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Determination of biogenic amines as their benzoyl derivatives after cloud point extraction with micellar liquid chromatographic separation

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Abstract

The advantages of micellar cloud point extraction combined with a surfactant-assisted separation in a HPLC system are presented as a method for the effective separation and determination of nine biogenic amines in fish substrates. Benzoyl derivatives of the amines are extracted inside the micelles of a non-ionic surfactant, Triton X-114, and separated with gradient elution micellar liquid chromatography. Quantification was performed by measuring the UV absorbance of the benzene ring at 254 nm. Detection limits of the nine biogenic amines were in the vicinity of 0.01 mg l^{-1} which are ~10 times lower than those of the conventional method (HPLC–UV) and 100 times lower than those of micellar electrokinetic capillary chromatography. The correlation coefficients of determinations were 0.9911–0.9996. The method was applied for the determination of putrescine, cadaverine, agmatine, tyramine, tryptamine, phenylethylamine, spermine, spermidine and histamine in trout samples. Recovery of the proposed method ranged from 95 to 103.5%.

Keywords: Cloud point extraction; Extraction methods; Micellar liquid chromatography; Gradient elution; Biogenic amines

1. Introduction

Biogenic amines (BAs) are low-molecular-mass organic bases of aliphatic, aromatic and heterocyclic structure. Their occurrence in foodstuffs is abundant especially in meat and fish products either as physiological constituents or as a result of bacterial growth and spoilage. Their primary source is the decarboxylation of free amino acids, thus the level of BAs in a food product is often considered as a marker of spoilage during storage and therefore a quality index [1–4]. Several BAs (e.g. histamine) are regarded as toxic when exceeding a certain level and are consequently accused of causing food poisoning. Both the European Union (EU) and the US Food and Drug Administration (FDA) have set limits to prevent BA intoxication by spoiled food [5–7].

The simultaneous determination of a group of biogenic amines is usually performed by a chromatographic method such as thin-layer chromatography (TLC) [8], gas chromatography (GC) [9], high-performance liquid chromatography (HPLC) [10–12] and even micellar electrokinetic chromatography (MECC) [13,14].

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Nevertheless, direct application of a single chromatographic approach is not feasible for the simultaneous determination of BAs because of the lack of a common chromophore or fluorophore in their structure. Therefore a derivatization step usually precedes chromatographic separation and detection, introducing an easily detectable label group within the amine structure.

Dansyl chloride [15], dabsyl chloride [16], fluorescamine [17] and *o*-phtalaldehyde [18,19] are some derivatizing agents used for the determination of BAs with satisfactory results, even though benzoyl chloride is a common reagent. In most of the published work the determination of BAs as their benzoyl derivatives is regarded as ''standard procedure'' because both benzoylation and consequent separation is significantly less time consuming than other processes [20,21].

In almost all approaches the derivatized amines have to undergo extraction in a suitable organic solvent, evaporation to dryness and re-dissolution before injection in the HPLC system. Thus, the risk of sample loss and contamination is introduced along with extensive analysis time. Furthermore, the chromatographic conditions result either in insufficient separation or prolonged analysis which may require longer than an hour.

In the proposed approach, the well-established cloud point extraction technique is introduced in order to replace the use of an organic solvent (usually diethyl ether). In aqueous solutions the solubility of most anionic, non-ionic and zwitterionic surfactants decreases rapidly producing turbidity when heated above a temperature referred to as the cloud point (CPT). At higher temperatures, two distinct phases are formed; one consisting almost entirely of the surfactant and the other containing a small portion of the surfactant equal to the critical micellar concentration (CMC). During formation, the surfactant micelles have proven to entrap several hydrophobic substances, isolating them from the bulk aqueous solution. The mechanistic procedure resembles the cleaning effect displayed by soaps and detergents. Mere centrifugation and discharge of the aqueous phase can easily separate the two phases. The surfactant-rich phase can subsequently be subjected to classical analysis such as flow injection analysis (FIA) and HPLC coupled with a variety of detectors [22–26].

In this perspective, the hydrophobic benzoyl derivatives—which are stable upon heating as well as their precursor amines—of the target nine BAs are entrapped within the micelles of the non-ionic surfactant Triton X-114 and separated by centrifugation. The surfactant-rich phase is re-dissolved in methanol and injected in the HPLC.

The separation of the BAs was achieved employing micellar liquid chromatography (MLC) with gradient elution where the mobile phase consisted of a 0.40 *M* sodium dodecyl sulfate (SDS) solution and acetonitrile (ACN).

Although surfactants have been extensively used for both the extraction of hydrophobic organic molecules (polycyclic aromatic hydrocarbons, vitamins, herbicides etc.) and the chromatographic separation of biogenic amines and amino acids in MECC, it seems that no attention has been paid to their potential usefulness in the extraction of BAs by cloud point extraction (CPE) and their subsequent separation by MLC.

2. Experimental

2.1. Apparatus and software

The liquid chromatograph consisted of a Shimadzu 10AD series for HPLC equipped with a UV–visible variable wavelength detector (Shimadzu) set at 254 nm. A LiChrospher 100 RP-18 [244×4.4 mm I.D., 5 μm column linked to a LiChrospher guard precolumn (10×4.6 mm I.D.)] and thermostated at 30 °C in a CTO-10A Shimadzu column oven, was used for all separations. Data collection and manipulation were performed by means of CLASS-VP Shimadzu automated software for chromatography. A Vortex Velp Scientifica mixer was used for thorough mixing of solutions. A Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments) was used for the separation of BAs from the fish samples. A thermostated bath maintained at the desired temperatures was used for cloud point temperature experiments and phase separation was assisted using a centrifuge (Hettich, Universal).

2.2. Reagents

All reagents were of analytical grade or of the

highest grade available. Tryptamine hydrochloride, 2-phenylethylamine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, histamine dihydrochloride, tyramine hydrochloride and agmatine sulfate salt were obtained from Sigma–Aldrich (USA). Suitable amounts of each amine salt were dissolved in 10 ml of distilled water to prepare 1 mg/ml stock solutions. Working solutions of each amine and of the amine mixture were prepared daily by appropriate dilutions of the standard solutions. Water and acetonitrile (Merck, Darmstadt, Germany) used for chromatographic separation were HPLC grade. Benzoyl chloride (Merck) used for the derivatization was ''*for synthesis*''. Triton X-114 (Aldrich, catalog no. 36,934-9) was used, without further purification, to prepare a 100 g l^{-1} aqueous solution. SDS 98% (Aldrich, catalog no. 86,201-0) and NaH_2PO_4 were used to prepare the micellar carrier.

2.3. General procedure

2.3.1. Sample preparation

Fish samples used in shelf life experiments were cut in small pieces. Five grams of each sample were ground in a Waring blender for 3 min and thoroughly homogenized with 10 ml trichloroacetic acid (TCA) 6% (w/v). The homogenates were centrifuged (12 000 rev./min, 20 min, 4 °C) to allow precipitation and filtered twice through Whatman No. 2 filter paper. The filtrates were transferred to 10-ml volumetric flasks and diluted with 6% (w/v) TCA to the mark.

The benzoyl derivatives of amines were prepared according to Yen and Hsieh [21]. Two ml of the standard or sample solution and 1 ml of a 2 *M* NaOH solution were added into a Hach centrifugal vial, followed by 10 μl of benzoyl chloride and 50 μl of a 100 g l^{-1} Triton X-114 solution. The mixture was shaken vigorously in a vortex mixer and allowed to stand for 15 min in a water bath at 30 °C. Two ml of a saturated NaCl solution were added to stop the benzoylation. The temperature was raised to 60 °C for 5 min in order for the micelles to form and effectively encapsulate the benzoyl derivatives. Separation of the surfactant-rich phase from the bulk aqueous solution was achieved by centrifugation at 4000 rev./min for 5 min. Then the vials were placed in an ice-bath to increase the viscosity of the micellar phase. Subsequently the aqueous phase was easily decanted by inverting the tubes. The remaining surfactant-rich phase was re-dissolved in 200 μl of HPLC-grade methanol, filtered through Millipore 0.22 μm poly(vinylidene difluoride) (PVDF) hydrophilic microfilters, and 20 μl of the filtrate were injected in the HPLC loop for subsequent analysis. Fig. 1 shows a schematic representation of the analytical procedure.

2.4. Separation of the benzoyl derivatives by MLC

A micellar liquid chromatographic separation approach was attempted for the effective and rapid separation of the nine BAs. The mobile phase consisted of a 0.40 *M* SDS aqueous solution buffered to pH 3.0 with a 0.02 *M* phosphate buffer solution and of HPLC-grade ACN. The gradient elution program adopted was set at 1.1 ml/min starting with

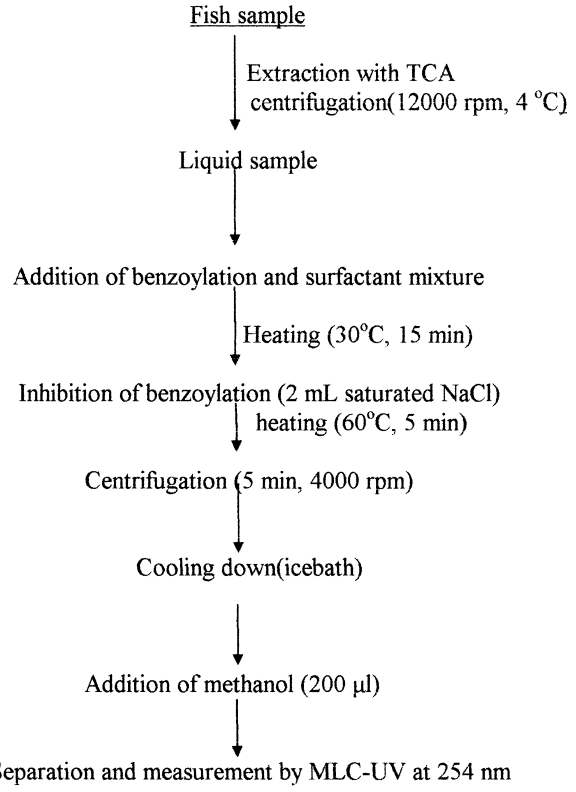

Fig. 1. Schematic representation of the major steps of the overall procedure for the extraction, separation and determination of the nine biogenic amines.

an acetonitrile–SDS mixture (30:70, v/v) for 1 min. The program proceeded linearly to acetonitrile–SDS (42:58, v/v) over 1 min and remained for another 2 min. This was followed by linear increase to acetonitrile–SDS (50:50, v/v) over 2 min and the same composition was maintained for another 2 min. Then the solvent mixture assumed its primary composition acetonitrile–SDS (30:70, v/v) within a 1-min period.

Determination and quantification of the amines was performed by measuring the absorbance of the benzene ring at 254 nm with a UV–Vis flow spectrophotometer and integrating the resulting peaks with a suitable software program.

3. Results and discussion

The conditions for the benzoylation procedure are almost the same as described in previous work [20,21]. Compared to the standard method, similar results were obtained by heating the benzoylation mixture for only 15 min instead of 40. This is attributed to the presence of the surfactant which creates an environment with both hydrophilic and hydrophobic properties allowing for a homogeneous rather than a heterogeneous reaction mixture between aqueous amines and hydrophobic benzoyl chloride.

The surfactant amount required for optimum extraction was tested with regard to CPE efficiency. It was found that 50 μl of a 100 g l^{-1} solution of Triton X-114 can effectively preconcentrate and extract up to 50 mg of BAs. Smaller amounts are not recommended because the presence of benzoyl chloride suppresses the clouding capacity while larger amounts demand larger volumes of methanol in order for the surfactant-rich phase to be re-dissolved thus reducing the preconcentration factor and the detection limits of the method.

After selecting the optimum conditions for the benzoylation and the subsequent cloud point extraction, the influence of chromatographic conditions on the separation of the nine biogenic amines (Phe, Put, Cad, Spd, Spm, Agm, Tyr, Tpm, and Him) derivatized with benzoyl chloride was an important target of this investigation. The derivatives, although hydrophobic and electrically uncharged, still contain several functional groups which can modify their retention capacity. Under this perspective, micellar liquid chromatography was used for separation. The nature of the surfactant phase was examined for its ability to separate the target amines. Although cationic surfactants are expected to have greater affinity than anionic or non-ionic surfactants for amine groups—due to their positive charge—through Lewis acid–base interactions, their use as solvents in MLC results in extensive retention times making analysis time-consuming. In contrast, anionic surfactants under acidic conditions exhibit not only shorter retention times but a higher resolution of the chromatographic peaks as well. Of the several anionic surfactants tested, SDS was chosen as the most suitable one due to its low cost and wide commercial availability. With regard to analytical performance, the majority of anionic surfactants gave similar results.

The acidity of the micellar solution is also important. Since extreme acidic or alkaline conditions are not recommended in HPLC, it was found that within a pH range of 2–10, a pH value below 5 produces satisfactory results. A pH value of 3 was finally selected for producing optimum resolution and analytical response. Such behavior is expected because in acidic solution the amine groups of the benzoyl derivatives are in the form of positively charged alkylammonium salts which interact with SDS anionic species either electrostatically or through hydrogen bonding. Since each derivative has a different K_a value and therefore different protonation behavior, it is logical to expect that this property would significantly contribute to their resolution by MLC [27]. The concentration of SDS was also evaluated. A 0.40 mol l^{-1} solution was finally adopted throughout the experiments for producing optimum results. Compared to the SDS concentrations used in conventional MLC or even MECC, the value of 0.40 exceeds the ordinary concentrations used. This is dictated by the need for the surfactant phase to retain its micellar structure even in an acetonitrile gradient of 30% (v/v) [28,29].

The chromatographic profile obtained under the aforementioned conditions is depicted in Fig. 2. All nine amines were effectively separated within 8 min with good peak resolution, sharpness, symmetry and reproducibility. The retention time of each amine is depicted in Table 1. The peaks of benzoyl chloride

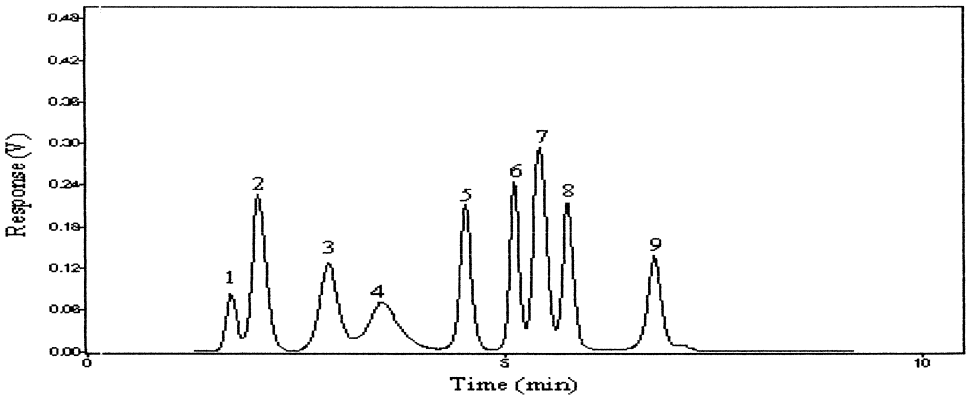

Fig. 2. Chromatogram of a standard solution of the benzoyl derivatives of the nine biogenic amines obtained after MLC separation (the order of the chromatographic peaks is that depicted in Table 2).

Table 1
Retention times of the nine biogenic amines separated by the proposed MLC gradient elution program

Peak no.	Biogenic amine	Retention time[a] (min)
1	Cadaverine (Cad)	2.00 (±0.12)
2	Tyramine (Tyr)	2.35 (±0.18)
3	Putrescine (Put)	3.10 (±0.10)
4	Agmatine (Agm)	3.75 (±0.30)
5	Spermidine (Spd)	4.75 (±0.20)
6	Tryptamine (Tpm)	5.33 (±0.15)
7	Phenylethylamine (Phe)	5.65 (±0.10)
8	Spermine (Spm)	5.97 (±0.19)
9	Histamine (Him)	7.01 (±0.22)

[a] Average of five runs.

and of Triton X-114 are eluted within the first minute in the chromatogram. Thus no interfering peaks appear. By selecting to collect data between minute 2 and 9 of the run no other peak appears on the chromatogram except those of the nine amine derivatives.

Standard curves of the nine amines were prepared by running both separate amine solutions and mixtures giving similar results. The analytical merits of the calibration curve for each amine are highlighted in Table 2. The results are satisfactory and the detection limits (0.01–0.1 mg l^{-1}) are a 10-fold improvement on those obtained by other HPLC

Table 2
Analytical features for the quantification of the nine biogenic amines after CPE and MLC separation with UV detection at 254 nm

Amine	Linear equation	R^a	LOD[b] (mg l^{-1})	LOQ[c] (mg l^{-1})
Agmatine	$S^d = 50\,002C$ (mg l^{-1}) + 25 620	0.9992	0.040	0.120
Cadaverine	$S = 39\,652C$ (mg l^{-1}) + 19 199	0.9996	0.022	0.060
Histamine	$S = 19\,903C$ (mg l^{-1}) + 6144	0.9994	0.033	0.100
Phenylethylamine	$S = 21\,949C$ (mg l^{-1}) + 13 471	0.9963	0.100	0.300
Putrescine	$S = 10\,143C$ (mg l^{-1}) + 4604	0.9911	0.015	0.050
Spermidine	$S = 30\,389C$ (mg l^{-1}) + 63 438	0.9971	0.080	0.250
Spermine	$S = 28\,043C$ (mg l^{-1}) + 26 244	0.9990	0.045	0.140
Tryptamine	$S = 30\,694C$ (mg l^{-1}) + 16 043	0.9993	0.055	0.180
Tyramine	$S = 28\,717C$ (mg l^{-1}) + 15 590	0.9994	0.050	0.150

[a] Correlation coefficient.
[b] Limit of detection defined as three times the signal-to-noise ratio.
[c] Limit of quantification defined as 10 times the signal-to-noise ratio.
[d] Peak area (arbitrary units).

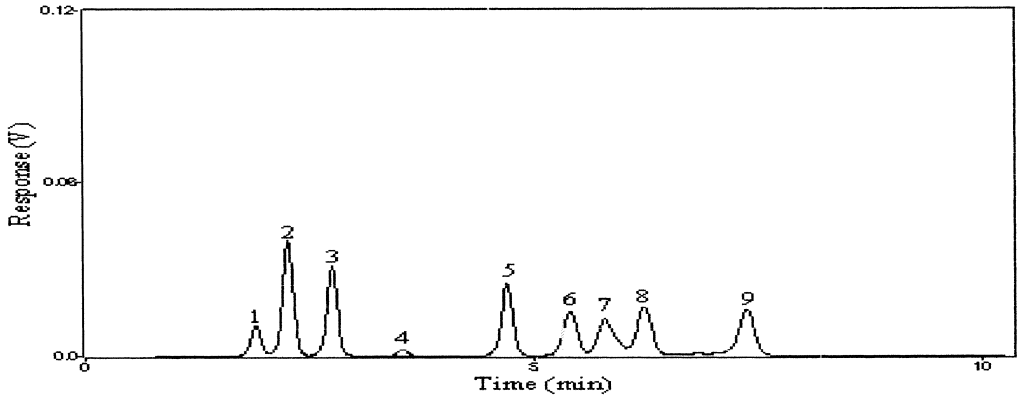

Fig. 3. Typical chromatogram of the benzoyl derivatives of the nine biogenic amines from the trout sample after CPE and MLC.

methods employing spectrophotometric quantification of the benzoyl derivatives [10–14].

The proposed method was applied for the determination of biogenic amines in whole and filleted trout samples which were employed in an experiment targeted on the effects of specific storage conditions on shelf life extension of trout. A typical chromatogram of the analyzed amines in a trout sample is presented in Fig. 3. The results are depicted in Table 3. It is obvious that all amines are detected and quantified effectively with the proposed method. Agmatine although detectable, cannot be quantified because its peaks are below the respective quantification limit. This is expected because agmatine mainly appears in squid and other invertebrata [1,2]. During the first week of fish storage the amine levels are low and they are not usually quantified because even contemporary spectrophotometric methods do not reach such low detection limits. The amines found in every sample are spermine and spermidine. This is expected due to the fact that these amines are natural products of live fish metabolism [1,2]. From the remaining amines the most abundant and frequently present are putrescine and cadaverine which are terminal products of the post mortem decomposition of organic matter [1,2]. On the other hand, histamine

Table 3
Biogenic amine content of whole and filleted trout samples stored in ice as a function of storage time

Storage time[a]	Putrescine[b]	Cadaverine	Tyramine	Spermidine	Tryptamine	Phenylethylamine	Spermine	Histamine
Filleted trout[c]								
15	24.50	3.50	3.27	10.31	2.30	1.39	4.67	1.10
18	33.10	6.00	6.19	14.40	7.33	7.82	5.74	1.34
Whole trout[c]								
15	16.90	0.28	2.56	7.79	0.25	1.01	5.01	1.56
18	23.10	2.67	2.97	13.59	0.69	2.21	5.13	1.61
Filleted trout[d]								
15	25.10	3.50	3.35	9.95	2.35	1.43	4.54	1.03
18	32.45	6.12	6.52	15.03	7.17	7.69	5.82	1.40
Whole trout[d]								
15	17.80	0.27	2.74	8.01	0.25	0.98	5.08	1.58
18	23.53	2.55	3.14	13.12	0.69	2.32	5.24	1.74

[a] Days of storage in ice.
[b] Results expressed in μg g^{-1} of fish sample.
[c] Analyzed after MLC separation–UV detection (proposed method).
[d] Analyzed after HPLC separation–UV detection (conventional method).

Table 4
Recoveries of biogenic amines spiked into water and trout fillet extracts

Amine	Initial content ($\mu g\ g^{-1}$)	Recovery of spiked amine (%)			
		0.5 $\mu g\ g^{-1}$		5.0 $\mu g\ g^{-1}$	
		CPE	Solvent extraction	CPE	Solvent extraction
Putrescine	18.6	99.0	101.0	100.0	100.0
	0	98.5	100.5	100.0	100.0
Cadaverine	1.4	98.0	105.0	99.5	102.0
	0	97.0	103.5	100.0	100.5
Tyramine	1.64	95.0	90.0	96.5	91.5
	0	99.0	92.0	99.0	94.0
Spermidine	7.37	96.0	95.5	96.0	98.0
	0	98.5	97.0	98.5	99.0
Tryptamine	0.68	97.0	83.0	98.0	85.5
	0	100.0	84.5	99.0	86.0
Phenylethylamine	0.97	97.5	83.0	99.0	87.5
	0	100.5	85.0	100.0	88.0
Spermine	3.50	99.0	90.5	100.0	90.0
	0	101.0	95.0	100.5	92.5
Histamine	0.83	103.5	88.0	103.0	92.0
	0	102.0	92.0	101.5	94.0
Agmatine	0	95.5	85.5	97.0	88.0

levels are low even after 18 days of storage and remain below the FDA's tolerance level of 100 mg kg^{-1} revealing that no histamine poisoning is induced during normal trout storage.

Comparison of the results was performed using the standard HPLC procedure appearing in numerous articles in the literature [10–12,20,21]. The obtained results presented in Table 3 offer a satisfactory match.

In order to assess the extraction efficiency of the proposed cloud point extraction approach, recovery experiments were also performed in samples which were spiked with standard amine solution. The results presented in Table 4 show that CPE gives almost quantitative extractions in contrast with solvent extraction in which a recovery above 85% is considered adequate.

4. Conclusions

The cloud point technique was applied for the first time as an alternative for the extraction of biogenic amines in aqueous samples after their derivatization with benzoyl chloride. The derivatives were separated with gradient elution micellar liquid chromatography. Combination of these two techniques results in the proposed method with a detection and quantification limit in the area of 0.01 mg l^{-1} which is almost 10 times lower than that of conventional detection by HPLC–UV [10–12,15,16] and 100 times lower than that of MECC [13,14]. Due to the nature of CPE, more than 20 samples can be benzoylated and extracted within 0.5 h depending on the capacity of the centrifuge. Thus the average time of analysis is greatly reduced (~12 min) compared to the conventional extraction with diethyl ether (almost 30 min). The extraction recoveries are also improved reaching almost 100%. Moreover, MLC offers a more rapid chromatographic analysis (8 min against more than 15 min) while enhancing the resolution of some peaks that show overlapping in conventional HPLC. Compared to MECC which also employs a surfactant mobile phase, the proposed method has lower detection limits and it also allows the determination of tyramine which cannot be detected by MECC. In conclusion, the proposed method is a simple, rapid and effective alternative to the conventional HPLC method for the simultaneous determination of biogenic amines. Similar attempts can be

further made for the differentiation of other biologically attractive substances.

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