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ARTICLE

In-Capillary Derivatization and Stacking Electrophoretic Analysis of γ-Aminobutyric Acid and Alanine in Tea Samples To Redeem the Detection after Dilution To Decrease Matrix Interference

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An in-capillary derivatization and stacking capillary electrophoresis (CE) technique has been applied to redeem the detection of dilute analytes in the analysis of γ-aminobutyric acid (GABA) and alanine (Ala) in tea samples. Extracts from samples were diluted to eliminate matrix interference before introduction into the CE system. GABA and Ala in the diluted sample zone were derivatized with *o*-phthaldialdehyde/2-mercaptoethanol (OPA/2-ME) to form fluorescence-labeled products in the stacking process, and the labeled derivatives were then enriched by online stacking. Optimal conditions for the stacking, such as the concentration of the background buffer solution, the matrix of the sample zone (sample solution), and the volume of the sample injection, were investigated and then applied to real sample analysis. Under optimum conditions, the detections were linear in the range of 5.0 nM−2.5 μM with the square of correlation coefficients (R^2) of 0.9995 and 0.9992 for GABA and Ala, respectively. Detection limits were found to be 0.7 and 0.8 nM for GABA and Ala, respectively. Tea samples were analyzed with recoveries between 92.33 and 97.87% and between 94.36 and 96.46% for GABA and Ala, respectively. This method is a rapid, convenient, and sensitive process for determining GABA and Ala in complicated matrix samples such as tea samples.

KEYWORDS: γ-Aminobutyric acid; capillary electrophoresis; derivatization; in-capillary stacking; matrix interference

INTRODUCTION

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian brain and is widely distributed throughout the nervous system. It helps regulate neuron activity and also helps keep nerve cells firing normally (*1*, *2*). Moreover, it is considered to have the potential to maintain the balance of blood pressure in individuals with hypertensive cardiovascular disease (*3*). Therefore, GABA-containing drinks brewed from teas have recently become a popular daily drink among health-conscious individuals in Asian countries (*4*). A convenient analytical method is thus required to determine such content in teas for the investigation of their biofunction.

Reverse-phase high-performance liquid chromatography (HPLC) (*5*−*7*) and capillary electrophoresis (CE) (*8*−*11*) have been applied to determine GABA in human biological fluids and tissues or in tea leaves and beverages, and a labeled derivatization is often used to enhance detection (*12*). The in-capillary derivatization method was developed into a versatile derivatization technique for CE or capillary electrochromatography (CEC) in the analysis of amino acids in samples (*13*−*20*), due to the instability of derivatives and the adverse effect of dilution volume from precolumn manipulation and the requirement of a complex instrument for a postcolumn derivatization. In our previous study, the concentration of GABA and Ala in tea samples was successfully measured by a CE system with an in-capillary labeling derivatization technique using *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (2-ME) as derivative agents (*20*). Nonetheless, the CZE system with an in-capillary labeling derivatization technique has been confirmed as being more sensitive and faster than the MEKC method in GABA detection (*20*). However, the interaction of analyte with sample matrix species would affect the interaction of analyte with separation media and, consequently, detection sensitivity. Moreover, sample matrix contributes to detection noise and consequently can decrease detection sensitivity. The levels of interference depend on the complexity of the sample matrix.

Sample stacking is a general method in CE used for online concentration of diluted analytes, which provides better separation efficiency and detection sensitivity. During the stacking process, analytes present at low concentrations in a long injected sample zone are concentrated into a short zone (stack) through various mechanisms (*21*). The stacked analytes are then separated, and individual zones are detected. Stacking techniques have been popularly used for the analysis of physiological samples (*22*, *23*) and applied in combination with in-capillary derivatization for enhancing detection sensitivity of amino acids

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in capillary electrophoresis (*24*, *25*). Therefore, the sample matrix interferences in the electrophoretic determination of GABA in tea samples can be improved through the dilution of a sample solution and then redemption of detection sensitivity by using stacking and in-capillary derivatization.

In this paper, we determined the concentration of GABA and Ala in tea samples using matrix dilution, in-capillary derivatization, and stacking coupled to a CE system using an acetonitrile/salt mixture as sample solvent and OPA/2-ME as derivative agents. The conditions for stacking were optimized, in addition to the conditions for labeling derivatization and separation we optimized before (*20*). Our results showed that this analytical procedure is a rapid, convenient, sensitive, and eco-friendly way to analyze GABA and Ala in tea samples.

MATERIALS AND METHODS

Reagents and Solutions. A Barnstead Nanopure water system (Barnstead, NY) was used to produce deionized water for all aqueous solutions. All chemicals used were of ACS reagent grade. Sodium tetraborate for the preparation of buffers was obtained from Wako (Tokyo, Japan). The electrophoretic buffer was composed of 50 mM sodium tetraborate buffer (pH 10) and was prepared daily from a stock solution of 0.1 M borate and adjusted with 1.0 M NaOH or 1.0 M HCl to the required pH. Standard stock solutions of 100 mM GABA and Ala (Sigma-Aldrich, St. Louis, MO) were prepared by dissolving 0.1031 g of GABA and 0.0891 g of Ala individually in 10 mL of 50 mM sodium tetraborate buffer (pH 10). The solution was stored at 4 °C in a silanized brown glass bottle with a Teflon-lined cap for a maximum of 3 months. Fresh working solutions were prepared daily by diluting the stock solution with the mixture of acetonitrile and 0.1 mM NaCl aqueous solution in 2:1. 2-ME and OPA (chemical grade) were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany), respectively. These labeling reagents, 2-ME and OPA, were prepared as 1.0 M in methanol daily and diluted to the desired concentration with the mixture of acetonitrile and 0.1 mM NaCl aqueous solution in 2:1. The NaH_2PO_4, HCl, and NaOH were also obtained from Merck (Darmstadt, Germany) for the preparation of buffer solution or adjustment of the pH. All solutions were filtered through a 0.45 μm poly(vinylidene difluoride) (PVDF) membrane filter or a 0.45 μm syringe filter. The jasmine green tea, sweet osmanthus−oolong tea, and GABA-rich tea were obtained from local supermarkets.

Apparatus. The capillary electrophoresis system used in this study was the model Prince four-tray system (Prince Technologies, Emmen, Netherlands) equipped with a tunable-wavelength fluorescence detector (Argos 250B, Flux Instruments, Basel, Switzerland). The Peak-ABC Chromatography Data Handling System (JiTeng Trading Pte., Ltd., Singapore) was used to control the operation of CE, obtain the electropherogram, and perform data calculations.

Sample Preparation. The sweet osmanthus−oolong tea, jasmine green tea, and GABA-rich tea samples were ground to powder. A powder sample (ca. 0.25 g) was added to boiling water (20 mL), and the mixture was maintained for 20 min and then filtered by a 0.45 μm filter. After the solution had cooled to room temperature, deionized water was added to a total volume of 25 mL. The sample solution was then diluted with the mixture of acetonitrile and 0.1 mM NaCl aqueous solution at 2:1 for CE analysis.

Sample Analysis. Separations were carried out with uncoated fused silica capillary tubings (50 μm i.d., 365 μm o.d., Polymicro Technologies, Phoenix, AZ) with an effective length of 65 cm and a total length of 80 cm. Before each analysis run, the capillary tubing was flushed with 0.1 M NaOH and then equilibrated with the separation buffer for 3 min. The electrophoresis buffer solution consisted of 50 mM sodium tetraborate buffer (pH 10). Separations were carried out at a continuously applied voltage of 21 kV and 23 °C, and the fluorescence detector was arranged in the cathodic end of the capillary to detect analytes at 495 nm (excitation in 240−400 nm) after in-capillary labeled derivatization and stacking.

In-Capillary Labeled Derivatization and Stacking. In-capillary labeled derivatization and stacking were achieved by the successive introduction of sample solutions and the labeling agents (2-ME and OPA) to the anodic end of the capillary column for 0.3 and 0.1 min at 50 mbar, respectively. In the electrophoresis process, derivatization occurred while both analytes and labeling agents were overlapping, and stacking occurred due to the difference of ionic strength between the sample solution and the buffer solution for separation.

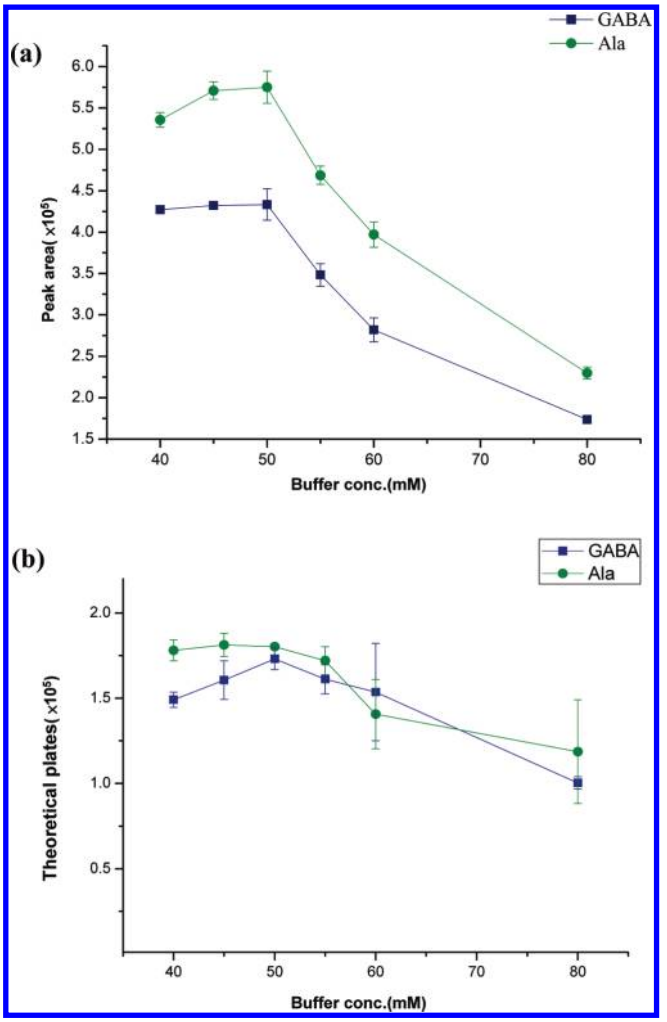

Figure 1. (**a**) Influence of background buffer concentration on the detection of derivatives after stacking: sample concentration, 1 μM GABA and 1 μM alanine in the SZ solution; derivative agents, 20 mM OPA/26.67 mM 2-ME in SZ solution; applied potential, 21 kV; background buffer concentration, 50 mM sodium tetrabroate at pH 10.0; injection, 50 mbar, 0.3 min for sample solution and 0.1 min for derivative agents. (**b**) Influence of background buffer concentration on the theoretical plates.

Spiked Samples. Quantities of the analytes (GABA and Ala) were dissolved in methanol, then mixed with samples (each of jasmine green tea, sweet osmanthus−oolong tea, and GABA-rich tea), and purged with nitrogen to dryness in a hood.

RESULTS AND DISCUSSION

Because the interaction between analyte and interferant decreases when sample solution is dilute and different compounds do not stack at the same ratio, in the proposed method, the matrix interferences were eliminated through dilution, and the detection of dilute analytes was redeemed through stacking and in-capillary labeled derivatization in CE. Therefore, parameters that affect stacking, such as the concentration of background buffer solution, matrix of sample zone (sample solution), and injection volume of sample solution, were investigated thoroughly, in addition to the in-capillary labeled derivatization we discussed previously (*20*).

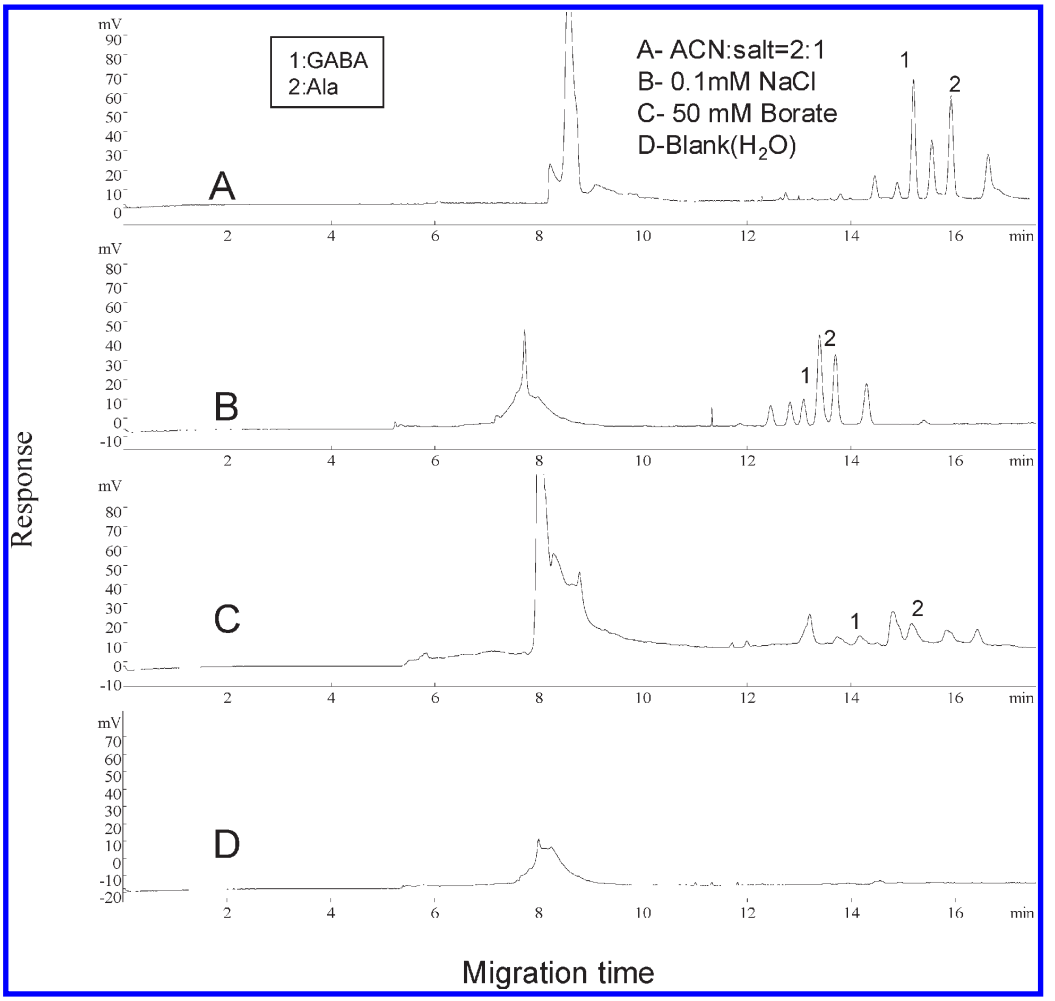

Figure 2. Influence of the sample zone solutions on the stacking: background buffer concentration, 50 mM sodium tetrabroate at pH 10.0; time for sample injection, 0.5 min; peak 1, GABA adduct; peak 2, Ala adduct.

Influence of Background Buffer on the Detection of Derivatives after Stacking. In our previous studies (*20*), a buffer concentration of 30 mM borate at pH 10 was applied in the separation after the optimization of the background buffer (BGB) on the labeled derivatization of analytes (GABA and Ala), the migration time, and the resolution, individually. It is well-known that the concentration of BGB affects the stacking of analytical species in addition to the migration and thus the separation in a CE process. In this study, the labeled derivatization of GABA and Ala occurred in the high-field stacking process because the mixture of labeling agents was prepared in the same solution of sample (SZ). Labeling is usually influenced by the field potential, which in turn is affected by the BGB concentration. In this study, the detection signals increased slightly with the increase in the BGB concentration from 40 to 50 mM, became optimal at 45−50 mM, and then declined sharply, as demonstrated in **Figure 1a** in the investigation of BGB concentration (40−80 mM sodium tetraborate buffer at pH 10) applied to detection with 0.3 min sample introduction and 0.1 min labeling agents containing 20 mM OPA and 26.67 mM 2-ME at 21 kV. The decline of detection signals after 50 mM was due to the field strength increased in SZ with the increase in BGB concentration, which increased electrophoretic velocities of analytes and the length of time that overlapping analytes and labeling agents decreased, and thus a smaller peak area was reached. By considering the influence of BGB concentration on the theoretical plates of GABA and Ala derivatives demonstrated in **Figure 1b**. The BGB with concentration of 50 mM (borate buffer at pH 10) was thus selected for the CE in the study.

Selection of Sample Zone Solution. It is well-known that stacking of analytes can be easily achieved by dissolving the sample in a solution with lower ionic strength than the electrophoresis buffer. For the purpose of stacking, Shihabi (*26*) used a mixture of 2 volumes of acetonitrile and 1 volume of 1% sodium chloride to dissolve sample to achieve peptide stacking and achieved a pseudo-isotachophoresis (p-ITP) in the separation of iohexol and theophylline (*27*). However, in our studies, 0.1 mM NaCl was better than 0.1 M NaCl as the salt in the sample solution for the stacking. **Figure 2** demonstrates the electropherograms of the samples and labeling agents dissolved in (**A**) acetonitrile/salt solution at 2:1, (**B**) 0.1 mM NaCl solution, (**C**) 50 mM borate solution, and (**D**) reagent water (as the blank). Obviously, both the acetonitrile/salt solution and the 0.1 mM NaCl solution, as the solution for the sample and labeling agents, have stacking phenomena. The acetonitrile/salt solution offered a better stacking result and was thus selected. Similar to Shihabi's results (*26*), the ratio of acetonitrile to 0.1 mM NaCl aqueous solution at 2:1 has the best stacking efficiency among the mixtures of acetonitrile/NaCl solution at different ratios. Therefore, the working solutions of sample and labeling agents were prepared in the mixtures of acetonitrile/0.1 mM NaCl solution at 2:1.

In-Capillary Labeled Derivatization and Stacking. It is well-known that labeled derivatization has been achieved through the overlap of analytes (GABA and Ala) and labeling agents

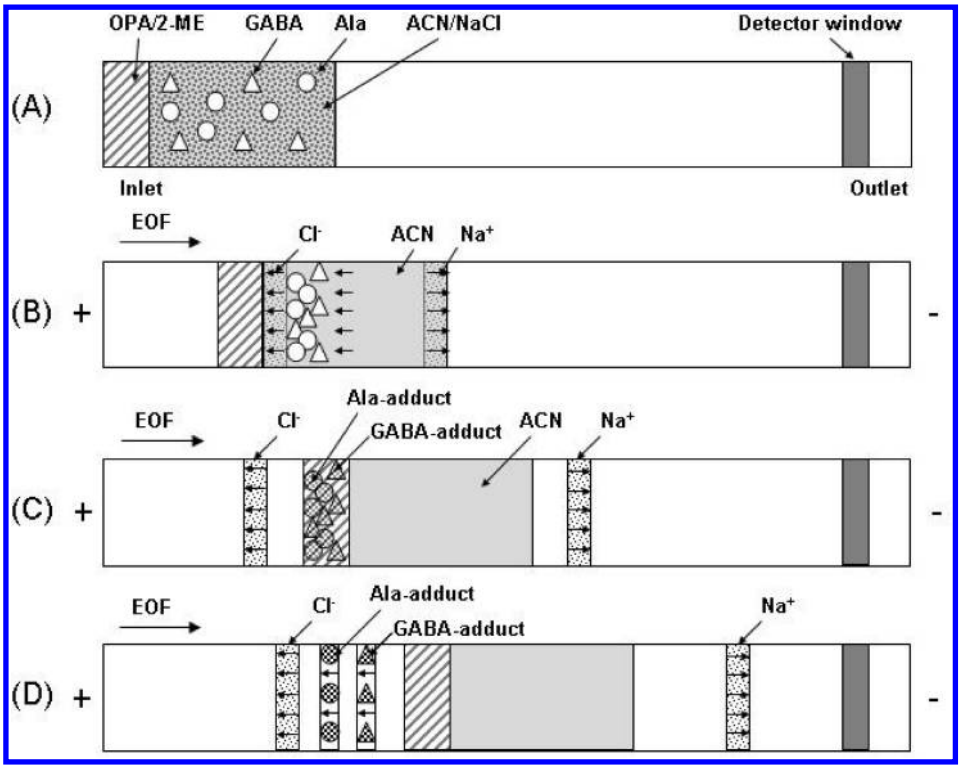

Figure 3. Schematic drawing of the in-capillary labeled derivatization and stacking in ACN/salt solution: (**A**) working sample solution and labeling agents were introduced successively to the anodic end of the capillary column; (**B**) labeled derivatization occurred through the overlap of analytes and labeling agents in the stacking process of analytes; (**C**) stacking of the labeled derivatives occurred due to their migration velocity reduced in the background buffer solution; (**D**) once the stacked ions left the sample zone solution, a capillary zone electrophoresis (CZE) process began.

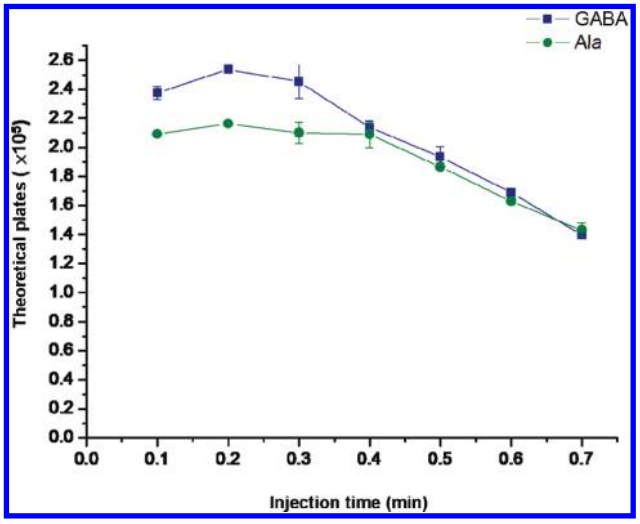

Figure 4. Influence of the injection quantity of sample solution on the theoretical plates: sample concentration, 1 μM GABA and 1 μM alanine in the SZ solution; derivative agents, 20 mM OPA/26.67 mM 2-ME in the SZ solution; injection, 50 mbar, 0.1 min for derivative agents.

(2-ME and OPA) due to their different migration rates in the capillary column (*20*). As described above, the mixture of acetonitrile/0.1 mM NaCl solution (2:1) was applied to achieve stacking on the basis of the difference of migration velocity of labeled products (GABA-adduct and Ala-adduct) between the SZ solution and the BGB solution. A schematic drawing of in-capillary derivatization and stacking is given in **Figure 3**. Working sample solution and labeling agents were introduced successively to the anodic end of the capillary column as shown in (**A**). At the initial CE operation, the SZ solution had low viscosity and

Table 1. Optimal Conditions for the In-Capillary Derivatization and Stacking CE

capillary column	50 μm i.d., 365 μm o.d.; total length = 80 cm; effective length = 65 cm
applied voltage	21 kV
background buffer solution	50 mM sodium tetraborate buffer at pH 10
sample zone solution	acetonitrile/0.1 mM NaCl solution at 2:1
injection time of sample solution	0.3 min
labeling agent	20 mM OPA/26.67 mM 2-ME in sample zone solution
injection time of labeling agent	0.1 min

Table 2. Calibration Parameters of Standard GABA and Alanine

	GABA	alanine
concentration range	0.005−2.5 μM	0.005−2.5 μM
linear equation[a]	$Y = 1555071X + 9042$	$Y = 1774352X + 44134$
R^2	0.9995	0.9992
mean slope (intraday)	1765467	1796972
%RSD	2.48	1.83
mean slope (interday)	1576740	1609170
%RSD	1.79	0.96
LOD	0.7 nM	0.8 nM

[a] Y is the response of peak area, and X is the concentration of analytical species in micromolar units.

conduction due to the 66.7% ACN. Once the voltage was applied, the negatively charged GABA and Ala in the SZ solution migrated quickly in the direction opposite the EOF as shown in (**B**). Because both GABA and Ala are negative (pH > p*I*) and labeling agents (2-ME and OPA) are neutral in the SZ solution, the labeling agents moved with EOF and overlapped with the analytes in the migration process. The labeled derivatization was achieved through the overlap of the analytes and the labeling

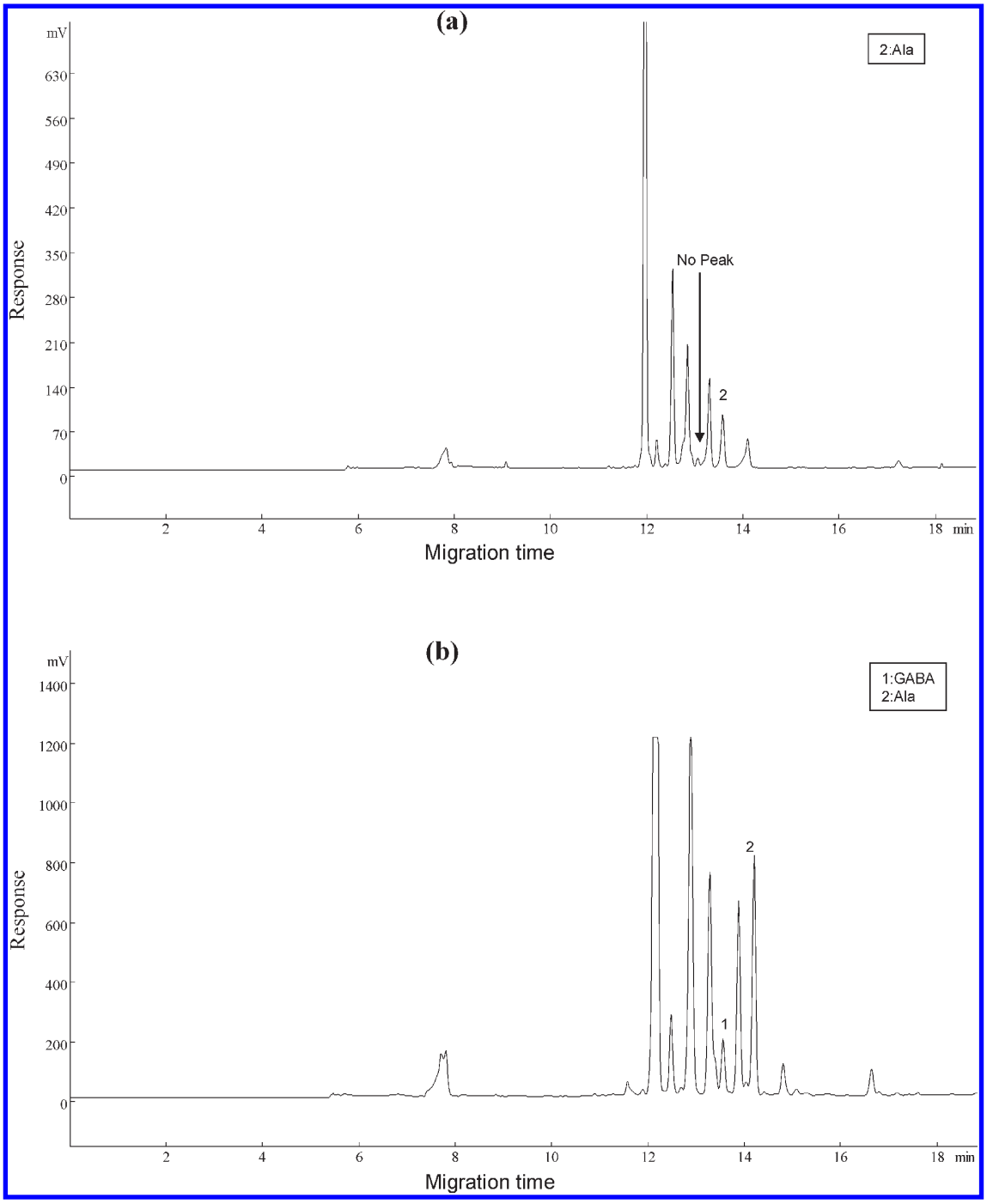

Figure 5. Electropherograms of GABA and alanine in jasmine green tea sample: (**a**) undiluted; (**b**) via stacking. Peaks: 1, GABA adduct; 2, Ala adduct.

agents. Because the labeled products were in a negatively charged form, they still moved quickly to the direction opposite to the EOF. When the labeled derivatives reached the BGB solution, they were stacked and concentrated as their migration velocity reduced in the BGB solution as shown in (**C**). Once the stacked ion zone left the SZ solution, a capillary zone electrophoresis (CZE) process began. The well-stacked ions were separated because of their different mobilities and pushed toward the outlet of the capillary by EOF as shown in (**D**).

Influence of Injection Quantity of Sample Solution. The acetonitrile/salt stacking method (*26*−*28*) permits a larger volume injection of sample (about 10−30% of the capillary volume) to enhance detection sensitivity, but the peaks would be broadened and theoretical plates would decrease significantly due to the wider spread of analyte in the sample zone and partially due to the decrease in the separation segment length of the capillary as a consequence of an increase in the sample size. Therefore, the influence of injection time (related to injected amount of sample solution) on the theoretical plates was examined from 0.1 to 0.7 min at 50 mbar (3.13−21.96 mm in the length of capillary column). **Figure 4** demonstrates that theoretical plates decreased significantly if the injection was >0.3 min (9.41 mm in length), although the theoretical plates with 0.2 min (6.27 mm in length) injection were slightly higher than that of 0.3 min injection. By considering the concentration levels of both analytes in samples and the resolution efficiency in electropherograms, an injection of sample solution for 0.3 min at 50 mbar was selected.

Repeatability, Linearity, and Detection Limit. The optimal conditions for in-capillary labeled derivatization and stacking CE are listed in **Table 1**. The in-house repeatability was examined

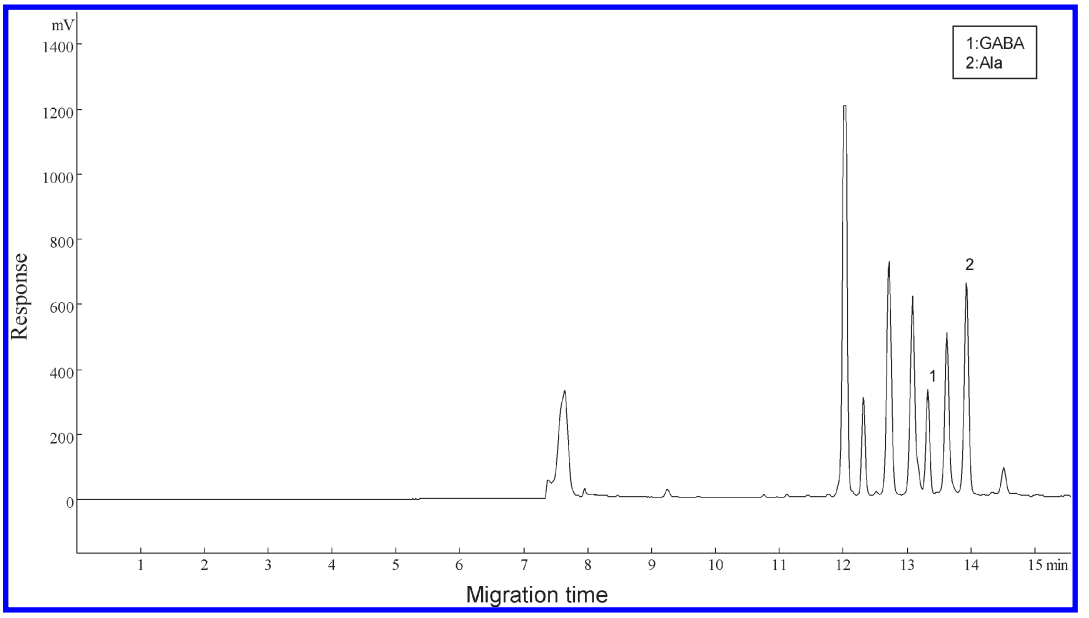

Figure 6. Electropherograms of GABA and alanine in sweet osmanthus−oolong tea with the proposed method after 10-fold dilution and stacking. Peaks: 1, GABA adduct; 2, Ala adduct.

Table 3. Content and Recovery of GABA and Alanine in Tea Samples

		GABA and alanine in samples (mg/100 g)	sample weight (g)	amount in sample (μg)	spiked quantity (μg)	detected quantity (μg)	recovery (%)
sweet osmanthus−oolong tea	GABA	6.02	0.2613	15.73	12.89	28.20	96.74
	alanine	13.33	0.2613	34.83	11.17	45.52	95.70
GABA-rich tea	GABA	157.24	0.2526	397.19	386.70	754.24	92.33
	alanine	51.76	0.2526	130.75	334.09	453.00	96.46
jasmine green tea	GABA	2.496	0.2537	6.33	25.78	31.56	97.87
	alanine	22.73	0.2537	57.66	22.34	78.54	94.36

by repeat injections (n = 7) of GABA and Ala standard solutions at a concentration of 1 μM. The relative standard deviation (RSD) of migration time and peak area of labeled GABA were 0.99 and 3.3%, respectively, within-day (n = 7), and 3.22 and 3.51%, respectively, between days (n = 5). For labeled Ala, the within-day RSD (n = 7) values were 0.96 and 5.12% for migration time and peak area, respectively; the RSD values between days (n = 5) were 3.39 and 4.45% for migration time and peak area, respectively. Calibration plots by this method were built up over the ranges of 0.005−2.5 μM (number of concentrations = 8) for GABA and Ala, respectively, with parameters, which are listed in **Table 2**. The linear relationships between the peak area (Y) and the spiked quantity (X) for GABA and Ala showed good agreement with the square of correlation coefficients (R^2) of 0.9995 and 0.9992 for GABA and Ala, respectively. The detection limits were calculated by dividing 3 times the average background noise by the detection sensitivity (slope of calibration plot); the detection limits were 0.7 and 0.8 nM for GABA and Ala, respectively, which were lower than that in our previous studies (4 and 20 nM for GABA and Ala, respectively) (*20*).

Analysis of GABA and Ala in Tea Samples. The present method was applied to the analysis of GABA and Ala in tea samples. After the sample preparation process, described under Materials and Methods, sample solutions were analyzed according to the proposed method. In our previous studies (*20*), GABA and Ala were analyzed by CE through a 1000-fold dilution of GABA-rich tea to compromise the detection signals of the analytes and peak resolution. However, jasmine green tea could not be diluted due to low contents of analytes, and only Ala with extremely trace amounts of GABA was detected in jasmine green tea as shown in **Figure 5a** (*20*). However, after the stacking process with the present method, both GABA and Ala in jasmine green tea were observed in the electropherograms as demonstrated in **Figure 5b**. Obviously, GABA became detectable and Ala concentrated visibly with efficiency of 10-fold. The area ratios of GABA/Ala peak in **Figure 5** before and after stacking are 0.18 and 0.24, respectively. This indicates that different compounds do not stack at the same ratio and GABA had greater stacking efficiency than Ala in the stacking process. The peaks of GABA and Ala in electropherograms were reverified with the standard addition method by standards spiked into sample solutions to confirm their migration time and estimate recovery. The proposed method was also applied to analyze both GABA and Ala in a sweet osmanthus−oolong tea sample, which contained low levels of both analytes in a complicated sample matrix. **Figure 6** shows the electropherogram of GABA and Ala in sweet osmanthus−oolong tea (after 10-fold dilution) with the proposed method. It is obvious that the dilution of the sample solution followed by the stacking in CE permitted the detection of analytes in a complicated matrix sample. **Table 3** lists the analytical results for GABA and Ala in sweet osmanthus−oolong tea, GABA-rich tea, and jasmine green tea samples. These results reflect that GABA and Ala actually exist in the sweet osmanthus−oolong tea and jasmine green tea, although the contents of GABA and Ala are not as great as in GABA-rich tea. When spiked samples, with quantities of GABA and Ala as listed in **Table 3**, were analyzed with the proposed method, the recoveries were from 92.33 to 97.87% and from 94.36 to 96.46% for GABA and Ala, respectively.

From previous results, it has been indicated that the proposed method has been successfully applied to determine GABA and Ala contents in tea samples within 15 min. In the proposed method, the dilution of the extracts from complicated matrix samples is applied to eliminate matrix interference, and a stacking technique in CE is used to redeem the detection of dilute analytes. This method has the potential to be employed to analyze if target species exist in a complicated matrix sample, as well as to measure GABA and Ala content in tea samples.

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