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High-Performance Liquid Chromatographic Determination of Biogenic Amines in Poultry Carcasses

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Biogenic amines, produced by bacterial decarboxylation of amino acids, have been associated with toxicological symptoms in broilers fed various poultry byproducts. A reversed-phase high-performance liquid chromatographic method is described for the quantitation of eight biogenic amines (tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine) in chicken carcasses. Amines were extracted with perchloric acid, derivatized with dansyl chloride, separated using gradient elution (methanol and water), and detected by fluorescence. Benzylamine was used as the internal standard. Linearity, repeatability, and recovery of the method were evaluated. The method was linear for all of the amines studied at concentrations ranging from 0.05 to 25 μg/mL. Average recoveries ranged from 92.6% to 96.8% for all amines except for histamine, which was 74.6%.

KEYWORDS: Biogenic amines, poultry meal, high-performance liquid chromatography, chicken carcasses

INTRODUCTION

Biogenic amines are naturally occurring organic bases of low molecular weight found at low levels in plants, animals, and microorganisms. These substances can be produced in feedstuffs as a result of bacterial decarboxylation of amino acids, e.g., tyramine (TY) from tyrosine, histamine (HI) from histidine, cadaverine (CA) from lysine, tryptamine (TR) from tryptophan, and phenylethylamine (PE) from phenylalanine. Putrescine (PU) is obtained from decarboxylation of arginine via ornithine, and it serves as a precursor to spermine (SPM) and spermidine (SDM) (*1*, *2*). High concentrations of different biogenic amines have long been used as indicators of spoilage (*3*, *4*).

Elevated levels of biogenic amines in broiler diets have been associated with poor performance and a condition named "necrotic cellular debris" (*5*). Poole (*6*) reported biogenic amine toxicity in broilers fed poultry meal made from raw poultry materials that were held over a weekend before being rendered. Keirs and Bennett (*5*) characterized biogenic amines from a number of field samples of poultry meal as low (1.8 ppm), average (343.9 ppm), and high (938 ppm) (as total biogenic amines).

Several chromatographic methods are used to quantify biogenic amines in biological material, including gas (*7*), ion-exchange (*8*), thin-layer (*9*), and reversed-phase high-performance liquid chromatography (HPLC), which is widely used because of its high sensitivity and wide range of linearity (*10*). For HPLC, quantitation involves extraction of the amines from the matrix (considered critical in terms of obtaining adequate recovery of all the amines) and analyte determination. Several solvents have been tested for their extraction capacity including trichloroacetic acid (*11*), perchloric acid (*12*), hydrochloric acid (*13*), and methanol (*8*). Reviewing HPLC methods for biogenic amine analysis of different biological materials, Hurst (*14*) found perchloric acid the solvent of choice for extraction from chicken samples, confirming the earlier recommendation of Zee et al. (*8*). After initial extraction, further purification is obtained by liquid−liquid extraction in which a salt, e.g., sodium bicarbonate or sodium chloride, is added and the amines are extracted into an organic solvent such as butanol, chloroform, or ether (*1*). Detection of the amines can then be done using ultraviolet (UV) or fluorescence detection.

Sander et al. (*15*) developed a method for biogenic amine analysis in feedstuffs including poultry meal, fish meal, and meat and bone meal. However, neither details of the accuracy and precision of the method nor its applicability to less processed or more heterogeneous material was described. Therefore, the present work describes the optimization and standardization of that HPLC-based method for the quantitation of eight biogenic amines in poultry carcasses.

MATERIALS AND METHODS

Reagents. The biogenic amine standards TR, PE hydrochloride, PU hydrochloride, CA dihydrochloride, HI dihydrochloride, TY hydrochloride, SDM trihydrochloride, and SPM tetrahydrochloride, along with benzylamine (BN) hydrochloride, were obtained from Sigma (St. Louis, MO).

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Standard Solutions. A stock solution (25 mg/mL) of each amine as a free base was prepared in 1 mM HCl. Working solutions, also in 1 mM HCl, were then prepared from the stock solution to yield the following concentrations: 5, 50 and 500 μg/mL. Benzylamine was also prepared at a concentration of 25 mg/mL and then diluted to 125 μg/mL. Stock solutions were prepared monthly and stored at 4−8 °C, while working solutions were prepared daily.

HPLC System. HPLC analysis was performed using a Shimadzu 6-A system (Shimadzu, Columbia, MD), equipped with a dual pump solvent delivery system with gradient capacity, a SIL 6A autoinjector, a SCL-6A controller, a CR-6A integrator, and an RF-535 fluorescence detector. Detection of amines was accomplished at an excitation wavelength of 350 nm and an emission wavelength of 520 nm. Separation was performed on a reversed-phase C_{18} Luna column (Phenomenex Inc., Torrance, CA) (15 cm length, 4.6 mm internal diameter, and 5 μm particle size) with a 4 × 3 mm Security Guardcartridge guard column (Phenomenex Inc., Torrance, CA).

Sample Preparation. Procedures described by Sander et al. (*15*) and Bennett (personal communication) were used with minor modifications. Whole chicken carcasses with feathers were ground in a commercial food processor (Robot Coupe, Model R-15, France) into a course puree, which contained different size fragments of feather, bone, muscle fiber, etc. Because of the physical nature of ground whole carcass material, the sample size needed for reproducible results was evaluated. In the original description of the procedure, 5 g was reported adequate for the analysis (*15*). In this study, from a single lot of ground chicken material, four 5 g samples and four 30 g samples were prepared and analyzed. Samples were accurately weighed into beakers, 150 mL of distilled water was added and mixed, and 150 mL of 0.4 M perchloric acid was added (note: for 30 g samples, the volumes of water and perchloric acid were increased proportionately to maintain constant ratios). The mixture was stirred for 30 min and then filtered through Whatman no. 1 filter paper (Fisher Scientific, Pittsburgh, PA) prior to derivatization.

Derivatization. A 0.5 mL aliquot of the filtrate was transferred into a test tube, and 20 μL of benzylamine (0.125 mg/mL) was added. A 1.5 mL volume of saturated sodium bicarbonate solution (excess sodium bicarbonate maintained in suspension by continuous stirring) was added to make the solution alkaline and mixed, and 1.0 mL of dansyl chloride in acetone (5 mg/mL) was added. The tubes were vortexed and incubated at 60 °C for 30 min. One hundred microliters of sodium glutamate (50 mg/mL) in saturated sodium bicarbonate was added, and the tubes were mixed and incubated at 60 °C for another 15 min. Distilled water (1.0 mL) was added, and the acetone was evaporated by heating (40 °C) under a stream of nitrogen. Finally, the mixture was extracted three times with 3.0 mL of diethyl ether. For each extraction, 3 mL of ether was added to the test tubes, and the tubes were vortexed for 30 s and then centrifuged (Labofuge 400R; Heraeus Instruments, South Plainfield, NJ) for 10 min at 3600 rpm (1469 relative centrifugal force). The ether layer was aspirated, and ether extracts were combined and evaporated to dryness under a stream of nitrogen with heating at 40 °C. The residue was then dissolved in 1 mL of methanol for HPLC analysis. In these analyses, the biogenic amine conversion to dansyl derivatives was assumed to be complete, although this has not been verified (*16*).

Chromatography. The mobile phases were water (A) and methanol (B). Before use, solvents were filtered through 0.1 μm Magna nylon membrane filters (Fisher Scientific, Pittsburgh, PA) and degassed under vacuum. Amines were eluted by the gradient shown in **Table 1** with a flow rate of 2 mL/min.

Efficiency. *(A) Standard Curves and Linearity.* A five-point standard curve for each amine was prepared. Standard solutions, prepared in triplicate and containing all amines at 0.05, 0.25, 2.5, 5, 10, 15, or 25 μg/mL, were extracted, derivatized, and quantitated as described above. Calibration curves were created by plotting the concentration of each amine against the ratio of the standard peak area to that of the internal standard (*17*). Simple linear regression analysis was performed to calculate the slope and intercept. The correlation coefficient (r) for each amine was also determined (*16*). Biogenic amine concentrations (micrograms per gram) in chicken samples were then obtained by calculation of the ratio of sample peak area to internal standard and using the regression equations from the standard curves for each amine.

Table 1. HPLC Gradient Elution Program for Biogenic Amine Analysis[a]

time (min)	water (%)	methanol (%)	time (min)	water (%)	methanol (%)
0	45	55	22	20	80
7	35	65	27	0	100
14	35	65	35	0	100
18	30	70	35	45	55
20	25	75	40	45	55

[a] Flow rate = 2 mL/min.

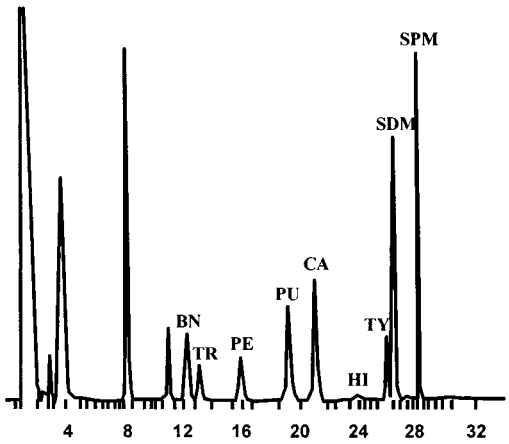

Figure 1. Typical chromatogram of a biogenic amine standard solution. Abbreviations: BN = benzylamine; TR = tryptamine; PE = phenylethylamine; PU = putrescine; CA = cadaverine; HI = histamine; TY = tyramine; SDM = spermidine; SPM = spermine.

(B) Repeatability. To evaluate precision, repeatability of both the instrument and the analytical procedure was determined. For instrumental repeatability, a standard solution containing the amines at 2.5 μg/mL (histamine at 25 μg/mL) was prepared, derivatized, and injected 10 times into the system. Means ($\bar{x}$, standard deviations (SD), and relative standard deviation (RSD) of the calculated concentrations were determined. To determine the repeatability of the entire method, 10 aliquots of the same chicken sample were extracted, derivatized, and analyzed separately. Means, standard deviations, and relative standard deviation were determined (*16*, *18*).

(C) Recovery. The standard addition method was used to determine the recovery of the method (*17*, *18*). A sample of ground poultry carcasses was extracted and analyzed. From the same material, another sample was extracted and then spiked with 5 μg of each amine and analyzed following the same procedure. This test was performed in triplicate.

RESULTS AND DISCUSSION

In preliminary tests, three compounds that do not occur naturally in chicken samples were evaluated as potential internal standards: benzylamine, isopropylamine, and hexylamine. Isopropylamine coeluted at 9.2 min with an unidentified peak; hexylamine (retention time = 16.5 min) eluted close to PE (retention time = 16.05 min), while benzylamine (retention time = 12.5 min) eluted ahead of any of the amines and was well separated from the closest biogenic amine, TR (retention time = 13.2 min).

Different gradient programs were evaluated to achieve good resolution of all the biogenic amines in the shortest time. The eight biogenic amines plus the internal standard were well resolved with the gradient described in **Table 1**. **Figure 1** shows a chromatogram typical of a standard solution, and **Figure 2**

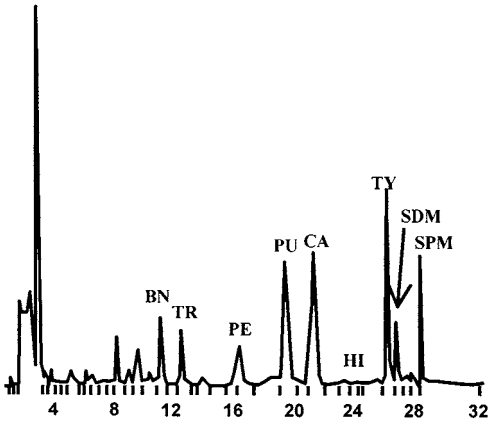

Figure 2. Typical chromatogram of a chicken carcass sample with feathers. Abbreviations: BN = benzylamine; TR = tryptamine; PE = phenylethylamine; PU = putrescine; CA = cadaverine; HI = histamine; TY = tyramine; SDM = spermidine; SPM = spermine.

Table 2. Linear Regression Equations and Correlation Coefficients for Calculation in Biogenic Amine Analyses

amine[a]	retention time[b] (min)	linear equation slope	linear equation intercept	correlation coeff (*r*)
TR	13.20	0.2386	–0.1106	0.9961
PE	16.05	0.3573	–0.0446	0.9989
PU	19.31	0.7351	0.0554	0.9854
CA	21.10	0.8051	–0.0381	0.9840
HI	23.91	0.2864	–0.1252	0.9144
TY	25.96	0.2864	0.0126	0.9980
SDM	26.51	0.8052	0.1009	0.9853
SPM	28.10	0.7823	0.2134	0.9858

[a] Abbreviations: TR = tryptamine; PE = phenylethylamine; PU = putrescine; CA = cadaverine; HI = histamine; TY = tyramine; SDM = spermidine; SPM = spermine. [b] Retention time for internal standard (benzylamine) = 12.5 min.

Table 3. Instrumental Repeatability

amine[a]	$\bar{x}$[b]	SD	RSD[b] (%)
TR	2.99	0.03	1.00
PE	2.78	0.02	0.72
PU	2.72	0.02	0.73
CA	2.85	0.03	1.05
HI	18.50	6.80	36.76
TY	2.52	0.03	1.19
SDM	2.55	0.09	3.53
SPM	2.58	0.10	3.87

[a] Abbreviations: TR = tryptamine; PE = phenylethylamine; PU = putrescine; CA = cadaverine; HI = histamine; TY = tyramine; SDM = spermidine; SPM = spermine. [b] $\bar{x}$ = mean of 10 injections of the same standard solution (2.5 μg/mL for all amines except histamine at 25 μg/mL). RSD = relative standard deviation.

shows that of a chicken sample. Amines were identified on the basis of retention time by comparison with standard solutions.

Method reliability, in terms of linearity, repeatability of the HPLC system, repeatability of the analytical method, and analyte recovery, was studied. **Table 2** summarizes the regression analyses for the eight biogenic amines. The method was linear for all of the biogenic amines for the concentrations studied (between 0.05 and 25 μg/mL). The correlation coefficient was greater than 0.98 for all amines except for HI (r = 0.9144). The HPLC instrumental repeatability was good for all amines (RSD < 3.87%) except for HI (RSD = 36.76%) (**Table 3**). The data suggested that the histamine derivative was not stable over time. The first injection returned the highest assay value (2.84 μg) of HI, but this level decreased with time, so that by the last injection (~9 h later) the level of detectable HI (1.13 μg) had dropped more than 50%.

Table 4. Analytical Repeatability

amine[a]	$\bar{x}$[b]	SD	RSD[b] (%)
TR	1.37	0.11	8.03
PE	12.98	0.97	7.47
PU	22.83	2.24	9.81
CA	51.66	4.77	9.23
HI	1.02	0.31	30.39
TY	20.99	1.14	5.43
SDM	4.97	0.51	10.26
SPM	2.64	0.13	4.92

[a] Abbreviations: TR = tryptamine; PE = phenylethylamine; PU = putrescine; CA = cadaverine; HI = histamine; TY = tyramine; SDM = spermidine; SPM = spermine. [b] $\bar{x}$ = mean (μg/g) of 10 preparations of the same sample material. RSD = relative standard deviation.

Table 5. Analytical Recovery

amine[a]	sample analysis[b] unspiked (μg)	sample analysis[b] spiked[c] (μg)	recovery (%)
TR	1.13 ± 0.01	5.93 ± 0.88	96.75
PE	8.34 ± 0.85	12.59 ± 1.21	94.38
PU	10.02 ± 0.81	14.10 ± 1.73	93.87
CA	29.11 ± 0.15	32.46 ± 3.63	95.16
HI	0.87 ± 0.07	4.38 ± 1.28	74.60
TY	13.42 ± 1.38	17.84 ± 2.15	96.85
SDM	2.41 ± 0.15	6.86 ± 1.70	92.58
SPM	1.56 ± 0.38	6.23 ± 0.90	94.97

[a] Abbreviations: TR = tryptamine; PE = phenylethylamine; PU = putrescine; CA = cadaverine; HI = histamine; TY = tyramine; SDM = spermidine; SPM = spermine. [b] Values are the means (±SD) of three chicken samples with or without spiking. [c] 5 μg of each amine was added to each sample.

Table 6. Effect of Sample Size on Precision

amine[a]	30 g $\bar{x}$[b]	30 g SD	30 g RSD[b] (%)	5 g $\bar{x}$[b]	5 g SD	5 g RSD[b] (%)
TR	1.15	0.10	8.70	0.70	0.20	28.57
PE	26.41	1.84	7.00	24.90	8.00	32.13
PU	80.03	4.81	6.01	81.31	23.80	29.27
CA	144.54	3.81	2.60	118.18	15.10	12.77
HI	50.10	18.30	36.52	30.74	20.40	66.36
TY	55.05	3.47	6.30	49.95	4.60	9.21
SDM	1.23	0.22	17.89	1.92	0.99	51.56
SPM	2.26	0.24	10.62	1.75	0.80	45.71

[a] Abbreviations: TR = tryptamine; PE = phenylethylamine; PU = putrescine; CA = cadaverine; HI = histamine; TY = tyramine; SDM = spermidine; SPM = spermine. [b] $\bar{x}$ = mean (μg/g) of three ground chicken samples that were prepared in the designated sample size. RSD = relative standard deviation.

Repeatability of the entire analytical procedure was tested, and results are summarized in **Table 4**. For trace analysis, repeatability with an RSD ≤ 10% is considered acceptable (*17*). The method had good repeatability for all amines except HI (RSD = 30.39%), which reflects the degradation of this compound suggested earlier. Therefore, when histamine levels are of particular importance, it is suggested that the samples be injected immediately after preparation.

Recovery of the amines was tested by the standard addition procedure. The recovery for all amines except HI was satisfactory (92.58−96.85%); the calculated recovery of HI was 74.6% (**Table 5**). This result is also consistent with the conclusion that the histamine derivative degrades quickly.

Table 6 shows the results of the sample size comparisons. For all of the amines, standard deviations were lower for the

30 g samples than for the 5 g samples. This result is attributable to the coarse nature of the sample. Since bone fragments, feathers, etc. are relatively refractory to typical grinding methods, homogenization did not resolve the variability. However, increasing sample size tested did reduce the error.

The proposed analytical method described for the determination of the eight biogenic amines has been demonstrated to be adequate with the modifications used, which address the physical nature of the material and which produced good linearity, repeatability, and recovery. Good precision and recovery were achieved for all of the amines except histamine, which will require special consideration. However, the data presented show that the method is appropriate for the analysis of biogenic amines in coarse, heterogeneous chicken samples. Further, the method would also be useful for related feed ingredients such as poultry meal, meat and bone meal, and others, in which the problem of heterogeneity is not as great as it is for the material tested in this study.

ABBREVIATIONS USED

TY, tyramine; PU, putrescine; PE, phenylethylamine; CA, cadaverine; HI, histamine; TR, tryptamine; SPD, spermidine; SPM, spermine; BN, benzylamine.

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