Application note | 000755

Food safety

HPLC determination of biogenic amines in beer

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Keywords
Vanquish Core HPLC, fluorescence detection, FLD, beer, food safety, food quality, derivatization, AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

Application benefits

- A sensitive reversed-phase HPLC method for the analysis of nine biogenic amines in beer
- Simple and reproducible sample preparation procedure
- Accurate quantification of seven biogenic amines in beer

Goal

To develop a reversed-phase method for analysis of biogenic amines in beer, based on sample derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

Introduction

Biogenic amines are naturally occurring organic compounds that are formed or degraded by the normal and physiological metabolism of microorganism, plants, and animals.[1] Biogenic amines belong to the group of biotoxins.[2] In food, they may occur naturally in small amounts but are more commonly formed secondarily by bacterial degradation. An unintentional microbial decomposition of proteins, for example in fish or meat, can lead to spoiled food. If this decomposition is intentional, it is usually referred to as fermentation. In this case, the decomposition takes place in a controlled manner, as in alcoholic fermentation or the ripening of cheese. The most commonly occurring biogenic amines in foods and beverages are histamine, tyramine, cadaverine, phenylethylamine, spermine, and spermidine.[1] Fresh foods contain, on average, a natural level of biogenic amines up to 40 mg/kg. In case of spoilage, this level is much higher, for example in fish up to 5,000 mg/kg. Therefore, they can serve as spoilage indicators and can provide indication on the microbic contamination and hygienic conditions during the processing of food and beverages.

Analysis of biogenic amines can be performed by HPLC. Like their biological precursors, amino acids, most biogenic amines lack a chromophore and cannot be detected by UV absorption. To overcome this limitation, a typical approach is to chemically label the amines with fluorescent molecules. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is a powerful reagent for pre-column derivatization because of the stability of AQC-derivatized components and the reproducibility of the derivatization reaction with both primary and secondary amines.[3] The AQC fluorescent derivatives are more hydrophobic than the native molecules, therefore, the highly hydrophilic amines can be effectively retained in reversed-phase mode.

In this work, a mixture of nine biogenic amines labeled with AQC were separated on a Thermo Scientific™ Accucore™ aQ C18 polar endcapped column using a Thermo Scientific™ Vanquish™ Core HPLC system. The Vanquish Core HPLC delivers outstanding retention time precision for confident peak identity assignment based on retention time. The injection volume precision enables accurate quantitation even for components close to the limit of quantification of the method. External standard calibration curves were generated for each amine, using a concentration range suitable for the analysis of the amines in non-spoiled food and beverages. The method was applied to the quantitation of amines in beer samples.

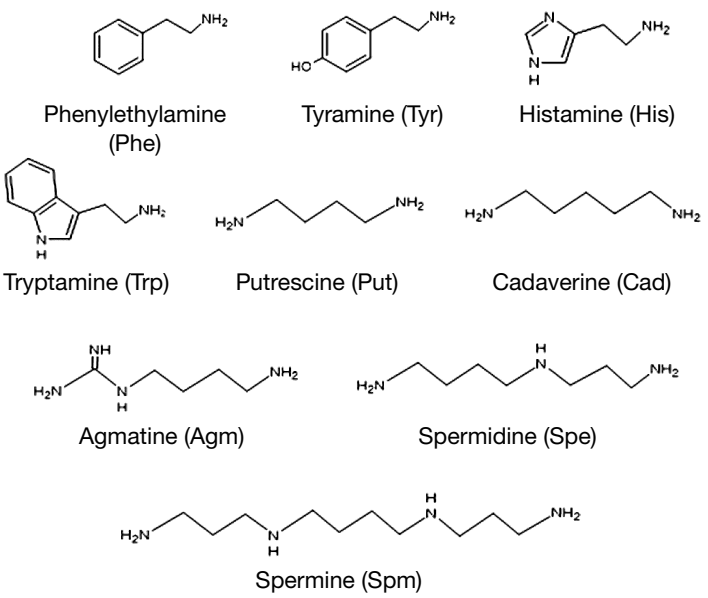

Figure 1. Chemical structure of biogenic amines analyzed in this work

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Acetonitrile, Optima™ LC/MS grade, Fisher Chemical™ (P/N A955-212)
- Ammonium acetate, ≥ 99.9%, Fisher Scientific™ (P/N A/3446/50)
- Acetic acid, Optima™ LC/MS grade, Fisher Chemical™ (P/N A113-50)
- Borate buffer 20×, Thermo Scientific™ Pierce™ (P/N 28341)
- AQC, Synchem (P/N S041)
- 1,4-Diaminobutane (putrescine), 99%, Thermo Scientific™ (P/N 112120250)
- 1,5-Diaminopentane (cadaverine), 98%, Thermo Scientific™ (P/N 112320050)
- Histamine dihydrochloride, 99%, Thermo Scientific™ (P/N 150620050)
- Agmatine sulfate, Thermo Scientific™ (P/N H55363)
- Tryptamine, Thermo Scientific™ (P/N A11116)
- 2-Phenylethylamine hydrochloride, Merck Life Science (P/N P6513)
- Tyramine, Merck Life Science (P/N 80345)
- Spermidine, Merck Life Science (P/N 85558)
- Spermine, Merck Life Science (P/N 55513)

Sample handling

- Thermo Scientific™ Digital Heating Cooling Drybath (P/N 88880030)
- Sartorius™ Minisart™ NML Standard Syringe Filter (P/N 16555)
- Fisherbrand™ Mini Centrifuge (P/N 12-006-901)
- Thermo Scientific™ Orion 3 Star™ pH Benchtop Meter (P/N 13-644-928)
- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ 11 mm Amber Glass Crimp/Snap Top Vial (P/N 10326542)
- Thermo Scientific™ 11 mm Autosampler Snap-It Caps (P/N 11518180)
- Thermo Scientific™ Finnpipette™ F1 Variable Volume Single-Channel Pipettes (various volumes), Thermo Scientific™ Finntip™ Flex™ Pipette Tips (P/N 11458479, P/N 94060310, P/N 11863420)

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Instrumentation

- Thermo Scientific Vanquish Core Quaternary HPLC system consisting of:
  - Vanquish System Base (P/N VC-S01-A)
  - Vanquish Quaternary Pump C (P/N VC-P20-A)
  - Static mixer 350 µL (P/N 6044.5310)
  - Vanquish Split Sampler CT (P/N VC-A12-A)
  - Vanquish Column Compartment C (P/N VC-C10-A-03)
  - Vanquish Diode Array Detector CG (P/N VC-D11-A)
  - Standard flow cell, SST, 10 mm (P/N 6083.0510)
  - Vanquish Fluorescence Detector F (P/N VF-D51-A)
  - Micro flow cell, 2 µL (P/N 6079.4330)

Standards and sample preparation

Sample derivatization

1 M borate buffer was diluted to 0.2 M by adding 200 µL buffer to 800 µL water. 4 mg of AQC was weighed into a vial and diluted to 1 mL with anhydrous ACN.

70 µL borate buffer diluted solution were mixed with 20 µL AQC solution and 10 µL of sample or standard. The mixture was immediately placed in a heating block and heated at 55 °C for 10 minutes. It was then removed and cooled to room temperature. Finally, the solution was diluted 1:5 with water and stored at room temperature.

Standard solutions

Stock solutions of the amines at concentration range 7.5–60 mg/L were prepared by dissolving the compounds in water. Due to poor solubility in water, tryptamine stock solution was prepared by dissolving the compound in water/acetonitrile 8/2 (v/v) at a concentration of 40 mg/L. Aliquots from the stock solutions were derivatized according to the procedure described above, yielding the final concentration levels described in Table 1.

Beer samples

Beer bottles were purchased from a local supermarket and opened one day before analysis. 50 mL of each beer were cooled in the refrigerator at + 4 °C for one day. Subsequently, 20 mL were transferred to a beaker and degassed for 20 minutes in an ultrasonic bath. Afterwards, 2 mL were filtered through a membrane filter. The filtered beer was diluted with 2 mL water.

Mobile phase A preparation

Ammonium acetate (3.925 g) was dissolved in 1 L water and pH adjusted to 5.0 with acetic acid.

Table 1. Concentration in mg/L of the biogenic amines calibration standards

Calibration standard (mg/L)						
Amine	**Level 1**	**Level 2**	**Level 3**	**Level 4**	**Level 5**	**Level 6**
Histamine	0.400	0.200	0.100	0.0300	0.0209	--
Agmatine	1.20	0.600	0.300	0.0900	0.0449	0.0226
Tyramine	0.800	0.400	0.200	0.0600	0.0299	0.0150
Putrescine	--	0.600	0.300	0.0900	0.0449	0.0226
Cadaverine	--	0.600	0.300	0.0900	0.0449	0.0226
Phenylethylamine	0.150	0.0750	0.0375	0.0112	0.00786	--
Spermine	--	0.350	0.175	0.0525	0.0262	0.0132
Spermidine	--	0.350	0.175	0.0525	0.0116	0.0132
Tryptamine	0.800	0.400	0.200	0.100	0.0720	0.0624

Table 2. Chromatographic conditions

Column	Thermo Scientific Accucore aQ C18 2.1 × 100 mm, 2.6 µm (P/N 17326-102130)	
Flow rate	0.45 mL/min	
Mobile phase	A: 50 mM ammonium acetate, pH 5.0 with acetic acid B: ACN	
Gradient	Time (min)	B (%)
	0.000	4.0
	18.370	39.0
	26.040	39.0
	26.041	100.0
	30.040	100.0
	30.050	4.0
	45.000	4.0
Column temperature	20 °C (forced air), passive pre-heater	
Autosampler temperature	20 °C	
Pump and autosampler rear seal wash	10% methanol in water	
Injection volume	1 µL	
FLD detector parameters	Excitation at 248 nm, emission at 298 nm, sensitivity 6, filter wheel auto, data collection rate 100 Hz, standard lamp mode, response time 0.05 s	
UV detector parameters	Detection at 248 nm, data collection rate 10 Hz, response time 0.5 s	

Recovery estimation

Recovery was measured by spiking 500 µL of beer H1 with aliquots of amines stock solution. Spiked and unspiked samples were derivatized as triplicate and injected as triplicate. The average amount of each amine measured in the spiked beer was subtracted by the average amount measured in unspiked beer. Then, the measured amount was divided by expected amount to calculate recovery.

Chromatography Data System and data processing

The Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS) was used for data acquisition and analysis. Noise for the signal-to-noise (S/N) calculation was measured from blank injection on a 30-second retention window centered on the expected retention time of the component. Limit of quantitation (LOQ) was calculated as 10 times the signal-to-noise ratio for a specific component.

Results and discussion

Quantitative method for biogenic amines

A method was developed to resolve the nine amines as described in Table 2. Chromatograms of labeled amines samples are shown in Figure 2. Peak identity was assigned by injection of single amine standard (data not shown). The fluorescence detection provided the highest signal-to-noise ratio for all amines except tryptamine. Tryptamine could be detected by UV. Poor signal response of tryptamine with fluorescent detection can be ascribed to fluorescence quenching phenomena.[4] The hypothesis is supported by the structural similarity of tryptamine with tryptophan, also known to be affected by fluorescence quenching when AQC labeling is applied. Based on the excellent run-to-run repeatability and sample preparation reproducibility, the method was further used for quantitative analysis of the biogenic amines.

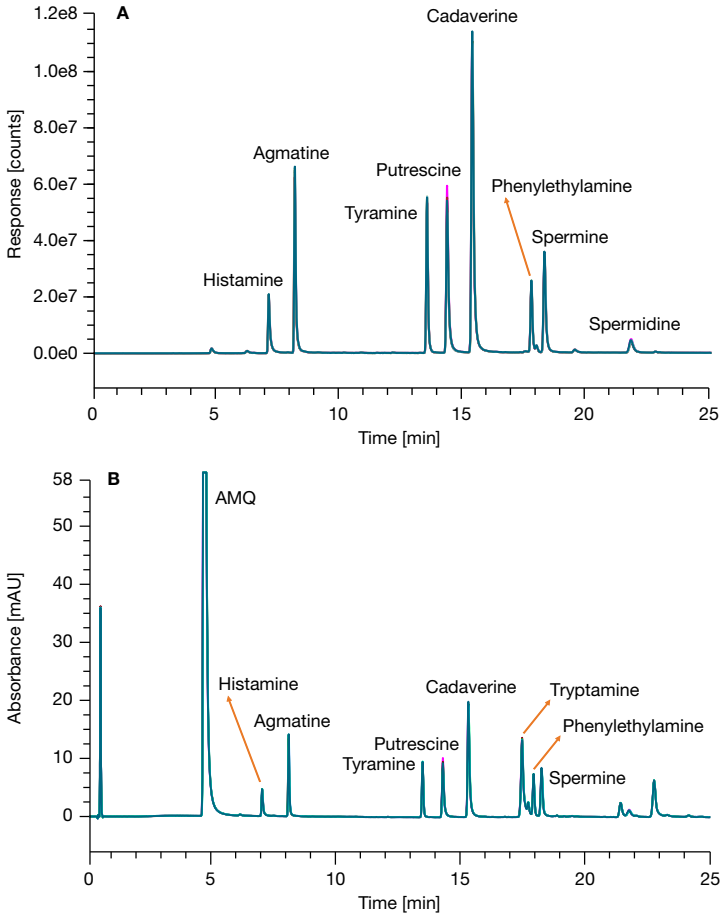

Figure 2. Overlaid chromatograms of three individual sample preparations of biogenic amines standard. Fluorescent chromatogram (A), and UV chromatogram (B). Each sample was injected in duplicate. Concentration of the amine standard: histamine 0.4 mg/L, agmatine 1.2 mg/L, tyramine 0.8 mg/L, putrescine 0.48 mg/L, cadaverine 0.48 mg/L, phenylethylamine 0.15 mg/L, spermine 0.28 mg/L, spermidine 0.28 mg/L.

Standard samples listed in Table 1 were used to prepare the calibration curves. Linear regression was calculated based on peak area of the fluorescence chromatogram for all amines except tryptamine. Good linearity was measured for all amines, and limits of quantitation were calculated in the range of 0.004 to 0.05 mg/L (Table 3). Sensitivity was sufficiently high to monitor biogenic amines in beer and fermented beverages.

Application example of analysis of amines in beer

The beers analyzed in this work are lager beers and wheat beers, all produced in Bavaria (Germany). The lager beers belonged to the *helles* type, *i.e.,* bottom-fermented malt beer from south Germany. For simplicity, the brand is omitted, and *helles* and wheat beers are denoted H1–H5 and W1–W5, respectively.

Figure 3 shows two examples of beer samples labeled with AQC. Peak identity assignment was based on retention time comparison with the standard injection and was confirmed by spiking the beer samples with individual amine standards (data not shown). Several unidentified components were detected. Those components are not present in the underivatized beer; therefore, it can be assumed they are molecules capable of binding to AQC, hence molecules that contain primary or secondary amino groups. Spermidine and tryptamine could not be detected in any of the beers. Histamine eluted in the tail of an unidentified component (Figure 4). The interference was present with strong signal in all beers except W3. In this beer, the interference signal was low, and the histamine peak could be resolved. Histamine in W3 was below the LOQ. Inspection of the shoulder of the interference peaks in Figure 5 indicated that the histamine level was very low in all samples. In this method, histamine content is reported as the sum of the actual histamine and interference peak area and thus must be interpreted as a substantial overestimation.

Table 3. Results of the external linear calibration based on standards of Table 1. Calibration curve equation: (Peak Area) = b*(Amount) + a. The LOQ estimation is based on 10 times the signal-to-noise ratio. Calibration of tryptamine is based on the UV signal; all other amines are calibrated based on the fluorescence signal.

Amine	Retention time relative standard deviation (%)	Calibration curve parameters			LOQ ± SD (mg/L)
		a	b	R^2	
Histamine	0.61	5.370E6	-1.078E4	0.9991	0.0102 ± 0.0011
Agmatine	0.39	4.329E6	-3.902E4	0.9998	0.0100 ± 0.0003
Tyramine	0.23	5.656E6	1.026E4	0.9998	0.0055 ± 0.0001
Putrescine	0.18	1.195E7	1.228E5	0.9962	0.0184 ± 0.0007
Cadaverine	0.16	1.367E7	1.283E4	0.9998	0.0200 ± 0.0003
Phenylethylamine	0.12	1.64E7	1.865E4	0.9988	0.0038 ± 0.0029
Spermine	0.15	6.682E6	-3.097E4	0.9984	0.0097 ± 0.0010
Spermidine	0.36	2.447E6	6.683E3	0.9969	0.0488 ± 0.0005
Tryptamine	0.02	9.020	2.796E-02	0.9998	0.0340 ± 0.0040

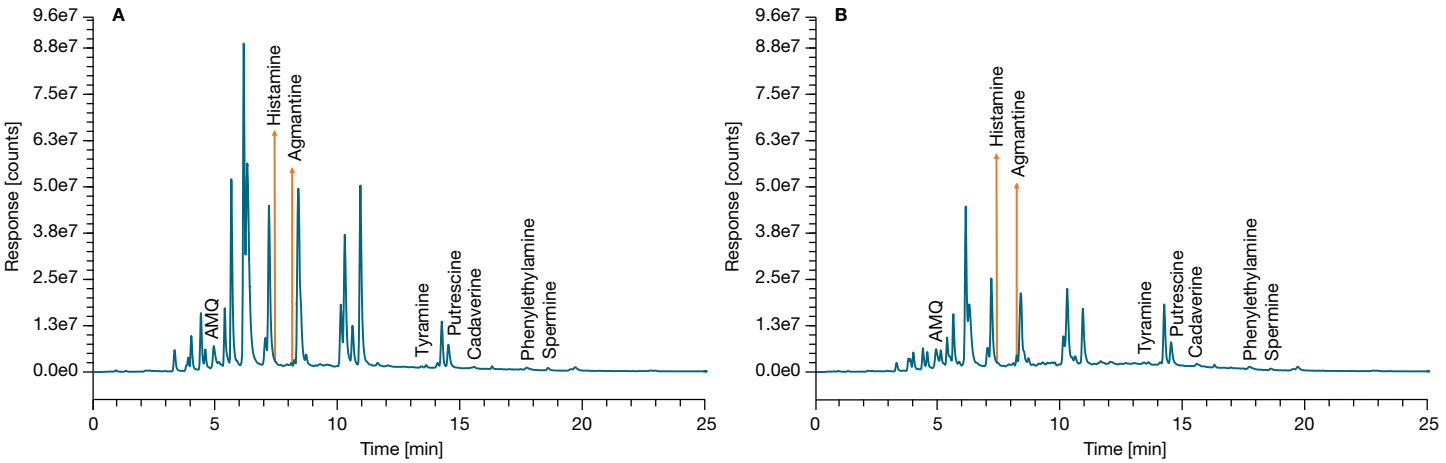

Figure 3. Example chromatograms of the lager and wheat beers; lager H3 (A) and wheat beer W1 (B)

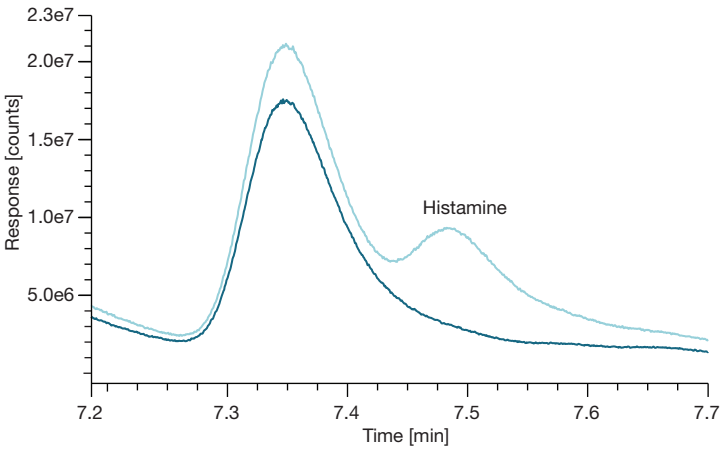

Figure 4. Beer H1 spiked with 0.13 mg/L histamine (light blue trace) and unspiked beer (dark blue trace). Histamine peak elutes in the tail of a strong matrix interference.

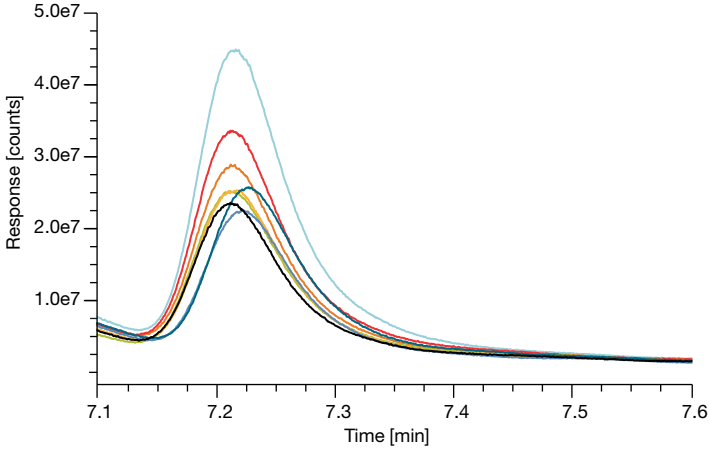

Figure 5. Detailed view of overlaid chromatograms of derivatized beer samples (all beers except H1 and W3), showing matrix interference peaks for all beers except H1 and W3. Histamine is expected to elute between 7.4 and 7.6 minutes. Histamine is either absent or at very low level.

Table 4. Concentration of biogenic amines in beer ± standard deviation in mg/L (n = 3). The concentration was corrected by recovery. The amount of histamine reported is an overestimation except for W3.

Amine	Recovery (%)	H1	H2	H3	H4	H5
Histamine	86.0	< 36.1	< 3.9	< 98.7	< 60.2	< 73.4
Agmatine	126.0	9.07 ± 0.07	3.17 ± 0.02	2.77 ± 1.30	3.97 ± 0.01	2.77 ± 0.03
Tyramine	93.7	< LOQ (0.5)	0.69 ± 0.08	1.01 ± 0.06	0.95 ± 0.06	1.33 ± 0.03
Putrescine	117.9	3.03 ± 0.03	3.52 ± 0.12	4.94 ± 0.17	5.09 ± 0.09	4.62 ± 0.06
Cadaverine	104.5	< LOQ (2.0)	< LOQ (2.0)	4.93 ± 0.06	< LOQ (2.0)	< LOQ (2.0)
Phenylethylamine	89.6	< LOQ (0.38)	< LOQ (0.38)	< LOQ (0.38)	< LOQ (0.38)	< LOQ (0.38)
Spermine	124.1	< LOQ (0.97)	0.84 ± 0.02	1.19 ± 0.03	0.94 ± 0.06	1.21 ± 0.10

Amine	Recovery (%)	W1	W2	W3	W4	W5
Histamine	86.0	< 54.4	< 55.0	< LOQ (1.0)	< 85.9	< 55.6
Agmatine	126.0	4.54 ± 0.04	4.91 ± 0.08	6.49 ± 0.06	5.73 ± 0.00	4.09 ± 0.01
Tyramine	93.7	0.83 ± 0.05	0.62 ± 0.09	0.72 ± 0.03	1.03 ± 0.06	1.40 ± 0.08
Putrescine	117.9	4.35 ± 0.05	4.31 ± 0.17	4.31 ± 0.07	4.55 ± 0.09	6.76 ± 0.33
Cadaverine	104.5	< LOQ (2.0)	< LOQ (2.0)	< LOQ (2.0)	< LOQ (2.0)	< LOQ (2.0)
Phenylethylamine	89.6	< LOQ (0.38)	< LOQ (0.38)	< LOQ (0.38)	< LOQ (0.38)	< LOQ (0.38)
Spermine	124.1	0.81 ± 0.01	1.79 ± 0.07	< LOQ (0.97)	1.15 ± 0.04	< LOQ (0.97)

Tryptamine and spermidine were not detected in any of the samples. The rest of the amines were detected in all beers. In many instances the amount was very low and below the LOQ of the method (Table 4). For instance, phenylethylamine was always below the LOQ, and cadaverine was above the LOQ only in beer H3. The findings are in agreement with results from literature.[5]

Conclusion

- An HPLC method was developed for separation of AQC-derivatized biogenic amines in beer.
- The dynamic range of the calibration curves and limit of quantitation are suitable for quantitation.
- The Vanquish Core HPLC system delivered outstanding run-to-run repeatability for confident peak identity assignment and precise quantitation.
- The Accucore aQ C18 column with polar end-capping delivered excellent selectivity and could resolve biogenic amines from each other and matrix interferences. Only histamine could not be resolved completely from an interference.
- Ten beers were analyzed. Biogenic amine contents were consistent with values reported in the literature.

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