Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1095 (2005) 185–188
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

# Selective determination of γ-aminobutyric acid, glutamate and alanine by mixed micellar electrokinetic chromatography and fluorescence detection

Li-Yao Zhang, Meng-Xiang Sun *

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

Received 2 August 2005; received in revised form 10 September 2005; accepted 22 September 2005
Available online 10 October 2005

**Abstract**

A mixed micellar electrokinetic chromatography method with fluorescence detection was developed to simultaneously monitor γ-aminobutyric acid (GABA), glutamate (Glu) and alanine (Ala) in biological samples. Amino acids were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA). The separation of three NDA-labeled isomers (GABA, α-ABA, β-ABA) was studied in detail with different micelles solutions such as sodium dodecyl sulfate (SDS), β-cyclodextrin (β-CD) and sodium cholate (SC). Simultaneous resolution of GABA, Glu and Ala from 21 amino acids was achieved within 5 min using 20 mM phosphate buffer at pH 8.7 containing 24 mM SC and 26 mM SDS. The detection limits were $4.0 \times 10^{-8}$, $1.1 \times 10^{-8}$ and $1.3 \times 10^{-8}$ M, for GABA, Glu and Ala, respectively, with S/N = 2. The method was applied to monitor the changes of amount of GABA, Glu and Ala in tobacco leaf in response to cold and dark stress.


*Keywords:* Mixed micellar electrokinetic chromatography; GABA; GABA isomers; Glutamate; Alanine

## 1. Introduction

γ-Aminobutyric acid (GABA), a non-protein amino acid, is a significant component of the free amino acid pool, which was produced through the decarboxylation of L-glutamate catalysed by glutamate decarboxylase (GAD, EC 4.1.1.15) [1]. GABA is an important inhibitory amino acid neurotransmitter in the central nervous system and essential for brain metabolism and function in animal [2]. While in plant, it has been treated merely as a metabolite for decades, but the recent evidence points towards a new possible role of GABA as a signal molecule in response to stress and carbon:nitrogen metabolism [3]. A number of analytical methods have been developed till now to meet the requirements of the research of the bioactive function of GABA, such as magnetic resonance spectrometry [4], electrochemical biosensor [5], gas chromatography [6], high-performance liquid chromatography (HPLC) [2], etc.

Capillary electrophoresis (CE) has emerged as a powerful analytical tool due to many advantages over HPLC. Mainly, there are two frequently used modes in CE: capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). The introduction of the latter one by Terabe [7,8] largely extended the application of CE in the field of analytical biochemistry, especially to the analysis of total amino acids. And the most popular surfactant in MEKC for the separation of amino acids is sodium dodecyl sulfate (SDS) [9,10]. Shou et al. [10] successfully used SDS micelles buffer solution to separate 18 NDA-labeled amino acid including α-ABA, β-ABA and GABA using a 10 μm i.d. separation capillary. Some other surfactant, sodium cholate (SC) [11,12], or additives, β-cyclodextrin analogues [13], were also used. Bowser and Kennedy [13] successfully applied hydroxypropyl-β-cyclodextrin modified CE to resolve *o*-phthaldehyde (OPA) labeled amine neurotransmitters, and the separation of α-ABA, β-ABA and GABA using this system was also investigated.

In this work, we present a mixed MEKC method for analysis of GABA and GABA-related amino acids: glutamate (Glu) and alanine (Ala), which are involved in the synthesis and metabolism of GABA, respectively. NDA was used as the derivatization reagent. The mixed SDS and SC were employed in MEKC for separation. Under the optimum conditions, GABA, Ala and Glu can be resolved from the other 21 amino acids including the two isomers of GABA, 16 protein amino acids and 3 non-protein amino acids. And the proposed method was

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

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.09.050

applied to monitor the changes of amount of GABA, Ala and Glu in tobacco leaves in response to cold and dark stress.

## 2. Experimental

### 2.1. Apparatus and chemicals

The setup used in this work is the same as we reported previously [14], except the uncoated capillary is 37–38 cm (26–27 cm length to the detector window) × 50 μm i.d. × 365 μm o.d.

α-ABA (also called 2-ABA) and β-ABA (also called 3-ABA) were purchased from Fluka. All other amino acids were purchased from Sigma (St. Louis, MO) and prepared in ultrapure water purified with a Milli-Q system (Millipore, Bedford, MA, USA), and stored in a refrigerator. SDS was purchased from Serva (Germany). SC and β-cyclodextrin (β-CD) were purchased from Sigma. NDA was obtained from Aldrich. NDA ($1.0 \times 10^{-2}$ M) stock solution was prepared in methanol and diluted to the desired concentration in methanol, and stored in refrigerator. Potassium cyanide (KCN) was purchased from molecular probes. Other chemical reagents were of analytical grade and used without further purification. Carrier electrolyte for capillary electrophoresis was prepared daily with ultrapure water.

### 2.2. Derivatization procedure

Sixty microlitres of a mixed amino acids solutions, 10 μl of $1.0 \times 10^{-2}$ M borate buffer (pH 9.1), 10 μl of $3.0 \times 10^{-2}$ M cyanide and 15 μl of $2.0 \times 10^{-3}$ M NDA solution was added to a small centrifugal vial sequentially and then thoroughly mixed. The resulting solution was allowed to stand for 30 min at room temperature prior to injection.

### 2.3. Capillary electrophoresis

A new capillary should be pre-treated with 1.0 M NaOH, water for 30 min sequentially. Each day before analysis, the capillary was rinsed with 0.1 M NaOH, water for 5 min and preconditioned with running buffer for 10 min at room temperature. The electrophoresis buffer consisted of 20 mM $Na_2HPO_4$ buffer (pH 8.7) containing 24 mM SC and 26 mM SDS. Sample injection was performed by hydrodynamic mode with sampling height at 9 cm for 20 s. Separations were carried out at 20–21 kV.

### 2.4. Plant materials and sample preparation

Sample 1: the fresh tobacco (Nicotiana The tabacum cv. SRI) leaf was sniped, and 7 mg of leaf was collected and immediately grinded in the presence of 1 ml methanol in the mortar for 5 min, the extract was transferred to a centrifugal tube, and then 1 ml methanol was added to the mortar to dissolve and dilute the remaining extract, and transferred to the tube, the procedure was repeated with water for several times, and the total extract finally was diluted with water to 20 ml.

Sample 2: 7 mg of leaf was collected from the same leave as the sample 1, then stored in a refrigerator at 0 °C for about 30 min, and restored at room temperature for 10 min, then treated with the same procedure as for sample 1 to extract amino acids. The extracts of sample 1 and 2 were centrifuged at 8000 rpm for 10 min, respectively. The supernatant was derivatized by NDA/$CN^-$ as the above derivatization procedure.

## 3. Results and discussion

### 3.1. Choice of derivatization conditions

NDA was used as the derivatization reagent due to the high reactivity, low background fluorescence and the high stability of derivatives [15]. In our experiments, the final concentration of $CN^-$ and NDA were about $3.2 \times 10^{-3}$ and $3.2 \times 10^{-4}$ M respectively, and the pH value for derivatization was 9.1, similar as previously reported [15,16]. The examination of effect of reaction time revealed that the reaction of NDA/$CN^-$ with GABA was quicker than with α-ABA and β-ABA, probably due to the lower space hinders of GABA, similar to that obtained for derivatization of histamine and histidine with NDA/$CN^-$ [14]. Considering the reactions of other amino acids, 30 min was chosen for derivatization.

### 3.2. Optimization of CE separation parameters

It is essential to resolve GABA from α-ABA and β-ABA to exclude the potential interference for analysis [13]. However, it is difficult to separate them by using simple running buffers, so additives or surfactants modified CE buffer should be used. Firstly, SDS was employed. It was found that β-ABA and GABA co-elute with SDS at 10–60 mM, though α-ABA can be partly identified with SDS at 30–60 mM. It was noted that in this case, a capillary with 50 μm i.d. was used to increase the sensitivity, though reducing the resolution compared to that reported with 10 μm i.d. capillary [10]. Similar results were obtained with β-CD or β-CD modified SDS as the additive, β-ABA and GABA could not be resolved either.

Then SC, a bile salt, was tested, which could produce micelles with hydrophobic surface and hydrophilic interior [17]. The baseline separation of three isomers was almost achieved when 20 mM SC was used. However, GABA could not be identified from other amino acids with single SC micelles, indicating the amino acids present in the biological samples might interfere the analysis of GABA. During the experiments, it was found that the reaction of cysteine with NDA/$CN^-$ is very slow, no fluorescence was detected within 30 min even in high concentration of cysteine ($1.0 \times 10^{-2}$ M), and proline cannot react with NDA/$CN^-$, so 24 amino acids (18 protein amino acids, α-ABA, β-ABA, GABA, taurine, citrulline and norvaline) were studied in this work.

The mixed SDS/SC micelle has been demonstrated as an effective micelles system to resolve complex compounds [18,19]. In this work, it was found that the resolution of the three isomers was greatly improved by mixed micelles, probably due to the extended elution range [12,20]. The further experimental results revealed that GABA could be resolved from other amino acids with SDS at 30 mM, but cannot be resolved with SDS at

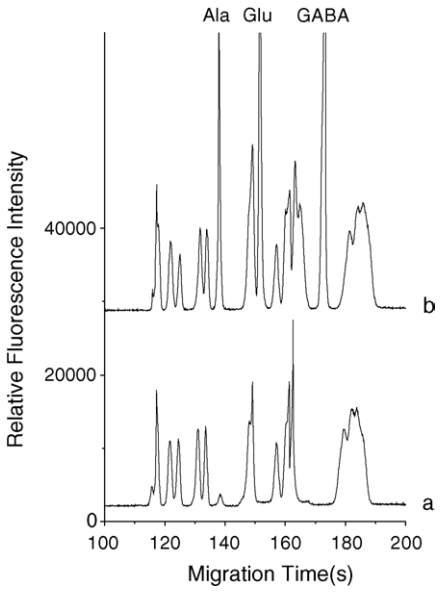


Fig. 1. Electropherograms of 21 amino acids without GABA, Glu and Ala (A) and 24 amino acids with GABA, Glu and Ala (B), using 20 mM phosphate buffer (pH 8.7) containing 24 mM SC and 26 mM SDS. The current in capillary was 72 μA. The composition of 24 amino acids were mentioned in the text; hydrodynamic injection, 9 cm (height) for 20 s; separation voltage, 20.2 kV.

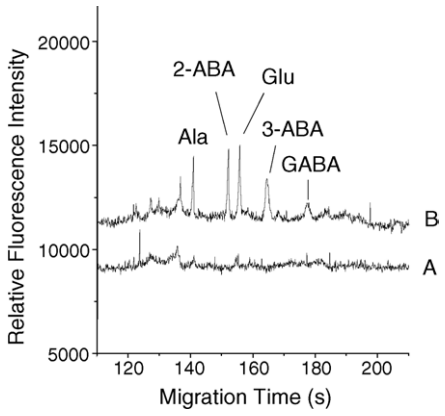


Fig. 2. Separation of NDA-labeled Ala, Glu, α-ABA, β-ABA and GABA under the optimum conditions. The concentration of Ala, Glu, α-ABA, β-ABA and GABA in (B) were $5.0 \times 10^{-8}$, $5.3 \times 10^{-8}$, $4.0 \times 10^{-8}$, $4.0 \times 10^{-8}$ and $4.0 \times 10^{-8}$ M, respectively. (A) It shows the blank derivatization. The current in capillary was 72 μA. The conditions were same as in Fig. 1.

10, or 20, or 40, or 50 mM in 25 mM SC micelles solutions, and SC higher than 30 mM with SDS fixed at 30 mM would make the resolution worse. And the examination of electrophores is behavior of Glu and Ala under the condition of 25 mM SC and 30 mM SDS showed that Glu could not be resolved. Whereas when 24 mM SC and 26 mM SDS was used, the three amino acids could be resolved with other 21 amino acids simultaneously (Fig. 1). It is noted that Ala was interfered by a small peak in Fig. 1, probably caused by impurity, since it is obviously smaller than peak of amino acids.

### 3.3. Typical electropherogram, reproducibility, linearity and detection limit

Fig. 2 shows the separation of five NDA-labeled amino acids (GABA, Glu, Ala, α-ABA and β-ABA) under optimal conditions. The reproducibility was evaluated by repeating injections ($n = 5$) of GABA, Ala, Glu, α-ABA and β-ABA standard solution at the concentration of $2.0 \times 10^{-6}$, $2.5 \times 10^{-6}$, $2.65 \times 10^{-6}$, $2.0 \times 10^{-6}$ and $2.0 \times 10^{-6}$ M. The relative standard deviation (RSD) of peak height and the migration time of five amino acids were found to be less than 6.0 and 2.0%. Using the fluorescence intensity versus sample concentration, the linear calibration curve was obtained for amino acids (number of concentration for each amino acid = 6). For Glu and Ala, the concentration range was $5.0 \times 10^{-8}$ to $5.0 \times 10^{-6}$ M (Glu $R = 0.998$, Ala $R = 0.998$); for α-ABA, β-ABA and GABA, it was $4.0 \times 10^{-8}$ to $4.0 \times 10^{-6}$ M (α-ABA $R = 0.998$, β-ABA $R = 0.997$, GABA $R = 0.996$). Based on the data of Fig. 2B, the detection limits for Ala, Glu, α-ABA, β-ABA and GABA are calculated to be $1.3 \times 10^{-8}$, $1.1 \times 10^{-8}$, $8.4 \times 10^{-9}$, $1.5 \times 10^{-8}$ and $4.0 \times 10^{-8}$ M, respectively, with S/N = 2.

### 3.4. Application

Typically, GABA levels in plant tissues are low, but increase several fold in response to many stimuli, including cold stimulation, hypoxia, etc. [1]. Fig. 3 showed the changes of amount of

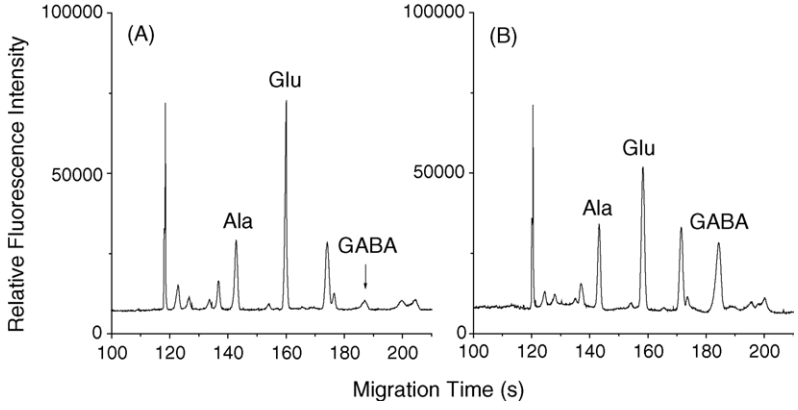


Fig. 3. Illustration of the changes of amount of Glu, Ala and GABA in tobacco leaf in response to cold and dark stress. (A) Without stress; (B) with stress. The current in capillary was 72 μA. The conditions were same as in Fig. 1.

GABA, Glu and Ala in tobacco leaf with cold and dark stress. To ensure the reliability, same amount of samples were collected from the symmetric position of the same leaf. It can be seen that the amount of GABA in tobacco leaf was obviously increased with stress, and eight-fold increase of the amount of GABA can be obtained. The amount of Glu obviously decreased with stress, indicating that glutamate decarboxylase was very active in the tissue of the tobacco leaf since Glu was a synthetic precursor of GABA. The amount of Ala increased slightly with this treatment, and it has been found that the cold and dark stress could induce the increase of Ala and GABA, and the decrease of Glu in soybean leaf [21]. The observed phenomena were similar to their results.

## Acknowledgements

This research is supported by the National Natural Science Foundation of China (30370743, 90408002), National Outstanding Youth Science Fund (30225006) and “PCSIRT”.

## References

[1] B.J. Shelp, A.W. Bown, M.D. McLean, Trends Plant Sci. 4 (1999) 446.
[2] M.Y. Khuhawar, A.D. Rajper, J. Chromatogr. B 788 (2003) 413.
[3] N. Bouche, H. Fromm, Trends Plant Sci. 9 (2004) 110.
[4] M. Hajek, M. Burian, M. Dezortova, Magn. Reson. Mater. Phys. Biol. Med. 10 (2000) 6.
[5] F. Mazzei, F. Botre, G. Lorenti, F. Porcelli, Anal. Chim. Acta 328 (1996) 41.
[6] E.A. Strays, W.S. Guerand, H.J. ten Brink, C. Jakobs, J. Chromatogr. B 732 (1999) 245.
[7] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[8] S. Terabe, K. Otsuka, T. Ando, Anal. Chem. 57 (1985) 834.
[9] S. Valerie, P. Sandrine, B. Nadia, B.M. Eva, R. Bernard, D. Luc, Electrophoresis 24 (2003) 3187.
[10] M. Shou, A.D. Smith, J.G. Shackman, J. Peris, R.T. Kennedy, J. Neurosci. Methods 138 (2004) 189.
[11] S. Hu, P.C.H. Li, J. Chromatogr. A 876 (2000) 183.
[12] S. Terabe, M. Shibata, Y. Miyashita, J. Chromatogr. 480 (1989) 403.
[13] M.T. Bowser, R.T. Kennedy, Electrophoresis 22 (2001) 3668.
[14] L.Y. Zhang, M.X. Sun, J. Chromatogr. A 1040 (2004) 133.
[15] P. de Montigny, J.F. Stobaugh, R.S. Givens, R.G. Garlson, K. Krinivasachar, L.A. Sternson, T. Higuchi, Anal. Chem. 59 (1987) 1096.
[16] F. Robert, L. Bert, L. Denoroy, B. Renaud, Anal. Chem. 67 (1995) 1838.
[17] R.O. Cole, M.J. Sepaniak, J. Chromatogr. 557 (1991) 113.
[18] M.C. Boyce, E.E. Spickett, J. Agric. Food Chem. 47 (1999) 1970.
[19] S.K. Wiedmer, M.L. Riekkola, Anal. Chem. 69 (1997) 1577.
[20] M.G. Khaledi, J. Chromatogr. A 780 (1997) 3.
[21] W. Wallace, J. Secor, L. Schrader, Plant Physiol. 75 (1984) 170.