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Analytical Methods

Simultaneous determination of amino acids in tea leaves by micellar electrokinetic chromatography with laser-induced fluorescence detection

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ABSTRACT

A rapid and effective method of micellar electrokinetic chromatography with laser-induced fluorescence detection was developed for the simultaneous determination of amino acids in tea leaves. Pre-column derivatization of the analytes used 4-chloro-7-nitrobenzofurazan (NDB-Cl). Optimal separation was achieved at +20 kV using an uncoated fused silica capillary (40.0 cm effective length, 50.2 cm total length, 75 μm internal diameter), as well as 20 mM sodium borate (pH 8.5), 20 mM Brij 35, and acetonitrile 10% (v/v) as running buffers. Within 11 min, 15 amino acids were separated completely. The optimized method demonstrated good linearity ($r^2 \geqslant 0.9990$), precision ($\leqslant$6.65%), accuracy (85.50–112.74%), and sensitivity (0.1 ng/mL–100 ng/mL). The method successfully determined the quantity of amino acids in five different tea leaves; furthermore, theanine was identified as the most abundant amino acid in teas. The proposed method showed great potential in further investigations on the biofunctions of different tea samples.

1. Introduction

Tea is one of the most popular and widely consumed beverages in the world owing to its attractive aroma, taste, and healthy effects (Kuo et al., 2005). Tea is made from the leaves of the plant, *Camellia sinensis* L. According to the degree of fermentation, tea can be grouped into three categories, namely, unfermented (green tea), semi-fermented (oolong tea), and fully fermented tea (black tea) (Zhao, Chen, Huang, & Fang, 2006). Tea is beneficial to human health because of the presence of compounds that include polyphenols, amino acids, vitamins, carbohydrates, and purine alkaloids. Free amino acids are important taste components in terms of tea quality (Horie & Kohata, 2000). More than 26 varieties of amino acids have been identified in tea (Tan, Tan, Zhao, & Li, 2011). Theanine, comprising 50% of the total amino acid content, is the most important amino acid in tea and is primarily responsible for giving tea its delicate taste. Furthermore, various studies have demonstrated that theanine also plays an important role in many biological activities, such as promoting relaxation, decreasing norepinephrine and serotonin levels in the brain, reducing blood pressure, and enhancing anti-tumor activity (Kimura, Ozeki, Juneja, & Ohira, 2007; Sugiyama & Sadzuka, 2003; Yamada & Terashima, 2009). Therefore, the rapid and effective analysis of free amino acids in tea has great biological and clinical significance.

The total amount of free amino acids in tea extracts can be determined according to the colorimetric methods with ninhydrin or 2, 4-dinitrofluorobenzene (Chen, Chen, Zhang, & Wan, 2009; Sun, Lin, Weng, & Chen, 2006). Several chromatographic approaches have been proposed to analyse the free amino acid composition in teas, including anion exchange chromatography (Ding, Yu, & Mou, 2002), high performance liquid chromatography (HPLC) (Alcázar et al., 2007; Syu, Lin, Huang, & Lin, 2008; Tan et al., 2011; Wang et al., 2010), and capillary electrophoresis (CE) (Aucamp, Hara & Apostolides, 2000; Fu, He, Wang, & Wang, 2007; Hsieh & Chen, 2007; Kato, Gyoten, Sakai-Kato, & Toy?oka, 2003). Most amino acids have no significant ultraviolet (UV), visible, or fluorescence absorption capabilities. To analyse amino acids, they should be detected either at low UV wavelengths or follow derivatization to produce chromophores. To date, most of the studies using HPLC separation of amino acids in teas have been conducted through derivatization. Different labeling agents, including phenylisothiocyanate (PITC), dansyl chloride or o-phthalaldehyde (OPA), have been employed for the assay of amino acids with UV or fluorescence detection. Among these, OPA is the most common labeling agent used. Unfortunately, these HPLC methods have been limited to long analysis periods and often require cleanup procedures to reduce interference in the sample matrix.

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By contrast, CE is a modern analytical technique that has attracted significant interest in the determination of amino acids in teas because of the following advantages: short analysis time, high separation efficiency, and minimal solvent consumption. For the best use of CE in the investigation of amino acids in tea, various detection and analytical techniques, including micellar electrokinetic chromatography (MEKC)/UV (Aucamp et al., 2000), CE/indirect UV (Fu et al., 2007), microchip/FD (Kato et al., 2003) and CE/LED-IF (Hsieh & Chen, 2007), have been employed for this purpose. However, all the existing CE methods also have some drawbacks. For example, the direct detection of amino acids at short UV wavelengths (200 nm) is plagued by interference and poor sensitivity. The indirect UV detection method, in which background reagents are used in the running buffer, may contaminate the buffer solution, thereby constraining the separation ability. Additionally, the microchip method has a short analysis time but can only separate six amino acids, and some of the amino acids are not baseline separated in the CE/LED-IF method (Kato et al., 2003).

In recent years, laser-induced fluorescence (LIF) detection has become one of the most sensitive methods available for detection in CE. MEKC is an important tool that is used to separate the neutral and charged analytes. To the best of our knowledge, the MEKC-LIF technique in CE for the analysis of various amino acids in tea extracts has not yet been reported in previous works. In our study, we used NBD-Cl as the labeling agent for the LIF detection to avoid time-consuming sample cleanup procedures. The labeling agent does not react with other water-soluble extracts in tea infusion such as polyphenols, carbohydrates, and purine alkaloid, among others. The choice of surfactant is the most important condition in obtaining optimum resolution in MEKC. Brij 35 is an alternative to the most widely used SDS, because it showed high separation efficiency and short analysis time. Therefore, a high sensitivity and excellent resolution method based on the optimization of CE is proposed for the separation of 15 amino acids. Moreover, the method performed under the optimized conditions has been validated and applied to successfully analyse amino acids in different kinds of tea infusions. The MEKC-LIF proved to be an excellent method in determining amino acids in tea.

2. Materials and methods

2.1. Chemicals and solutions

Lysine (Lys), phenylalanine (Phe), leucine (Leu), methionine (Met), valine (Val), theanine (Thea), histidine (His), γ-aminobutyric acid (GABA), threonine (Thr), alanine (Ala), serine (Ser), glycine (Gly), cysteine (Cys), glutamic acid (Glu), aspartic acid (Asp), and NBD-Cl were purchased from Acros (Geel, Belgium). Sodium borate and Brij 35 were from Sigma (St. Louis, MO, USA). All the reagents were of analytical grade. Deionized water was further purified by a synergy UV water system from Millipore (Milford, MA, USA).

2.1.1. Instrumentation and analysis conditions

A P/ACE MDQ CE system (Beckman Coulter Instrument, Fullerton, CA, USA) equipped with a LIF detector (excitation at 488 nm and emission at 520 nm, Beckman Coulter, Fullerton, CA, USA) was employed throughout the experiment. A computer with 32 Karat Software (version 8.0) was used for system control and data analysis. All separations were performed using an uncoated fused silica capillary (Yong-nian Optical Fiber Factory, Hebei, China) with 75 μm inner diameter, 375 μm outer diameter, 50.2 cm total length, and 40 cm effective length. Before use, the new capillary was washed consecutively with methanol for 5 min, 1 M NaOH for 10 min, and water for 10 min. At the beginning of each working day, the capillary was flushed with 1 M HCl for 3 min, 0.5 M NaOH for 5 min, water for 5 min, and running buffer for 5 min. In between runs, the capillary was rinsed with 0.5 M NaOH for 0.5 min, water for 2 min, and running buffer for 3 min. The capillary was kept at 25 °C. Samples were injected by applying a pressure of 0.5 psi for 3 s at the anodic end of the capillary, after which separations were carried out on a +20 kV voltage using a running buffer composed of sodium borate (20 mM, pH 8.5), Brij 35 (20 mM), and acetonitrile (10 %, v/v). Prior to analysis, all buffers were allowed to pass through a 0.45 μm membrane filter (AA-56313, ChuDing, Shanghai, China).

2.2. Procedures

2.2.1. Preparation of standard solutions

All standard solutions of 15 amino acids were 5 mg/ml (except for His, Cys, and Asp at 10 mg/mL each) in water. A mixed standard solution was prepared at appropriate concentrations for different amino acids (50 μg/mL for His and Cys; 10 μg/mL for Lys, Thea, Glu and Asp; 5 μg/mL for Leu, Met, Thr and Ser; 2.5 μg/mL for Phe, Val, GABA, Ala, and Gly) by mixing 15 standard solutions. Diluted standard mixtures used as calibration solutions were prepared with water to obtain the required concentrations. All the standard solutions were prepared weekly and stored at 4 °C.

2.2.2. Preparation of tea samples

Several commercial tea leaves purchased from a local market were analysed as tea samples. These included keemun black tea (Dali, Yunnan), superior jasmine tea (Chengdu, Sichuan), roasted green tea (Emei, Sichuan), biluochun tea (Suzhou, Jiangsu), and jinding maofeng tea (Chengdu, Sichuan). Each tea sample (1 g) was reflux extracted with 50 mL of hot distilled water (85 °C to 90 °C) for 30 min. Subsequently, the mixture was centrifuged at 3000 rpm for 5 min at room temperature. After allowing the mixture to pass through a filter with a 0.45 μm membrane, the resulting supernatant (50 μL) containing amino acids was directly subjected to derivatization as described below.

2.2.3. Derivatization

Derivatization of amino acids with NBD-Cl was performed as follows: 50 μL amino acid standard mixture (or 50 μL tea sample solution), 100 μL derivatization buffer (30 mM sodium borate, pH 8.5), and 100 μL NBD-Cl solution (40 mM) were added sequentially into a 0.5 mL microcentrifugation tube. After thorough mixing, the mixtures were kept in a 60 °C hot-water bath to react for 30 min. Afterwards, 250 μL water was added to the reaction tube to quench the labeling reaction. After cooling to room temperature, the derivative solution was diluted to the desired concentration with deionized water for analysis.

3. Results and discussion

3.1. Optimization of the derivatization reaction conditions

3.1.1. Influence of derivatization buffer concentration and pH

The optimization of the sample derivatization conditions is substantial for analysis (Szöko & Tábi, 2010). After studying the effect of sodium borate concentration on derivatization in the range of 10 mM to 50 mM, we found that the fluorescence intensity of the analytes increased when sodium borate concentration increased in the range of 10 mM to 30 mM and then decreased slightly from 30 mM to 50 mM. Therefore, 30 mM was selected as the derivatization buffer concentration.

pH is another important parameter with a significant influence on derivatization. The effect of pH on the derivatization of the labeled amino acids was investigated in the range of 8.0 to 8.5 with