Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 820 (2005) 211–219
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of histamine and polyamines by capillary zone electrophoresis with 4-fluor-7-nitro-2,1,3-benzoxadiazole derivatization and fluorescence detection

Li-Yao Zhang, Xing-Chun Tang, Meng-Xiang Sun *

Key Laboratory of MOE for Plant Development Biology, College of life Sciences, Wuhan University, Wuhan 430072, China

Received 10 November 2004; accepted 14 March 2005

Abstract

Capillary zone electrophoresis (CZE) with fluorescence detection was applied to the simultaneous determination of histamine and polyamines including spermine, spermidine, diaminopropane, putrescine, cadaverine, diaminohexane with 4-fluor-7-nitro-2,1,3-benzoxadiazole (NBD-F) as the fluorescent derivatization reagent. The seven NBD-F labeled amines was separated within 200 s using 85 mM phosphate running buffer at pH 3.0. The concentration limits of these amines ranged from 5.1×10^{-8} M for spermine to 2.1×10^{-8} M for histamine. The relative standard deviations for migration time and peak height were less than 1.5% and 6.0%, respectively. The method was successfully applied to the analysis of biogenic amines in the lysate of tobacco mesophyll protoplasts, and spermidine and putrescine were detected in the lysate with satisfying recovery.

Keywords: Capillary electrophoresis; 4-Fluor-7-nitro-2,1,3-benzoxadiazole; Histamine; Polyamines; Tobacco protoplast

1. Introduction

Biogenic amines are a group of low-molecular-mass active organic bases that usually synthesized by the decarboxylation of amino acid or by the amination and transamination of aldehyde and ketones. They are wildly found in diverse biological systems [1,2]. Of them, histamine is well known as an important neurotransmitter, and a causative agent of scombroid fish poisoning [3]. Meanwhile, putrescine, spermidine, spermine, and to a lesser extent cadaverine, are the major cellular polyamines in the living organisms. These biogenic amines are involved in cellular growth and differentiation, regulation of nucleic acid and protein synthesis, stabilization of lipids, brain development, nerve growth and regeneration [4]. In plant, they also have been found to associate with membrane stability, pH and thermic or osmotic stress responses, delay senescence, and may function as allelochemical compounds and as components of the chemical and physical defenses against herbivores and pathogens [5].

In order to deeply understand the role of these biogenic amines in biological processes, it is essential to develop simple and sensitive analytical techniques for detecting and quantifying these compounds in biological fluids. So far, many analytical methods have been exploited for the determination of histamine and polyamines in food or plant materials, such as gas chromatography [6,7], thin-layer chromatography [8], high-performance liquid chromatography (HPLC) [9–19] and capillary electrophoresis (CE) [1–4,20–25]. Among them, HPLC is by far the mostly frequently reported technique for biogenic amines separation and quantification. And CE has emerged as a powerful analytical tool in the field of analytical biochemistry since it offers higher column efficiency, shorter analysis time and lower running costs compared to HPLC. Especially, capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are the most widely used separation modes in CE, which have been frequently applied to the

* Corresponding author. Tel.: +86 27 68752378; fax: +86 27 68756010.
E-mail address: mxsun@whu.edu.cn (M.-X. Sun).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.03.033

analysis of many chemical and biological reagents in different samples [26–30]. Recently, the analysis of amines in plant material, the electromigration methods for amino acids, biogenic amines and aromatic amines in CE were extensively reviewed by Bouchereau et al. [5] and Oguri [31], respectively.

CE with fluorescence detection has been a most popular analytical tool due to the higher sensitivity over electrochemical and ultraviolet detection. As biogenic amines do not exhibit strong fluorescence, they could not be detected directly in a sensitive manner. Thus, derivatization technique is most frequently used for the biogenic amines detection. Several reviews on the derivatization techniques in capillary electrophoresis have been given in recent years [32,33].

Till now, many fluorescence derivatization reagents have been used for the biogenic amines derivatization, such as fluorescein isothiocyanate (FITC) [1], 5-(4,6-dichloro-*s*-triazin-2-ylamino) fluorescein (DTAF) [25], *o*-phthalaldehyde (OPA) [18,19,21,22], naphthalene-2,3-dicarboxaldehyde (NDA) [26,27,34,35] and 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ) [2]. However, there are some disadvantages of these derivatization reagents. For instance, FITC reaction with amine is very slow, and more than five hours are needed. The reaction rate of DTAF with amines is more rapid than that of FITC with amines, but still needing at least 1 h. OPA can easily react with primary amines within about 30 s in the presence of a reducing reagent, such as *N*-acetylcyteine or 2-mercaptoethanol (2-ME), but the derivatives are not very stable. On the basis of the isoindole formation mechanism in the OPA/2-ME derivatizaion of primary amines, de Montigny et al. [35] developed an improved fluorogenic reagent, NDA, that react with primary amines in the presence of cyanide to produce *N*-substituted 1-cyanobenz[*f*]isoindole (CBI) derivatives. The reaction of NDA with primary amines in the presence of cyanide is simple and quick, and the derivatives are very stable. Recently, we successfully analyzed histamine by pre-column NDA derivatization and CE separation [26]. However, polyamines easily reacted with NDA to produce bis-derivatized polyamines, which exhibit severe reduced fluorescence than the mono-derivatized compounds due to intramolecular quenching [4,34]. Similar phenomena were also found in FQ derivatization with polyamines [4].

Since introduced by Imai and Watanabe [36–40], 4-fluor-7-nitro-2,1,3-benzoxadiazole (NBD-F) has been a popular fluorescent reagent due to its good reactivity with both primary and secondary amines, and high reaction rate [41]. Until now, NBD-F has been identified to be a useful and sensitive fluorescent reagent for HPLC separations of amino acids [38,39], polyamines [40], catecholamines [42], sympathomimetic amines [43], opines [44], agmatine [45], domoic acid [46] and ABT-089, a neutronal cholinerglic channel modulator [47], and CE separations of amino acids [41], methamphetamine [48] and dipeptide [49]. However, to the best of our knowledge, no study has been reported on the analysis of the histamine and polyamines by NBD-F derivatization using CE.

In this work, we report the use of CE and lamp-induced fluorescence detection for the rapid and selective determination of histamine and polyamines with NBD-F as the derivatization reagent to label these amines. Under the optimum conditions, the seven biogenic amines derivatives can be separated within 200 s. This method was successfully applied in the analysis of biogenic amines in the lysate of tobacco protoplasts.

2. Materials and methods

2.1. Apparatus

CE was carried out on a laboratory-built system based on an upright fluorescence microscope (Olympus BH2, Japan), a photo-multiplier tube (PMT), a ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, China) and a uncoated fused-silica capillary of 44–45 cm (34–35 cm length to the detector window) × 50 μm i.d. × 365 μm o.d. (Yongnian Optical Conductive Fiber Plant, China). A 100 W high-pressure mercury lamp was used as the excitation radiation. The optical sub-system in the microscope consisted of a 40× objective, a NIB excitation cube including an excitation filter (EX 400–490 nm), a dichroic mirror (DM 510 nm) and a barrier filter (BA 515 nm). The signal from the PMT was monitored using photon-counting device (Beijing Bingsong photon technological corporation, China) and collected by a computer (Inter PIII550) with photon-counting software, and processed with Origin software packages.

2.2. Chemicals

The tetrahydrochloride salt of spermine (Spm), the trihydrochloride salt of spermidine (Spd), the dihydrochloride salts of histamine, diaminopropane (Pro), putrescine (Put), cadaverine (Cad), and diaminohexane (Hex) purchased from Sigma (St. Louis, MO) were prepared at the concentration of 1.0×10^{-2} M in pure water, diluted to the desired concentration and stored in a refrigerator. NBD-F was obtained from Aldrich. 1.0×10^{-2} M NBD-F stock solution was prepared in ethanol and diluted to the desired concentration in ethanol, then stored in refrigerator at −20 °C. Enzyme solution consisted of 0.6% cellulase, 0.5% pectinase and 9% mannitol at pH 5.7. Other chemical reagents were of analytical grade and used without further purification. All aqueous solutions were prepared from de-ionized water purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Derivatization procedure

Ten microliters of mixture of standard biogenic amines, 10 μl of 5.0×10^{-3} M borate buffer (pH 7.2), and 10 μl of

7.0×10^{-4} M NBD-F solution was added sequentially and thoroughly mixed. Then the mixture was heated at 65 °C for 15 min under dark. After cooling to room temperature, the mixture was directly applied to injection for CE without dilution.

2.4. Capillary electrophoresis

A new capillary was pre-treated with 1.0 M NaOH and water for 30 min sequentially. Each day before analysis, the capillary was rinsed with 0.1 M NaOH, water for 10 min and preconditioned with running buffer for 5 min at room temperature.

The electrophoresis buffer was phosphate buffer solution (PBS) which consisted of 85 mM sodium dihydrogen and adjusted to pH 3.0 with H_3PO_4. Sample injection was performed by hydrodynamic mode with sampling height at 11 cm for 80 s. Separations were carried out at a constant voltage of 20 kV.

2.5. Protoplast lysate preparation

The fresh tobacco (*Nicotiana* The *tabacum* cv. W38) leaves were collected and surface were sterilized according to Sun et al. [50]. The upper epidermis was torn off and the leaves were put in 10 ml of the enzyme solution at 25–26 °C for 4–5 h in the dark. The suspension, containing a large number of protoplasts, was filtered through a 120 μm filter to remove large pieces of plant material. The resulting filtrate solution was centrifuged at $100 \times g$ for 4 min. The suspension enzyme solution was removed with plastic pipettes. At last the pellet was resuspended in 9% mannitol solution. The resuspension cells were carefully overlaid on 15% sucrose solution and centrifuged for 3.5 min at $100 \times g$. Then, the intact protoplasts formed in interphase, were carefully collected. The protoplast solution was centrifuged again at $100 \times g$ for 3 min. And the suspension protoplast solution was transferred into another tube. The number of sedimentary protoplast was calculated to be 2.6×10^4 by contrast method. Then some water was added into the protoplast pellet to lyse the protoplast. The final volume of lysate was 200 μl. The lysate solution was centrifuged at $1400 \times g$ for 3 min. The supernatant was labeled by NBD-F following the procedure mentioned above.

3. Results and discussion

3.1. Separation conditions

In our preliminary experiment, it was found that the amine derivatization reaction with NBD-F is slower than that with NDA and the main reaction products of NBD-F labeled polyamines were monosubstituted derivatives under the mild reaction conditions, which could be separated by CZE. Generally, in CZE separation, the pH of running buffer is a dominant factor for the separation of charged compounds because of its effect on the electroosmotic flow as well as the over-all charges of the analytes. Here, Fig. 1 shows the effect of pH of running buffer ranged from 5.8 to 3.0 on the separation of seven NBD-F labeled biogenic amines. It can be seen that the resolution is strongly dependent on the running buffer pH value. When the pH value was equal to or higher than 5.8, the resolution of the seven labeled amines was very bad due to the high electroosmotic flow. Under this condition, the labeled Spm exhibited a strongly tailing peak, leading to the reduced detection limit. When the pH value was 5.0, the labeled histamine and Hex could not be separated, and the labeled Spm also exhibited a wide peak, which reduced its detection sensitivity. When the pH value was 4.0, the labeled histamine and Put could not be separated either. When the pH value was 3.0, all seven NBD-F-labeled biogenic amines could be well separated and the labeled Spm exhibited a sharp peak. Therefore, pH 3.0 was chosen as the most optimum condition. The peak 1′ was probably the bi-substituted derivative of Spm. The peak 2′ existed in all conditions was probably caused by the impurity of Spd reagent as it co-existed with Spd peak 2. The peak a in Fig. 1D is probably the bi-substituted product of Spd since it was found that the peak co-existed with peak 2 and 2′ by the experimental demonstration. The peak b in Fig. 1D is probably due to reaction side products: NBD-OH and NBD-NH_2 [39,41] as this peak was also found in the background experiment (data not shown). The reason for the different migration order of histamine with diamines with the change of buffer pH was probably the weak dissociation constant of histamine (the dissociation constant of imidazole of histamine are pK_a 6.14 [51]). It was noted that all the peak identification was performed with standard addition method.

The ion strength of running buffer is an important factor in CE separation due to its effect on the electroosmotic flow and the interaction with analytes. Fig. 2 compares the effect of different concentration of phosphate buffer on the separation of seven NBD-F labeled amines. It can be seen that the resolution was improved upon increasing the concentration from 20 to 85 mM. Complete resolution of seven labeled amines is almost achieved when the concentration is 85 mM. However, the resolution between labeled histamine and cadavescine become worse when concentration is 100 mM. This may be due to the increased Joule heating effect. In the experiments, we also tried to further improve the resolution of Put, histamine and Cad, by decreasing the electroosmotic flow by the addition of the triethylamine or cation surfactant, cetyl trimethylamine bromide (CTAB), but unsuccessful. Therefore, 85 mM phosphate buffer (pH 3.0) was the suitable choice for the separation of seven NBD-F labeled amines. Using the optimized conditions, the seven biogenic amines of interest were separated within 200 s. This value is the shortest analysis time ever reported in CE analysis of these biogenic amines. Selectivity of the method was also tested. It was found that the reagent peaks (NBD-OH and NBD-NH_2) and some other biogenic amines derivatives,

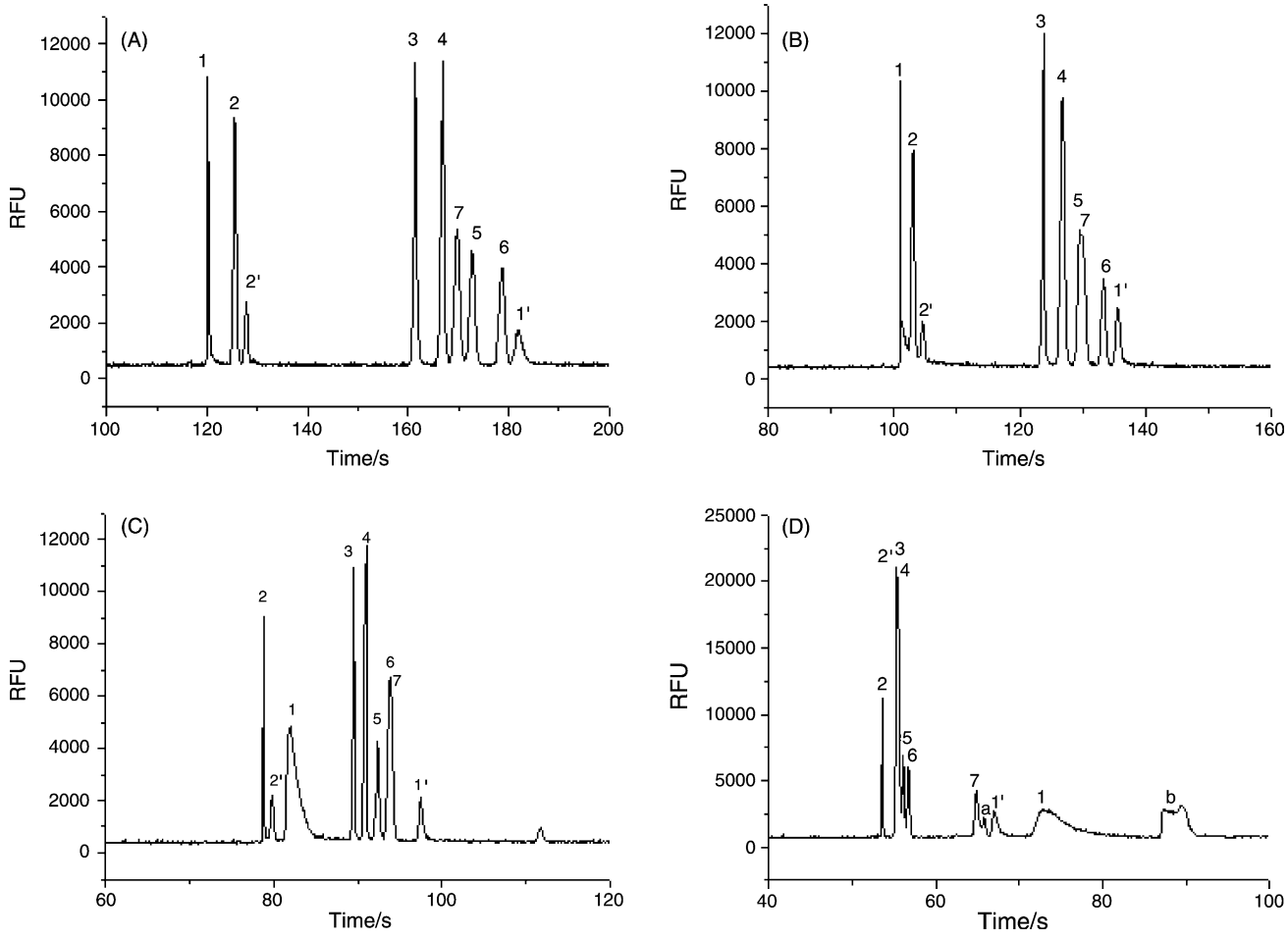

Fig. 1. Electropherograms of NBD-F labeled seven biogenic amines in running electrolytes of different pH values: (A) 3.0, (B) 4.0, (C) 5.0 and (D) 5.8. Capillary, 45 cm × 50 μm i.d.; hydrodynamic injection, 11 cm (height) for 10 s; separation voltage, 20 kV; electrophoresis buffer, 85 mM PBS. Peak identification: (1) Spm; (2) Spd; (3) Pro; (4) Put; (5) Cad; (6) Hex and (7) histamine.

including tryptamine, tyramine, phenylethylamine, catecholamines, and the 20 amine acids derivatives were not detected in the above seven amines separation window of 200 s. Therefore, the method could show better anti-interference capability for the determination of these biogenic amines. This can be explained as the reagent product, the most other biogenic amine derivatives and amino acid derivatives were neutral or anionic in the above separation conditions (85 mM, pH 3.0 phosphate buffer).

3.2. Optimization of derivatization conditions and the injection time

As the derivatization reaction is the typical nucleophilic reaction, the pH of derivatization is an important factor in effecting the derivatization efficiency. The experiments indicated that the derivatization efficiency of seven amines strongly depends on the pH of derivatization, and the best pH value was different for different biogenic amines. For the Spm and Spd, which compose of both primary and secondary amine, pH 6.5–7.2 is the most suitable condition. However, for histamine and diamines, pH 7.2–7.5 is the best choice. With the comprehensive consideration, pH 7.2 was used in this work.

The sample ion strength plays a central role in the separation efficiency. The lower ion strength of sample compared with the running buffer, the more sample stacking effect. The effect of different concentration of derivatization buffer (5, 20 and 40 mM) on the resolution of biogenic amine was studied. It was found that the resolution of Put, histamine, Cad became worse with the increasing of the concentration of derivatization buffer, especially when the concentration was 40 mM. Considering that the low buffer concentration would decrease the buffer capacity we chose 5 mM as the optimal concentration of derivatization buffer.

For potential applications of the method to quantitative analysis, it is important to achieve a single fluorescent product [42]. This is particularly important for the polyamines since they consist of multiple derivatization sites. Spm and Spd have four and three potential derivatization sites, respectively, while diamines have two potential derivatization sites. Excessive amounts of NBD-F would accelerate the derivatization reaction, meantime it might also lead to the less mono-substituted derivatives, but more multi-substituted

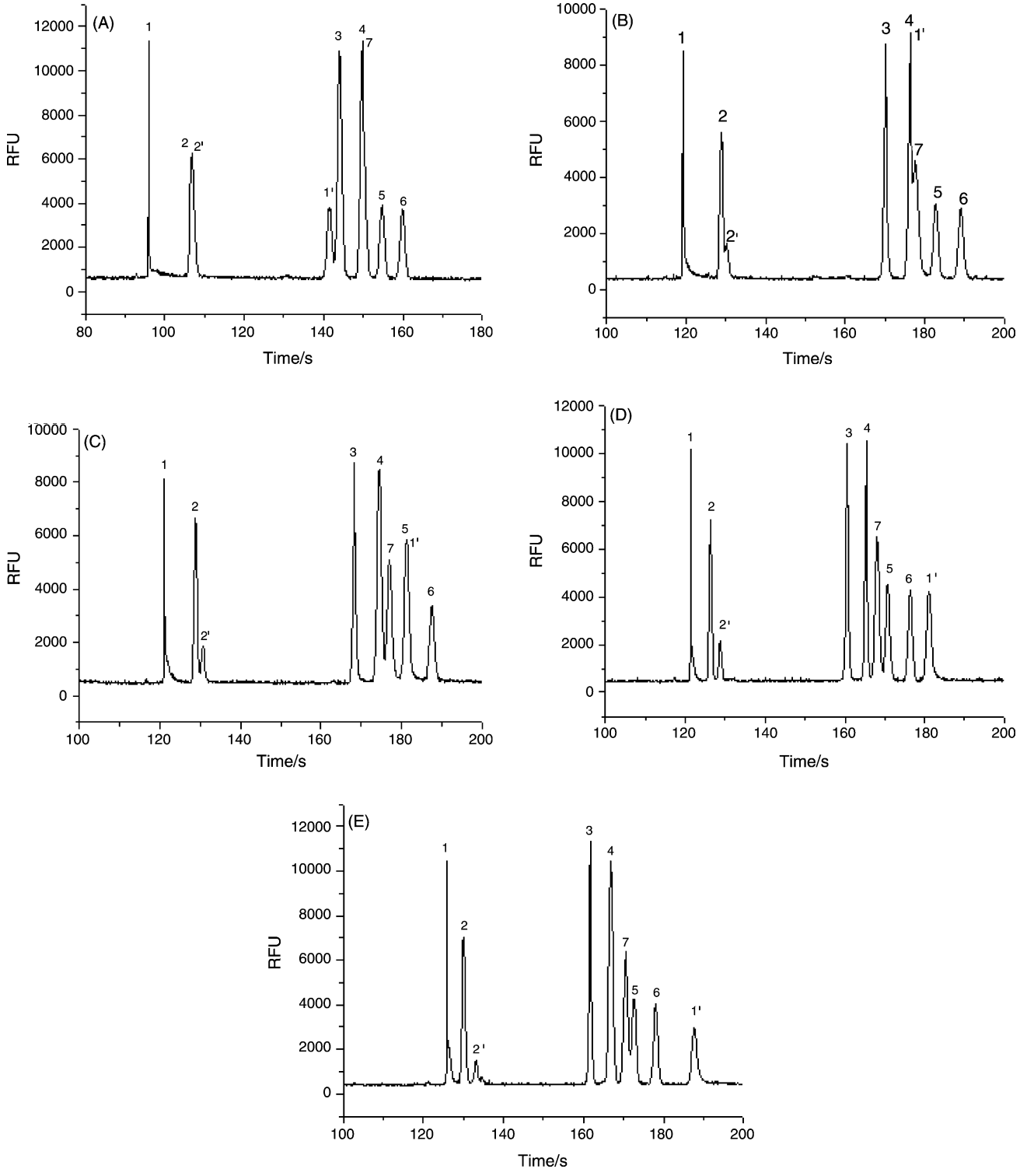

Fig. 2. Effect of electrophoresis buffer concentration on separation of NBD-F labeled seven biogenic amines: (A) 20 mM; (B) 40 mM; (C) 60 mM; (D) 85 mM and (E) 100 mM. Peak identification as in Fig. 1. Other conditions as in Fig. 1.

derivatives. The experiments demonstrated that with the increase of concentration of NBD-F over the range of 0.375–1.5 mM, the derivatization efficiency of diamine and histamine was increased. However, when concentration of NBD-F was beyond 0.75 mM, the mono-substituted derivatization efficiency of Spm and Spd decreased. Therefore, 0.70 mM was chosen for the derivatization reaction in this case.

The derivatization reaction was found active even at 25 °C. However, it was found that the reaction could be accelerated by increased temperature. The yields of diamine and

histamine derivatives were stable when the reaction temperature was between 60 and 90 °C. However, the yields of Spm and Spd derivatives decreased when the reaction temperature was higher than 70 °C. Thus, 65 °C is considered as suitable condition. The reaction time effect was also tested. It was found that the derivatization efficiencies of diamine and histamine increase with the increase of reaction time, and then level off when the reaction time reaches 15 min. However, the fluorescence signal of Spm and Spd became decrease when the reaction time is beyond 15 min, which may be due to the more formation of multi-labeled derivatives. Based on the comparison, 15 min was used for the derivatization. The stability of derivatization product was examined under the room temperature. It was found that the derivatization products of Spd, diamines and histamine were found to be stable at least 20 min. But for the mono-derivatization product of Spm, the fluorescence signal decreased slowly with the increase of time. The different optimal derivatization conditions between the Spm, Spd and diamines was probably due to the higher reactive of secondary amines with NBD-F than primary amine [40]. The sample amount is an important factor influencing the detection sensitivity. With the increase of sample amount, the sensitivity was improved. But excess amount would lead to overloading effect. In our experiments, when the sampling height was fixed at 11 cm, it was found that more than 90 s of injection time would lead to very worse resolution between Put, histamine and Cad. Thus, the injection time of 80 s was at the optimal condition.

3.3. Separation efficiency, reproducibility, linearity, and detection limits of biogenic amines

Fig. 3A and B shows the CZE separation of seven NBD-F labeled biogenic amines under optimal conditions. The separation time is within 200 s. The slightly different migration time compared with Fig. 2D was probably due to the little difference between the capillary lengths. In order to ensure the good reproducibility, the capillary must be washed by the running buffer between the injections, which can eliminate the residual of the last injection and the absorption of hydrophobic NBD-F onto the capillary wall. The analytical reproducibility of the present method was tested by repeating derivatization and injection ($n = 5$) of Spm, Spd, Pro, Put, histamine, Cad, Hex standard solution at concentration of 6.0×10^{-7}, 6.0×10^{-7}, 1.0×10^{-6}, 1.0×10^{-6}, 1.0×10^{-6}, 6.0×10^{-7} and 6.0×10^{-7} M, respectively, under the optimum conditions (Table 1). It can be seen that the relative standard deviations (R.S.D.) of the peak height and the migration time of seven biogenic amines were found to be less than 6.0 and 1.5%, respectively. The linear calibration range, regression equations and the detection limits of seven biogenic amines are listed in Table 1. The detection limits were calculated according to the peak height and noise in Fig. 3A. And the concentration limits of these amines ranged from 5.1×10^{-8} M for spermine to 2.1×10^{-8} M for histamine. The performance comparison of the proposed method with other HPLC and CE method using other reagent was shown in Table 2. It can be seen: NBD-F exhibits the higher reaction velocity compared to FQ, FITC, PSE, Dans-Cl with amines, but the lower reaction velocity compared to OPA and NDA; the separation time of this method is the smallest in all the methods summarized here; the sensitivity obtained with NBD-F was lower than that obtained by FQ, NDA and PSE, but similar to that obtained by OPA and FITC. And the maximum excitation wavelength of NBD-F labeled derivatives was 480 nm, close to argon ion laser line at 488 nm [45], it is expected that the sensitivity could be improved by LIF.

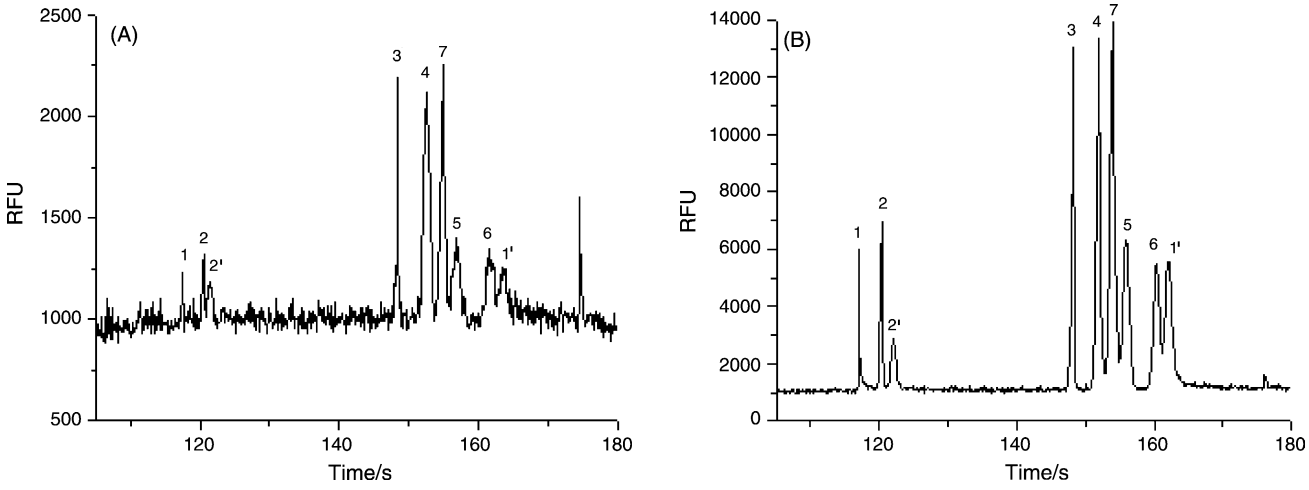

Fig. 3. (A and B) Electropherogram of NBD-F labeled seven biogenic amines under the optimum conditions. The concentration of Spm, Spd, Pro, Put, histamine, Cad, Hex in part (A) were 6.0×10^{-8}, 6.0×10^{-8}, 1.0×10^{-7}, 1.0×10^{-7}, 1.0×10^{-7}, 6.0×10^{-8} and 6.0×10^{-8} M, respectively. The concentrations of amines in part (B) are 10-fold of corresponding amines concentration in A. Conditions: Capillary, 44 cm × 50 μm i.d.; hydrodynamic injection, 11 cm (height) for 80 s; separation voltage, 20 kV; electrophoresis buffer, 85 mM PBS (pH 3.0). Peak identification as in Fig. 1.

Table 1
Linear ranges, reproducibility and the detection limit of amines of the proposed method

Amines	Linear range (μM)	Regression equation	r	R.S.D. (%)		Detection limit (μM)
				MT	PH	
Spermine	0.1–3.0	$Y = 9.57 \times 10^9 C - 731$	0.9996	1.1	5.8	0.051
Spermidine	0.05–3.0	$Y = 9.76 \times 10^9 C - 248$	0.9998	1.1	5.1	0.033
Diaminopropane	0.05–5.0	$Y = 1.04 \times 10^{10} C + 232$	0.9999	1.2	3.2	0.022
Putrescine	0.05–5.0	$Y = 1.04 \times 10^{10} C + 270$	0.9999	1.2	3.5	0.022
Cadaverine	0.05–5.0	$Y = 4.8 \times 10^9 C + 18$	0.9993	1.2	4.2	0.038
Diaminohexane	0.05–3.0	$Y = 6.32 \times 10^9 C + 64$	0.9996	1.3	3.8	0.035
Histamine	0.05–3.0	$Y = 1.03 \times 10^{10} C + 493$	0.9995	1.2	4.2	0.021

C: concentration of biogenic amines; MT: migration time; PH: peak height; Y: relative fluorescence intensity; R.S.D.: relative standard deviation.

Table 2
Comparison of the performance of HPLC and CE separation of biogenic amines with pre-column derivatization using different reagents

Separation method	Reagent[a]	Detection method[b]	Analyte	Reaction time	Separation time (min)	Detection limits[c]	Reference
HPLC	Dans-Cl	Fluor.	11 biogenic amines	30 min	35	0.1–2 ng	[13]
		Fluor.	Polyamines	30 min	10	50–80 nM	[15]
	ACCQ	Fluor.	Polyamines	20 min	40	660 fmol	[16]
	HSQC	Fluor.	Polyamines	20 min	40	30–66 fmol	[17]
	OPA	Fluor.	Polyamines	4 min	15	40–60 nM	[18]
		Fluor.	Histamines	4 min	8	18 nM	[19]
	NBD-F	Fluor.	Polyamines	1 min	30	76–1200 fmol	[40]
CE	FITC	Fluor.	8 biogenic amine	5–12 h	15	$\sim 10^{-8}$ M	[1]
	NDA	Fluor.	Histami	15 min	3	5.5 nM	[26]
	OPA	LIF	8 biogenic amines		25	250 nM	[22]
	PSE	LIF	Polyamines	40 min	10	6–13 nM	[4]
	FQ	LIF	8 biogenic amines	40 min	28	0.5–5 nM	[2]
	NBD-F	Fluor.	7 biogenic amines	15 min	3	51–21 nM	This work

[a] Dans-Cl: dansyl chloride; ACCQ: 6-aminoquinoyl-*N*-hydroxysuccinimidyl-carbamate; HSQC: *N*-hydroxysuccinimidyl-6-quinolinyl carbamate; OPA: *o*-phthalaldehyde; FITC: fluoresceine isothiocyanate; NDA: naphthalene-2,3-dicarboxaldehyde; PSE: 1-pyrenebutanoic acid succinimidyl ester; FQ: 3-(2-furoyl)quinoline-2-carboxaldehyde.
[b] Fluor.: fluorescence; LIF: laser induced fluorescence.
[c] The best data reported in the reference for histamine and polyamines.

3.4. Application

Tobacco protoplasts have been widely used as model material for plant tissue culture [52]. It is easy to control the cell developmental fate by varying culture condition. The cells can be induced to form callus or differentiate into embryos. Analysis of some biogenic amines in different cells with different developmental potential may offer a new clue to study the mechanism of cell fate determination and embryogenesis. As a preliminary study, the developed method has been

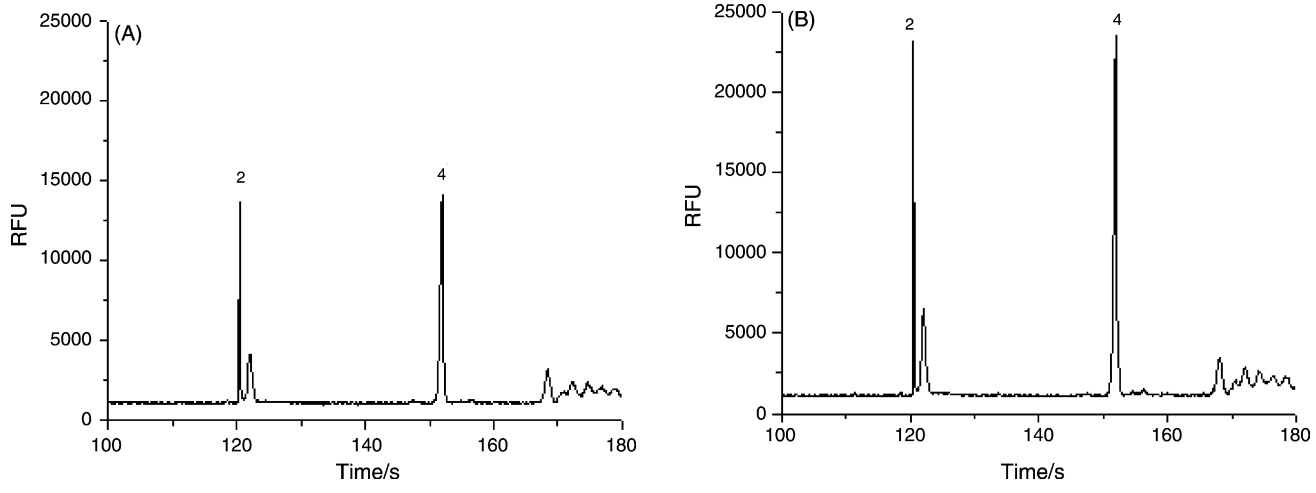

Fig. 4. (A) Electropherogram of biogenic amines analysis in lysate of tobacco mesophyll protoplast and (B) spiked with Spd and Put. Conditions as in Fig. 3.

Table 3
Analytical results of amines in lysate of tobacco leaf protoplast and recovery

Amines	Found (μM)	Added (μM)	Total amount (μM) ($n = 4$)	Recovery (%) ($n = 4$)
Spermidine	1.28	1.00	2.22	97.4
Putrescine	1.13	1.00	2.00	93.9

applied to determination of amines in the lysate of tobacco mesophyll protoplasts. Fig. 4A shows the electropherogram of the lysate of protoplast analysis. To identify the peak of the sample, a standard addition method was employed (Fig. 4B). It was observed that some amount of Spd and Put were detected in the protoplast lysate in this case. It was noted that there are some unknown peaks after migration time of 167 s in the sample analysis (Fig. 4A and B). The analytical results of Spd and Put in the lysate and the recovery were summarized in Table 3. Based on the lysate volume, the number of protoplasts and the lysate concentration, the average amount of Spd and Put in single protoplast was found to be 9.8 and 8.7 fmol, respectively. The blank test was also performed for the comparison, and biogenic amines are not detectable in the mannitol and enzyme solution.

4. Conclusions

The high resolving power of CE provides reliable separations of NBD-F labeled histamine and polyamines. Optimum separation was obtained using 85 mM phosphate buffer at pH 3.0 with separation time within 200 s. A detection limit of 10^{-8} M was obtained for the amines, which is equivalent to or better than other CE analytical methods without using laser as the excitation radiation. The method is suitable for the biological sample analysis by the selective experiment, and offered a potentially convenient technique for analysis of the role of these biogenic amines in both callus and embryos derived from protoplast culture.

Acknowledgements

This work is supported by the National Natural Science Fund of China (30370743, 90408002), the National Outstanding Youth Science Fund (30225006) and the Postdoctoral Fund of China.

References

[1] I. Rodriguez, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 745 (1996) 255.
[2] X. Liu, L.X. Yang, Y.T. Lu, J. Chromatogr. A 998 (2003) 213.
[3] S.C. Su, S.S. Chou, P.C. Chang, D.F. Hwang, J. Chromatogr. B 749 (2000) 163.
[4] R.E. Paproski, K.I. Roy, C.A. Lucy, J. Chromatogr. A 946 (2002) 265.
[5] A. Bouchereau, P. Guenot, F. Larher, J. Chromatogr. B 747 (2000) 49.
[6] B. Dorhout, A.W. Kingma, E. de Hoog, F.A.J. Muskiet, J. Chromatogr. B 700 (1997) 23.
[7] K. Pittertschatscher, R. Hochreiter, J. Thalhamer, P. Hammerl, Anal. Biochem. 308 (2002) 300.
[8] A.R. Shalaby, Food. Chem. 65 (1999) 117.
[9] R. Romero, D. Gazquez, M.G. Bagur, M. Sanchez-Vinas, J. Chromatogr. A 871 (2000) 75.
[10] J. Kirschbaum, K. Rebscher, H. Bruckner, J. Chromatogr. A 881 (2000) 517.
[11] O.O. Lasekan, W.O. Lasekan, Food Chem. 69 (2000) 267.
[12] V. Frattini, C. Lionetti, J. Chromatogr. A 809 (1998) 241.
[13] Z. Loukou, A. Zotou, J. Chromatogr. A 996 (2003) 103.
[14] O. Busto, M. Miracle, J. Guasch, F. Borrull, J. Chromatogr. A 757 (1997) 311.
[15] S. Fu, X. Zou, X. Wang, X.X. Liu, J. Chromatogr. B 709 (1998) 297.
[16] S. Merali, A.B. Clarkson, J. Chromatogr. B 675 (1996) 321.
[17] T. Weiss, G. Bernhardt, A. Buschauer, K.W. Jauch, H. Zirngibl, Anal. Biochem. 247 (1997) 294.
[18] M. Venza, M. Visalli, D. Cicciu, D. Teti, J. Chromatogr. B 757 (2001) 111.
[19] M. Previati, A. Raspadori, L. Bertolaso, A. Parmeggiani, D. Bindini, C. Vitali, I. Lanzoni, E. Corbacella, M. Saviano, F. Fagioli, G. Blo, S. Capitani, J. Chromatogr. B 780 (2002) 331.
[20] L. Arce, A. Rios, M. Valcarcel, J. Chromatogr. A 803 (1998) 249.
[21] S. Oguri, S. Watanabe, S. Abe, J. Chromatogr. A 790 (1997) 177.
[22] K.B. Male, J.H.T. Luong, J. Chromatogr. A 926 (2001) 309.
[23] M. Krizek, T. Pelikanova, J. Chromatogr. A 815 (1998) 243.
[24] S. Oguri, Y. Yoneya, M. Mizunuma, Y. Fujiki, K. Otsuka, S. Terabe, Anal. Chem. 74 (2002) 3463.
[25] M. Molina, M. Silva, Electrophoresis 23 (2002) 2333.
[26] L.Y. Zhang, M.X. Sun, J. Chromatogr. A 1040 (2004) 133.
[27] L.Y. Zhang, Y.M. Liu, Z.L. Wang, J.K. Cheng, Anal. Chim. Acta 508 (2004) 141.
[28] K. Otsuka, S. Terabe, J. Chromatogr. A 875 (2000) 163.
[29] T. Watanabe, S. Terabe, J. Chromatogr. A 880 (2000) 295.
[30] B.F. Liu, H. Hisamoto, S. Terabe, J. Chromatogr. A 1021 (2003) 201.
[31] S. Oguri, J. Chromatogr. B 747 (2000) 1.
[32] H.A. Bardelmeijer, H. Lingeman, C. de Ruiter, W.J.M. Underberg, J. Chromatogr. A 807 (1998) 3.
[33] W.J.M. Underberg, J.C.M. Waterval, Electrophoresis 23 (2002) 3922.
[34] B.K. Matuszewski, R.S. Givens, K. Srinivasachar, R.G. Carlson, T. Higuchi, Anal. Chem. 59 (1987) 1102.
[35] P. de Montigny, J.F. Stobaugh, R.S. Givens, R.G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Anal. Chem. 59 (1987) 1096.
[36] K. Imai, Y. Watanabe, Anal. Chim. Acta 130 (1981) 377.
[37] Y. Watanabe, K. Imai, Anal. Biochem. 116 (1981) 471.
[38] Y. Watanabe, K. Imai, Anal. Chem. 55 (1983) 1786.
[39] Y. Watanabe, K. Imai, J. Chromatogr. 239 (1982) 723.
[40] T. Toyo'oka, Y. Watanabe, K. Imai, Anal. Chim. Acta 149 (1983) 305.
[41] S. Hu, P.C.H. Li, J. Chromatogr. A 876 (2000) 183.
[42] X. Zhu, P.N. Shaw, D.A. Barrett, Anal. Chim. Acta 478 (2003) 259.
[43] O. Al-Dirbashi, N. Kuroda, K. Nakashima, Anal. Chim. Acta 365 (1998) 169.
[44] D. Zhang, R. Shelby, M.A. Savka, Y. Dessaux, M. Wilson, J. Chromatogr. A 813 (1998) 247.

[45] S. Zhao, Y. Feng, M.H. LeBlanc, J.E. Piletz, Y.M. Liu, Anal. Chim. Acta 470 (2002) 155.
[46] K.J. James, M. Gillman, M. Lehane, A. Gago-Martinez, J. Chromatogr. A 871 (2000) 1.
[47] P.D. Bryan, M.L. Emry, T.A. El-Shourbagy, J. Pharm. Biomed. Anal. 20 (1999) 49.
[48] N. Kuroda, R. Nomura, O. Al-Dirbashi, S. Akiyama, K. Nakashima, J. Chromatogr. A 798 (1998) 325.
[49] I. Beijersten, D. Westerlund, J. Chromatogr. A 716 (1995) 389.
[50] M.X. Sun, H.Y. Yang, C. Zhou, Acta Bot. Sinica 36 (1994) 489.
[51] J.A. Dean, Lange's Handbook of Chemistry, 11th ed., McGraw-Hill Book Company, New York, 1973.
[52] E. Frioozabady, Plant Sci. 46 (1986) 127.