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Capillary Electrophoretic Analysis of γ-Aminobutyric Acid and Alanine in Tea with In-Capillary Derivatization and Fluorescence Detection

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The aim of this study was to investigate an in-capillary derivatization capillary electrophoresis (CE) technique that was performed to determine the concentration of γ-aminobutyric acid (GABA) and alanine (Ala) in tea after being derivatized with *o*-phthaldialdehyde/2-mercaptoethanol (OPA/2-ME) to form fluorescence-labeled products. The conditions of labeled derivatization and CE separation were optimized and then applied to real sample analysis. The labeled derivatization with 20 mM OPA and 26.67 mM 2-ME (mol ratio = 0.75) at pH 10 offered the most sensitive detection, and the separation with 30 mM sodium tetraborate buffer (pH 10.0) under 21 kV achieved good selectivity within 14 min. The detections were linear in the range of 0.05−5 μM with correlation coefficients (R^2) of 0.9995 and 0.9964 and with detection limits of 0.004 and 0.02 μM for GABA and Ala, respectively. The recoveries were 94.22% (3.58% RSD) and 93.54% (6.46% RSD) for five determinations of GABA and Ala, respectively. This method is a fast, convenient, sensitive, and eco-friendly way to determine the GABA and Ala in tea samples from different manufacturing processes.

KEYWORDS: Capillary electrophoresis; in-capillary derivatization; GABA; alanine

INTRODUCTION

GABA (γ-aminobutyric acid) 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*). GABA is the product of a biochemical decarboxylation of glutamic acid by the vitamin pyridoxal, as well as by decarboxylase (*3*). Because the accumulation of GABA is markedly stimulated under anaerobic conditions (*4*, *5*), GABA content is rare in the traditional manufacturing process of tea. Thus, GABA-rich tea was prepared by fermenting fresh green tea under nitrogen (*6*). The consumption of GABA-rich green tea by salt-sensitive, hypertensive rats has been shown to significantly lower their blood pressure (*7*) and is considered to have the potential to maintain the balance of blood pressure in individuals with hypertensive cardiovascular disease. GABA-rich tea has recently become a popular drink for health-conscious individuals in Asian countries (*8*). Because the GABA content in tea depends on the manufacturing process, a convenient analytical method is required to determine such content in tea. Alanine (Ala) may be found in tea after the transamination of GABA with pyruvate in the GABA shunt (*9*).

Reverse-phase HPLC (*10*−*13*) and capillary electrophoresis (*14*−*16*) have been performed to determine amino acids, including GABA, in human biological fluids or tissues. Because the direct detection of amino acids provides low sensitivity due to their weak chromophores, a labeled derivatization is applied to enhance the detection **(***17*). Precolumn derivatization is the most popular method for this purpose (*18*). However, precolumn manipulation may lack precision due to the instability of derivatives and the adverse effect of dilution volume, whereas postcolumn derivatization requires a complex instrument. Thus, the in-capillary derivatization method was developed into a versatile derivatization technique as an alternative method for CE or capillary electrochromatography (CEC) to analyze amino acids in samples (*19*−*25*).

In modern amino acid analysis, several fluorogenic reagents are available, among which *o*-phthalaldehyde (OPA)/thiol based reagents play the most significant role (*26*, *27*). The most important advantages of OPA are that it does not fluoresce intrinsically and that it reacts rapidly with primary amines in the presence of a thiol co-reactant under alkaline conditions. On the other hand, it has the disadvantage of the instability of resulting fluorophores. This instability can be minimized by on-column labeling such as the use of *N*-acetylcysteine (NAC) in CE (*28*, *29*) and 2-ME in CEC (*24*, *25*).

To determine GABA and Ala in plant tissues, Zhang and Sun (*14*) applied mixed micellar electrokinetic chromatography and fluorescence detection after 30 min of off-column naphthalene-2,3-dicarboxaldehyde(NDA)-labeled derivatization. To the best of our knowledge, there have not been any studies that have measured GABA in plant tissues by CE with in-capillary labeling derivatization.

Because 2-ME (as a co-reactant) is neutral, the resulting fluorophores of GABA and Ala have a lower negative mobility

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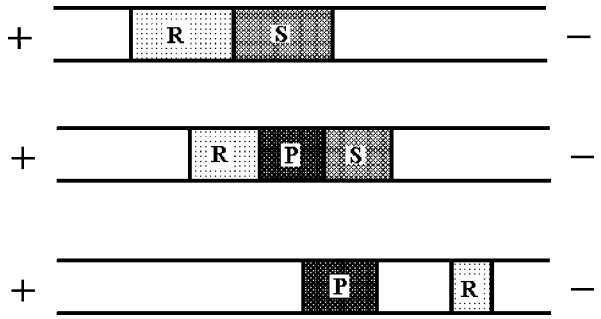

Figure 1. Scheme of the in-capillary-labeled derivatization. S: Sample solution containing analytes (GABA and Ala); R: labeling reagents containing OPA and 2-ME; and P: labeled products.

than those using an anionic co-reactant and thus have shorter migration times. Moreover, 2-ME has the lowest odor threshold among liquid mercaptans. Therefore, we applied OPA and 2-ME to achieve in-capillary labeling derivatization in the proposed protocol.

In this paper, we determined the concentration of GABA and Ala in tea samples using an in-capillary derivatization technique coupled to a CE system using OPA/2-ME as derivative agents. The conditions for labeling derivatization and separation were optimized. Our results showed that this analytical technique is a fast, convenient, eco-friendly way to analyze GABA and Ala in tea.

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 was obtained from Wako (Tokyo, Japan) for the preparation of buffers. The electrophoretic buffer was composed of 30 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 1000 mg/L γ-aminobutyric acid (GABA) and alanine (Ala) (Sigma-Aldrich, St. Louis, MO) were prepared by dissolving 0.100 g of GABA and Ala individually in 90 mL of 30 mM sodium tetraborate buffer (pH 10) and were diluted to 100 mL with the same buffer as the stock solution. 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 30 mM sodium tetraborate buffer. 2-Mercaptoethanol (2-ME) and *o*-phthaldialdehyde (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 water. NaH_2PO_4, HCl, and NaOH were obtained from Riedel-deHaën (Hanover, Germany) for the preparation of buffer solution or adjustment of the pH. All solutions were filtered through a 0.45 μm polyvinylidene difluoride (PVDF) membrane filter or a 0.45 μm syringe filter. GABA-rich A and B and jasmine green teas were obtained from a local supermarket.

Apparatus. The capillary electrophoresis system used in this study was the Model Prince 4-tray system (Prince Technologies, Emmen, The 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. Tea (1.0 g) leaves were put into boiling water for 20 min and then filtrated by a 0.45 μm filter. After the tea had cooled to room temperature, water was added to a total volume of 100 mL. The solution was then diluted with water for CE analysis.

Sample Analysis. Separations were carried out with uncoated fused-silica capillary tubings (50 μm i.d. × 360 μm o.d., Polymicro Technologies, Phoenix, AZ) with an effective length of 65 cm and a total length of 80 cm. Before each run of analysis, 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 30 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 on-column derivatization.

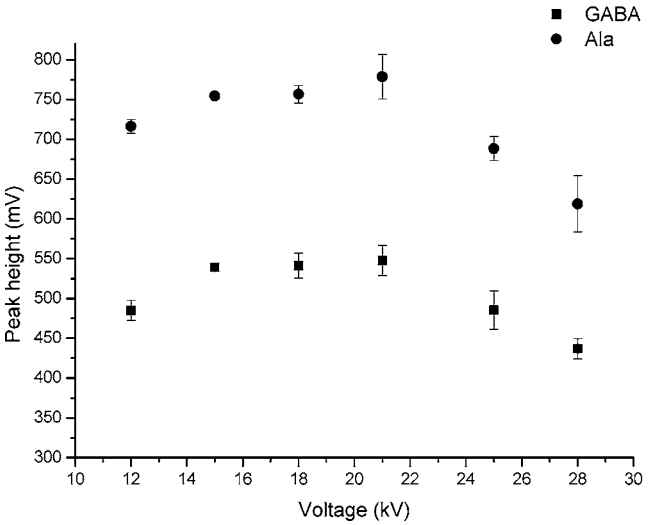

Figure 2. Effect of applied voltage on the quantitative detection CE conditions: 30 mM sodium tetraborate, pH 10, 20 mM OPA/2-ME (mol ratio = 0.75). Sample injection: 50 mbar, 0.1 min.

In-Capillary-Labeled Derivatization. In-capillary-labeled derivatization was achieved by successive introduction of sample solution and the labeling agents (2-ME and OPA) to the anodic end of the capillary column for 6 s at 50 mbar. In the electrophoresis process, derivatization occurred while both analytes and labeling agents were overlapping.

Spiked Tea Samples. Quantities of analytes (GABA and Ala) were dissolved in methanol, then mixed with tea and purged with nitrogen to dryness in a hood.

RESULTS AND DISCUSSION

In-Capillary-Labeled Derivatization Reaction. Labeled derivatization was achieved through the overlap of analytes and labeling agents due to their different migration rates in the capillary column. Sample solution and labeling agents were introduced successively to the anodic end of the capillary column. Because both analytes (GABA and Ala) are negative (pH > pI) and labeling agents (2-ME and OPA) are neutral in the buffer system, the labeling agents move faster than analytes and will overlap each other in the migration process. The derivatization reaction occurs while both zones are overlapping. Because the labeled products are in a negatively charged form, they move slower than the labeling agents. Thus, the peaks of labeled products in the electropherogram are free from the interference of labeling agents. The scheme of the in-capillary-labeled derivatization is shown in **Figure 1**.

Influence of Applied Voltage. It is well-known that the applied potential affects the migration and thus the separation as well as the labeled derivatization in a CE process. In this study, the detection signals increased initially with the increase in applied voltages from 12 to 15 V and slightly after 15 V, became optimal at 21 kV, and then declined, as demonstrated in **Figure 2**, in the investigation of voltage (12−28 kV) applied to detection with 30 mM sodium tetraborate buffer (pH 10) and

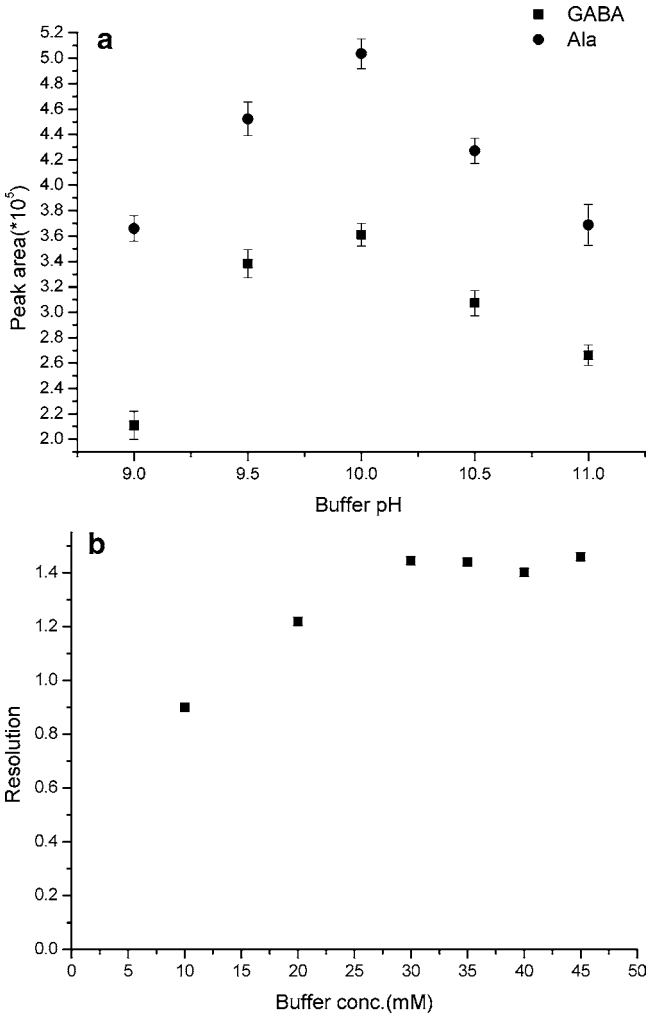

Figure 3. Influence of buffer on quantitative detection and resolution. (**a**) Effect of the buffer pH on the quantitative detection CE conditions: 30 mM sodium tetraborate, 20 mM OPA/2-ME (mol ratio = 0.75), 21 kV. (**b**) Effect of the buffer concentration on the resolution CE conditions: sodium tetraborate (pH 10), 20 mM OPA/2-ME (mol ratio = 0.75), 21 kV.

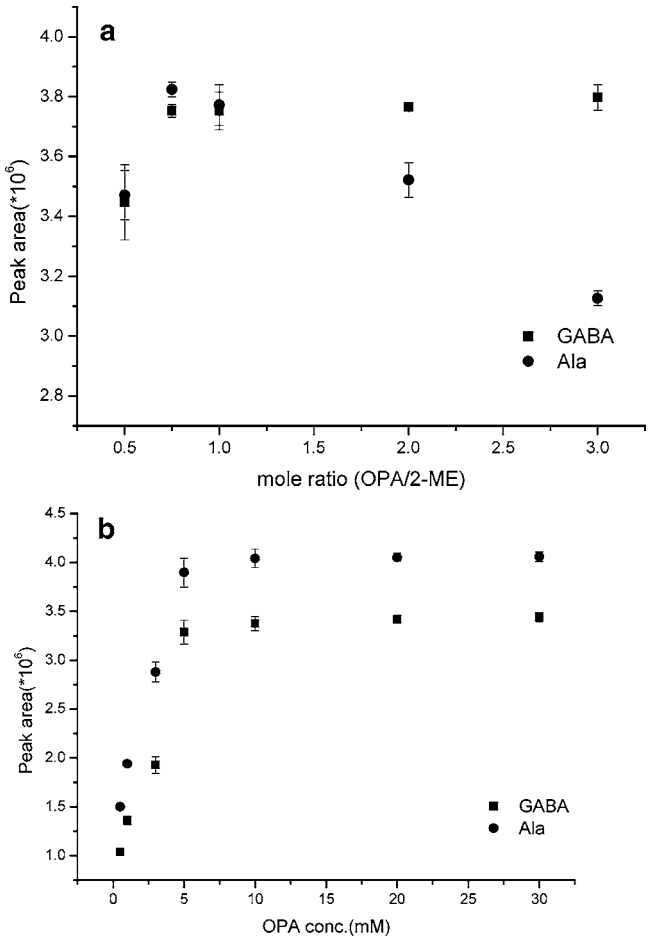

Figure 4. Influence of the OPA/2-ME mol ratio and OPA/2-ME concentrations. (**a**) Effect of OPA/2-ME ratio on labeled derivatization. CE conditions: 30 mM sodium tetraborate (pH 10), 20 mM OPA/2-ME, 21 kV. (**b**) Effect of the concentration of labeling agents on labeled derivatization CE conditions: 30 mM sodium tetraborate (pH 10), OPA/2-ME (mol ratio = 0.75), 21 kV.

labeling agents containing 20 mM OPA and 26.67 mM 2-ME (mol ratio = 0.75). The decline of detection signals after 21 kV was due to the length of time that overlapping amino acids and labeling agents decreased with the increase in applied potential, and thus a smaller peak area was reached. The applied voltage of 21 kV was thus selected.

Influence of Buffer on Quantitative Detection and Resolution. In this study, the labeled derivatization was influenced by the pH of the buffer solution. The peak areas of both GABA and Ala increased with buffer pH and reached optimal level at pH 10 and then declined as shown in **Figure 3a**. This result indicated that an alkaline matrix can accelerate the labeled derivatization; however, analytes are degraded significantly at extreme pH (higher than 10). Although the buffer concentration did not significantly affect the labeled derivatization in this study, it obviously influenced the migration time and thus the resolution because of its influence on the electro-osmotic flow and electrolyte viscosity. The effect of borate concentration (at pH 10) on the separation from 10 to 45 mM with labeling agents (20 mM OPA and 26.67 mM 2-ME) at 21 kV is demonstrated in **Figure 3b**. The resolution was apparently poor at low buffer concentration and increased with increasing buffer concentration, then became flat (R_s = 1.5) at 30 mM. Therefore, a buffer concentration of 30 mM borate at pH 10 was applied in the separation.

Influence of the OPA/2-ME Mol Ratio, OPA/2-ME Concentration, and Analyte Quantities. OPA and 2-ME acted as labeling agents in the fluorescence-labeled reaction. The effect of the OPA/2-ME ratio on the labeled derivatization was investigated, and the highest yield of derivatization occurred at the OPA/2-ME ratio of 0.75, as demonstrated in **Figure 4a**. In a quantitative aspect, the peak areas of both labeled products apparently initially increased with the labeling agents and then became constant at 10 mM OPA (13.33 mM 2-ME), as shown in **Figure 4b**. To obtain the maximum response under dynamic conditions, we selected 20 mM OPA and 26.67 mM 2-ME as the labeling agents in the analytical protocol. As to the effect of the injected amount of sample, the peak areas of both labeled products increased with the injection time of the sample solution, but the peaks were broadened, and theoretical plates were decreased significantly. By considering the concentration levels of both analytes in tea samples and the resolution efficiency in electropherograms, an injection of sample solution for 6 s at 50 mbar was selected.

Typical Electropherogram, Repeatability, Linearity, and Detection Limit. Figure 5a shows the electropherogram of standard labeled GABA and Ala under optimal conditions. The

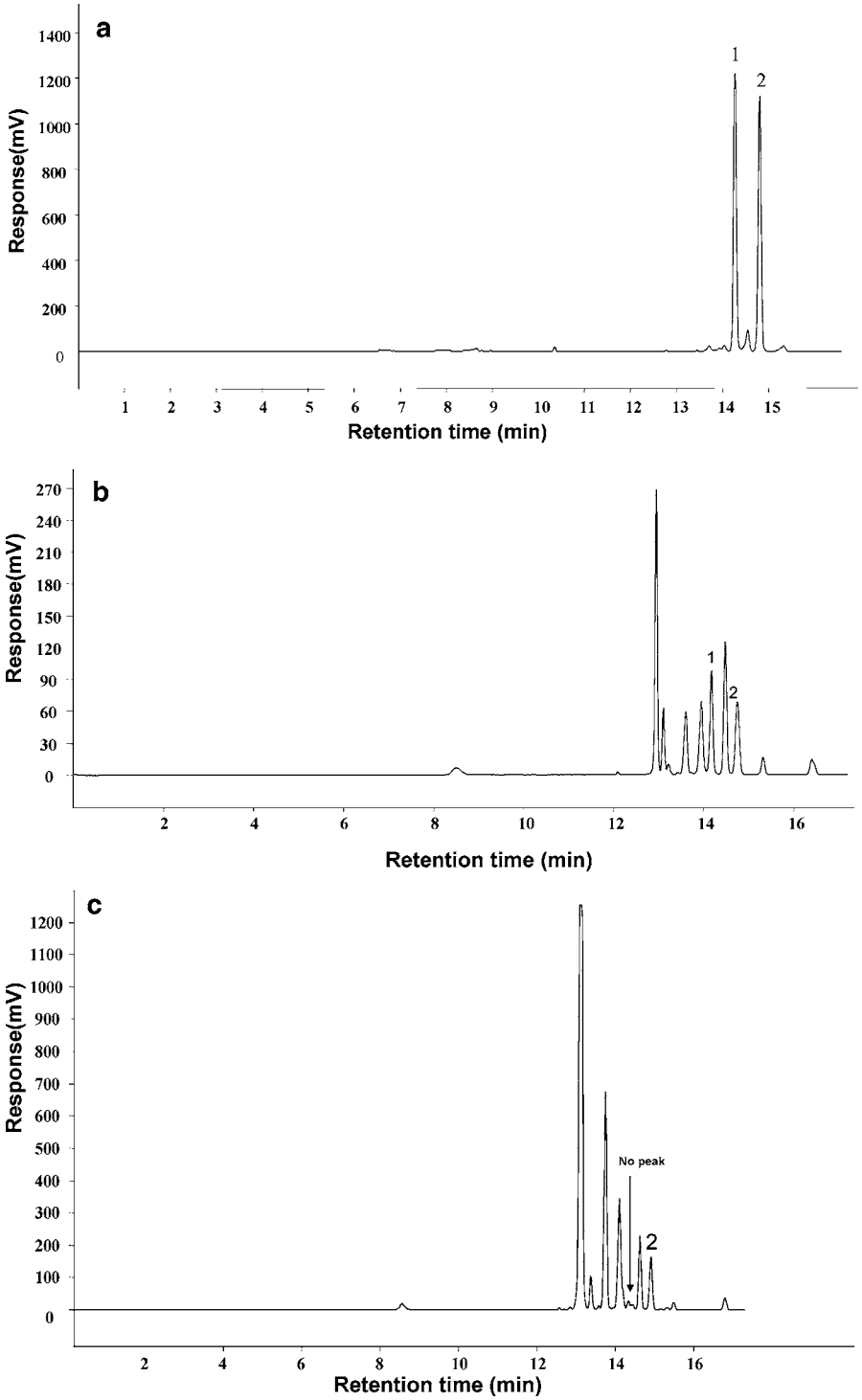

Figure 5. Electropherograms of GABA and Ala in (**a**) standard sample solution (5 μM GABA and 5 μM alanine); (**b**) GABA-rich tea (diluted 1000 times); and (**c**) jasmine green tea (undiluted). CE conditions: 30 mM sodium tetraborate (pH 10), 20 mM OPA/2-ME (mol ratio = 0.75), 21 kV. Peak 1: GABA and peak 2: Ala.

in-house repeatability was examined by repeat injections (n = 5) of GABA and Ala standard solutions at a concentration of 2 μM. The relative standard deviation (RSD) of migration time and peak area of labeled GABA were 0.88 and 3.51%, respectively, within-day (n = 7) and 0.61 and 3.76%, respectively, between days (n = 5). For labeled Ala, the within-day RSD (n = 7) were 0.83 and 3.26% for migration time and peak area, respectively, and the RSD of between days (n = 5) were 0.56 and 2.36% for migration time and peak area, respectively.

Calibration plots by this method were built up over the ranges of 0.05−5.0 μM (number of concentrations = 8) for GABA and Ala, respectively, with parameters, which are listed in **Table 1**. The linear relationships between the peak area (Y) and the spiked quantity (X) for GABA and Ala showed good agreement with correlation coefficients (R^2) of 0.9995 and 0.9964 for GABA and Ala, respectively. The detection limits were calculated by dividing three times the average background noise by the detection sensitivity (slope of calibration plot), and the detection limits were 0.004 and 0.02 μM for GABA and Ala, respectively.

In comparison with the performance of mixed micellar electrokinetic chromatography (MEKC) and fluorescence detec-

Table 1. Calibration Parameters of Standard GABA and Alanine

	concentration range ($n = 8$) (μM)	linear equation[a]	R^2
GABA	0.05–5	$Y = 1025479X + 30113$	0.9995
alanine	0.05–5	$Y = 831715X + 285511$	0.9964

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

Table 2. Analytical Results of GABA and Alanine in Tea Samples

	GABA-rich tea (A) (mg/g)	GABA-rich tea (B) (mg/g)	jasmine green tea (mg/g)
GABA	1.59[a]	1.08	trace
alanine	0.66	0.52	0.08

[a] Marked as 1.50 mg per gram.

tion methods for the determination of GABA and Ala (*14*), although the MEKC method achieved the separation of amino acids within 5 min, it took at least 30 min for the off-column derivatization prior to injection to the CE system. However, the present method takes a total 14 min to achieve the separation and in-capillary derivatization, and it is more sensitive than the MEKC method in GABA detection.

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 in the Materials and Methods, sample solutions were analyzed by the proposed method. Because the baseline of the electropherogram was interfered by the sample matrix, the tea sample solution was diluted to find a better resolved electropherogram. After a series of tests, a 1000-fold dilution of GABA-rich tea was selected by compromising the signals of the analytes and peak resolution. However, jasmine green tea was not diluted due to low contents of analytes and acceptable resolution. The electropherograms of GABA and Ala in GABA-rich tea (diluted 1000 times) and jasmine green tea (undiluted) are shown in **Figure 5b**,**c**, respectively. The peaks of GABA and Ala in electropherograms were re-verified with the standard addition method. Both GABA and Ala were found in GABA-rich tea, but only Ala with extremely trace amounts of GABA was observed in jasmine green tea. **Table 2** lists the analytical results for GABA and Ala in tea from three commercial products. These results reflect the dependence of the GABA content in tea on the manufacturing process. The GABA content in GABA-rich tea (A) was 1.59 mg per gram of tea marked as 1.50 mg per gram.

Five milligrams of spiked GABA and 2.5 mg of spiked Ala were added to 10 g of GABA-rich tea (B) and then analyzed with the proposed method. The recoveries were 94.22% (3.58% RSD) and 93.54% (6.46% RSD) for five determinations of GABA and Ala, respectively.

From the previous results, it is indicated that the proposed method has been successfully used to determine the GABA and Ala content in teas within 15 min. This method has the potential to be employed to monitor the GABA and Ala content in the GABA-rich tea manufacturing process for quality control purposes.

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