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Direct separation and detection of biogenic amines by ion-pair liquid chromatography with chemiluminescent nitrogen detector

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ABSTRACT

Analysis of biogenic amines is critical to pharmaceutical and food industry due to their biological importance. For many years, the determination of biogenic amines has relied on high performance liquid chromatography (HPLC) coupling with pre-, on-, or post-column derivatization procedures to enable UV or fluorescent detections. In this study, 14 biogenic amines were separated on a Phenomenex Luna® Phenyl-Hexyl column by an ion-pair liquid chromatography method using perfluorocarboxylic acids as ion-pair reagents and detected by a chemiluminescent nitrogen detector (CLND). This direct separation and detection HPLC method eliminated the time consuming and cumbersome derivatization procedures. Compared with HPLC–UV (post-column derivatization with ninhydrin) and HPLC-charged aerosol detector (CAD) methods, this HPLC–CLND technique provided narrower peaks, better baselines, and improved separations and detections. Excellent linearity was acquired by CLND for each of the 14 biogenic amines ranging from less than 1 ng to about 1000 ng (on-column weights). The relative response factors determined by this LC–CLND method were proportional to the numbers of nitrogen atoms in each compound, which has been the characteristic of the equimolar determinations by CLND. In addition, a number of samples including beer, dairy beverage, herb tea, and vinegar were analyzed by the LC–CLND method with satisfactory precision and accuracy.

1. Introduction

As products of decarboxylation of amino acids, biogenic amines play diverse roles in cellular growth and metabolism. Due to their biological importance, biogenic amines had been determined by a variety of analytical techniques in plants [1]; foods–fruits [2], vegetable products [3], meat [4], fish [5] and milk products [6]; beverages–beer [7,8] and wine [9,10]; biological samples – fluids [11], tissues [12,13], and urines [14]. As depicted in Fig. 1, putrescine (1,4-diaminobutane/butanediamine), cadaverine (1,5-diaminopentane), spermidine, and spermine have no chromophores or fluorophores; and β-alanine (3-aminopropionic acid), γ-aminobutyric acid (GABA, 4-aminobutanoic acid), and agmatine have very weak UV absorbance – not suitable for low level detections. In addition, these small, polar amines hardly retain on a reversed phase HPLC column. Therefore, liquid chromatography coupling with pre or post-column derivatization had been the most common practices in analyzing variety of samples containing biogenic amines.

Dansyl chloride [15] and 9-fluorenylmethyl chloroformate (FMOC-Cl) [10,11,16] are often used in pre-column derivatization to convert biogenic amines into adducts to improve the on-column separation and spectroscopic responses. Depending on the complexity of derivatization and sample matrix, multiple sample preparation steps may have to be carried out prior to HPLC analysis. Thus, recovery studies are warranted to demonstrate the accuracy and precision of sample preparation processes. Pre-column derivatization is nevertheless preferred in a number of cases as it is less demanding on instrumentation and system setups.

Post-column derivatization, on the contrary does not require the extra sample preparations as biogenic amines can be derivatized online after HPLC separation prior to detection. O-phthaldialdehyde (OPA) and ninhydrin [17] had been widely used in post-column derivatization. The post-column approach did provide the benefits of automatic online derivatization – eliminating the extra sample manipulations which could have brought in imprecision and prolonged sample preparations. However, post-column derivatization requires the addition of extra chromatographic components, stable supply of derivatization reagents with acceptable purities, and delicate setups. The turbulence generated during the online mixing must be controlled to maintain a satisfactory chromatographic baseline. It is inevitable that the increased system dwell volume and instrument bandwidth will

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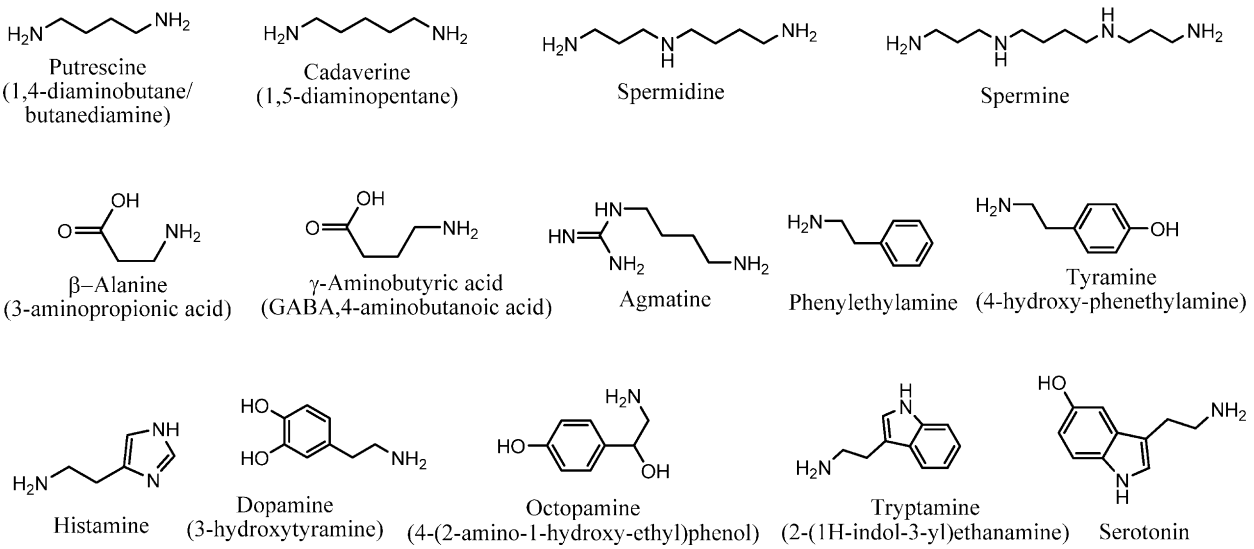

Fig. 1. Structures of 14 biogenic amines.

bring about peak broadening and sample dilution. This has been the inherent drawback of post-column derivatization method and creates formidable challenges for a HPLC practitioner improving resolution, signal-to-noise ratio, and peak capacity.

A variety of new techniques had been developed over time to overcome the inconveniences and imperfections of pre and post-column derivatizations such as on-column fluorescent derivatization method [18], capillary electrophoresis with pulsed amperometric detection [19], ion-exchange chromatography with conductivity detector [20] and integrated square-wave electrochemical detection [21]. Based on our extensive literature searches so far, no work was published demonstrating direct separation and detection of biogenic amines by a LC–CLND method.

Rather than develop and validate another LC-derivatization method for a new sample matrix with the familiar approaches, the aim of our work is to develop a simple chromatographic separation method with a direct detection of biogenic amines to eliminate the derivatization and other drawbacks. Petritis et al. [22] had extensively studied the compatibilities of a number of long perfluorinated carboxylic acids as ion-pair reagents with various detectors in the determinations of amino acids by HPLC and concluded that electrospray mass spectrometry in tandem mode and CLND are the most promising ones due to their higher sensitivity and specificity. Given that CAD was not included in their detector comparison and no derivatization was required for the amino acids LC–UV method, we decide to focus our efforts on identifying the most suitable chromatographic conditions that enables the detections of the 14 biogenic amines by UV derivatization, CAD, and CLND. In our study the impacts of perfluorocarboxylic acids as ion-pair reagents, their concentrations, and the column temperatures were closely examined. As there are no adjacent nitrogen atoms present in any of biogenic amines, equimolar determinations by CLND should be expected and the performances of the three detection techniques can be compared. Finally LC–CLND method was applied in the determination of biogenic amines in a variety of samples to demonstrate its precision and accuracy.

2. Materials and methods

2.1. Samples

Beer, herb tea, dairy beverage, and vinegar were purchased from retail stores in California, USA. All samples were filtered through a 0.2 μm cellulose acetate membrane filter prior to HPLC analysis. The contents in the herb teabag were extracted by hot drinking water. The dairy beverage was first centrifuged at 13,000 rpm for 5 min to facilitate the filtration.

2.2. Chemicals

The water used in all experiments was obtained from Milli-Q system (Millipore, Billerica, MA, USA). The other solvents/reagents used were HPLC grade methanol, isopropyl alcohol (IPA) and trifluoroacetic acid (TFA) from J.T. Baker (Phillipsburg, NJ, USA); long-chain perfluorocarboxylic acids including pentafluoropropionic acid (PFPA, 97%), heptafluorobutyric acid (HFBA, 99%), nonafluoropentanoic acid (NFPA, 97%, synonyms: perfluoropentanoic acid or nonafluorovaleric acid), and undecafluorohexanoic acid (UFHA, 97%, synonyms: perfluorocaptroic acid or perfluorohexanoic acid) from Sigma–Aldrich (St. Louis, MO, USA); Trione ninhydrin reagent T100 from Pickering Laboratories (Mountain View, CA, USA). All 14 biogenic amines: spermidine (SD), spermine (SM), phenethylamine (PHE) hydrochloride, dopamine (DO, synonym: 3-hydroxytyramine) hydrochloride, serotonin (SE) hydrochloride, octopamine (OC, synonym: 4-(2-amino-1-hydroxy-ethyl)phenol) hydrochloride, histamine (HI) dihydrochloride, agmatine (AG) sulfate, γ-aminobutyric acid (GABA, synonym: 4-aminobutanoic acid), putrescine (PU, synonym: 1,4-diaminobutane/butanediamine), cadaverine (CA, synonym: 1,5-diaminopentane), β-alanine (BAL, synonym: 3-aminopropionic acid), tyramine (TY, synonym: 4-hydroxy-phenethylamine), and tryptamine (TR, synonym: 2-(1H-indol-3-yl)ethanamine) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Stock standard solution preparation

0.2% TFA in water was prepared as sample diluent by adding 2 mL TFA in 1 L water. All biogenic amines except TR have good solubility in the 0.2%TFA. To make an approximately 800 μg/mL stock standard solution of it, about 20 mg TR was weighed and transferred into a 25 mL volumetric flask, added 1 mL methanol to dissolve and diluted to volume with sample diluent. As for the other 13 biogenic amines, about 20 mg equivalent free base of each amine was weighed and dissolved separately in 25 mL sample diluent. The free base concentration of each amine in each stock standard solution was ~800 μg/mL. To make a mixing stock standard solution, 1 mL

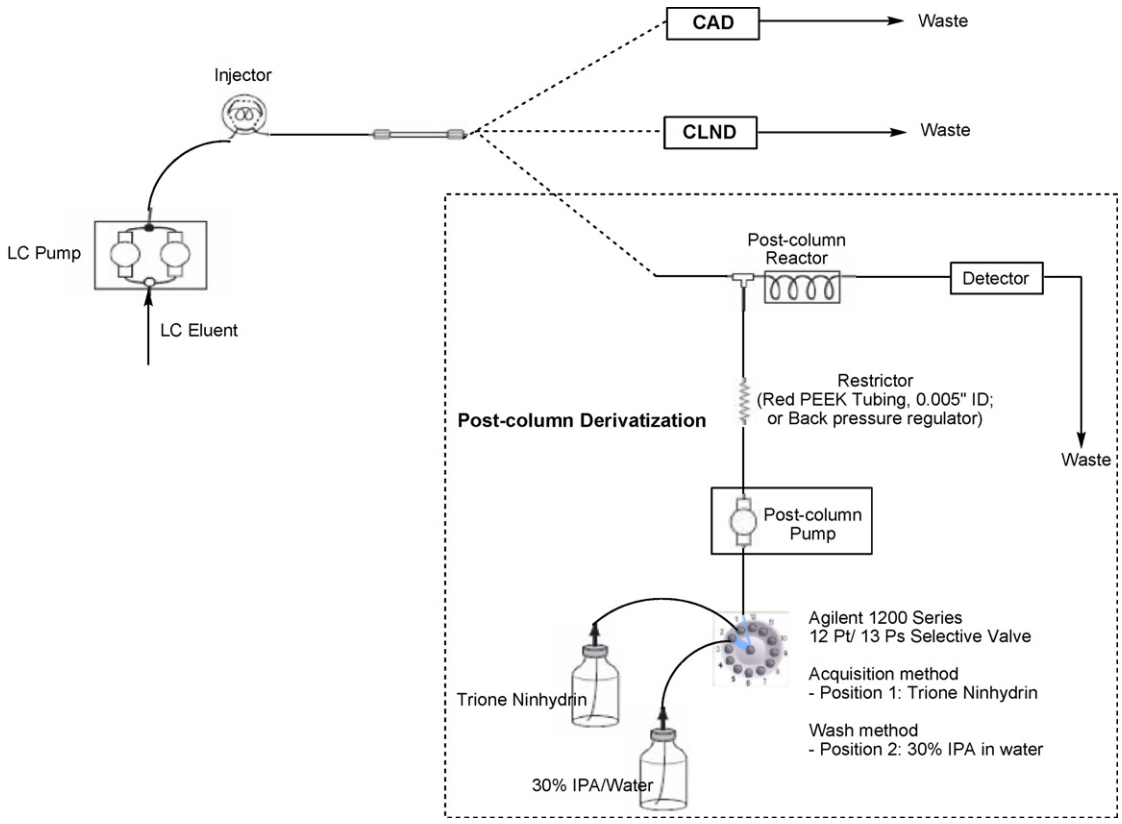

Fig. 2. Instrument setups of LC–UV post-column derivatization, LC-CAD, and LC–CLND methods.

each of the 14 stock standard solutions was pipetted into a 20 mL volumetric flask and diluted to volume with 0.2% TFA. This mixing stock standard solution contained ~40 μg/mL of each amine. In the linearity study, the mixing stock standard solution was further diluted 10 times and 100 times with sample diluent to achieve a concentration of ~4 μg/mL and ~0.4 μg/mL respectively for each amine.

2.4. Equipment

1100 and 1200 series binary pumps, autosamplers, thermostated column compartments, diode array UV detectors (Agilent, Santa Clara, CA, USA) were used in all experiments. Data acquisition and processing were controlled by ChemStation. In the post-column derivatization method, AXP pump (Dionex, Sunnyvale, CA, USA) was used as a post-column pump to deliver ninhydrin reagent, which was connected to a zero dead volume tee installed between the column outlet and a CRX400 post-column reactor (Pickering Laboratories, Mountain View, CA, USA) prior to the UV detector. In the non-derivatization methods, the following detectors were used: Antek 8060 chemiluminescent nitrogen detector (PAC, Houston, TX, USA); Charged aerosol detector (ESA – a Dionex company, Chelmsford, MA, USA). The setup details of above methods are depicted in Fig. 2. Notes for the post-column derivatization setup: (1) to minimize baseline noises, it was necessary to operate the post-column pump at a pressure ≥1000 psi, which can be achieved by installing a back pressure regulator or a 'red' PEEK tubing (1/16″ OD, 0.005″ ID) of appropriate length between the post-column pump and the tee. (2) Agilent 1200 series 12 ports/13 positions selective valve controlled by ChemStation was used to automatically flush the HPLC system with 30% IPA in water after the data acquisition runs with ninhydrin were complete (due to solvent evaporation, ninhydrin reagent tends to plug the system if flow stops and it stay on for a long period of time).

2.5. Chromatographic conditions

Luna Phenyl-Hexyl, 3 μm, 150 × 2 mm column (Phenomenex, Torrance, CA, USA) was used in the final methods. 14 biogenic amines were eluted by a linear gradient program ramping the mobile phase compositions from A:B 90:10 to A:B 5:95 in 35 min. A 10-min post run (A:B 90:10) was applied to equilibrate the column to the initial condition. Mobile phases A (0.3% v/v NFPA in water) and B (0.3% v/v NFPA in methanol) were prepared by adding 3 mL NFPA into 1 L of water and into 1 L of methanol respectively. The HPLC flow rate was set at 0.25 mL/min, column temperature at 20 °C, and autosampler injection volume at 10 μL. During method development, other columns were evaluated including Luna C18(2), 2.0 × 150 mm, 3 μm (Phenomenex, Torrance, CA, USA) and Xbridge™ Phenyl, 3.5 μm, 100 × 3 mm (Waters, Milford, MA, USA). Gradients, perfluorocarboxylic acids and their concentrations in mobile phases, flow rates, and column temperatures were varied and studied as well. In the linearity study, various injection volumes were used.

The critical operating parameters associated with detection techniques are described as below: In the post-column derivatization method, the ninhydrin reagent was pumped at 0.25 mL/min, the post-column reactor was thermostated at 110 °C and the UV–Vis detection was set at 570 nm. In the LC–CLND method, the CLND was operated with 280 mL/min of oxygen, 50 mL/min of argon, 25 mL/min of ozone, 1050 °C furnace temperature, 750 V on photomultiplier tube (PMT), 5 °C PMT cooler temperature, 1 V output, amplifier gain = 100 (high gain) × 1 (select). In the LC-CAD method, the nitrogen pressure was set at 35 psi, range = 100 pA, no filter.

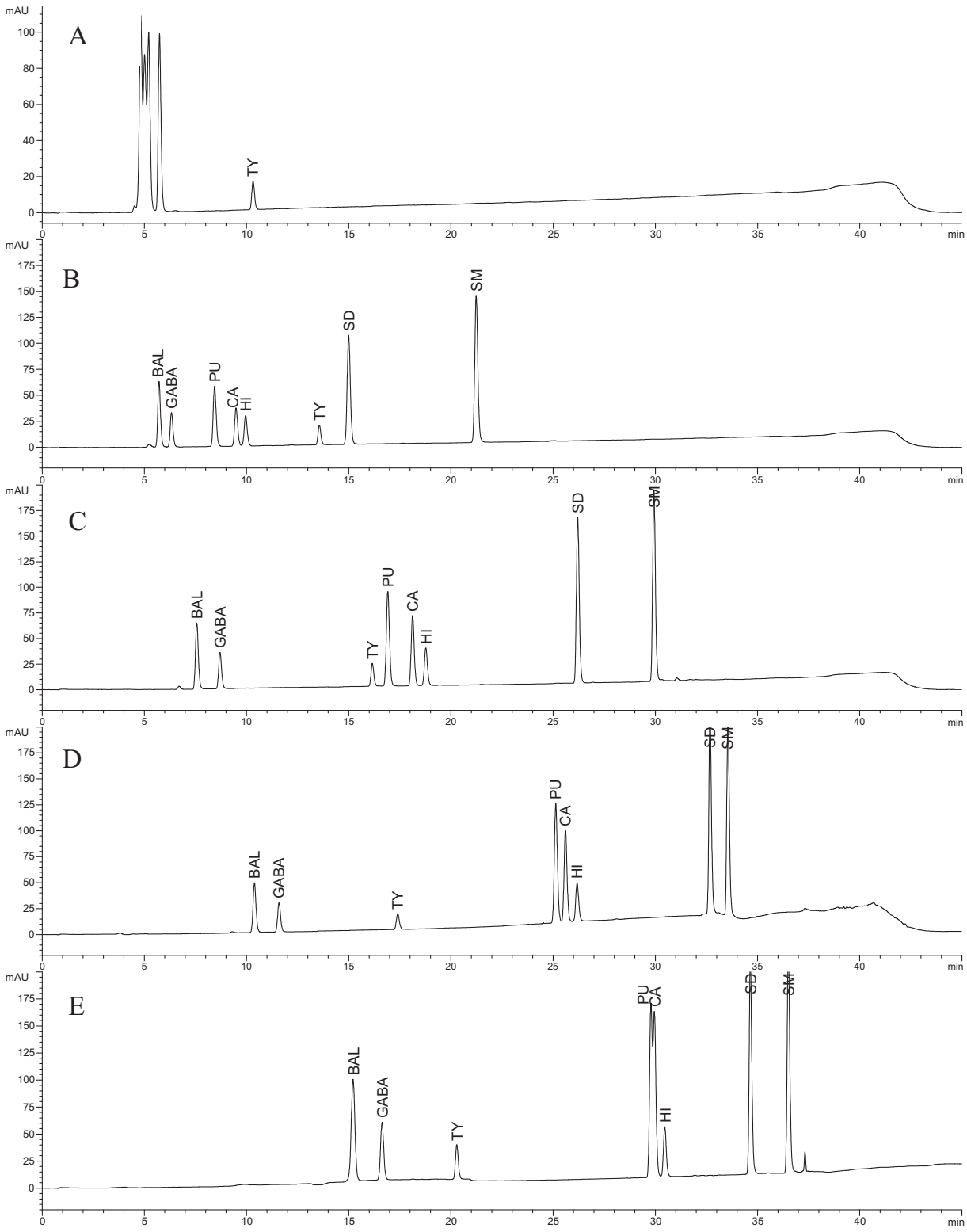

Fig. 3. Chromatograms of the mixing solution of 8 amines obtained by LC–UV derivatization method with 0.6% TFA (A), 0.6% PFPA (B), 0.6% HFBA (C), 0.6% NFPA (D), and 0.6% UFHA (E) in mobile phases.

3. Results and discussion

The response of CLND is very much structure dependent: Yan et al. [23] had demonstrated that when analytes contain isolated nitrogen atoms, the response is close to quantitative with a variation about 10–20% depending on structures; when adjacent nitrogen atoms are connected by a double bond, the response is near zero, indicating the strong tendency of N_2 formation; when connected with a single bond, without substitution on nitrogen or in the molecule, very low responses were obtained (0.0–0.08/nitrogen atom) in comparison with molecules only have isolated nitrogen atoms. For the 14 biogenic amines, none of them

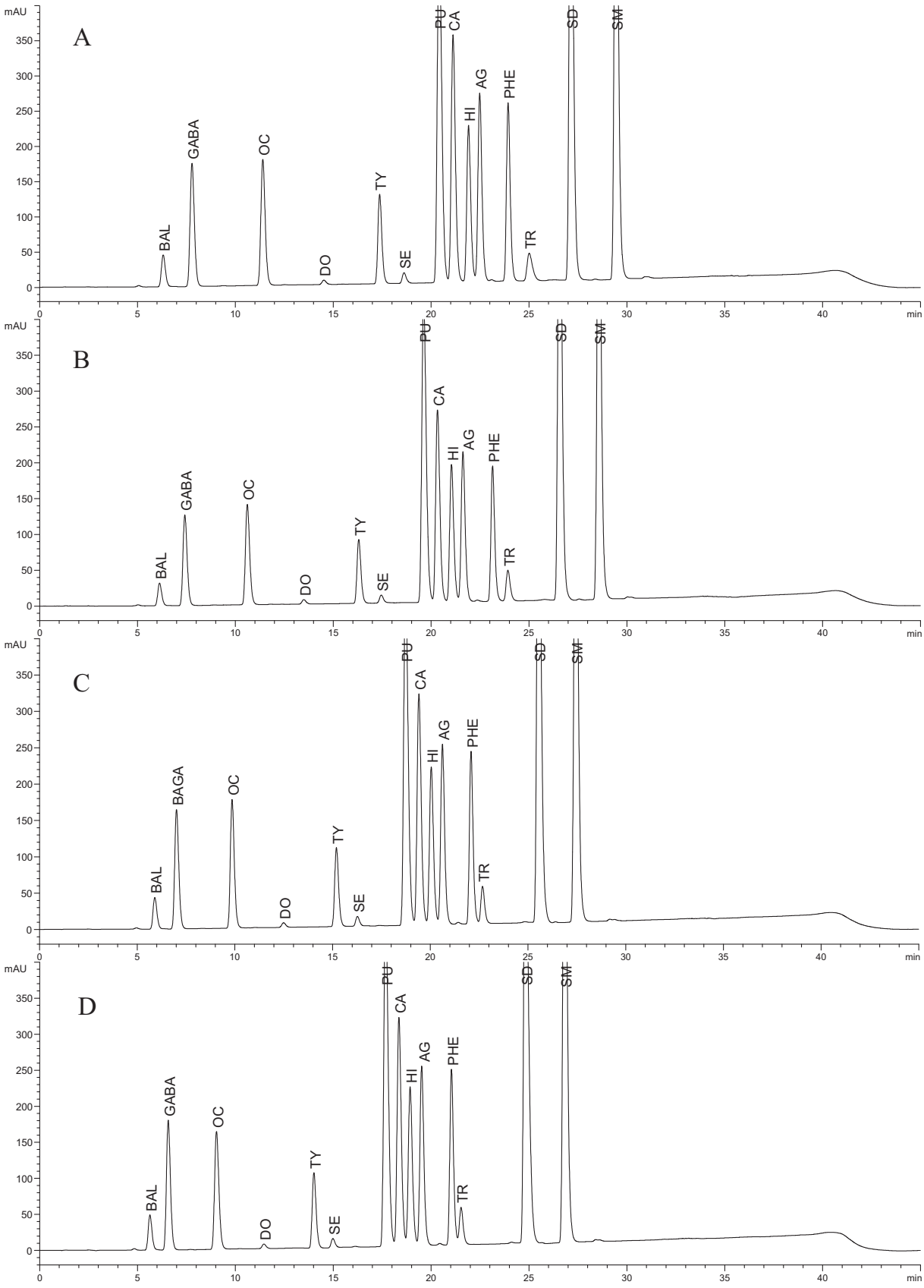

Fig. 4. Chromatograms of the mixing solution of 14 amines obtained by LC–UV derivatization method with 0.3% NFPA in mobile phases and column temperature of 20 °C (A), 30 °C (B), 40 °C (C), and 50 °C (D).

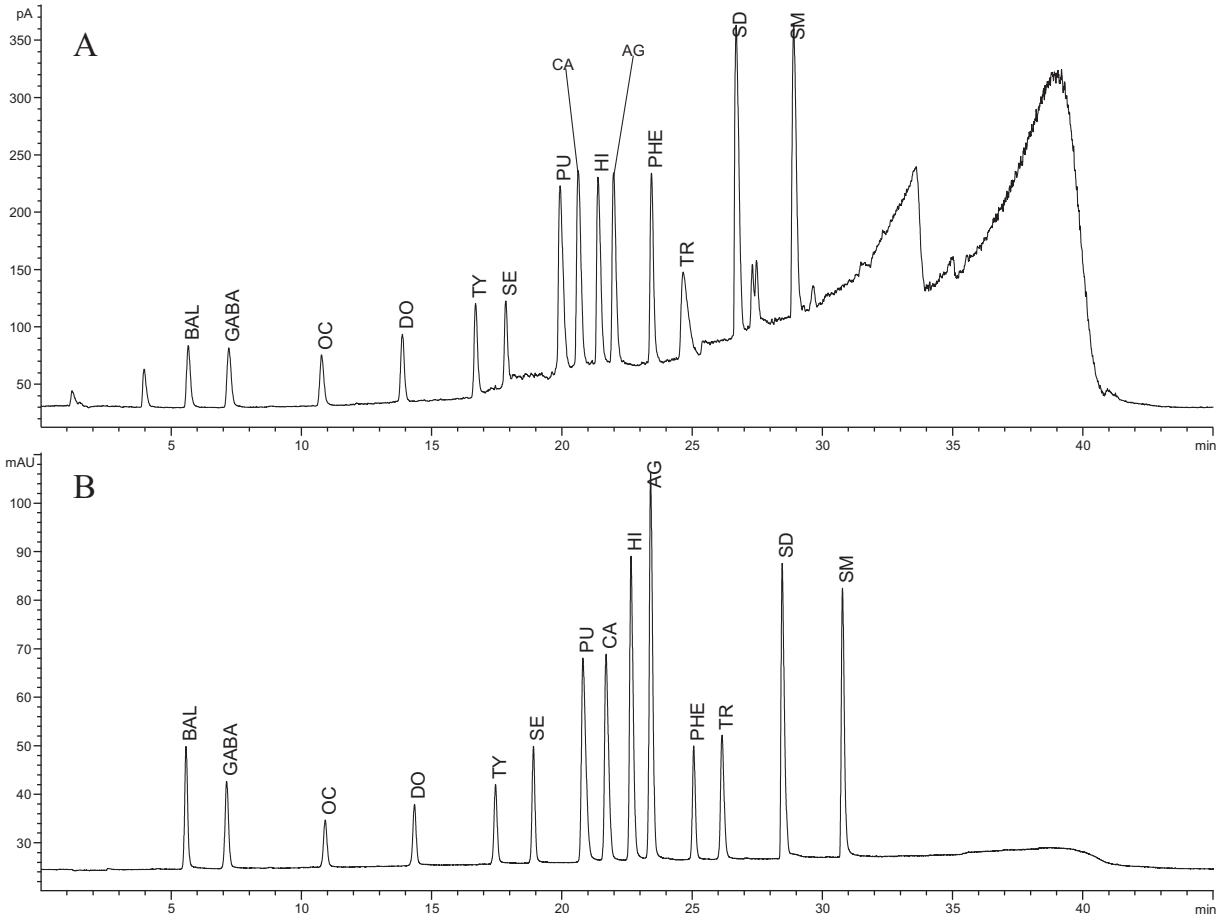

Fig. 5. Chromatogram of the mixing solution of 14 amines obtained by LC-CAD (A) and LC–CLND (B).

contains adjacent nitrogen atoms and 8 contain two or more nitrogen atoms (Fig. 1), which means strong CLND responses should be obtained. However, there are restrictions when a CLND is used with HPLC: the mobile phases must be volatile and nitrogen free. This has created challenges for direct separation and detections of these 14 amines by LC–CLND methods. Without pre-column derivatization, some of the small, polar amines will not retain on a reversed phase HPLC column. Ion-pair reagents help retain them but they are not compatible with CLND, such as sodium octanesulfonate, or heptanesulfonate and octylamine. Our initial approach to this challenge is utilizing a post-column derivatization method to identify relatively volatile and nitrogen free ion-pair reagents capable of retaining and separating all 14 amines in a single chromatographic run. Once the ion-pair reagents are identified and HPLC method is finalized, CLND and other detectors such as CAD can be used for direct detections.

3.1. Method development and optimization

Volatile perfluorocarboxylic acids of long n-alkyl chain had been used as ion-pair reagents in the analysis of underivatized amino acids because of their compatibility with a number of detection techniques [22]. Comparing with the 20 amino acids, the structure and the number of nitrogen atoms are dramatically different among the 14 biogenic amines. The performances of the perfluorocarboxylic acids and their compatibility with LC and CLND can be compromised by the impurities or contaminants from their synthetic processes.

In an initial experiment intended to identify the most suitable ion-pair reagents for a LC–CLND method, a series of mobile phases A and B were prepared by adding 0.6% (v/v) TFA, PFPA, HFBA, NFPA, and UFHA in water and methanol respectively. A mixing solution containing 8 amines – BAL, GABA, TY, PU, CA, HI, SD, and SM at 10 μg/mL level was injected on a Luna C18(2), 2.0 × 150 mm, 3 μm column, eluted by the 5 sets of mobile phases (HPLC and ninhydrin flow rates = 0.35 mL/min, column temperature = 40 °C), derivatized with ninhydrin, and detected at 570 nm. The chromatograms acquired by these conditions are overlaid and compared in Fig. 3. As expected, Fig. 3 clearly shows that the longer the perfluorinated n-alkyl chain becomes the stronger retentions. 0.6% TFA failed to provide sufficient retentions at all; on the other hand 0.6% UFHA delayed the elution of every compound so much that it 'pushed' putrescine into cadaverine. PFPA, NFPA, especially HFBA looked very promising as ion-pair reagents for the biogenic amines. A mixing solution containing all 14 amines was then injected using the same chromatographic conditions with 0.6% HFBA in mobile phases. However, in the resulting chromatogram SE co-eluted with PU and TR co-eluted with PHE. Meanwhile, the mixing solution of 8 amines was also separated and detected by LC–CLND method with a decreased flow rate of 0.3 mL/min and the 3 sets of mobile phases containing 0.6% PFPA, HFBA, and NFPA. The retention behaviors of the 8 amines by the LC–CLND method were similar to those by the LC–UV post-column derivatization method (Fig. 3), but with pronounced baseline drift and weak chemiluminescent responses. Most likely the baseline drift was caused by the impurities of ion-pair reagents. The purity of a perfluorocarboxylic acid was limited by its synthetic and purification process, for example, HFBA with 99% purity is readily obtained, however, the NFPA of more than 97% purity is not commercially available at all. When eluted at high percent mobile phase B (strong phase),

Table 1
Summary of peak widths, resolutions, and plate numbers of 14 amine peaks obtained by the three detection methods.

	Peak width (min)[a]			Resolution[b]			Plate numbers		
	UV	CAD	CLND	UV	CAD	CLND	UV	CAD	CLND
BAL	0.22	0.16	0.12	–	–	–	4632	7384	11,780
GABA	0.23	0.17	0.15	3.8	5.5	6.8	6188	9783	13,402
OC	0.24	0.17	0.15	8.9	12.0	15.0	12,997	23,146	30,691
DO	0.23	0.15	0.14	7.8	11.4	14.0	22,937	46,748	61,773
TY	0.22	0.14	0.13	7.4	11.4	13.6	35,840	77,496	102,480
SE	0.22	0.13	0.12	3.3	5.0	6.8	41,161	108,087	141,445
PU	0.25	0.20	0.18	4.5	7.6	7.2	37,680	56,900	72,705
CA	0.23	0.18	0.16	1.7	2.2	2.9	47,492	71,406	97,776
HI	0.22	0.17	0.14	2.0	2.6	3.5	54,939	91,183	136,832
AG	0.22	0.16	0.14	1.5	2.2	3.1	59,584	104,624	158,650
PHE	0.20	0.14	0.12	4.0	5.7	7.3	76,366	157,804	241,651
TR	0.29	0.30	0.14	2.5	3.3	4.8	40,195	38,713	184,432
SD	0.23	0.18	0.13	4.7	5.0	9.6	76,809	12,4071	252,399
SM	0.23	0.18	0.13	5.8	7.2	10.4	94,324	145,507	335,818

[a] Width at half height.
[b] Resolution to the preceding peak by tangent method.

the small percentage of impurities can produce dramatic baseline shifting. Similar observations were also recorded by other researchers, for example separating peptides by using long n-alkyl chained perfluorinated carboxylic acid as ion pairing agents in a LC–UV method [24]. Based on the above results, additional method developments were conducted primarily with HFBA and NFPA of lower concentrations in mobile phases to mitigate the drifting baselines and improve the nebulization efficiency of CLND. It was found after extensive studies that 0.3% NFPA, Luna Phenyl-Hexyl column (3 μm, 150 × 2 mm), with a flow rate of 0.25 mL/min performed best in separating all 14 amines. Column temperature study indicated low temperature will offer better baseline separations – the run time shortens and separation worsens as temperature increases, thus 20 °C was set in the final method. The chromatograms obtained by the final method at different temperatures are shown in Fig. 4.

3.2. Comparison of the three methods

The final chromatographic conditions described in Section 2.5 were applied in the LC-CAD and LC–CLND methods. Fig. 5 shows the chromatograms of 14 amines separated and detected by both methods. These chromatograms clearly show that the LC-CAD method had the worst baseline drifting (calculated a 7.5 pA/min increase) relative to the LC–UV method (0.6 mAU/min) and to the LC–CLND method (0.1 mAU/min). Fig. 5 also shows that the LC–CLND method has the least interferences toward the detections of biogenic amines as CLND is a nitrogen specific detector. In the contrast, CAD, a universal detector, can detect any charged particles – virtually any non-volatile and semi-volatile compounds. When used with HPLC, CAD responses to the existing impurities or contaminants in mobile phases, sample diluents, or residues on a HPLC system. Thus, compared with the other two methods, LC-CAD method had the noisiest and drifting baselines, which could give interferences that were not present in the other two methods and were problematic for integrations.

Table 1 summarizes the performances of the three methods in terms of peak width, resolution, and plate numbers. Due to the large system dwell volume and increased instrument bandwidth, the peaks detected by the post-column derivatization method were broad and can lose baseline separation as column temperature increases (Fig. 4). With column temperature set at 20 °C, the peak widths at half height by LC–UV method range from 0.20 to 0.29 min, which were significant larger comparing to the corresponding peak widths acquired by LC-CAD method (except TR peak) and by LC–CLND method. Narrowest peaks (0.12–0.18 min) exhibited in LC–CLND analysis. As a consequence, the resolutions were dramatically improved. For example, the resolutions of PU-CA pair and HI-AG pair increased from 1.7 and 1.5 by LC–UV to 2.2 and 2.2 by LC-CAD, and to 2.9 and 3.1 by LC–CLND methods respectively. The same trend observed in plate numbers: as shown in Table 1, the plate numbers of each amine peak in the LC–CLND chromatogram are more than doubled relative to that in the LC–UV chromatogram. A number of factors can influence plate numbers including column properties, flow rate, mobile phase viscosity, column temperature, analyte properties, and extracolumn effects [25]. With the addition of extra hardware components to conduct

Table 2
Summary of linearity and equimolar detection results of the 14 biogenic amines by the LC–CLND method.

Compound name	Linear range	Linear equation	R^2	RRF[a]	Deviation[b] (%)
SM	2–944 ng	$y = 1.2366x - 14.309$	0.999	4.3	7.1
AG	0.8–793 ng	$y = 1.9782x - 14.809$	0.999	4.4	10.3
SD	0.8–842 ng	$y = 1.3148x - 11.043$	0.999	3.3	9.0
HI	0.8–789 ng	$y = 1.7871x - 13.742$	0.999	3.4	13.4
PU	2–851 ng	$y = 1.347x - 12.61$	0.999	2.0	1.7
CA	1–998 ng	$y = 1.2134x - 11.797$	0.999	2.1	6.2
TR	4–776 ng	$y = 0.6875x - 8.396$	0.998	1.9	5.7
SE	4–787 ng	$y = 0.5852x - 8.452$	0.998	1.8	11.7
TY	4–817 ng	$y = 0.4121x - 4.4788$	0.998	1.0	3.2
GABA	4–852 ng	$y = 0.5453x - 5.844$	0.999	1.0	3.7
BAL	2–836 ng	$y = 0.6553x - 6.9706$	0.998	1.0	0.0
PHE	4–823 ng	$y = 0.4998x - 6.201$	0.998	1.0	3.7
DO	4–807 ng	$y = 0.3599x - 4.1256$	0.998	0.9	5.6
OC	4–827 ng	$y = 0.3382x - 3.6267$	0.999	0.9	11.3

[a] RRF = RF of each amine/RF of BAL; RF in mAu * s/nmole = slope of linear curve in mAU * s/ng × MW of biogenic amine.
[b] % Deviation = absolute value of (RRF – number of nitrogen atoms)/number of nitrogen atoms × 100.

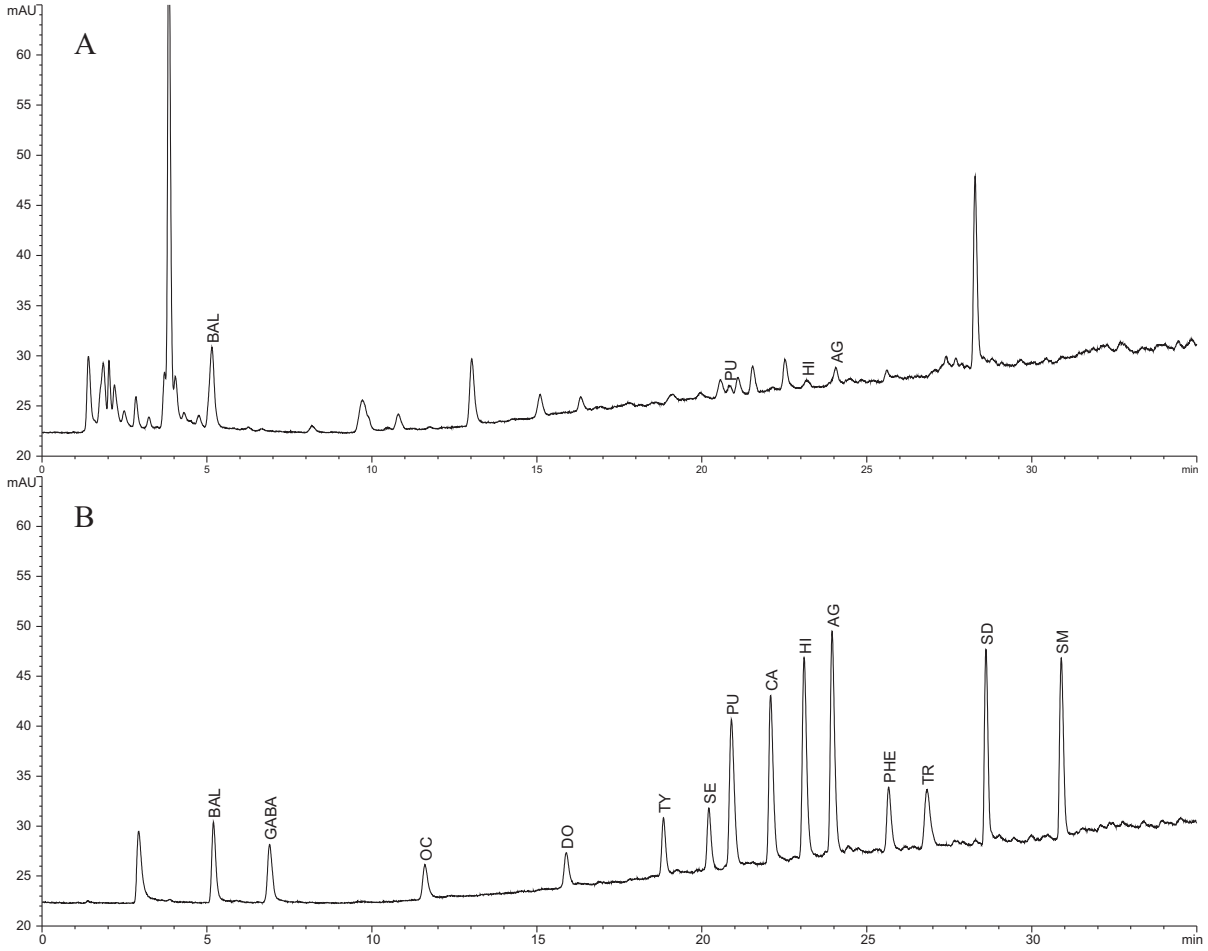

Fig. 6. Chromatograms of dairy beverage sample solution (A) and working standard solution (B).

online derivatization, it is no surprise that LC–UV derivatization system has the largest extracolumn volumes and lowest plate numbers among the three methods. The plate numbers in Table 1 also demonstrate the superior performances of LC–CLND over LC-CAD method in the determination of biogenic amines. In addition, the responses of this LC–UV post-column derivatization method varied substantially among the biogenic amines (Fig. 4) as the derivatization reactions are highly structure dependent. Their sensitivities toward certain biogenic amines especially DO and SE were not satisfactory. At an on-column weight level of 400 ng (~40 μg/mL by 10 μL), a signal-to-noise ratio of 26.9 and 65.8 were determined for DO and SE peak respectively, which will not meet the challenges of low level detection. In the LC-CAD method, for the late eluting peaks, the capability of quantitation can be compromised by the drifting baseline and blank interferences.

In summary, among the three detection techniques, CLND had shown excellent peak shapes, narrow peak widths, ideal baselines and better responses with baseline separations.

Table 3
Precision and accuracy of the LC–CLND method for the determination of 14 biogenic amines in dairy beverage, beer, herb tea, and vinegar.

Component name	Dairy beverage			Beer		Herb tea		Vinegar	
	Conc (μg/mL)	%Rec	%RSD (n = 6)	Conc (μg/mL)	%Rec	Conc (μg/mL)	%Rec	Conc (μg/mL)	%Rec
BAL	53.1	98.3	0.7	51.2	101.7	15.6	99.2	ND	100.4
GABA	ND	99.2	2.8	45.4	99.4	13.6	101.4	ND	100.4
OC	ND	100.2	2.2	ND	100.6	ND	99.2	ND	101.2
DO	ND	99.4	3.1	ND	101.7	ND	99.0	ND	100.6
TY	ND	100.0	0.7	ND	101.3	ND	100.4	ND	100.3
SE	ND	98.7	1.2	8.7	102.9	ND	101.9	ND	98.9
PU	3.2	99.0	2.2	10.0	101.7	ND	99.0	3.2	100.7
CA	ND	99.6	1.1	ND	99.9	ND	99.2	ND	99.6
HI	1.9	98.8	1.1	ND	101.0	ND	101.4	ND	101.0
AG	3.1	99.3	2.5	9.9	100.8	1.6	101.9	ND	99.5
PHE	ND	99.6	1.5	ND	99.1	ND	102.8	ND	99.4
TR	ND	101.3	2.2	ND	99.2	ND	100.8	ND	99.4
SD	ND	100.1	0.4	ND	99.9	ND	100.9	ND	99.9
SM	ND	100.5	1.4	ND	101.6	ND	99.1	ND	100.3

Note: ND = not detected.

3.3. Linearity, detection limit, and equimolar detection

Although it has never been used in this type of applications, the LC–CLND method reported here clearly showed dominating performances comparing with LC-CAD method and LC–UV post-column derivatization method in separating and detecting biogenic amines. The linearity of this LC–CLND was studied for each of the 14 amines in 0.2% TFA.

In the linearity study, three standard solutions with concentration levels of 40 μg/mL, 4 μg/mL, and 0.4 μg/mL were prepared. The on-column concentrations/weights of the 14 biogenic amines were achieved by varying injection volume from 1 μL, 2 μL, 5 μL, 10 μL to 20 μL at each concentration level. The linear curves were obtained by plotting peak area responses against on-column weights (concentration × injection volume, in nanogram). In cases two peak areas were acquired for the same on-column weight, for example, 40 μg/mL by 1 μL and 4 μg/mL by 10 μL, the average peak area was used in plotting linearity curves. The linearity results are summarized in Table 2. The slope of the linear curve in mAU * s/ng was then used to calculate response factor in mAu * s/nmole of each biogenic amine. In Table 2, the relative response factor (RRF) of each amine was calculated against the response factor of BAL.

The results in Table 2 show that the LC–CLND method provides wide linear dynamic ranges (1–1000 ng, $R^2 \geq 0.998$), close to equimolar detections (deviations are within 15%) and competitive sensitivities toward biogenic amines, especially to those without any chromophores or fluorophores such as PU, CA, SD, and SM. The detection limit can be obtained from the lowest on-column weight of the linear regression curve. With an injection volume of 10 μL, the detection limit is 0.1 μg/mL for CA, HI, AG, and SD; 0.2 μg/mL for BAL, PU, and SM; 0.4 μg/mL for GABA, OC, DO, TY, SE, PHE, and TR.

3.4. Precision

After the dairy beverage sample was centrifuged and filtered, 0.5 mL of the resulting solution was mixed with 0.5 mL of sample diluent (0.2% TFA in water) in a HPLC vial to prepare the dairy beverage sample solution. The spiked sample solution was prepared by adding 0.5 mL of the mixing stock standard solution (containing about 40 μg/mL of each amine) instead of the sample diluent. Six 5 μL injections of each solution were made and amine contents were measured against a working standard solution, which was prepared by centrifuging and filtering the mixing stock standard solution and diluting it by half with the sample diluent. To accommodate the early eluting peaks of sample matrix, a 2 min holding section of A:B 95:5 were added to the gradient program. The resulting chromatograms of dairy beverage and working standard solutions are shown in Fig. 6. The amine contents, their recoveries, and %RSD of six injections are summarized in Table 3. Table 3 shows that the RSD of 14 biogenic amines in spiked dairy beverage sample solution of six injections ranges from 0.4% to 3.1%. Without standard addition, four biogenic amines were detected in dairy beverage sample, BAL, PU, HI, and AG. A RSD of 1.1%, 3.6%, 3.3%, and 1.5% were obtained respectively for each of the four amines. For all amines, the RSD of six replicate injections were always less than 5%. It is no surprise that the RSD of PU and HI in the unspiked sample solution were over 3% and relatively higher than that in the spiked sample solution as their peak area were very small before the standard addition.

3.5. Accuracy

Beer, herb tea, and vinegar samples were first filtered, the sample solutions and spiked sample solutions were then prepared and analyzed by using the same procedures described in Section 3.4. As shown in Table 3, five biogenic amines, BAL, GABA, SE, PU, AG were detected in beer; three in herb tea, BAL, GABA, AG; and one in vinegar, PU. For all biogenic amines, the recovery of standard addition ranges from 98.3% to 101.3% in dairy beverage, 99.1% to 102.9% in beer, 99.0% to 102.8% in herb tea, and 98.9% to 101.2% in vinegar. In all samples the recovery of all biogenic amines is larger than 98% but less than 103%.

4. Conclusion

The outcomes of this study demonstrated that coupling with ion-pair liquid chromatography, CLND provides a new, simple and yet sensitive method for the analysis of biogenic amines without derivatization. The volatile long chain perfluorocarboxylic acids, which are compatible with many detectors including CLND, can play important roles as ion-pair reagents in retentions of small, polar or charged compounds. The applications of perfluorocarboxylic acids in ion-pair liquid chromatography with various detectors should be further explored and utilized beyond amino acid analysis. It can be concluded that the LC–CLND method explored here can serve as a better surrogate for the traditional analytical procedures requiring pre-, on- or post-column derivatizations and can potentially be used to determine a wide range of nitrogen-containing compounds in pharmaceutical, foods, plants, and biological samples.

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