

LC/MS/MS Analysis of Biogenic Amines in Foods and Beverages

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ABSTRACT

This study describes the development of an LC/MS/MS analysis of 11 biogenic amines that are indicators of food freshness. They are also associated with Scombroid fish poisoning. They are produced naturally in food by enzymatic decarboxylation of free amino acids. We have examined several new generation HILIC and fluorinated columns, and found that Pinnacle[®] DB PPPF column interfaced to an API 3200[™] LC/MS/MS system gives an LOD about 100 times better than US-FDA specifications for histamine in fish products. Analysis can be completed in 6 min using a 1.9-µm column, 8 min using a 3-µm column and 12-min with a 5-µm column.

INTRODUCTION

Biogenic amines are a group of biologically active organic compounds produced by decarboxylation of free amino acids. They are found in bacterially contaminated food, particularly in fish and are therefore potential quality indicators. They can, in sufficient concentrations, pose a threat to human health⁽¹⁾. Histamine is the main causative agent in Scombroid fish poisoning. The other biogenic amines such as putrescine, cadaverine and tyramine are also of great interest as their presence enhances the toxicity of histamine. Biogenic amines can also react with nitrites to form potentially carcinogenic nitrosamines. Analysis of these amines is usually carried out by ELISA at a detection limit of low – medium ppm. Analysis by traditional RP-HPLC is difficult because of poor retention. Derivatization methods are time-consuming, ion-pairing agents can inhibit LC/MS analyses, and both can adversely affect method reproducibility. In 2006 we investigated the use of cation-exchange column coupled with tandem mass spectrometry detection to analyze biogenic amines in seafood⁽²⁾. Although the method worked well, it requires column regeneration, ion suppressor, etc., and is not suited for high throughput analysis.

With the introduction of new LC phases, we wanted to see if HILIC or fluorinated packing material can handle the direct analysis in one injection of these polar biogenic amines: cadaverine, histamine, 2-phenylethylamine, putrescine, serotonine, spermidine, spermine, tryptophan, tyramine, tyramine and uroacanic acid (Figure 1). We examined several LC columns and found that Pinnacle[®] DB PPPF (pentafluorophenyl phase with a propyl spacer) works well with 0.1% trifluoroacetic acid (TFA) for all of these amines with detection limits (based on S/N=100) of low ppb. The current method indicates the permitted level for histamine to be 500 ppm based on toxicity and 50 ppm defect action level, because histamine is generally not uniformly distributed in a decomposed fish. If 50 ppm is found in one section, there is the possibility that other parts of the fish may exceed 500 ppm⁽³⁾. Therefore, our new method must meet the requirement for routine screening for these compounds. The analysis time including column regeneration is 6 min using a 1.9-µm, 50 x 2.1 mm PPPF column, whereas 5-µm, 150 x 2.1 mm PPPF column requires 12 min or less per sample. The LC used for this study was a rapid separation system and requires a short time for re-equilibration. Over 200 various foods and beverages have been tested in triplicate injections for reproducibility and robustness of this new method. This method was also applied to a study of time, storage condition and concentration of these biogenic amines.

MATERIALS AND METHODS

Chemicals: cadaverine, histamine, 2-phenylethylamine, putrescine, serotonine, spermidine, spermine, tyramine, tyramine, triptophan, uroacanic acid, ammonium formate and trifluoroacetic acid were purchased from Sigma-Aldrich of St. Louis, MO. Histmine- α , β , β -D(2HCl) was procured from C/D/N isotopes of Pointe-Claire, Quebec. HPLC solvents were manufactured by Mallinckrodt Baker Inc. of Phillipsburg, N.J. Water was made in house using a Millipore Milli-Q gradient/ELIX 3 system (Bedford, MA).

Samples: Liquid samples such as wine, soy milk and beer were filtered using a 0.45-µm filter paper and transferred to a 1.7-mL amber auto-sampler vial. Solid samples were cut into small pieces and shaken with 20 mL of 70% methanol + 30% water for 20 min., then centrifuged at 7,000 rpm at 4°C for 20 min. The supernatant was transferred to a 1.7-mL amber auto-sampler vial for LC/MS/MS analysis. The salmon samples used in the storage time study (freshly chilled) as well as commercial salmon purchased from a local supermarket) were each filleted including skin and homogenized in a Waring blender. 5 g tissue aliquots from each fish type were weighed and placed in 50-mL Falcon centrifuge tubes. 4-6 aliquots per fish type were stored at four different temperature conditions: -20°C, 4°C, ambient and 37°C. Aliquots were removed at periodic intervals and extracted. The aliquots were extracted at 0, 3, 5, 7, 10 and 14 days for all the fish types at the different storage conditions except for those kept at ambient and 37°C temperatures. The sampling for those two temperatures did not extend to 10 and 14 days as the fish would normally be rejected due to the faster putrefaction rate.

The deuterated internal standard was used to spike Atlantic mackerel to study recovery. A total of 222 food and beverage samples were examined for any interference and compatibility with our new method.

Figure 1: Chemical Structures of Biogenic Amines

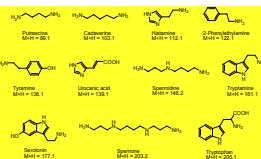


Table 1: Time program for 3-µm 50 x 2.1 mm PPPF column

Time (min)	% A	%B	Flow rate
0.00	100	0	0.6 mL/min
1.00	100	0	0.6 mL/min
5.00	0	100	0.6 mL/min
6.00	0	100	0.6 mL/min
6.10	100	0	0.6 mL/min
8.00	100	0	0.6 mL/min

In order to increase sensitivity, acetonitrile was added to the LC eluent at 0.4 mL/min at the Turbo[™] source's "Tee" piece. It helps to form dry spots at the early part of time program where water content is high. For 1.9-µm and 5-µm particle sizes slightly modified time programs were used to obtain optimum results. Mobile phase A = water + 0.1 (or 0.05%) TFA; B = acetonitrile + 0.1 (or 0.05%) TFA

LC/MS/MS: A new Dionex UltiMate[™] 3000 Rapid Separation LC(RS)LC system consisting of a pump, vacuum degasser, auto-sampler, column compartment, variable wavelength detector that can operate up to 800 bar was used in combination with an Applied Biosystems/MDS Sciex API 3200[™] mass spectrometer. After examination of several columns, we selected Pinnacle[®] DB PPPF, 5-µm, 150 x 2.1 mm or 1.9-µm, 50 X 2.1 mm for fast analysis. Later a 3-µm 50 x 2.1 mm column was packed and this was found to be the best format for speed and robustness. Two ion pairs per chemical species were monitored under positive ion, multiple-reaction monitoring (MRM) mode (See Table 2). Results were transformed into a summary table using a new "reporter" template developed for this work. For inter-laboratory comparison of the method the NRC group ran the same set of salmon samples on an Agilent 1200 HPLC system interfaced to an API 4000[™] using the same method, but slightly different lens parameters.

Table 2: MRM table for LC/MS/MS analysis of 11 biogenic amines

Name Formula	m/z	Q3	Dwell time	DP	EP	CEP	CE	CXP	Retention min
Putrescine	88	50.0	50.0	21.5	5.5	7.2	27.0	27.5	74.0
C ₄ H ₁₂ N ₂ ⁺	88	72.0	50.0	21.5	5.5	7.2	14.0	14.5	3.0
Cadaverine	103	69.0	50.0	21.0	5.5	6.1	22.0	22.5	5.2
C ₅ H ₁₄ N ₂ ⁺	103	86	50.0	21.0	5.5	6.1	13.0	13.5	1.9
Histamine	112	49.0	50.0	22.0	6.0	6.0	17.0	17.5	0.76
C ₆ H ₁₂ N ₂ ⁺	112	55	50.0	23.0	6.0	6.0	21.0	21.5	0.3
β-Histamine	116	72.0	50.0	23.0	6.0	6.0	21.0	21.5	0.76
β-Histamine	116	99.0	50.0	23.0	6.0	6.0	21.0	21.5	0.76
2-Phenylethylamine	122	77.0	50.0	21.0	5.5	4.5	11.0	11.5	0.3
C ₉ H ₁₀ N ₂ ⁺	122	105	1.0	21.0	5.2	11.0	16.0	16.5	6.1
Tyramine	139	77.0	50.0	20.0	6.1	7.2	18.0	20.0	9.8
C ₉ H ₁₀ N ₂ ⁺	139	121.0	50.0	20.0	6.1	12.0	15.0	15.5	3.3
Uroacanic acid	138	121.0	50.0	20.0	6.1	12.0	15.0	15.5	3.3
C ₈ H ₁₀ N ₂ ⁺	138	121.0	50.0	20.0	6.1	12.0	15.0	15.5	3.3
Spermidine	146	72.0	50.0	20.0	5.6	7.0	23.0	23.5	2.9
C ₁₀ H ₁₆ N ₃ ⁺	146	112	50.0	20.0	5.6	7.0	19.0	19.5	1.4
Tyramine	151	117	50.0	20.0	6.0	6.0	22.0	22.5	9.1
C ₁₀ H ₁₆ N ₃ ⁺	151	145	1.0	20.0	6.0	12.0	16.0	16.5	6.1
Spermidine	177	113	50.0	16.0	10.0	10.0	22.0	22.5	2.6
C ₁₁ H ₁₆ N ₃ ⁺	177	141	50.0	16.0	10.0	10.0	18.0	18.5	6.9
Spermine	191	113	50.0	16.0	6.0	10.0	18.0	18.5	1.54
Spermine	205	129	50.0	16.0	6.0	10.0	19.0	19.5	25.1
Tyramine	205	145	1.0	20.0	6.2	10.0	22.0	22.5	6.1
C ₁₁ H ₁₆ N ₃ ⁺	205	181	1.0	20.0	6.2	11.0	22.0	22.5	3.99

Detection limits in ng/mL were obtained by triplicate injections of a series of standard solutions, and calculated by 10 σ (standard deviation) of 20-30 background readings. Actual numbers may vary due to sample sticking on plumbing or presence of interfering peaks in real-life samples.

RESULTS AND DISCUSSION

Figure 2 shows a typical ion chromatogram obtained from a 50 x 2.1 mm, 3-µm PPPF column with a matching guard column. Uroacanic acid gave a slightly broad peak or twin peaks (see inset, depending on gradient profile and perhaps pH or ionic strength of medium?) due the presence of 2 stereoisomers around the double bond.

Figure 2: Extracted Ion Chromatogram of Biogenic Amines

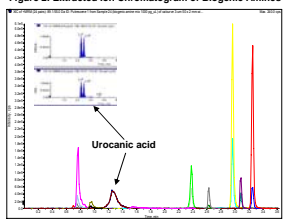


Figure 3: XIC of Slightly Spoiled Salmon

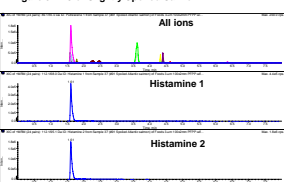
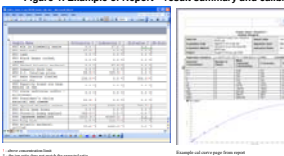


Figure 4: Example of Report – result summary and calibration curve

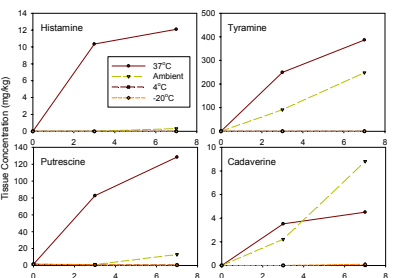


Over 800 injections of standard solutions as well as extracts of various food and beverages were made, and the column maintained good reproducibility throughout the analysis. 222 foods and beverages were examined for any potential interferences: uncooked or cooked rice, cheese, soy products, sausages, spices, mushrooms, pickled vegetables and fish, meat, wines, liquor, fruit juices, beer, nuts, pizza, spreads, sardines, salmon, squid, Arctic surf clams, shrimp, tuna, mackerel, herring, red snapper, cod, mahi-mahi, amberjack, kingfish, eel, pollock, oyster, sea bream, swordfish, scallop, Alaskan king crab, Atlantic lobster, sweet fish, anchovy, haddock, octopus, bonito, dogfish, Indian white pomfret, turbot, butterfish, jagg fish, golden thread fish, mullet fish, many dairy and meat products. Few interference peaks were observed, but the confirmation ion helped us in interpreting the data.

One of our coworkers purchased Atlantic salmon fillets kept on ice at a local grocery store. She did not notice any foul smell at the time of purchase. She kept it in a refrigerator. The next day, she tried to cook it and noticed odour and slimy texture. She brought the fish to our laboratory for analysis. The sample was processed and injected in triplicate to produce results shown in Figure 3. It indicated the presence of putrescine(0.5±0.1), cadaverine (9.5±0.5), histamine(2.9±0.1), 2-phenylethylamine (0.07±0.01), tyramine (2.1±0.2), uroacanic acid (0.62±0.05), spermidine (1.55±0.03), spermine (0.37±0.09) and tryptophan(4.6±0.2). All concentrations are in ppm or mg/kg fish.

Figure 4 is a portion of Report where a result table generated by the Analyst[®] 1.5 software is automatically converted into MS Word document for general use. It tabulates all the ion pairs used for quantification, ion pair ratio information, and calibration curve that spans a wide range of concentration. Because the system is sensitive, responses sometime follow quadratic curves.

Figure 5: Some of the results from a fresh salmon stored at different temperatures



Quantitation of the various biogenic amines in freshly culled salmon samples stored at different temperatures and time (in days) revealed some very interesting changes in concentration. The most interesting results for 4 compounds are shown in Figure 5. At room temperature the level of cadaverine and tyramine were significantly higher after 3 days when compared to the other biogenic amines. The best indicator of fish freshness might be tyramine which gives the most sensitive indication of storage time and temperature. This set of fish samples was run at both NRC Halifax and AB/MDS AT laboratories and gave good agreements.

CONCLUSIONS AND FUTURE WORK

- It has been demonstrated that 11 biogenic amines often found in spoiled foods and beverages can be analyzed in 6 – 12 min depending on LC column size and flow rate. The use of fast resolution HPLC system produces good speed and reproducibility. This new method comfortably meets the FDA histamine analytical requirement of 50 ppm defect action level and 500 ppm based on toxicity.
- This method has been applied to the analysis of 222 various foods, spices and beverages. Except for a few samples (such as vinegar), the method worked well. Pinnacle[®] DB PPPF column worked well with these samples.
- Controlled decay fish samples were examined and results show good agreement with our previous data using IC-MS/MS. Inter-laboratory data are in a good agreement.
- More food samples, especially seafoods, should be run in a typical food inspection environment. Automation of sample preparation may be very useful in applying this technique.

REFERENCES

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 [3] U.S.FDA Fish and Fisheries Products Hazards and Control Guidance: 3rd ed., June 2001, Appendix E: FDA & EPA Safety Levels in Regulations and Guidance, and <http://www.cfsan.fda.gov/~comm/haccp4x5.html>

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