Scientific paper

Determination of Biogenic Amines at Low μg L^{-1} Levels as Acetylacetone Derivatives by RP-HPLC with UV-Visible Detection in Expired Apple Juice Samples

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Abstract

An accurate and sensitive method for trace quantification of three biogenic amines in expired apple juice samples based on reversed-phase liquid chromatography (RP-HPLC) with UV-visible detection is described. Biogenic amines including cadaverine, histamine, and tyramine, were converted to their acetylacetone derivatives in water-methanol medium. The proposed method involves a pre-column derivatization of species followed by RP-HPLC separation with Thermo Hypersil Gold reversed-phase column and UV detection at 315 nm. A flow rate of 0.9 mL min^{-1} was used in the proposed method. An efficient separation of biogenic amines was successfully performed in 11 min with a good resolution using 35:65% (*v/v*) acetonitrile-water mixture as the mobile phase. Detection limits of 0.03, 0.23, and 0.08 μg L^{-1} were obtained for cadaverine, histamine and tyramine, respectively. The proposed method has been successfully applied for the analysis of two different commercially available expired apple juice samples. Recovery rates between 98.78 and 102.12% were obtained with an RSD of 0.16–1.65% for the analysis of 20 mL of expired apple juice samples indicating that the recoveries of biogenic amines were very satisfactory.

Keywords: Biogenic amines; acetylacetone; RP-HPLC; UV-visible spectrophotometry; expired apple juice

1. Introduction

Biogenic amines are aliphatic, alicyclic or heterocyclic organic bases of low molecular weight which arise as a consequence of metabolic processes in animals, plants, and microorganisms. These amines are found in a variety of foods and beverages. Their production occurs during a ripening or fermentation process. Biogenic amines can also occur naturally in foods and beverages.[1] Biogenic amines are also important for their role as indicators of quality and/or acceptability in some foods.[2] Furthermore, they have important metabolic roles in living cells; for instance, polyamines and putrescine are essential for cell growth, and other amines such as histamine, tyramine, and serotonin are involved in the central nervous system functioning for the regulation and control of the blood pressure.[3]

Biogenic amines generally do not cause any risk to human health unless they are ingested in large amounts or the natural mechanism for their catabolism is genetically defective or inhibited.[4] The consumption of food products containing 80–100 mg L^{-1} biogenic amine level causes a variety of disorders. Thus, the formation of biogenic amines in food products should be controlled for the general public health. In addition, the storage time and temperature of food products may also bear a health risk for consumers. Therefore, the inhibition of effective microorganisms and decarboxylase enzyme activities are essential for the formation of biogenic amines.

The chemical structure of biogenic amines can be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, phenylethylamine), or heterocyclic (histamine, tryptamine).[5] Histamine and cadaverine are classified as diamines containing two nitrogen groups in their structures. They are derived from decarboxylation of histidine and lysine, respectively. Tyramine is a monoamine compound containing one nitrogen in its structure and is derived from decarboxylation of tyrosine.

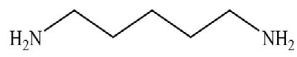
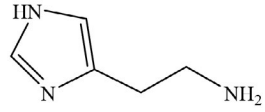
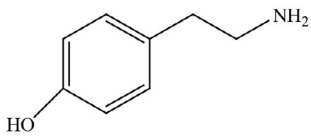
Histamine poisoning is a significant concern for the food safety. The ingestion of foods containing high levels of histamine affects human health, and the symptoms of histamine poisoning include difficulties in breathing, itching, rashes, vomiting, fever, and flushing.[6] The European Food Safety Authority confirmed histamine and tyramine as the most toxic and particularly relevant for food safety and the products with high contents of biogenic amines may be harmful for susceptible individuals.[7] Several methods for the determination of biogenic amines in foods have been developed and the most applied ones involve high performance liquid chromatography (HPLC) coupled with different detectors.[8–23] However, HPLC generally suffers from the matrix effects. Therefore, extraction and purification steps must be undertaken prior to chromatographic analysis. These steps are the most critical aspects in terms of obtaining an adequate recovery for each amine. The aim of the extraction and purification steps is to remove interfering compounds from the matrix, but during these steps losses of biogenic amines must be kept as small as possible. The extraction of biogenic amines from a food matrix is generally carried out using hydrochloric acid, perchloric acid or trichloroacetic acid (TCA).

Biogenic amines have been detected in numerous kinds of foods and beverages, e.g. cheese, fish, vegetable, meat, wine, and beer.[24] In the literature there is no study of biogenic amine determination in expired apple juice samples. In the present study, biogenic amines were analyzed in expired apple juice samples. Various types of bacterial degradation in apple juice samples with the past expiration date can result in the formation of some amines and their derivatives. Biogenic amines produced by microbial contamination are mainly bacterial, e.g. cadaverine, histamine and tyramine.[25] Biogenic amines, especially histamine, tyramine, putrescine and cadaverine have been suggested as indicators of spoilage of some foods, such as fresh fish, meat and vegetables.[26] Biogenic amine concentrations are normally lower in non-fermented food (e.g., fruits, vegetables, meat, milk and fish), but higher in fermented foods (e.g., cheese, soybean products) and beverages as a result of a contaminating microflora exhibiting amino acid decarboxylase activity.[15] Therefore, three biogenic amines (cadaverine, histamine and tyramine), which have the most important role in foods and life, have been identified and included in the study.

The quantification of this group remains challenging due to the variation in the physico-chemical properties and potential matrix effects from other substances present within the sample. This problem has been addressed using a derivatization process, with pre- and post-column approaches in high-performance liquid chromatography most widely employed at present.[24] The pre-column derivatization technique is used more frequently than the post-column derivatization because of more sensitive detection. Derivatization may be a cause for the extended sample preparation time, but is often required to complement the analysis. The determination of biogenic amines is commonly achieved by chromatographic methods such as thin-layer chromatography (TLC), gas chromatography (GC), capillary electrophoresis (CE) and high performance liquid chromatography (HPLC). TLC does not have adequate sensitivity. CE requires complex operations. GC method is not so often applied for the determination of biogenic amines due to their low volatility. GC-MS and LC-MS/MS methods are generally non-derivative methods. But, MS detectors are very expensive and require a specially trained operator. On the other hand, there is difficulty in introduction of the small size sample into the high vacuum system. Therefore, it is higly expensive and requires technical skills and it is not widely preferred. Whereas, UV detectors are cheap devices and good enough for detection of biogenic amines. The most popular of all these methods is the HPLC for the determination of biogenic amines.

For HPLC analysis using spectrophotometric detectors the determination of biogenic aminse needs a derivatization because most biogenic amines lack the chromophore. This derivatization, that occurs via amino groups with different tagging, or reagents helps to improve the selectivity and sensitivity of the methodology. In order to increase their absorption intensities, biogenic amines

Table 1. Biogenic amines studied

Name	Abbreviation	Structure	Molecular weight (g mol^{-1})	p$Ka^{20,40}$
Cadaverine	CAD		102.2	pKa_1 : 11.0 pKa_2 : 9.9
Histamine	HIS		111.2	pKa_1 : 9.8 pKa_2 : 6.0
Tyramine	TYR		137.2	pKa_1 : 9.6

should be converted to corresponding compounds using an organic chelator. The resulting compounds will allow the indirect determination of biogenic amines by UV-vis spectroscopy. *Ortho*-phthalaldehyde (OPA),[18,27–32] dansyl chloride,[13–15,33–37] and benzoyl chloride[16–17] have been reported as the derivatizing agents for the determination of biogenic amines using HPLC. Acetylacetone has also been utilized by Nishikawa[38] and Asan and Isildak[39] in the derivatization of primary amines and aliphatic diamines.

In this study, we propose a procedure for the determination of biogenic amines which cannot be directly identified due to their low absorption intensities by UV-vis spectroscopy. Initially, biogenic amines reacted with acetylacetone to form derivatives and thus their absorption intensities were increased. Then, the chromatographic conditions and wavelength of detection were optimized in order to maximize the sensitivity of the procedure. Under the optimized conditions, the chromatographic separation of cadaverine, histamine and tyramine derivatives was carried out in a reverse-phase column. Finally, the proposed method was applied for the quantification of biogenic amines in expired apple juice samples. The biogenic amines analysed are shown in Table 1.

2. Materials and Methods

2. 1. Chemical and Reagents

All chemicals and solvents used were of analytical and chromatographic grade, respectively. The biogenic amine compounds (cadaverine, histamine and tyramine) were provided by Fluka. HPLC-grade acetonitrile and HPLC-grade methanol were obtained from Sigma–Aldrich. Acetylacetone was supplied by Merck. Dipotassium hydrogenphosphate (K_2HPO_4) was purchased from Merck. Trichloroacetic acid was obtained from Kanto Chemical Co. Inc. Perchloric acid ($HClO_4$) was obtained from Sigma–Aldrich. Ultrapure water with conductivity 18.2 μS cm^{-1} was used for the preparation of all aqueous solutions.

2. 2. Apparatus

The RP-HPLC analysis was performed on a Shimadzu HPLC system (Kyoto, Japan) equipped with an LC-20 AD pump, a SPD-M 20A DAD detector system, and a CTO 20 AC column oven. The instrument has a DGU 20A degassing system. The system operates at 315 nm for cadaverine, histamine and tyramine. Termo Hypersil Gold C18 (2.5 μm, 175 mm × 5 mm) was used as stationary phase at 31 °C. The maximum operating pressure on the system was 400 bar. Mettler Toledo (Greifensee, Switzerland) MA 235 pH/ion analyser with Hanna HI 1332 Ag/AgCl combined glass electrode (USA) was used for pH measurements. Heidolph was used as the evaporator. Ultrapure water was obtained using a Zeneer Power I water system (Human Corp. Korea).

2. 3. Preparation of Standard Solutions of Biogenic Amines

Stock solutions of cadaverine, histamine and tyramine were prepared by dissolving each biogenic amine in 10% (*v/v*) methanol-water. The final concentrations were 137, 102 and 111 μg L^{-1} for cadaverine, histamine, and tyramine, respectively. Standard samples with lower concentrations were prepared by appropriate dilution in deionized water-methanol solution of the same ratio. All biogenic amine solutions were stored refrigerated at +4 °C and protected from light.

2. 4. Derivatization Procedure

The acetylacetone derivatives of the biogenic amines were prepared following the procedure described by Asan and Isildak[39], after a major modification. Firstly, all parameters for the derivatization reaction between the biogenic amines and acetylacetone were optimized. An appropriate amount of each biogenic amine was added to a 100 mL solution containing 10 mL methanol, 1.0 g K_2HPO_4, and 1.0 mL of acetylacetone were added under vigorous shaking for 10 min to complete the reaction at room temperature. Then, the reaction mixture was evaporated. The final concentration of each biogenic amine derivatives was 50 μg L^{-1}. The residue was redissolved in 1 mL of mobile phase and 20 μL of each sample was injected onto the RP-HPLC column. The biogenic amines were identified qualitatively from their retention times and they were determined quantitatively by their peak areas.

For sample analysis, firstly the expired apple juice samples were filtered through a membrane of 0.45 μm pore and 47 mm diameter. The homogenized sample was diluted to 50 mL with 5% trichloroacetic acid.[31] And then, 20 mL of each sample was added to a mixture of 100 mL of 10% (*v/v*) methanol-water containing 1.0 g K_2HPO_4, 10 mL methanol, and 1.0 mL of acetylacetone. The resulting mixture was shaken for 10 min to complete the reaction. The mixture was evaporated and the residue was redissolved in 1 mL of mobile phase and 20 μL of each sample was injected onto RP-HPLC system.

2. 5. Chromatographic Procedure

To determine the biogenic amines in expired apple juice samples, an analytical method was established using reversed-phase high performance liquid chromatography coupled to diode-array detector. Firstly, chromatographic conditions (mobile phase, flow-rate. etc.) were optimized using standard biogenic amine solutions. The chromatographic separation of biogenic amines was carried out using a Termo Hypersil Gold C18 (2.5 μm, 175 mm x 5 mm) reversed-phase column. The flow-rate in RP-HPLC system was 0.9 mL min^{-1}. The separation of acetylacetone derivatives of biogenic amines was suc-

cessfully performed with a good resolution in 11 min using acetonitrile-water mixture (35:65%, *v/v*) as the mobile phase and detected spectrophotometrically at a wavelength of 315 nm.

3. Results and Discussion

3. 1. Analytical Characteristics of the Method

Optimal conditions for the separation of biogenic amines by HPLC were determined by using Thermo Hypersil Gold (reversed-phase column) as the stationary phase and 35:65% (*v/v*) acetonitrile-water mixture as the mobile phase at the column temperature of 31 °C and the flow-rate of 0.9 mL min^{-1}. Under these conditions, acetylacetone derivatives of biogenic amines were eluted for *ca.* 11 min. Fig. 1 shows the chromatogram of the standard biogenic amine solution where a good resolution for peaks relating to three biogenic amines examined was observed in a quite short analysis time. The analytical method was validated by determination the linear range, limit of detection (LOD) and limit of quantification (LOQ), precision and recovery. Results are summarized in Table 2 and Table 3. Linearity of the calibration curves was established by injecting five concentrations of the biogenic amines standard mixtures (1.02–21.95 μg L^{-1}). Good linearity (r^2: 0.9925–0.9982) was obtained between peak area and analyte concentration.

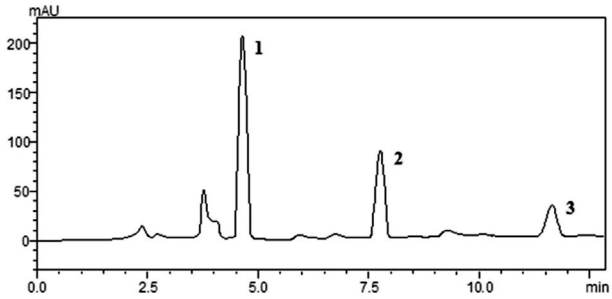


Figure 1. RP-HPLC chromatogram of the acetylacetone derivatives of biogenic amines. Amount of the biogenic amines: (1) histamine (15.78 μg L^{-1}); (2) tyramine (18.95 μg L^{-1}); (3) cadaverine (10.35 μg L^{-1}). Mobile phase: acetonitrile-water (35:65%, *v/v*); Flow-rate: 0.9 mL/min; Injection: 20 μL; Termo Hypersil Gold C18 column (175 mm × 5 mm)

The LOD was determined from the minimum concentration of the amine required to give a signal to noise ratio of 3 while the LOQ was determined with a signal to noise ratio of 10. The sensitivity of the method, as reflected by the LOD and LOQ values, is comparable to the previously reported data in the literature.[41–42]

3. 2. Recovery Studies

The repeatability and reproducibility of the RP-HPLC method were assessed by the injection of the each standard mixture for five times on the same day (intra-day) and over six days (inter-day), respectively. Good reproducibility of both the peak area (RSD ≤ 2.85%) and the retention times (RSD ≤ 0.89%) were found (Table 3). Intra-day repeatability (same analyst, apparatus and reagent) was assessed by injecting a mixture containing all the analytes five times during the same chromatographic run. Inter-day repeatability (the same analyst and apparatus but different reagents) was assessed by injecting individual samples of a standard mixture over seven days.

The proposed method of analysis was applied for recovery studies in order to examine the effect of sample matrices as the composition of apple juice samples is extremely complex. For this purpose, two concentrations (low and high) of cadaverine, histamine and tyramine

Table 2. Linearity and sensitivity data of the developed RP-HPLC method for the determination of the biogenic amines.

Biogenic amine	Retention time (min)	Regression equation[a]	Linear range (μg L^{-1})	R^2	LOD[b] (μg L^{-1})	LOQ[c] (μg L^{-1})
CAD	11.3	y = 0.345x + 0.471	1.02–16.35	0.9925	0.03	0.09
HIS	4.7	y = 0.307x + 0.544	1.11–17.78	0.9961	0.23	0.71
TYR	7.8	y = 0.251x + 0.551	1.37–21.95	0.9982	0.08	0.13

[a]y = bx + a; y:area/$area_1$* (areas obtained for concentrations 1.02 μg L^{-1} for cadaverine, 1.11 μg L^{-1} for histamine and 1.37 μg L^{-1} for tyramine)
[b]S/N = 3; [c]S/N = 10

Table 3. Method accuracy and precision

	Intra-day (RSD%) (n = 5)		Inter-day (RSD%) (n = 5)		Recovery (%) ± RSD (%) (n = 5)	
	t_R	Area	t_R	Area	Low	High
CAD	0.89	0.41	0.49	1.72	98.78 ± 0.16	101.35 ± 1.65
HIS	0.74	0.32	0.35	2.23	101.07 ± 1.07	99.61 ± 0.68
TYR	0.47	0.58	0.64	2.85	100.33 ± 0.47	102.12 ± 0.22

Recovery (in%) found in the analysis of two different apple juice samples spiked at low (5 μg L^{-1}) and high (15 μg L^{-1}) levels; RSD, relative standard deviation; t_R, retention time

were added to the samples for the analysis using the proposed procedure. The expired juice sample was derivatized as previously described. The mixture was evaporated and the precipitates were redissolved in 1 mL of mobile phase and then 20 μL injected into the system. The recovery values were obtained from the regression equation.

The data obtained showed that cadaverine, histamine and tyramine produced peak heights as for their standard solutions at previously determined retention times on the chromatogram of the standard mixture. The accuracy of the method was evaluated from the calculation of recoveries after spiking expired apple juice at two different concentration ranges for each biogenic amine. Table 3 shows the corresponding results for cadaverine, histamine and tyramine with recoveries in the range from 98 to 102%. Consequently, these biogenic amines can be quantified using the proposed method with high accuracy.

The results showed that the accuracy, precision and reproducibility of the proposed procedure is excellent for the determination of biogenic amines in samples.

3. 3. Determination of Biogenic Amines in Expired Apple Juice Samples

The proposed procedure has been applied to expired apple juice samples for the determination of biogenic amines. The developed method was applied to expired apple juice samples since they contain biogenic amines due to the passing of the usage period. Two different brands of expired apple juice samples were purchased from local markets in Samsun (Turkey). After the optimization of chromatographic method, it was used to determine the biogenic amines in the expired apple juice samples. Fig. 2 shows typical chromatograms of biogenic amines in 20 mL of expired apple juice samples. The results are given in Table 4. The chromatogram of the expired apple juice sample indicates the sensitivity of the method. As may be seen from the chromatograms of the real samples, histamine and cadaverine were not detected in any of the two different brands of samples. The experimental results showed that tyramine at low concentration occurred in two expired apple juice samples. Thus, it was concluded that more tyramine was formed than the other biogenic amines resulting from degradation of apple juice. The peaks appearing for tyramine correspond to 5.16 and 2.21 μg L^{-1} for sample 1 and sample 2, respectively. These results proved that low ppb levels of biogenic amines in real samples could successfully be determined.

3. 4. Comparison with the Reported Methods

The comparison of the proposed method with the previously reported methods (published over the period 2009–2017) is presented in Table 5. The proposed method enabled the LOD 1.02–21.95 μg L^{-1} for biogenic amines without complex pre-treatment. The LOD obtained by the proposed procedure are well compared to the previously reported methods presented in Table 5.

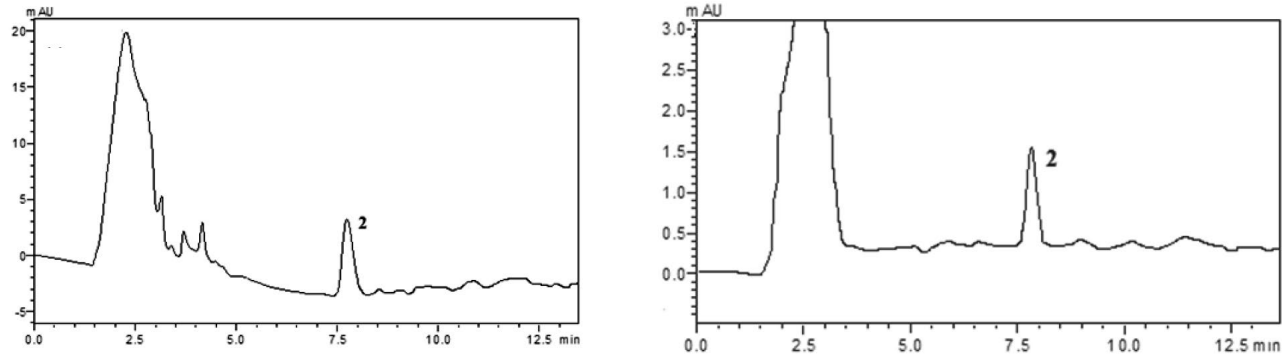


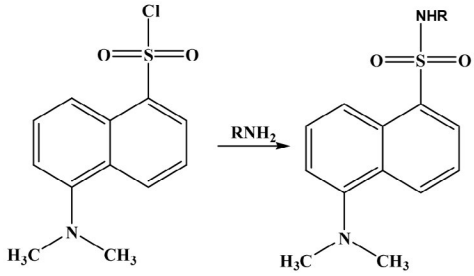
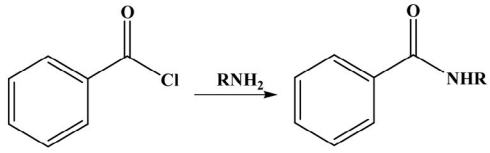
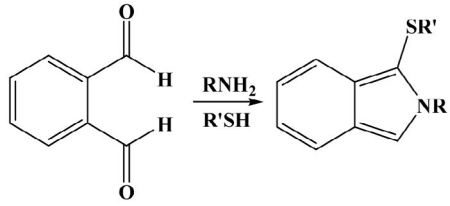
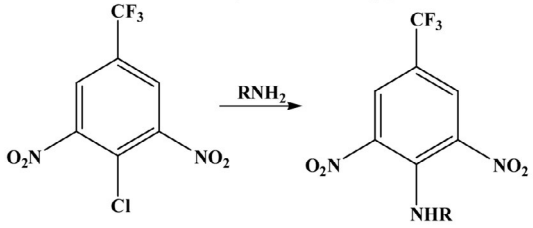
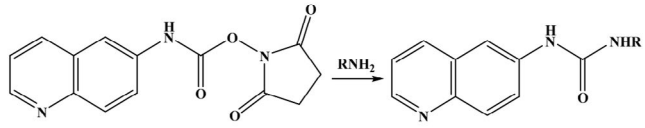
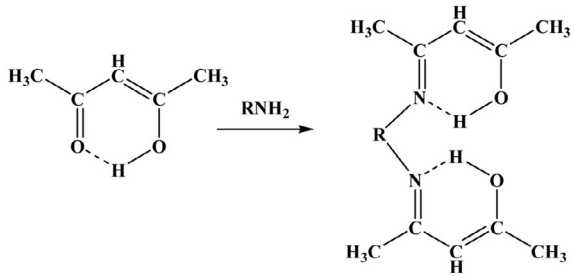
Figure 2. RP-HPLC chromatogram of biogenic amines obtained for the apple juice samples; (a): Sample 1, (b): Sample 2; (2) tyramine. Chromatographic conditions were the same as in given Fig. 1. (see Table 3 for analytical results)

Table 4. Biogenic amine contents in two apple juice samples calculated as mean[a] and RSD (%)[b].

Apple Juice Samples	CAD (μg L^{-1})		HIS (μg L^{-1})		TYR (μg L^{-1})	
	Mean	RSD	Mean	RSD	Mean[a]	RSD[b]
Sample 1	BDL[c]	–	BDL	–	5.16	2.03
Sample 2	BDL	–	BDL	–	2.21	1.89

[a] $\mu = \bar{X} \pm \frac{t_s}{\sqrt{n}}$ (The results were obtained with 95% confidence level for n = 5) [b]Relative standard deviation
[c]Below the detection limit

Table 5. Comparison of literature HPLC methods (published over the period 2009–2017) for biogenic amines involving the derivatization with principle reagents.

Derivatizing reagent/reaction scheme	Method	Sample	LOD	Reference
Dansyl chloride 	HPLC-FD	Wine, fruit nectar	0.06–8 mg L^{-1}	[13]
	HPLC-FD	Fruit juices	0.006–0.0077 mg L^{-1}	[14]
	HPLC-UV	Tomato, ketchup, orange juice, soybeansauce, fish sauce	0.001–50 mg L^{-1}	[15]
Benzoyl chloride 	HPLC-UV	Wine	95.32–1433 mg L^{-1}	[16]
	HPLC-UV	Wine, beer	0.05–8 μg mL^{-1}	[17]
o-Phthaldialdehyde 	HPLC-FD	Fish	1.5 mg kg^{-1}	[18]
2-Chloro-1,3-dinitro-5-(trifluoromethyl)benzene 	HPLC-UV	Wine	0.09–9 mg L^{-1}	[19]
6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate 	HPLC-FD	Wine	0.027–0.070 mg L^{-1}	[20]
Acetlyacetone 	HPLC-UV	Expired apple juice	1.02–21.95 μg L^{-1}	This work

4. Conclusions

A reliable procedure was developed for the determination of biogenic amines in expired apple juice samples. Biogenic amines, cadaverine, histamine and tyramine, were converted to their acetylacetone derivatives in water-methanol medium. The proposed method involves a pre-column derivatization of species followed by HPLC separation with Thermo Hypersil Gold reversed-phase column and UV detection at 315 nm. The detection limits of 0.03, 0.23, and 0.08 μg L^{-1} were obtained cadaverine, histamine, and tyramine, respectively. The proposed procedure was applied to the analysis of two different brands of apple juice samples. The recovery rates obtained between 98.78 and 102.12% with RSD of 0.16–1.65% for biogenic amines from the analysis of 20 mL of expired juice samples were very satisfactory. The proposed chromatographic method presented here is simple, convenient and cost effective. The method is also accurate and precise for the determination of biogenic amines in real samples and it may have a potential for accurate determination of biogenic amines from other food and beverage samples.

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Povzetek

Opisujemo točno in občutljivo metodo za kvantifikacijo sledov treh biogenih aminov v vzorcih jabolčnega soka s pretečenim rokom, ki je osnovana na reverznofazni tekočinski kromatografiji (RP-HPLC) z UV-vidno detekcijo. Biogene amine: kadaverin, histamin in tiramin smo pretvorili v njihove acetilacetonske derivate v vodno-metanolnem mediju. V predlagani metodi predkolonski derivatizaciji zvrsti sledi RP-HPLC separacija na Thermo Hypersil Gold reverznofazni koloni in UV detekcija pri 315 nm. V predlagani metodi smo uporabili pretok 0,9 mL min^{-1}. Učinkovito ločbo biogenih aminov smo z uporabo 35:65% (*v/v*) mešanice acetonitril-voda kot mobilne faze izvedli v 11 min z dobro ločljivostjo. Meje zaznave so bile 0,03 μg L^{-1} za kadaverin, 0,23 μg L^{-1} za histamin in 0,08 μg L^{-1} za tiramin. Predlagano metodo smo uspešno uporabili za analizo dveh komercialno dosegljivih vzorcev jabolčnega soka s pretečenim rokom. Za analizo 20 mL vzorca smo dobili izkoristke med 98,78 in 102,12% z RSD 0,16–1,65%, kar kaže, da so bili izkoristki za biogene amine zelo zadovoljivi.