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Determination of biogenic amines by capillary electrophoresis with pulsed amperometric detection

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Abstract

The biogenic amines, putrescine, cadaverine, spermidine and spermine were separated and quantified by capillary electrophoresis with pulsed amperometric detection. Detection potential of the pulsed amperometric detection was optimized as 0.6 V. Optimal separation of the biogenic amines was achieved using a separation buffer of 30 m*M* citrate at pH 3.5, while keeping the buffer in the detection cell as 20 m*M* NaOH. Using these conditions, the four biogenic amines were baseline separated. Extrapolated limits of detection for putrescine, cadaverine, spermidine and spermine were 400, 200, 100 and 400 n*M* for the standard mixture (polyamines dissolved in running buffer), respectively. These are lower than ultraviolet detection and comparable or even lower than laser-induced fluorescence detection results as reported in the literature. The number of theoretical plates was maintained at the 10^5 level, which is absolutely higher than any reported method. When applying capillary electrophoresis–pulsed amperometric detection to milk analysis, only spermidine was found in amounts varying between 0.1 and 0.5 mg/kg.

Keywords: Electrochemical detection; Pulsed amperometric detection; Detection, electrophoresis; Biogenic amines; Amines

1. Introduction

Biogenic amines are formed during normal metabolic processes in all living organisms and are, therefore, present in food. Because of the diversity of the roles of biogenic amines in cellular metabolism and growth, the requirement for biogenic amines is particularly high in rapidly growing tissues. Indeed, the importance of putrescine, spermidine and spermine in tumour growth is widely recognized, and the inhibition of biogenic amines biosynthesis in tumour-bearing individuals is a major target of cancer therapy research [1,2]. A number of studies have indicated higher concentration of putrescine, spermidine and spermine or total biogenic amine contents (free and acetylated) in cancer patients compared to healthy subjects [3–5]. Owing to the health implications of the intake of high biogenic amines, a new direction being investigated to inhibit tumour growth is to limit biogenic amine intake. Biogenic amines are also responsible for food poisoning. Histamine, putrescine and cadaverine levels are used by the food industry as a measure of the degree of spoilage or aging in fish, meat and cheese [6,7]. The

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possibility of simultaneous detection of biogenic amines, such as putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spm) in food-stuffs is very important. There are several methods for determining biogenic amines in foods: thin-layer chromatography (TLC) [8,9], gas chromatography (GC) [10,11], liquid chromatography (LC) [12,13], and capillary electrophoresis (CE) [14,15]. However, high-performance liquid chromatography (HPLC), preceded by amine derivatization, is by far the most frequently reported technique for separation and quantification [16–18].

Biogenic amines do not exhibit any structural features that enable their direct detection in a sensitive manner. Most of the analytical procedures reported for the determination of biogenic amines include both a derivatization step and a separation step [19]. Unfortunately, chemical modifications can be time-consuming and unreliable, resulting in the dilution of the sample, affecting the separation process, and difficult to implement with very small sample volumes. Since pre- and post column derivatization are cumbersome, electrochemical detection has also been investigated following reversed-phase or cation-exchange chromatography separation, but these methods suffer from large reagent consume and possible organic reagent contact [20–22]. Therefore, the successful development of a simple, rapid and reliable method to determine biogenic amines in food is needed.

Capillary electrophoresis (CE) has developed enormously during the last decade. It offers many advantages over its counterpart, HPLC, in terms of higher column efficiency (10^5–10^6 theoretical plates), lower sample volume (~10 nl), and very high sensitivity with femtomole to zeptomole limits having been reported [23,24]. As for detection, electrochemical technique provides high selectivity and sensitivity. In this paper, pulsed amperometric detection (PAD) is presented for detection of biogenic amines after capillary electrophoresis. Because direct, sensitive, and reproducible detection of polar aliphatic compounds could be accomplished by pulsed electrochemical detection (PED), while constant applied potential electrochemical detection usually leads to diminishing response due to the electrode fouling [25]. CE–PED, including CE–PAD and CE–IPAD (integrated pulsed amperometric detection), has been successfully applied to the detection of amino acids [26–29], peptides [26–28,30], monosaccharides [27–29,31–33], oligosaccharides [30,34,35], thiols [26,27], disulfides [26,27], and inorganic ions [36]. To our knowledge, capillary electrophoresis in conjunction with pulsed amperometric detection of biogenic amines has never been reported. The primary advantage of PAD is that no derivatization is necessary for the detection of biogenic amines. In this paper, the variables involved in capillary electrophoresis separation and pulsed amperometric detection of four biogenic amines were optimized. This method was applied to the analysis of biogenic amines in milk. It is proved that CE–PAD exhibited a large number of theoretical plates and high sensitivity. It is superior to the CE with indirect ultraviolet detection in terms of detection limit and it indicated small sample volume and simple operation process than GC or HPLC for biogenic amines analysis.

2. Experimental

2.1. Apparatus

Cyclic voltammetry and pulsed amperometry were performed with CHI 832 electrochemical analyzer (CH Instruments, Cordova, TN, USA). The electrochemical detection cell was equipped with a 50-μm diameter gold disk working electrode, a Ag/AgCl reference electrode and a Pt wire counter electrode. Positive voltage was applied at the injection end of the capillary through a platinum wire in the sample/buffer reservoir using a ±30 kV high voltage power supply (Spellman CZE1000R, Plainview, NY, USA). The detection end of the capillary was grounded through a stainless steel tube. A 65-cm×25-μm I.D.×360-μm O.D. uncoated fused-silica capillary was used (Hebei Yongnian Optical Fiber Factory, Hebei, China). The capillary was rinsed with 0.1 *M* NaOH overnight each day before use. Before each sample injection, the capillary inner surface and gold electrode were pretreated by flushing with double-distilled water and buffer solution for 90 and 120 s, respectively, and running a cyclic voltammogram from −1.0 to +0.9 V simultaneously in order to achieve better reproducibility.

2.2. Reagents and solutions

All chemicals were used as received without further purification. Reagent grade putrescine, cadaverine, spermine and spermidine were purchased from Sigma (St. Louis, MO, USA). Reagent grade 1,6-hexanediamine was obtained from Shanghai Chemical Reagent Corp. Standard mixture of the five polyamines were dissolved in the running buffer and injected into the capillary without further pretreatment. The 70% perchloric acid, citric acid and sodium citrate were obtained from Beiing Chemical Reagent Factory (Beijing, China).

2.3. Electrode preparation

The gold electrode was fabricated by sealing a 1-cm length of 50 μm diameter gold wire into a 2-mm I.D. glass tube using epoxy, with one end protruded out of the tip. The glass tube had been pulled to a point in a Bunsen burner flame. The gold wire was connected with a Cu wire through graphite powder which had been introduced into the glass tube after epoxy curing. The gold disk electrode was finally polished with 0.05 μm α-Al_2O_3 on fine abrasive paper.

2.4. Sample preparation

An 10-g aliquot of fresh milk was accurately weighed and thoroughly mixed with 6 ml of 5% perchloric acid in a magnetic stirring plate for 30 min. Then, centrifugation at 5000 rpm for 10 min was carried out to separate the two phases. The centrifugation was repeated after re-suspending the resulting pellet in 4 ml of 5% perchloric acid, mixing it thoroughly for 20 min. The supernatants were combined and concentrated in room temperature to a final volume of 5 ml. For analysis, samples (200 μl) were taken and mixed with 50 μl of 0.5 m*M* 1,6-hexanediamine as internal standard.

3. Results and discussion

3.1. Selection of electrode material

Electrode material was essentially important because a suitable detector indicates good electrochemical responses and low background noise. We have tried copper microelectrode and carbon fiber microelectrode for detection of biogenic amines at constant voltage mode. The results obtained by cyclic voltammetry and current–time curves showed that (results not presented in this paper) only Cad exhibited favorable electroactivity at copper electrode, while Cad and Spd produced good current responses at carbon fiber electrode. For the Ni microelectrode under pulsed amperometry mode, very low to no electrochemical response of the four biogenic amines was obtained. However, the four biogenic amines can be oxidized simultaneously at the gold electrode and well-defined peaks were obtained under the pulsed amperometric detection mode at alkaline condition.

3.2. Optimization detection potential

The PAD waveform maintains electrode activity via a triple pulse sequence including detection, electrode cleaning, and electrode reaction potential. The representation of the PAD waveform in this study is shown in Fig. 1. This waveform consists of the application of a sufficiently positive potential

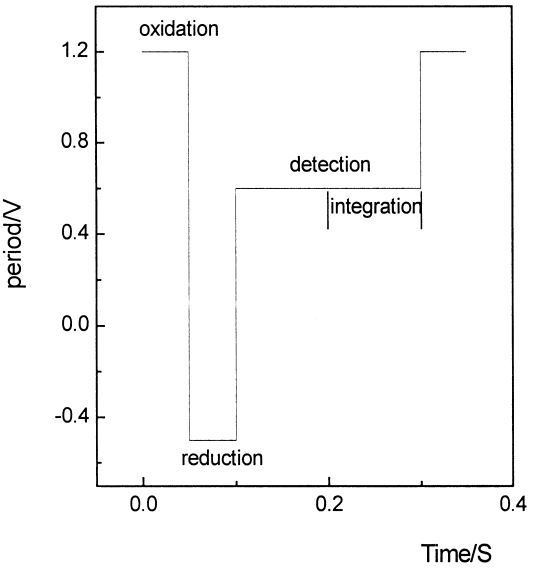

Fig. 1. PAD waveform. Parameters: oxidation at 1.2 V for 0.05 s, reduction at −0.5 V for 0.05 s, detection at 0.6 V for 0.2 s, data integration from 0.2 to 0.3 s.

+1.2 V to remove adsorbed molecules, which result in the formation of AuO layer on the electrode surface. Then a sufficiently negative potential −0.5 V was used to reduce the AuO layer and reactivate the surface for biogenic amine adsorption. Times for cleaning and activation were kept as short as possible to achieve a high sampling rate, which is mandatory for the registration of narrow peaks. There is no data acquisition during the oxidation and reduction step, the data are sampled at a later 1/2 period of detection step. A long sample interval will have better signal averaging and less noise. So we keep the times at 0.05, 0.05, and 0.2 s for the oxidation, reduction and detection step, respectively. Since the gold electrode was frequently used in alkaline solutions under pulsed mode, we adopted 20 m*M* NaOH solution as the test buffer to determine the optimal detection potential. A 0.1 m*M* solution of each analyte was evaluated at three different detection potentials to determine the optimal detection potential while keeping cleaning and activation steps of the potential-time function (Fig. 1) constant. The response for the PAD of four biogenic amines was recorded and the results are shown in Fig. 2. The optimized detection potential was determined to be 0.6 V, as the electrochemical response for all three compounds—Put, Spd, Spm—was the maximum at this potential, only Cad and Hex (internal standard) had a higher potential.

3.3. Optimization of separation conditions

In CZE, the optimization of the buffer composition plays a key role in method development. Buffer pH was absolutely critical for polyamine separation. Since at higher pH values (pH>9), such as borate or NaOH solutions, biogenic amines are neutral or negatively charged and tend to migrate at the same velocity, therefore no separation was observed. When using the neutral or very weakly acidic buffer system (pH 7–5) such as phosphate or citrate buffer system, biogenic amines were partly charged and exhibited small difference in migration velocity as judged by the overlapped peaks. Finally, citrate buffer system with pH of 3.5 was proved to be the most suitable for separation. The four biogenic amines together with the internal standard 1,6-hexane are baseline separated as shown in Fig. 3. The migration order was Put, Cad, Hex, Spd and Spm, since they are polycations under acidic conditions and exhibit net

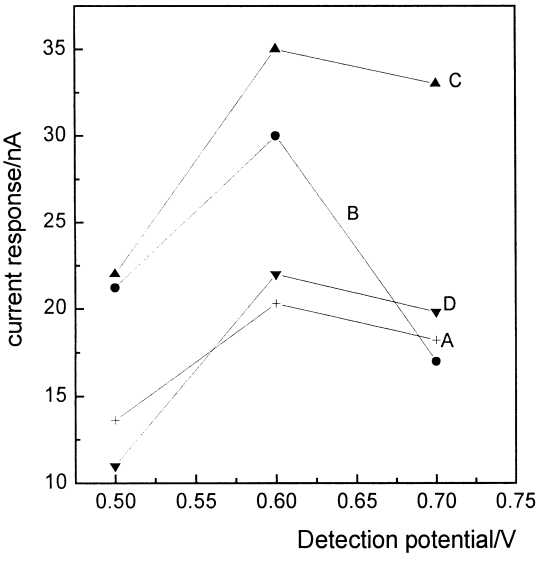

Fig. 2. Optimization of detection potential of the four biogenic amines. (A) 0.1 m*M* Put; (B) 0.1 m*M* Cad; (C) 0.1 m*M* Spd; (D) 0.1 m*M* Spm. Conditions: separation buffer, 20 m*M* NaOH; detection solution, 20 m*M* NaOH.

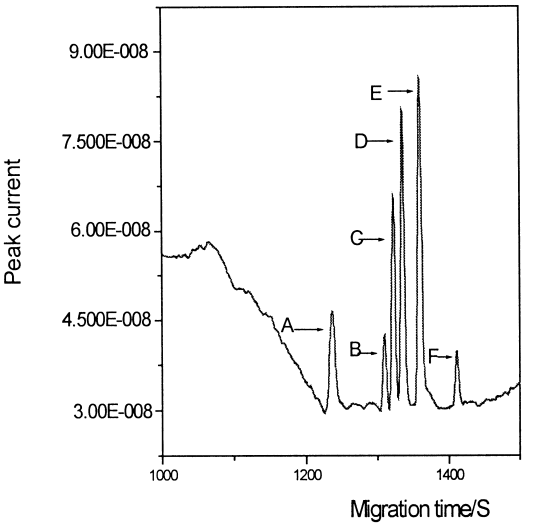

Fig. 3. Electropherogram of the standard solution of the four biogenic amines. (A) unknown peak; (B) 0.1 m*M* Put; (C) 0.1 m*M* Cad; (D) 0.1 m*M* Hex (internal standard); (E) 0.1 m*M* Spd; (F) 0.1 m*M* Spm. Conditions: separation buffer, 50 m*M* citrate with pH of 3.5; detection solution, 20 m*M* NaOH;.

charges close to 2+, 2+, 2+, 3+ and 4+, respectively. It should be pointed out that optimized pH of separation buffer was not consistent with that of the solution in the detection cell, which always remains as 20 m*M* NaOH. During the separation process the buffer additive, such as ethanol, acetonitrile, 18-crown-6, β-cyclodextrane, urea, Cu^{2+} were tested, but they cannot simultaneously improve the separation of the four biogenic amines.

The effect of buffer concentration in terms of peak current (i_p) and the number of theoretical plates (N) was investigated. N was calculated according to the following equation:

$$N = 5.54(t_m / W_{1/2})^2 \quad (1)$$

where t_m is the migration time and $W_{1/2}$ is the width at half height of the electrophoretic peak. As is shown in Fig. 4A, the i_p reaches maximum at 30 m*M*, then decreases gradually, and tends to level off at the buffer concentration higher than 100 m*M*. It was proved that 30 m*M* citrate buffer corresponds with the maximal current response without loss of resolution. From Fig. 4B, we can roughly conclude that higher buffer concentration results in lower N. So, 30 m*M* citrate buffer was acceptable since the best current response and moderate N were obtained at this concentration.

The detection limits of PAD are determined by the fluctuations of the background current (baseline noise). These fluctuations depend on the detection parameters (residual charging currents), on the presence of electroactive impurities, and on the pH of the eluent. Thus, the NaOH concentration used for detection has an effect on sensitivity and detection limits since it related closely to the background noise [37]. We investigated the effect of post-column NaOH concentration on detection sensitivity by varying the NaOH concentration from 20 to 100 m*M*. The results showed that, the values of background noise and peak current change little while resolution degrades with the increase of NaOH concentration. We keep the concentration as 20 m*M* as used previously.

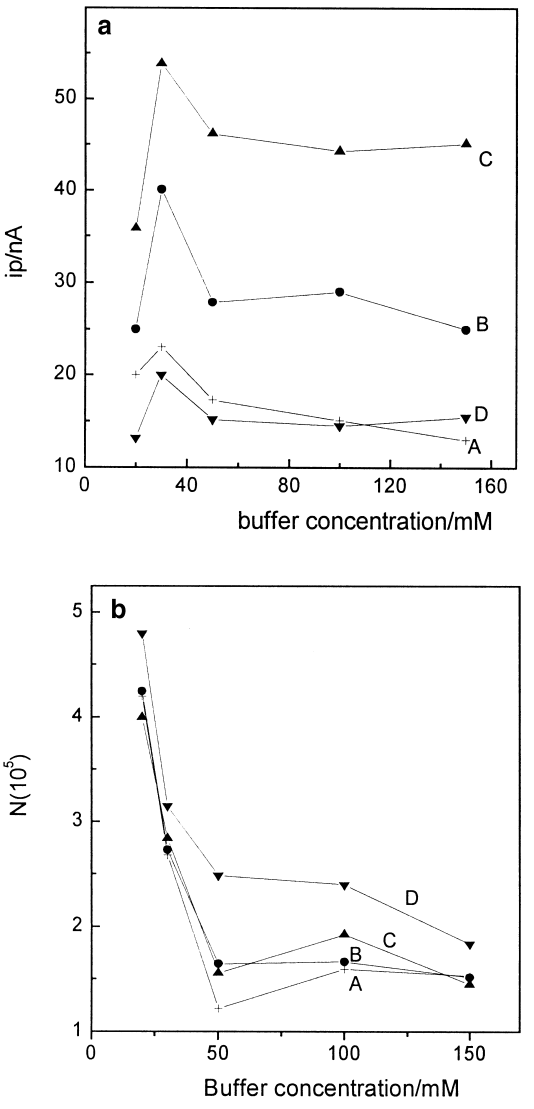

Fig. 4. (a) Effect of buffer concentration on i_p. (A) 0.1 m*M* Put; (B) 0.1 m*M* Cad; (C) 0.1 m*M* Spd; (D) 0.1 m*M* Spm. Conditions: separation buffer, pH 3.5; detection solution, 20 m*M* NaOH. (b) Effect of buffer concentration on *N*. (A) 0.1 m*M* Put; (B) 0.1 m*M* Cad; (C) 0.1 m*M* Spd; (D) 0.1 m*M* Spm. Conditions: separation buffer, 30 m*M* citrate of pH 3.5; detection solution, 20 m*M* NaOH.

3.4. Performance characteristics

Repeatability, linearity and sensitivity of the method were examined. The repeatabilities of the method expressed as relative standard deviation (RSD) of t_m and i_p are shown in Table 1. RSD of t_m and i_p did not exceed 3% in seven consecutive runs. The linearity data were collected for 12 different con-

Table 1
Repeatability, linearity and detection limits of biogenic amines

Amine	Put	Cad	Spd	Spm
t_m (RSD %) ($n=7$)	1.7	2.0	2.2	2.0
i_p (RSD %) ($n=7$)	2.0	2.5	1.5	1.3
N (10^5)	4.2	4.3	4.0	4.8
Linearity	$5\cdot10^{-4}$–$2\cdot10^{-6}$	$5\cdot10^{-4}$–$4\cdot10^{-6}$	$2\cdot10^{-4}$–$1\cdot10^{-6}$	$4\cdot10^{-4}$–$7\cdot10^{-6}$
LOD (M)	$4\cdot10^{-7}$	$2\cdot10^{-7}$	10^{-7}	$4\cdot10^{-7}$

Conditions: separation buffer, 30 mM citrate buffer with pH 3.5; detection solution, 20 mM NaOH.

centrations (from 10^{-7} to 10^{-3} M) of biogenic amine standards, using triplicate responses at each concentration. PAD response was concluded to be linear for about two orders of magnitude as illustrated in Table 1. The N values are extremely large, they are 10 times higher as compared to the micellar electrokinetic capillary chromatography [38,39] and 100 times higher than HPLC [40]. The limit of detection for Put, Cad, Spd, Spm was determined to be 400, 200, 100 and 400 nM for a signal-to-noise ratio of 3. These limits of detection are comparable or superior to those previously reported in the literature for UV detection [14,41], and were comparable or superior to the laser-induced fluorescence detection for some biogenic amines reported in recently published papers [42–44].

3.5. Milk analysis

The assay for biogenic amines was applied to milk to illustrate the analytical utility of the procedure. Electropherograms of standard sample and milk sample are shown in Fig. 5. In order to identify the corresponding peak, the pretreated milk samples, spiked with 0.1 mM Put, Cad, Spd and Spm, respectively, were injected into the capillary. Finally, only the peak for Spd was found except for the internal standard, Hex, while the other three biogenic amines were not detected which was consistent with previous reports [45]. Because of the complex matrix effect of the milk sample, the migration time of Hex, Spd became longer compared with the standard sample, but the reproducibility of migration time and peak current for a series of milk samples ($n=10$) was 2.5 and 3.2%, respectively. The Spd content was calculated using the internal standard of 0.1 mM Hex. By analyzing six milk samples obtained from different milk stores claimed to be collected on the same day, a wide range of Spd contents was found, from 0.1 to 0.5 mg/kg. In contrast, the available data were 0.16–0.18 mg/kg; this large difference may be due to quality problems [45].

4. Conclusions

The proposed method appears to be suitable for the easy determination of biogenic amines. Its analysis of biogenic amines was tested and found to be

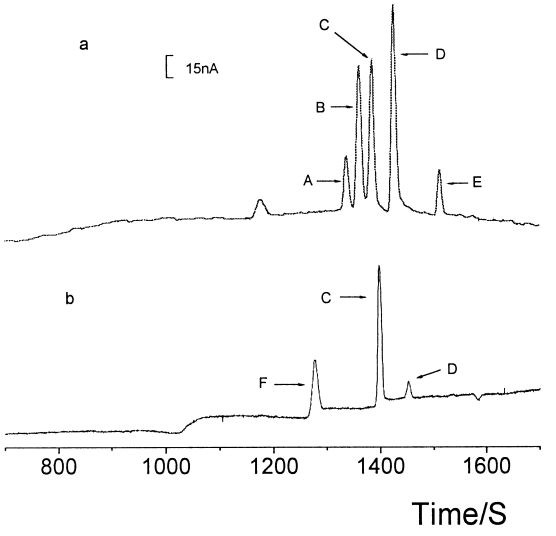

Fig. 5. Electropherograms of standard solution of the four biogenic amines and milk sample spiked with Hex. (a) Standard mixture of 0.1 mM Put (A), Cad (B), Hex (C), Spd (D) and Spm (E), respectively. (b) Milk sample. 0.1 mM Hex (C), Spd (D) and unknown peak (F). Conditions: separation buffer, 30 mM citrate buffer with pH 3.5; detection solution, 20 mM NaOH.

applicable. Under the optimized separation and detection conditions, the four amines are well resolved, and high sensitivity and theoretical plates were obtained. CE method in conjunction with pulsed amperometric detection appears to be a suitable method for the analysis of biogenic amines.

Acknowledgements

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