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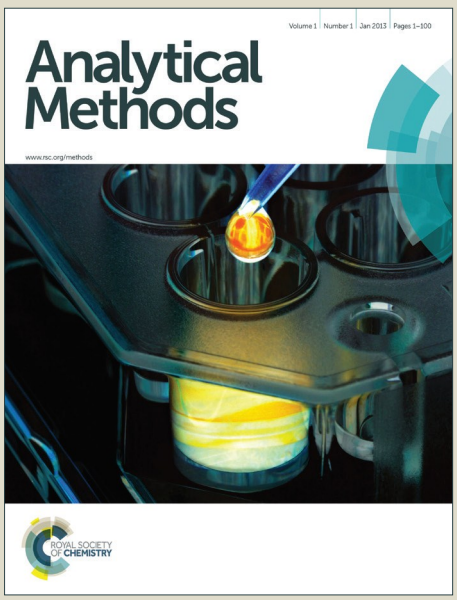

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Determination and Separation of Putrescine and Spermidine in Aquatic Products

Siyuan Chen[a], Zhuqiu Chen[b], Ruifang Wang[a], Li Wang[*a]

Abstract

A sensitive, simple and rapid CE-ECL method is established for the determination of putrescine and spermidine in aquatic products with a new luminous source material, PMo_{12}- $Ru(bpy)_3^{2+}$. This method is based on the electrochemical reaction of putrescine, spermidine and PMo_{12}- $Ru(bpy)_3^{2+}$ on a platinum electrode. The experimental conditions for the biogenic amines' separation and detection were optimized. Under optimal conditions, the detection limit (S/N=3) was 6.5×10^{-3} mg/L. For spermidine, the detection limit (S/N=3) was 9.8×10^{-3} mg/L. No spermidine was detected in the fresh ray samples, and for the determination of putrescine, the content was 0.019 mg/L. The relative standard deviation (RSD) of the peak height and migration time for putrescine were 1.19% and 1.11%, respectively, with a recovery of 90.0%-104.0%. The new luminous source could make the CE-ECL method cleaner and more effective on the premise of guaranteeing accuracy.

Keywords: capillary electrophoresis (CE); electrochemical luminescence (ECL); polyoxometalate (POMs)

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Introduction

Biogenic amines (BAs) are nitrogenous organic materials that have biological activity and are widespread in protein-rich food, such as aquatic products, meats, dairy products and alcohol[1, 2]. Amines are needed as precursors in biological synthesis of some proteins and nucleic acids. Free amino acid decarboxylation can play an important role in the formation of biogenic amines[3, 4]. The formation of BAs is associated with bacterial enzymes when food spoils[5]. For this reason, BAs might be a helpful indicator of food quality and freshness[6, 7]. Histamine, putrescine, cadaverine, tyramine, spermine and spermidine are most import BAs in food[7, 8].

Putrescine, also known as butyl diamine, commonly is found in aquatic products, livestock and poultry products. Low concentrations of putrescine lead to positive physiological activity, but if the content of putrescine in the human body is higher than needed, it will endanger the person's health by causing some diseases and certain tumors[9, 10]. In addition, the existence of putrescine and spermidine will further intensify the toxicity of histamine[8, 11]. Thus, the content of putrescine and spermidine should be considered when determining the freshness of food, and it is urgent to establish a highly sensitive and reliable analysis system for the rapid detection of the two BAs.

Biogenic amines in food are generally detected by high performance liquid chromatography (HPLC)[12, 13]. Other detection methods have also been used, including ion chromatography (IC)[14], thin-layer chromatography (TLC)[15, 16], gas chromatography (GC)[17, 18]and enzyme linked immunosorbent assay (ELISA). And an amperometric biosensor was also developed for the in vitro measurement of low concentration of putrescine in blood[19]. Most of them are effective, however, these conventional detection methods also have their own disadvantages, such as time-consuming, low sensitivity and they are not suitable for routine use. Capillary electrophoresis (CE) is a widely adopted separation detection technique with advantages including higher separation efficiency and higher sensitivity, and is widely applied in various fields. Biogenic amines exhibit no native absorbance and fluorescence, but the amine group can produce electrochemiluminescence (ECL) with

$Ru(bpy)_3^{2+}$. Moreover, there are many measures to improve the sensitivity of ECL system, such as using $Ru(bpy)_3^{2}$/formaldehyde ECL system in the detection of ozone[20]. Based on the above principle, a sensitive, simple and rapid CE-ECL method could be established for the determination of biogenic amines in aquatic products.

Polyoxometalate (POMs) is a type of anionic single molecule cluster with an excellent range of physical properties and the characteristics to form activity structures[21] using a transition metal atom, such as V, Nb, Ta, Mo and W[22]. POMs have special redox potential and an excellent ability for storage and transport of electrons and protons; therefore, they have gained extensive attention in many fields, such as in catalysis, electronics and medicine[23, 24]. POMs can also generate rapid, reversible and stepwise multielectron-transfer reactions with their own structures kept intact[25, 26]. In addition, most POMs are water soluble[27], which lead to the possibility of applications in detection cells of CE-ECL systems.

Because they are composed of two transition metal atoms (Mo and W centers), some lacunary POMs have a high oxidation ability[28]. As one type of Keggin-polyoxometalate, PMo_{12} can rapidly react in a series of single and double electron reduction processes that are reversible without changing the structure of the PMo_{12}. Based upon published literature, tris(2,2-bipyridine) ruthenium(II) and 12-molybdophosphate ($RuPMo_{12}$) appear to have outstanding stability, good reproducibility and the distinct advantage of surface-renewal when modified with carbon paste electrode[29]. Additionally, POMs can enhance the photoelectric conversion performance of solar cells[24], but there are few in depth studies regarding the application of PMo_{12} with $Ru(bpy)_3^{2+}$ as an effective luminous source in an ECL detection system. $Ru(bpy)_3^{2+}$ is still the primary luminous source for CE-ECL system detection for biogenic amine[30]. In this article, a new Keggin-polyoxometalate complex, PMo_{12}- $Ru(bpy)_3^{2+}$, was synthesized to replace the traditional luminous source, $Ru(bpy)_3^{2+}$. According to other researches, PMo_{12}-$Ru(bpy)_3^{2+}$ immobilized on electrode surface could enhance the sensitivity of an ECL sensor[31]. The application for

detection of aquatic products provides a theoretical basis for studying more efficient and sensitive electrochemical source materials.

Materials and methods

Reagents and instruments

The tris(2'2-bipyridy) ruthenium(II) was purchased from J&K technology Co., Ltd. The phosphomolybdic acid hydrate, sodium hydroxide, hydrochloric acid, perchloric acid, absolute ethyl alcohol, diethyl ether and potassium bromide were purchased from Sinopharm Chemical Reagent Co., Ltd. Putrescine and spermidine were acquired from Acros. Rays were bought in Jimei vegetable markets in Xiamen City, Fujian province, China.

A MPI-A-type capillary electrophoresis electrochemiluminescence detection system was obtained from Ruimai Analytical Instruments Co., Ltd in Xi'an province, China, including a computer numerical control (CNC) capillary electrophoresis high voltage power pack, an electrochemical analyzer and a chemiluminescence detector. Nicolet Avatar 330 FT-IR was acquired from Thermo Electron Corporation (USA). Lambda 35 UV/vis Spectrometer was purchased from PerkinElmer (USA).

Synthesis of PMo_{12}- $Ru(bpy)_3^{2+}$

$H_3PMo_{12}O_{40}$ solution was dripped slowly into $C_{30}H_{24}Cl_2N_6Ru \cdot 6H_2O$ solution and the liquid mixture was stirred for 1 hour at 80□ to form a reddish brown sediment. After suction filtration, the sediment was washed by ultrapure water, ethanol and ethyl ether and the final product was desirable.

The PMo_{12}-$Ru(bpy)_3^{2+}$ was characterized by UV spectrometry and FTIR spectra. Through analyzing, the synthesized compounds has polyoxometalate groups with Keggin structure as well as $Ru(bpy)_3^{2+}$, which is identical with the reported ones[31, 32].

Sample preparation

Fresh ray samples (5.00 g) were homogenized with 10 mL of 6% perchloric acid and were freeze centrifuged at 4□ at 5000 r/min after mechanical shaking and extraction for 30 min. The residue was homogenized with 10 mL perchloric acid and freeze centrifuged at 4□ at 5000 r/min after mechanical shaking and extraction for 30 min again. The supernatant fluid from two runs of the centrifugations was taken, and the volume was adjusted to 50 mL with perchloric acid. The sample liquid was stored fresh in the refrigerator and was filtered by a 0.22 μm cellulose acetate membrane before use.

Results and Discussion

Optimization of the detection conditions

Detection potential

<Fig.1>

The electrochemiluminescence (ECL) intensity of the analytes in a capillary electrophoresis-electrochemiluminescence (CE-ECL) system is significantly affected by the detection potential, which provides a crucial ECL co-reaction platform for the analyte and the ruthenium species[30]. The light intensity emitted depends on the chemical reaction rate, and chemical reaction rate is associated with the voltage on the working electrode. For the best detection results, the relationship between ECL intensity and detection potential was studied between 1.05 and 1.25 V. The ECL intensity of putrescine and spermidine peaked at 1.15 V at the same time as shown in Fig.1. In addition, excessive detection potential will increase the noise of the CE-ECL system, reduce the signal-to-noise ratio (S/N) and affect the detection sensitivity. Based on the above-mentioned factors, 1.15 V was chosen as the optimal detection potential.

Separation voltage

<Fig.2>

The separation voltage of the CE is another important factor that can impact the detection efficiency by affecting the transition time and separation efficiency of the samples. A low separation voltage could lead to an unclear outcome for the separation and give no criterion for judgment. An excessively high separation voltage could increase the Joule heat and baseline noise. And at the same time, excessively high separation voltage could also shorten the migration time and increase the flow velocity of analytes, which resulted in decreasing the concentration of the luminescent material and a low detection sensitivity[33]. As shown in Fig. 2, when the separation voltage is 16 kV, the ECL intensity of the two BAs both have an acceptable maximum value and migration time. When the separation voltage is higher than 16 kV, a large number of analytes drain out from capillary in a short period of time and then the concentration of PMo_{12}- $Ru(bpy)_3^{2+}$ on the surface of electrode is diluted, which reduces the separation efficiency of analytes. Therefore, 16 kV was chosen for the subsequent work.

The separation buffer concentrations and pH values

<Fig.3.A>

<Fig.3.B>

The separation buffer concentrations and pH values are important factors that can impact the CE-ECL luminous intensity. The results shown in Fig.3.A indicate that the ECL intensity increased from 5.00 to 7.00, and

the maximum value obtained was 7.00. When higher than 7.00, the ECL intensity of putrescine decreased with an increase in buffer pH, and the ECL intensity of spermidine fell sharply at first and then rose, which might be caused by inadequate hydroxyl activation of silicon in the capillary. Therefore, a phosphate buffer at a pH of 7.00 was used in the separation capillary in the further experiment.

Then, the effect of the running concentration was investigated from 10-90 mmol/L, and the results are shown in Fig.3.B. When concentration is lower than 50 mmol/L, the ECL intensity of putrescine and spermidine both increased when the buffer concentration increased. As the buffer concentration continued to increase, the two ECL intensities of the BAs decreased. However, when the buffer concentration was higher than 70 mmol/L, the ECL intensity of putrescine only increased slightly. A high running concentration resulted in an increase in the Joule heat of the capillary and caused a low signal-to-noise ratio (S/N). Thus, 50 mmol/L was used as a suitable running concentration.

Injection conditions

<Fig.4.A>

<Fig.4.B>

For the CE-ECL system using the electrokinetic injection, the ECL intensity depends on the injection voltage and injection time. Though higher voltage or a longer time increases the analyte reactions with $Ru(bpy)_3^{2+}$ on the electrode, which resulted in a higher ECL intensity and sensitivity, a broadened peak and a decreased number of theoretical plates are inevitable. In addition, too many analytes might reduce the detection sensitivity, and an overloading effect might take place[30]. Just as is shown in Fig. 4(A), the ECL intensity of putrescine and

spermine increased with the increase in injection voltage, while the theoretical plate number exhibited the opposite trend, indicating that the separation efficiency also decreased. The trend varied similarly when the injection time was investigated. When luminous intensity and separation efficiency were considered, as shown in Fig. 4(A) and (B), an injection voltage of 14 kV and an injection time of 12 s were chosen.

Limit of detection, linearity

Under the optimized conditions (detection potential at 1.15 V, 50 mmol/L separation buffer at pH 7.0, separation voltage at 16 kV, and electrokinetic injection 12 s at 14 kV) for the detection of putrescine, the ECL intensity was linear with a concentration in the range of 0.01-0.1 mg/L. The linear equation was I=623.41c+25.923, and the correlation coefficient was r=0.9980 (n=5). For spermidine, the ECL intensity was linear with a concentration of spermidine in the range of 1×10^{-3}-1×10^{-2} mg/L. The linear equation was I=15438c+14.808, and the correlation coefficient was r=0.9981 (n=5). With the application of the new luminous source material, PMo_{12}- $Ru(bpy)_3^{2+}$, the limits of detection (LOD, S/N=3) of putrescine and spermidine were 6.5×10^{-3} mg/L and 9.8×10^{-4} mg/L, respectively. I represents the ECL intensity of the samples, while c represents the concentration of the samples. The detection limits of the improved CE-ECL method were just above the LOD of the method using $Ru(bpy)_3^{2+}$ luminous source[30] but much lower than those for HPLC (0.5 μg/mL) (GT/B 5009.208, 2008).

Application

<Fig. 5>

The detection results for the ray samples are shown in Fig. 5. Two electropherograms were placed in the

same figure to compare the migration time and peak height between the two samples. The existence of other substances in the sample did not obstruct the detection of putrescine and spermidine. According to the migration time of putrescine and spermidine standard, the first peak in this diagram suggests that putrescine was detected, and spermidine was not detected or the concentration was lower than the detection limit of this method. Through determination, the content of the putrescine in fresh rays is 0.019 mg/L. The internal standard was added to three other samples at a concentration of 0.01, 0.03 and 0.05 mg/L. The ECL intensity of putrescine in the samples including the standard was significantly higher than samples without the standard, and the average recovery and RSD of peak heights and migration times are listed in Table 1 after 5 parallel experiments. The results are within error and are satisfactory.

<Table 1>

Conclusions

This work is based on the new luminous source material, PMo_{12}- $Ru(bpy)_3^{2+}$, which has been applied in a CE-ECL detection system. After the improvement of the system, the separation and detection of putrescine and spermidine in an aquatic product was implemented separately. Under the optimal conditions, the ECL intensity was linear with a concentration of putrescine ranging from 0.01-0.1 mg/L, a correlation coefficient of r=0.9980 (n=5), and a detection limit (S/N=3) of 6.5×10^{-3} mg/L. For spermidine, the ECL intensity was linear at the concentration of spermidine ranging from 0.001-0.01 mg/L. The correlation coefficient was r=0.9981 (n=5), and the detection limit (S/N=3) was 9.8×10^{-3} mg/L. The electrochemical luminescence has become a high-profile detection technology in recent years. For the traditional liquid chemiluminescence detection technology, $Ru(bpy)_3^{2+}$, which is the luminous source material, is expensive and readily causes pollution. By combining

$Ru(bpy)_3^{2+}$ with polyoxometalate, we produced a novel electrochemical luminescence luminous source, PMo_{12}-$Ru(bpy)_3^{2+}$, to solve the above-mentioned issues. The consumption of PMo_{12}-$Ru(bpy)_3^{2+}$ in one detection is lower than $Ru(bpy)_3^{2+}$, and the experimental results also presented better reproducibility[30]. The synthesis and application of PMo_{12}-$Ru(bpy)_3^{2+}$ in the detection of biogenic amines in aquatic products provides a theoretical basis for the study of more efficient and sensitive electrochemical source materials. Combined with some new findings, such as the application of FSN molecules in electrochemical potential control[34,35], the new luminous source could create the chance that the more stable and accurate sensors for biomolecular interaction analysis could occur.

Acknowledgments

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Figure captions

Fig.1. Effect of detection potential on the ECL intensity

Fig.2. Effect of the separation voltage on the ECL intensity and migration time

a:the ECL intensity of putrescine；b:the migration time of putrescine；

c:the ECL intensity of spermidine；d:the migration time of spermidine.

Fig.3. Effect of the running buffer pH (A) and the running buffer concentration (B) on the ECL intensity

Fig.4. Effect of the injection voltage (A) and the injection time (B) on the ECL intensity and theoretical plate number

a: the ECL intensity of putrescine； b: the theoretical plate numbers of putrescine；

c: the ECL intensity of spermidine； d: the theoretical plate numbers of spermidine.

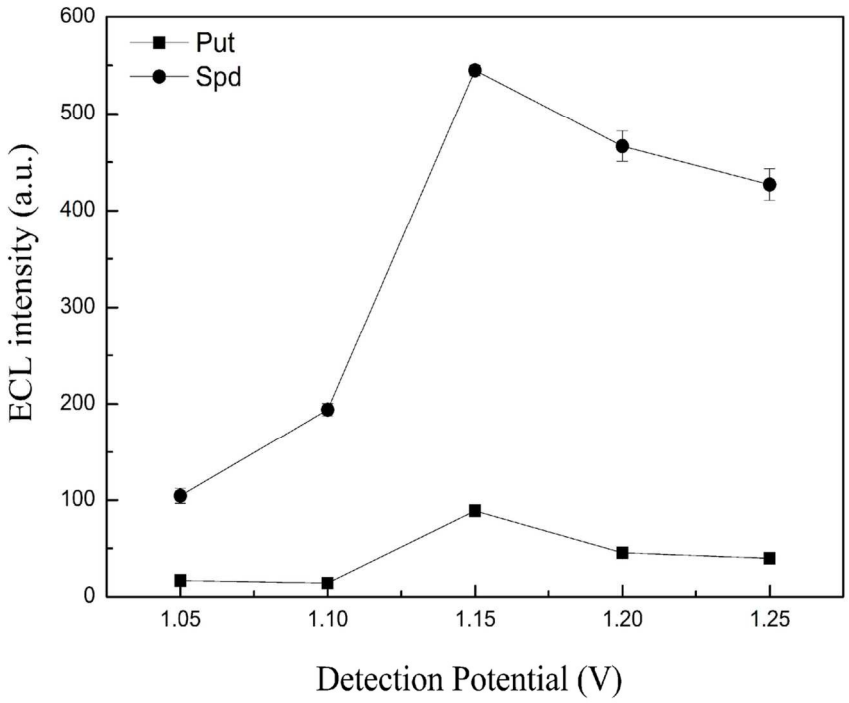
Fig.5. Electrophoresis of the ray sample (a) and after adding 0.03 mg/L of the standard ray sample (b)

Table captions

Table 1. The ECL intensity, the peak height and migration time of the four samples of putrescine with and without the standard

Table 1 The ECL intensity, the peak height and migration time of the four samples of putrescine with and without the standard (Spermidine was not detected in ray samples).

Sample	ECL (a.u.)	Putrescine (mg/kg)	Peak height RSD (%)	migration time RSD (%)	Recovery (%)
Without standard	38.0	0.019	1.19	1.11	-
Standard 0.01mg/L	43.4	0.028	1.83	0.99	90.0
Standard 0.03mg/L	57.1	0.049	2.52	0.67	100.0
Standard 0.05mg/L	70.4	0.071	0.85	0.84	104.0



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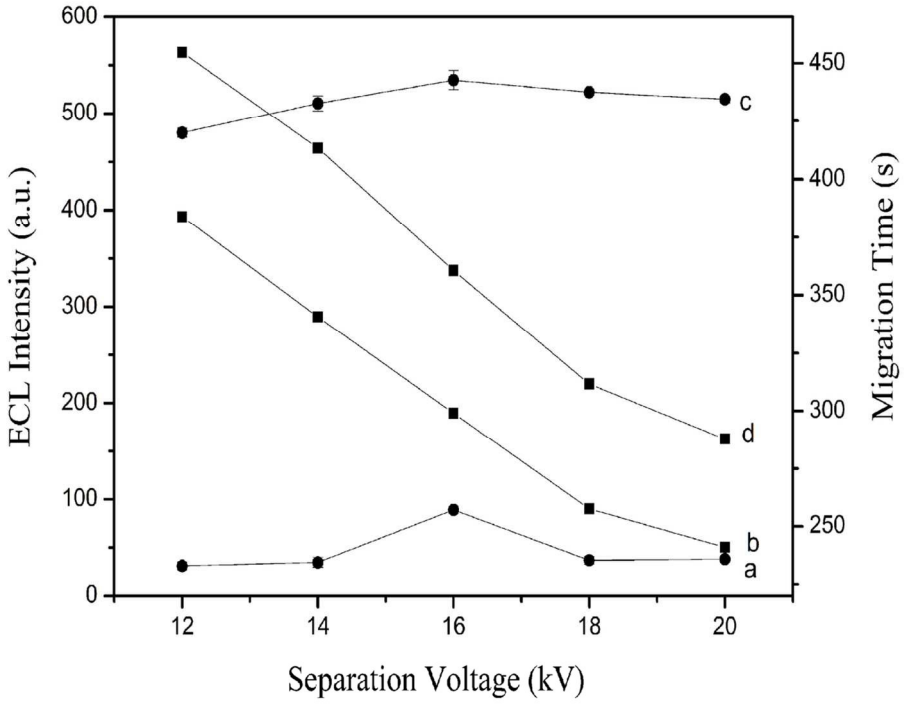

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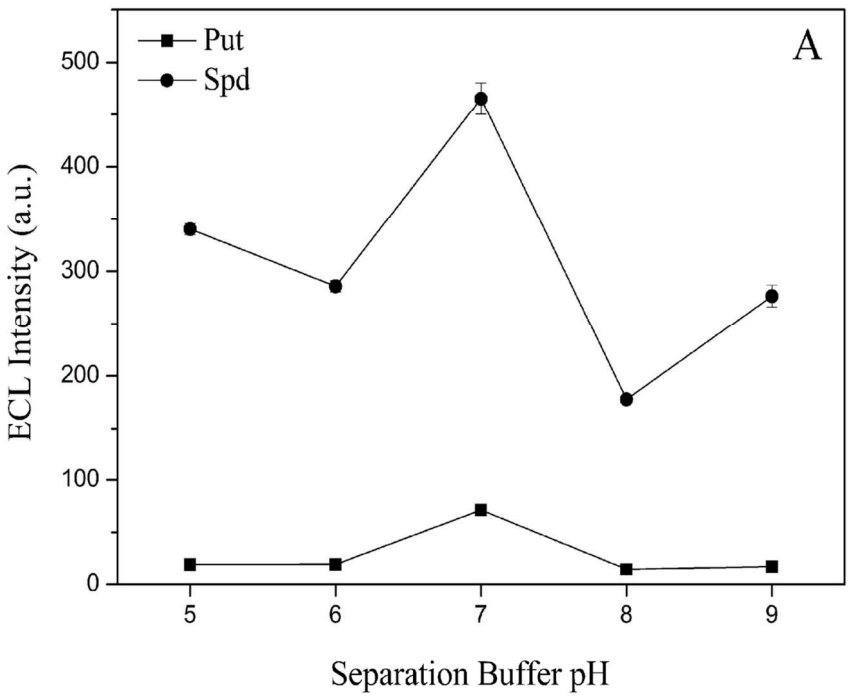

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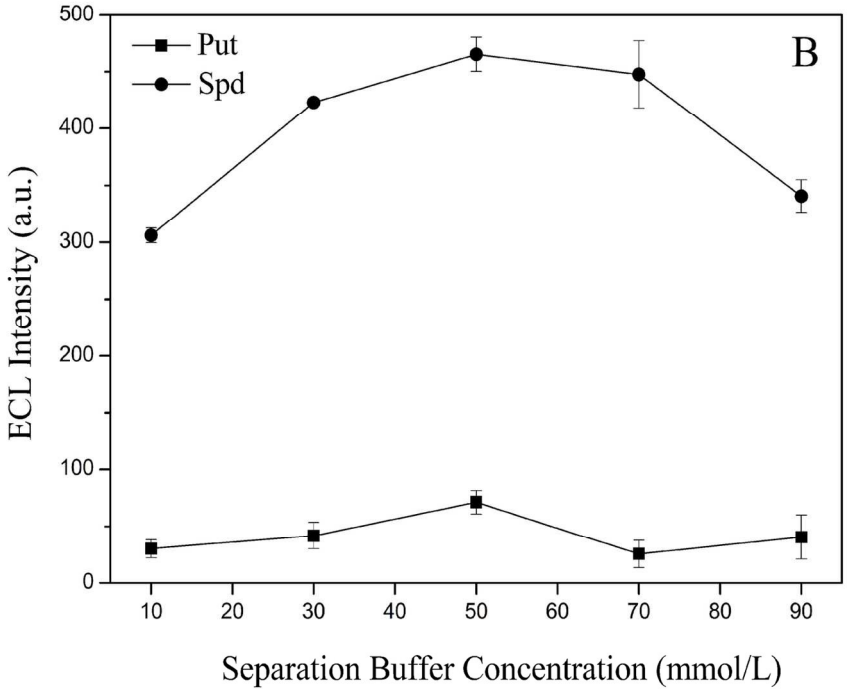

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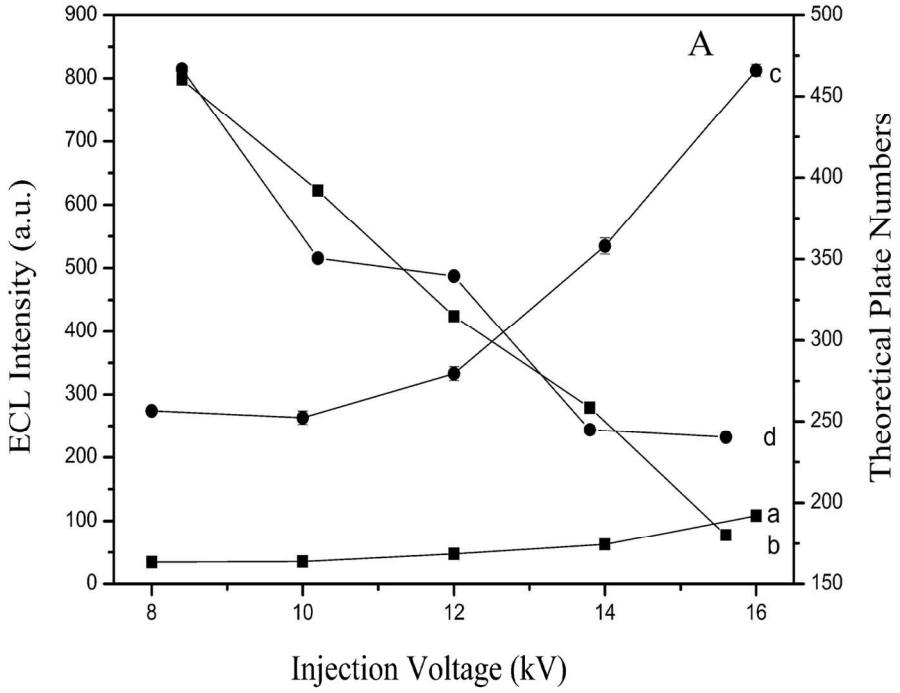

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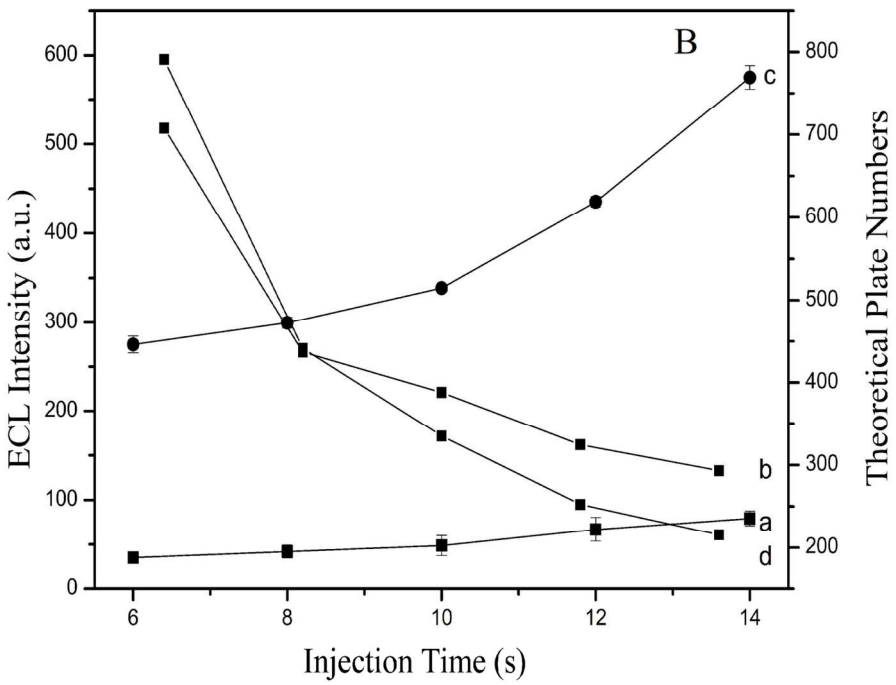

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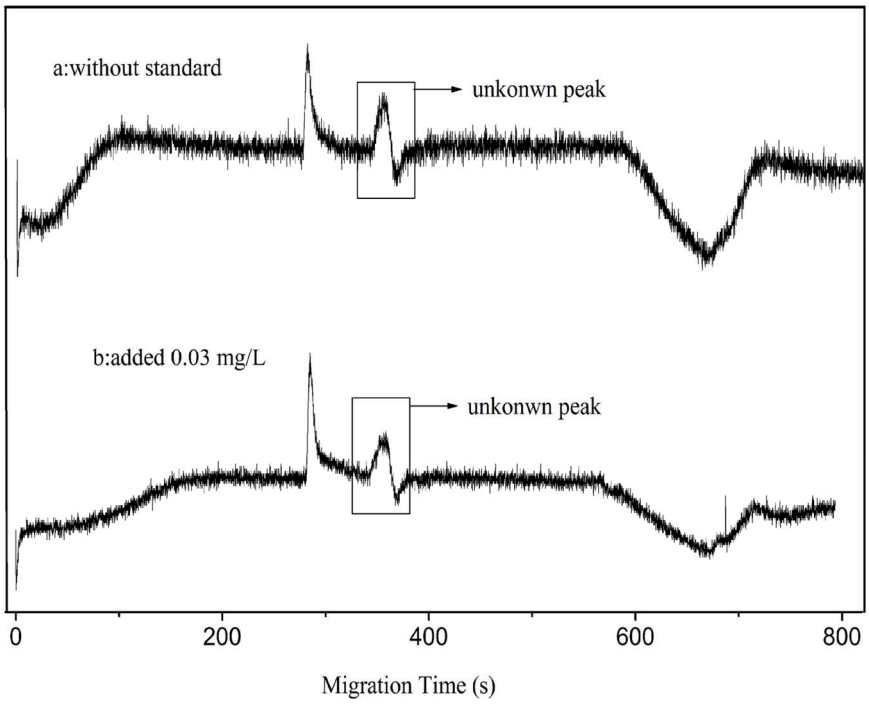

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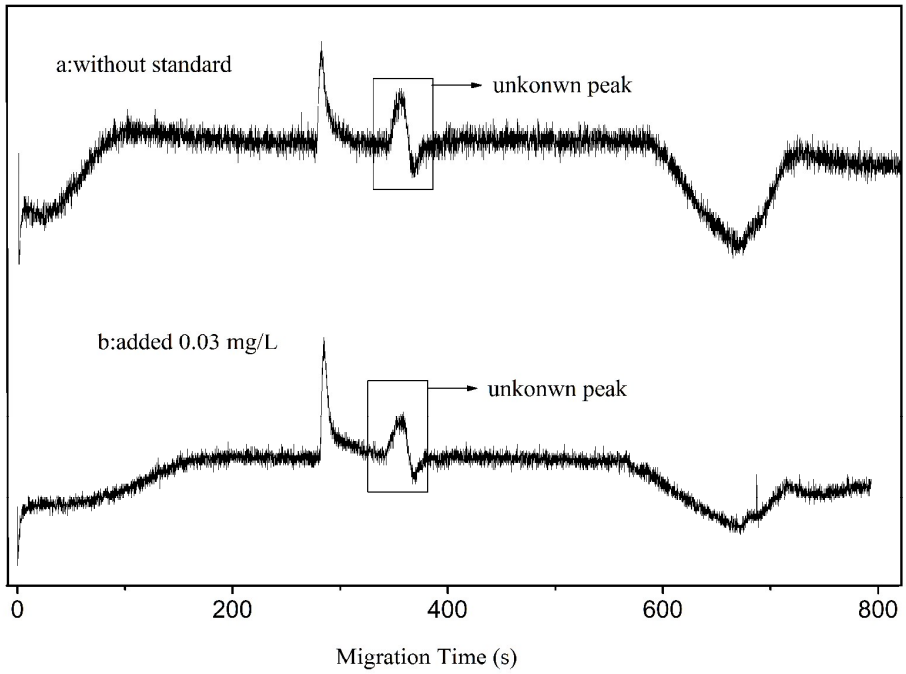

Electropherogrames of rays sample (a) and added 0.03mg/L standard herring sample (b)

As figure shown, the BAs were well separated within 10 min. According to the standard migration time of putrescine and spermidine, the first peak in this diagram represents the existence of the putrescine, while spermidine is not detected or the concentration is lower than the detection limit of this method.