl Chloride

Huaping Zhu, Shanshan Yang, Yan Zhang, Guozhen Fang and Shuo Wang*

Key Laboratory of Food Nutrition and Safety, Ministry of Education of China, Tianjin Key Laboratory of Food Nutrition and Safety, Tianjin University of Science and Technology, Tianjin 300457, China

*Corresponding author: Shuo Wang

Tel.: +86 22 60271689

Fax: +86 22 60912493

E-mail address: s.wang@tust.edu.cn

Abstract Simultaneous detection of many kinds of biogenic amines (BAs) are difficult because they have diverse structures. A HPLC method was established suitably for the simultaneous detection of fifteen biogenic amines in four types of animal-derived food products. The biogenic amines were derivatized with dansyl chloride, purified by Waters Sep-Pak C18 then separated on an ODS-2 Hypersil C18 column with a binary system using gradient elution. The derivatives were detected using wavelengths of 350 and 480 nm for excitation and emission, respectively. Limit of detection (LOD) for BAs ranged from 0.002 to 0.03 mg kg^{-1} and the linarites of linear regression equations for fifteen biogenic amines were good (R^2 between 0.9990 and 0.9999). The method was applied to detect BAs in pork, beef, carp and crucian carp. Recoveries ranged from 70.49 to 121.16% at three spiked levels (0.5, 1 and 2 mg kg^{-1}), with RSDs in a range from 0.71-15.99%. Intra- and inter-day precisions (RSD %) were in a range of 0.30%-4.60% and 4.62%-14.97%, respectively. These data indicated that the established method was capability for simultaneous and precise quantitation of fifteen biogenic amines in animal-derived products of potential physiological importance for human health.

Keywords Biogenic amines, Simultaneous detection, Animal-derived products, HPLC-FLD, Solid phase extraction

1. Introduction

Biogenic amines (BAs) are basic nitrogenous compounds with low molecular weights. Depending on their chemical structure, they can be divided into three groups: aliphatic (e.g. methylamine, ethylamine, putrescine and cadaverine), aromatic (e.g. 2-phenylethylamine and tyramine) and heterocyclic (e.g. tryptamine and 5-hydroxytryptamine).

BAs are significant components of bioorganic bodies and play an important physiological role. However, there is a risk that at high levels of intake, humans are unable to detoxify them; BAs can be harmful to humans, causing a variety of symptoms, damaging the nervous system and cardiovascular system and, in severe cases, causing death [1]. BAs are not equally toxic, while, histamine is the most toxic among BAs. Putrescine and cadaverine are able to react with nitrite to produce nitrosamines which are potentially carcinogenic[2].

BAs are generated mainly by decarboxylases produced by microorganisms, but also by the amination and transamination of aldehyde or ketone [3-4]. BAs are present in many foods, especially those rich in protein. The amount of BAs has been found to be associated with the degree of food freshness. Vinci et al [5] detected several BAs in beef and chicken meat after storage at 4°C for 36 days. They found that the concentration of cadaverine reflected the degree of spoilage in white and red meat and that the concentration of tyramine reflected the degree of freshness of beef during storage. Galgano et al[6] investigated BA contents as indicators of spoilage in fresh beef stored at 4 °C for 8 days. They concluded that the contents of cadaverine and tyramine were affected by storage time so could be used as spoilage indices for fresh beef. BAs are difficult to remove by cooking once they have been formed [7], so are of great concern regarding food quality and safety.

Due to the toxicity of BAs, many authorities have given advice on the maximum level of BAs

allowed in food products. The US FDA has formulated a guideline maximum level of histamine of 50 mg kg^{-1} in aquatic products, and levels of 500 mg kg^{-1} of histamine and 100 mg kg^{-1} of tyramine in other foods[8]. The European Union has restricted the level of histamine to 100 mg kg^{-1} in some fish species and other foods[9].

Determination of BAs is not easy due to their various structures and low levels in complex matrix samples. Several qualitative and quantitative analytical methods are available for BAs. Enzyme-linked immunosorbent assay (ELISA)[10-12] and thin layer chromatography (TLC)[13] were employed to detect the BAs, they can give quick results, but not accurately quantitative. Ion chromatography (IC) with a conductivity detector[14-16] or amperometric detection[15-16] can detect BAs without derivation, however, only some limited kinds of BAs can be analyzed. Gas chromatography - Mass Spectrometer (GC-MS)[17-19] and electrophoresis (CE)[20-23] can detect BAs with good results, but they require trained personnel and high capital expenditure. Due to its high selectivity and sensitivity, liquid chromatography was extensively used to determine BAs. Eva et al[24]determined 8 kinds of BAs derivatived with dansyl chloride in pork, beef, chicken and fish meat, cheese and edible mushrooms, using UHPLC coupled with diode array detector (DAD), with LODs and LOQs ranged between 0.36-1.12 mg kg^{-1}, and 1.2-3.7 mg L^{-1}. Wu et al[25] established a method for simultaneous determination of 7 kinds of BAs in beer, rice wine, cheese, yogurt and ham sausage using HPLC-FLD with LODs of 1.1-7.8 ng mL^{-1} and LOQs of 3.5-26.1 ng mL^{-1}. Lázaro et al[26] quantitatively determined 5 kinds of BAs in chicken meat via HPLC with ultraviolet detector (UV) with LODs and LOQs were respectively in the range of 0.03-1.25 and 0.15-5.00 μg L^{-1}.

All the previously reported analytical methods[24-28] only simultaneously analyzed less than 8 kinds of BAs, which couldn't meet with the practical use in real sample. To overcome this disadvantage, in

this paper, a rapid, simple and stable method was established and applied for the simultaneous detection of 15 kinds of BAs in animal-derived products, which was sensitive enough to evaluate the freshness of foods using BAs as possible indicators.

2. Experiments

2.1 Materials and Chemicals

Methylamine hydrochloride, ethylamine hydrochloride, tryptamine, butylamine, phenylethylamine, amylamine, putrescine dihydrochloride, cadaverine dihydrochloride, histamine dihydrochloride, octopamine, n-hexylamine, 5-hydroxy-tryptamine hydrochloride (serotonin), tyramine, spermidine, spermine (purity≥97%) were obtained from Sigma-Aldrich (St. Louis, MO,USA); HPLC-grade acetonitrile (ACN) used as the mobile phase, from Merck Company (Darmstadt, Germany); a Milli-Q water purification system from Millipore Corp. (Milford, MA, USA). Other chemicals were analytical reagent grade and obtained from local companies. Waters Sep-Pak C18 and HLB solid-phase extraction (SPE) cartridges (6 mL, 500mg sorbent) were purchased from Waters Corp. (Milford, MA, USA). Agilent Bond Elut C18 was purchased from Agilent Corp. (Santa, CA, USA), Agela ODS C18 was purchased from Agela Corp. (Tianjin, China).

2.2 Equipment

The BAs were analyzed using an HPLC system (Shimadzu, Kyoto, Japan) comprising an online vacuum degasser, binary pump and a thermostatically-controlled column, fluorescence detector compartment on an ODS-2 Hypersil C18 (5 μm), 4.6 × 250mm column (Thermo-Scientific, Waltham, MA, USA). A vortex mixer (HQ-60-II) (Kylin-Bell, Nantong, China), ultrasonic cleaner (UC-6200) (Ameritech, Los Angeles, CA, USA) and centrifuge (5804R) (Eppendorf AG, Hamburg, Germany) were used for extracting BAs from the food samples. A solid phase extraction (SPE) device Visiprep™

DL (Supelco, Bellefonte, PA, USA) was used for purifying the BAs.

2.3 Preparation of Standard Solutions

Standard solutions were prepared by dissolving BAs in 0.1M HCl to obtain 1000 mg L^{-1} individual stock solutions, and then was stored at 4°C in the refrigerator under dark for further dilution. Different concentration of standard working solutions were prepared using 0.1M HCl from individual stock standard solutions.

2.4 Sample Pretreatment

Raw, boneless and skinless pork, beef, carp and crucian carp were bought from Tesco supermarket in Tianjin. The meat was diced, thoroughly homogenized by a meat grinder, then was stored at -20°C no more than 7 days before use.

2.4.1 Extraction of BAs from Food Samples

The extractions of BAs were performed according to the reported studies[29-30], with minor modification. In brief, 5.00 g of meat samples were weighed into a tube, vortexed for 1 min with 10 mL 5% trichloroacetic acid (TCA), treated with ultra sound for 20 min then centrifuged at 10,000 g (4°C) for 10 min. The supernatant was filtered into a 25-mL volumetric flask. The extraction was then repeated. Finally, the supernatants were merged and set the volume to 25 mL with 5% TCA. Five milliliters of supernatant were pipetted into a 50-mL centrifuge tube then, 5 mL of n-hexane were added to eliminate fat then repeated again.

2.4.2 Derivatization of BAs

Because of the high reactivity with primary amines and secondary amines with dansyl chloride, and the derivatives possessing strong fluorescence following UV absorption, this reagent was selected to form derivatives of BAs.

One milliliter of the defatted extract was pipetted into a 5-mL flask then 200 µL of 2M NaOH, 300 µL of saturated Na_2CO_3 solution and 4 mL of 5 mg L^{-1} dansyl chloride solution were added. After mixing, the flask was placed at 60°C for 15 min, shaken once every 5 min. After derivatization, an aliquot of 200 µL of ammonia was added immediately to remove any unreacted dansyl chloride. After standing for 20 min to allow return to room temperature, the reaction mixture was aspirated to reduce its volume to 5 mL under a gentle flow of nitrogen at 40°C.

2.4.3 Solid-Phase Extraction (SPE)

The Sep-Pak C18 cartridges were first activated using 6 mL of methanol, followed by equilibration with 6 mL of water. The derivative sample solution was adjusted to a pH value of 9, then 1 mL was loaded into the cartridges. The cartridges were subsequently washed with 6 mL of aqueous 10% acetone, followed by drying using negative pressure. The BAs were eluted with 5 mL ethyl acetate into a 10-mL tube. Finally, the eluent was evaporated to dryness under a gentle flow of nitrogen and redissolved in 1 mL acetonitrile. After passing through a 0.22-µm filter, the sample was ready for analysis.

2.5 HPLC Conditions for Chromatographic Separation

Separations were performed using an ODS-2 Hypersil C18 (5 µm), 4.6×250 mm column (Thermo-Scientific). The column temperature was set at 40°C. The excitation wavelength was 350 nm, the emission wavelength 480 nm and the sample volume was 20 µL. An optimal separation was achieved using a binary mobile phase at a flow rate of 0.8 mL min^{-1} and a mobile phase gradient consisting of water (A) and acetonitrile (B). The gradient elution program was 0-10 min, 65-75% B; 10-20 min, 75-90% B; 20-25min, 90% B; 25-26 min, 90-65% B; 26-30 min, 65% B.

2.6 Method validation

The method was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ), recovery, precision and stability.

2.6.1 Linearity, LODs and LOQs

The regression equations were obtained by plotting a series of BA standard solutions over a wide concentration range versus the corresponding peak area with weighted least-square linear regression. The LODs and LOQs for standard solution of BAs were generated, based on a signal-to-noise ratio of 3:1 and 10:1, respectively.

2.6.2 Spike and Recovery

The recoveries of the established method were examined by analyzing pork, beef, carp and crucian carp with samples at three different spike levels (0.5, 1 and 2 mg kg^{-1}).

2.6.3 Precision and stability

Reproducibility of the proposed method was evaluated by carrying out five replicate quantitative determinations for 15 BAs spiked with 1 mg kg^{-1} in beef samples, on the same day, and five replicates on five consecutive days.

3. Results and discussion

3.1 Optimization of Derivative Conditions

3.1.1 The quantity of dansyl chloride

The quantity of dansyl chloride is an important factor for derivative reaction, so the volume of derivative reagent was optimized under certain concentration. **Fig.1** showed that the more derivative reagent, the higher response peak areas appeared, the peak areas of most biogenic amines were no longer enhanced except ethylamine, when 5 mL of dansyl chloride was used, indicating the derivative

products were no longer increased over a certain amount of derivative reagent. Therefore, 5 mL was the appropriate volume of dansyl chloride in this experiment.

3.1.2 Temperature and Time of Derivatization

Four groups of temperature and time (room temperature, 20 h; 40°C, 1 h; 60°C, 15 min and 70°C, 10 min) were designed based on the previous reports[11,30,32-33] to optimize the derivative conditions. The results showed that different temperature and time have a little influence on the derivative effect of BAs, except for the lower peak areas of BAs appeared under 70°C and 10min. It is possibly because that the derived structure of BAs were not stable under higher temperature. In order to get better results and save time, 60 °C and 15 min were chosen.

3.2 Optimization of HPLC Conditions

Fifteen types of derivatized BAs were used to optimize the HPLC conditions, including buffer composition, elution gradient, flow rate of mobile phase and oven temperature.

3.2.1 Mobile Phase

The most commonly used mobile phases for analyzing BAs are acetonitrile/water [31-35], methanol / water [36], methanol / sodium acetate [37] and methanol / ammonium acetate, formic acid [35]. According to Sun[38], ammonium acetate can protect derivative histamine from fluorescence quenching, so the effects of ultra-pure water and 10 mM of ammonium acetate on the separation of BAs were compared. The results showed that there was no difference in the chromatographic behavior of derivative histamine between the acetonitrile / water or acetonitrile / 10 mmol L^{-1} of ammonium acetate mobile phases. Therefore the acetonitrile / water combination was chosen as mobile phase for further optimization.

3.2.2 Gradient Elution Program

Given the need to separate a larger set of BAs than previously, three gradient elution programs were designed and investigated to improve the separation. **Fig.2** shows that under the gradient (a) program, the peaks were symmetrical and relatively sharp. However, not all of the BAs could be separated completely, with the peaks of amylamine and cadaverine overlapping each other with similar behavior for octopamine and hexylamine. Under the gradient (b) program, the retention times of the target BAs were often very close, especially from butylamine to octopamine. There was also a large interval between the last three BAs, thus prolonging the analysis time. All the BAs were completely separated with a resolution greater than 1.5 under the gradient (c) program and were fully eluted within 30 min. Thus, the gradient (c) program was selected as the best elution program.

3.2.3 Flow Rate of Mobile Phase

The flow rate of the mobile phase cannot change the eluting sequence of target compounds, but will change their retention time and degree of resolution. A better resolution was obtained by optimizing the flow rate of the mobile phase. The effect of flow rates (0.6, 0.7, 0.8, 0.9 and 1.0 mL min^{-1}) of the mobile phase on the chromatographic behavior of the BAs were examined. With an increase in flow rate, the analysis time became shorter. To ensure the separation of the desired compound in a shorter separation time, a flow rate of 0.8 mL min^{-1} was chosen.

3.2.4 Oven Temperature

The effect of column temperature was similar to that of the flow rate: the higher the column temperature, the shorter the retention time. The effect of oven temperature (30, 35, 40, 45 °C) on the chromatographic behavior of the BAs was determined. Methylamine could not be effectively separated at 30 °C. BAs were completely separated at 35 °C and 45 °C, while, the separation of trytamine was poor. At 45 °C, the retention times of BAs were too close. A better resolution was obtained at 40 °C

with good separation of methylamine and tryamine. The resolutions of the other BAs were also good, so 40 °C was chosen as the oven temperature.

3.3 Optimization of Solid Phase Extraction Procedure

To reduce the matrix effects of the samples and derivatization reagents, SPE was applied to purify and concentrate the BAs after extraction and derivatization. The SPE column type, the pH of the sample solution, washing solution, eluent reagent and eluent volume, were the primary factors affecting the efficiency of adsorption and elution from the SPE column. Standard mixture at a concentration of 2 mg L^{-1} was derived and then was used to optimize these parameters.

Four types of SPE column (Waters HLB, Agilent Bond Elut C18, Agela ODS C18 and Waters Sep-Pak C18) were evaluated. The Waters HLB column appeared to provide a better absorption of methylamine and ethylamine than the other BAs. This could be because that HLB has a better adsorption of polar compounds than non-polar compounds and the polarity of methylamine and ethylamine is the greatest of the BAs analyzed. The Agela ODS C18 column provided poor absorption of the derivatized BAs. However, an excellent absorption efficiency for BAs was provided by the Waters Sep-Pak C18 and Agilent Bond Elut C18 columns. Taking into account its higher stability, the Waters Sep-Pak C18 column was chosen for further experiments after these preliminary tests.

Derivatized BAs were relatively stable under alkaline conditions, so the effect of pH (8, 9, 10, 11, 12 and 13) on the adsorption efficiency was determined. The results showed that Waters Sep-Pak C18 had the greatest adsorption efficiencies for amylamine, cadaverine, hexylamine, 5-hydroxytryptamine, tyramine, spermidine and spermine at different sample solution pH values. The loss of the other BAs was 0.80-8.47% at pH 9; however, the loss increased to 3.63-41.78% at other pH values. Therefore a value of pH 9 was selected for the sample solutions.

Acetone was selected as the solvent for removing miscellaneous impurities, mainly because the derivatized solution contained unreacted dansyl chloride which dissolves in acetone. The effect of acetone concentration (0, 5, 10, 15, 20, 25 and 30%) on removing impurities was investigated, while preserving the target BAs that were adsorbed on the SPE column. Using water as the washing solution, the losses of all BAs were higher than for all concentrations of acetone solution. Different acetone concentrations had no obvious effect on the losses of butylamine. When using 5% acetone as the washing solution, the adsorption of cadaverine, histamine, octopamine, and tyramine was the same as for other solution concentrations, but for the other BAs, losses were higher, ranging between 5.12-23.82%. The losses of BAs using a 10% acetone solution (4.28-7.92%) were similar to those with a 30% acetone solution (4.56-9.46%). Taking into account environmental pollution, 10% acetone solution was chosen as the washing solution.

Elution was the final key step in the solid-phase extraction process. Different elution solvents (methanol, ethanol, ethyl acetate, acetonitrile and acetone) were optimized to improve the recovery and purity. The results, shown in **Fig. 3**, showed that the five eluents had no obvious difference on the recovery of various biogenic amines. In general, ethyl acetate appeared better than the other eluents. Except for methylamine, ethylamine, octopamine and tyramine, the deviations in recovery values of the other BAs were less than 6.0% when using ethyl acetate for elution, indicating better reproducibility. Therefore ethyl acetate was selected as the eluting solvent.

3.4 Performance of the Established Method

To evaluate the overall performance of our method, parameters such as the regression equation, linearity range, coefficient of determination and sensitivity were evaluated and listed in **Table 1**.

All BAs displayed good linearities from 0.025 to 5.0 mg L^{-1}, with coefficients of determination

(R^2) for the method exceeding 0.999.

The LOD for standard solution of BAs, based on a signal-to-noise ratio of 3:1, ranged from 0.002 to 0.03 mg kg^{-1}; for a signal-to-noise ratio of 10:1, LOQ ranged from 0.006 to 0.09 mg kg^{-1}, which was sufficient for determination of biogenic amines in real samples.

3.5 Application to Real Samples

Our analytical method was applied to the simultaneous detection of BAs in real food samples — pork, beef, carp and crucian carp. To evaluate the accuracy and stability of the method, each sample was spiked with three levels (0.5, 1, 2 mg kg^{-1}) of BAs. The results were listed in **Table 2**. The recoveries and RSDs ranged between 70.49-121.16% and 0.71-15.99%, respectively, indicating the high accuracy and reproducibility of the method. **Fig. 4** showed the HPLC chromatography of BAs in the pork sample (a), beef sample (b), carp sample (c) and crucian carp sample (d). Almost all concentrations of BAs found in these meat samples, ranged from 0 to 7.48 mg kg^{-1}, levels that were fortunately below the guideline maximum level[8-9, 39-40].

The intra- and inter-day precision ranged from 0.30% to 4.60% and from 4.62% to 14.97%, respectively showed in **Table 3**, indicating good reproducibility in the sample preparation and HPLC performance.

4 Conclusion

In the present study, we had developed a simple, sensitive and accurate method for the simultaneous quantitation of 15 important BAs at trace levels in animal-derived products. BAs could be extracted from these samples using trichloroacetic acid, followed by dansyl chloride derivatization, then purified using SPE, separated and, finally, quantitated using HPLC-FLD. Both the SPE procedure and HPLC conditions underwent systematic optimization to allow analysis of an extended range of Bas

required. Under these conditions, the full range of BAs was separated completely within 25 min with LOQs for all investigated compounds between 0.006 and 0.09 mg kg^{-1}, lower than those of previously reports[35, 41-42]. The recoveries and RSDs in the real samples ranged from 70.49-121.16% and 0.71-15.99%, respectively. Intra- and inter-day precision (RSD %) ranged from 0.30%-4.60% and from 4.62%-14.97%, respectively, which was somewhat less than that of R. Romero(3.2-10.3%) [42]. All the results indicated that the method established is capable of the simultaneous accurate quantification of the BAs commonly found in animal-derived products. Due to its high sensitivity, the established method can also be applied to estimate the freshness of food.

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Conflict of Interest

Huaping Zhu declares that he has no conflict of interest. Shanshan Yang declares that she has no conflict of interest. Yan Zhang declares that she has no conflict of interest. Guozhen Fang declares that she has no conflict of interest. Shuo Wang declares that he has no conflict of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

Not applicable.

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Table 1. Performance of the established method for the analysis of 15 types of biogenic amines

Analytes	Linear Equations X(μg L^{-1}) Y(mAU)	Coefficient of Determination (R^2)	Linear range (mg L^{-1})	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Methylamine	Y=1183.4x+28795	0.9994	0.05-2.5	0.015	0.05
Ethylamine	Y=1434.9x+27490	0.9995	0.05-2.5	0.015	0.05
Tryptamine	Y=218.86x-20149	0.9991	0.1-5.0	0.03	0.09
Butylamine	Y=348.31x+39379	0.9994	0.025-2.5	0.006	0.02
Phenylethylamine	Y=703.37x+4986.1	0.9992	0.025-2.5	0.005	0.02
Amylamine	Y=1684.5x-6931	0.9994	0.025-2.5	0.005	0.02
Putrescine	Y=1105.4x-11283	0.9990	0.025-2.5	0.006	0.02
Cadaverine	Y=1691.8x+141866	0.9992	0.05-2.5	0.01	0.03
Histamine	Y=82.072x+1029.9	0.9998	0.025-2.5	0.006	0.02
Octopamine	Y=109.91+411.36	0.9992	0.05-2.5	0.01	0.03
Hexylamine	Y=1263.5x+8833.6	0.9995	0.025-2.5	0.002	0.006
5-Hydroxytryptamine	Y=153.68x-7213.6	0.9991	0.1-2.5	0.02	0.06
Tyramine	Y=271.24x+23374	0.9996	0.05-2.5	0.01	0.03
Spermidine	Y=1053.5x-190.43	0.9999	0.025-2.5	0.002	0.006
Spermine	Y=853.1x+23374	0.9999	0.025-2.5	0.002	0.006

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Table 2. The BAs contents of meat samples (mg kg^{-1}) and the recovery of meat samples at different spiked levels (%, (RSD)) (n=3)

Analytes	Spiked levels (mg kg^{-1})	Pork	Beef	Carp	Crucian carp
	0	0.24	0.48	1.66	N.D[a]
Methyl-	0.5	115.08(7.23)	72.43(1.68)	104.83(7.13)	96.18(12.45)
amine	1	108.45(11.84)	83.38(6.72)	78.17(4.65)	87.87(12.02)
	2	119.47(5.67)	87.26(5.75)	81.62(2.94)	111.21(13.76)
	0	0.50	0.41	0.086	0.083
	0.5	92.91(7.61)	109.42(4.33)	99.32(5.89)	94.59(14.2)
Ethylamine	1	74.40(10.31)	103.92(4.59)	80.86(14.69)	98.09(3.68)
	2	105.09(12.17)	90.69(6.27)	92.36(10.50)	95.12(13.02)
	0	0.91	0.77	1.07	0.88
	0.5	70.49(6.77)	77.25(14.02)	99.31(1.27)	80.59(14.88)
Tryptamine	1	70.92(6.41)	82.59(14.90)	80.79(5.13)	99.85(8.01)
	2	104.22(14.36)	88.59(0.94)	91.45(5.02)	99.51(8.42)
	0	0.24	N.D[a]	1.10	N.D[a]
	0.5	77.44(9.69)	92.66(2.91)	70.88(10.67)	73.60(6.81)
Butylamine	1	79.49(7.39)	93.66(5.02)	75.84(13.57)	93.07(7.90)
	2	80.67(1.99)	90.16(11.16)	85.64(12.58)	81.94(4.28)
2-	0	0.55	0.24	N.D[a]	1.77
Phenylethy-	0.5	75.20(12.67)	83.06(8.99)	90.22(12.07)	83.99(6.10)

lamine	1	91.50(5.40)	94.04(4.02)	73.62(9.96)	74.13(12.28)
	2	110.61(6.95)	88.03(11.15)	88.27(14.59)	78.65(2.89)
Putrescine	0	1.74	1.82	4.52	1.56
	0.5	77.94(2.49)	77.51(15.84)	86.59(3.23)	84.62(6.51)
	1	81.26(3.29)	87.79(8.58)	79.25(9.48)	83.31(15.33)
	2	75.32(3.54)	97.31(11.58)	76.27(14.94)	85.17(1.63)
Amylamine	0	N.D[a]	N.D[a]	1.82	0.20
	0.5	88.99(10.02)	88.87(3.50)	80.01(8.99)	79.23(1.24)
	1	88.68(3.96)	82.03(13.61)	73.37(8.13)	86.85(10.75)
	2	84.35(9.22)	84.32(10.66)	92.48(14.48)	84.67(15.99)
Cadaverine	0	4.28	3.67	2.66	2.30
	0.5	118.16(7.01)	89.23(14.62)	102.87(5.89)	91.66(2.93)
	1	78.67(13.09)	107.63(13.38)	79.03(13.74)	76.08(11.98)
	2	117.84(6.19)	119.84(10.46)	85.82(13.26)	75.03(10.08)
Histamine	0	0.095	1.27	0.60	1.50
	0.5	107.80(8.01)	105.21(9.09)	108.98(2.84)	80.04(7.51)
	1	111.41(2.11)	104.93(7.06)	94.89(2.63)	89.83(3.33)
	2	114.12(2.84)	100.12(9.21)	115.21(10.89)	115.64(9.26)
Octopam-	0	0.15	N.D[a]	N.D[a]	0.24
	0.5	103.49(2.44)	86.78(6.12)	109.10(11.74)	80.11(9.15)
ine	1	77.57(1.42)	111.80(5.80)	98.03(10.28)	94.27(15.84)
	2	74.23(1.29)	113.46(2.00)	98.53(7.25)	70.55(1.69)

	0	N.D[a]	N.D[a]	0.14	N.D[a]
	0.5	86.85(3.32)	86.98(8.18)	73.43(3.52)	79.99(3.37)
Hexylamine	1	76.95(1.72)	92.85(4.54)	74.61(10.48)	78.24(1.43)
	2	82.95(5.78)	88.81(9.22)	80.49(11.05)	76.65(0.73)
	0	N.D[a]	N.D[a]	0.87	0.84
5-	0.5	81.53(6.25)	84.36(12.82)	75.50(12.06)	77.00(15.40)
Hydroxy-	1	87.25(0.71)	97.67(7.11)	73.47(6.16)	80.01(12.54)
tryptamine	2	76.27(1.39)	75.09(1.89)	71.16(14.13)	70.51(2.05)
	0	7.48	6.33	2.43	5.25
	0.5	103.96(2.93)	96.95(8.74)	108.56(1.94)	95.02(12.90)
Tyramine	1	81.48(2.98)	99.05(7.17)	85.98(7.32)	92.66(10.11)
	2	115.90(11.94)	81.88(15.74)	90.61(7.64)	81.34(4.71)
	0	1.14	2.30	2.92	2.06
	0.5	78.28(5.75)	94.56(13.39)	116.03(3.11)	121.16(7.19)
Spermidine	1	105.80(6.52)	104.16(6.43)	111.11(13.76)	85.51(7.03)
	2	89.85(13.66)	98.55(8.33)	89.03(11.22)	78.75(6.32)
	0	2.97	3.63	5.43	3.15
	0.5	101.23(15.92)	120.84(11.21)	109.86(8.71)	115.11(12.09)
Spermine	1	121.02(14.52)	103.03(15.62)	105.45(12.82)	79.71(7.85)
	2	111.52(9.92)	85.35(11.81)	84.51(14.38)	85.14(11.39)

N.D[a]: content was below the LOD

LODs of BAs in real food samples ranged from 0.01 to 0.15 mg kg^{-1}

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Table 3 The intra-day and inter-day precision of the established method for detection of fifteen biogenic amines in beef meat (n=5)

Biogenic amines	Intra-day precision		Inter-day precision	
	Peak Area		Peak Area	
	Mean±SD	RSD	Mean±SD	RSD
Methylamine	2212239±21363	0.97%	2738242±409914	14.97%
Ethylamine	3530300±18412	0.52%	3054834±213312	6.98%
Tryptamine	469529±9502	2.02%	476137±60529	12.71%
Butylamine	701337±3944	0.56%	653882±54343	8.31%
Phenylethylamine	1319614±12381	0.94%	1238287±89695	7.24%
Amylamine	3630903±21244	0.59%	3906518±316393	8.10%
Putrescine	1337724±1298354	4.60%	1609883±197764	12.28%
Cadaverine	12542523±91270	0.73%	1184614±922650	8.25%
Histamine	219523±4381	2.00%	240825±14216	5.90%
Octopamine	233149±1314	0.56%	262010±18624	7.11%
Hexylamine	2148666±11881	0.55%	2103919±177911	8.46%
5-Hydroxytryptamine	153559±3121	2.03%	744445±73545	9.88%
Tyramine	1149422±3469	0.30%	4564005±210902	4.62%
Spermidine	2608453±18423	0.71%	2368889±168017	7.09%
Spermine	7097291±50068	0.71%	6416264±470449	7.33%

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Figure captions

Fig. 1 Effect of quantity of dansyl chloride on the peak area of the fifteen biogenic amines.

Fig. 2 HPLC chromatograms obtained with three different gradient elution programs

Peak reference numbers: 1. Methylamine, 2. Ethylamine, 3. Tryptamine, 4. Butylamine, 5. Phenylethylamine, 6. Putrescine, 7. Amylamine, 8. Cadaverine, 9. Histamine, 10. Octopamine, 11. Hexylamine, 12. 5-Hydroxytryptamine, 13. Tyramine, 14. Spermidine, 15.Spermine. The gradient elution programs were: (a) 0-10 min: 55% B, 10-15 min: 55-65% B, 15-20 min: 65-80% B, 20-25 min: 80% B, 25-30 min: 80-90% B, 30-33 min: 90% B, 33-35 min: 90-55% B, 35-40 min: 55% B. (b) 0-5 min: 65-75% B, 5-10 min: 75-85% B, 10-13 min: 85-100% B, 13-19 min: 100% B, 19-20 min: 65% B, 20-30 min: 65% B. (c) 0-10 min: 65% B, 10-20 min: 65-90% B, 20-25 min: 90% B, 25-26 min: 90-65% B, 26-30 min: 65% B.

Fig.3 Effect of elution solvents on the recoveries of the fifteen biogenic amines.

Peak reference numbers: 1. Methylamine, 2. Ethylamine, 3. Tryptamine, 4. Butylamine, 5. Phenylethylamine, 6. Putrescine, 7. Amylamine, 8. Cadaverine, 9. Histamine, 10. Octopamine, 11. Hexylamine, 12. 5-Hydroxytryptamine, 13. Tyramine, 14. Spermidine, 15.Spermine.

Fig.4 HPLC chromatograms for fifteen biogenic amines in pork (a), beef (b), carp (c) and crucian carp samples (d).

Peak reference numbers: 1. Methylamine, 2. Ethylamine, 3. Tryptamine, 4. Butylamine, 5. Phenylethylamine, 6. Putrescine, 7. Amylamine, 8. Cadaverine, 9. Histamine, 10. Octopamine, 11. Hexylamine, 12. 5-Hydroxytryptamine, 13. Tyramine, 14. Spermidine, 15.Spermine.

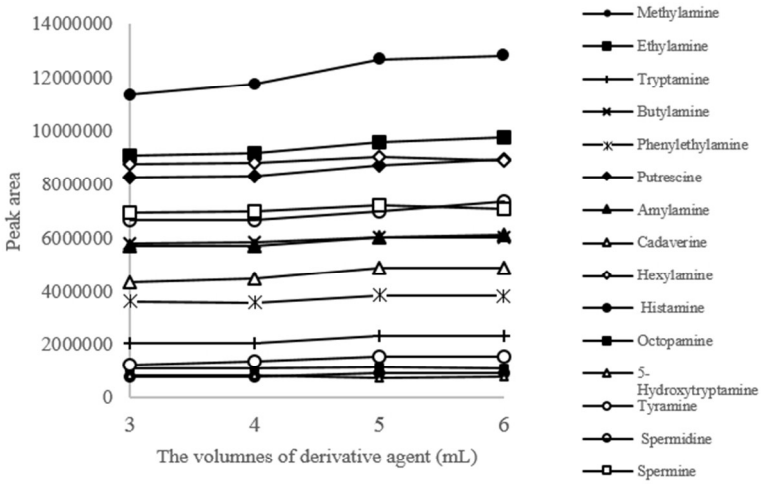


Fig. 1 Effect of the volume of dansyl chloride on the peak area of the fifteen biogenic amines
216x121mm (96 x 96 DPI)

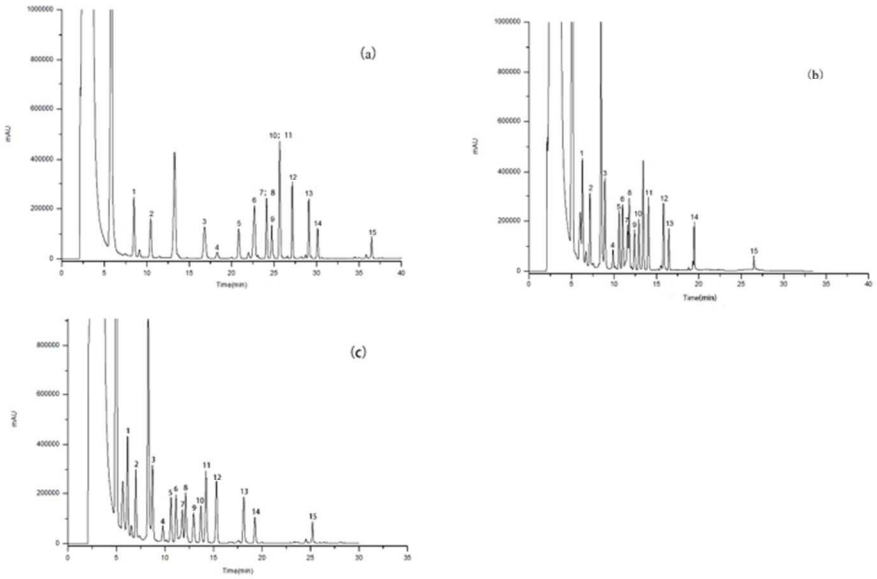


Fig. 2 HPLC chromatograms obtained with three different gradient elution programs
216x130mm (96 x 96 DPI)

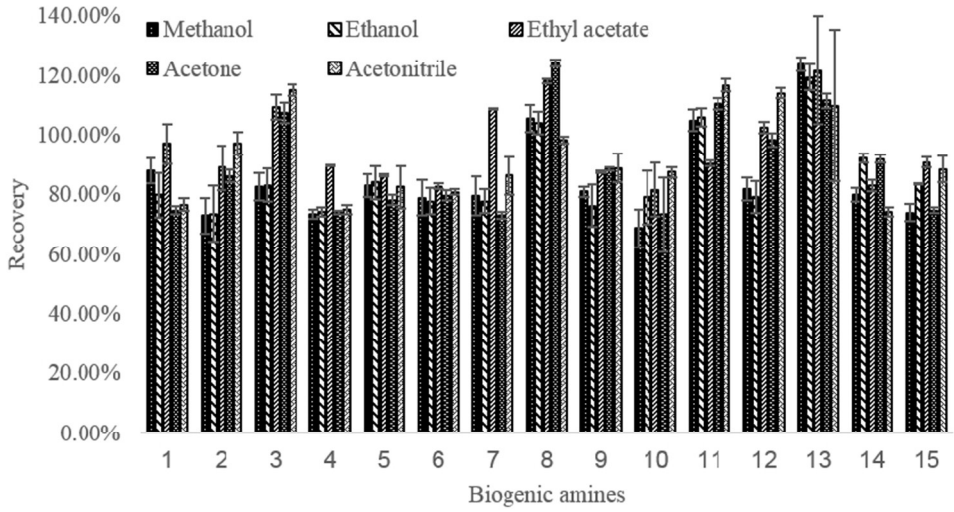


Fig.3 Effect of elution solvents on the recoveries of the fifteen biogenic amines.
232x128mm (96 x 96 DPI)

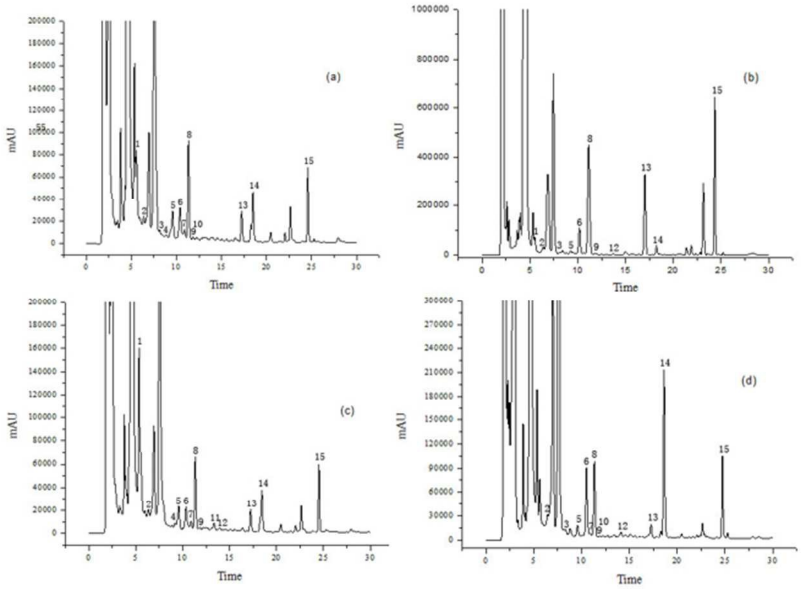
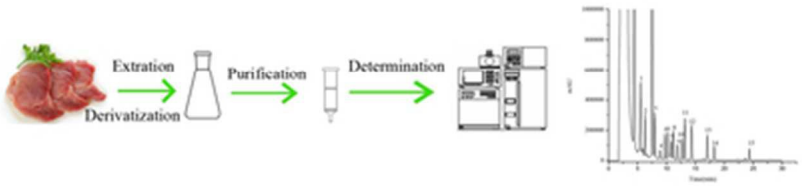


Fig.4 HPLC chromatograms for fifteen biogenic amines in pork (a), beef (b), carp (c) and crucian carp samples (d).
216x130mm (96 x 96 DPI)



After extraction, derivatization and purification, the fifteen kinds of biogenic amines in meat were separated and quantitated by HPLC-FLD.
39x19mm (300 x 300 DPI)