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Analytical Methods

Analysis of free amino acids in Chinese teas and flower of tea plant by high performance liquid chromatography combined with solid-phase extraction

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ABSTRACT

An effective liquid chromatographic method that involved precolumn derivatisation with *o*-phthaladehyde combined with solid-phase extraction has been developed for the determination of free amino acids in tea. Firstly, tea infusion was treated by C18 cartridge before derivatisation, resulting in great improvement of separation by Zorbax Eclipse XDB-C_{18} column. Then, the contents of free amino acids in Chinese green, black, and Oolong teas and the flower of plant *Camellia sinensis* have been determined. The results showed that theanine was the most abundant amino acid in teas, and green tea contained much higher amounts of free amino acids than fermented ones. While the contents and composition of free amino acids in tea flower were quite different from those of teas. The tea flower contained much higher content of free amino acids. Furthermore, although theanine was the most abundant amino acid in tea flower as tea, histidine became the second one.

1. Introduction

Tea is one of the most popular and widely consumed beverages in the world because of its refreshing taste, attractive aroma, and potential healthy benefits (Kuo et al., 2005). It is made from the leaves of the plant *Camellia sinensis* (L.). Generally, tea can be broadly classified according to the production method as unfermented tea (green tea), semi-fermented tea (Oolong tea), fully fermented tea (black tea) or post-fermented tea (pu-erh tea) (Zhao, Chen, & Huang, 2006). Black tea is consumed worldwide, while green and Oolong teas are consumed mainly in Asia and North Africa. Nowadays, a lot of epidemiological and preclinical studies have demonstrated that drinking tea may reduce the risk of cancer and cardiovascular disease (Khan & Mukhtar, 2007; Yang, Maliakal, & Meng, 2002). Moreover, other biological functions of tea have also been reported, such as anti-inflammation, anti-oxidation, anti-allergy, and anti-obesity (Fujimura, Tachibana, & Yamada, 2004; Khan & Mukhtar, 2007). These beneficial effects have been attributed to the presence of tea compounds such as polyphenols, amino acids, vitamins, carbohydrates, and purine alkaloids (Bolling & Chen, 2009).

Theanine, γ-glutamylethylamide or 5-*N*-ethyl glutamine, is a non-protein amino acid that was first discovered in tea leaves (Sakato, 1949). It is the main free amino acid in teas, representing as much as 50% of the total amino acids in black tea and 1–2% of the dry weight of green tea (Hara, Luo, Wikramasinghe, & Yamanishi, 1995). It not only plays an important role in the characteristic flavour and delicate taste of tea but also shows many biological effects. It involves in many biological activities such as promoting relaxation, inhibiting caffeine's negative effects, reducing blood pressure, and enhancing anti-tumor activity (Kimura, Ozeki, Juneja, & Ohira, 2007; Sugiyama & Sadzuka, 2003; Yamada & Terashima, 2009). Moreover, it has been reported to have physiological activities including neuroprotection and anti-obesity (Cho et al., 2008; Egashira et al., 2004; Zheng et al., 2005). Consequently, there is a demand for rapid and effective analytical methods for the analysis of free amino acids, which should be suitable across a wide range of research and practical applications.

A number of methods have been developed to determine the presence of various amino acids in teas. The total contents of amino acids in teas can be determined by the ninhydrin or 2,4-dinitrofluorobenzene colorimetric method (Chen, Chen, Zhang, & Wan, 2009). As for the analysis of free amino acid compositions in teas, various chromatographic methods including high performance liquid chromatography (HPLC), capillary electrophoretic and anion exchange chromatography have been reported (Alcazar et al., 2007; Aucamp, Hara, & Apostolides, 2000; Ding, Yu, & Mou, 2002; Ohtsuki, Kawabata, Kokura, & Taguchi, 1987; Pongsuwan et al., 2008; Syu, Lin, Huang, & Lin, 2008; Thippeswamy, Gouda, Rao, Martin, & Gowda, 2006; Ying, Ho, Chen, & Wang, 2005). Due to the lack of a suitable chromophore, it is necessary to label the amino acids by using labeling reagent, such as 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride), *o*-phthaladehyde

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(OPA), and phenylisothiocyanate. In such a case, it involves derivatisation with precolumn or postcolumn and detection by fluorescence or diode array detector (DAD). However, clear chromatograms are not obtained when the analysis of the composition of free amino acids in Chinese green teas was attempted, according to the reported methods by use of OPA as derivative reagent. In fact, tea infusion is usually prepared using distilled water. Accordingly, a number of other water-soluble extracts especially tea catechins are extracted out together with the free amino acids. They may affect the derivatisation or separation of the amino acids by HPLC. Therefore, it should be necessary to treat tea infusion before derivation by liquid–liquid or solid-phase extraction (SPE). As we know, SPE is becoming more used because it is rapid, economical, and sensitive. In addition, different cartridges with a great variety of sorbents can be used (del Alamo, Casado, Hernandez, & Jimenez, 2004; Perez-Magarino, Ortega-Heras, & Cano-Mozo, 2008). However, there is little information about the application of SPE for the sample preparation in the analysis of free amino acids in tea. In this report, therefore, we introduced SPE combined with HPLC-DAD for the determination of free amino acids including alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser), theanine (Thea), threonine (Thr), tryptohan (Trp), and tyrosine (Tyr) in Chinese teas. In addition, the contents of free amino acids in the flower of plant *Camellia sinensis* (L.) have also been analyzed by using the developed method.

2. Materials and methods

2.1. Chemicals and regents

Standards of amino acid, Ala, Arg, Asn, Asp, Gly, Ile, Leu, Lys, His, Met, Phe, Ser, Thr, Glu, Trp and Tyr, were purchased from Shanghai Kayon Biological Technology Co., Ltd. (Shanghai, China). A standard of Thea was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Standards of (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). OPA was obtained from Sigma (St. Louis, MO, USA). Hydrochloric acid, boric acid, formic acid, disodium phosphate and β-mercaptoethanol were obtained from Sinopharm Chemical Regent Co., Ltd (Shanghai, China). HPLC grade of acetonitrile and methanol were purchased from Hanbon Science and Technology (Jiangsu, China). The Sep-Pak-C18 cartridge was purchased from Waters Co., Ltd. (Milford, USA).

2.2. Tea samples and preparation of tea infusion

In the present study, green teas 1–3 were purchased from Hunan, Hubei, and Fujian in China, respectively. Black teas were obtained from Qimen Tea Factory (Anhui, China). Oolong teas 1 and 2 were purchased from Guangdong province in China, whereas Oolong tea 3 was obtained from Taiwan, China. Firstly, all of these teas were ground into tea powders by a mill. Then, tea infusions were prepared as follows: 0.250 g of tea powder was extracted with 10 ml of distilled water at 90 °C for 20 min. After the infusion was cooled to room temperature, it was made up to 10 ml with distilled water and filtered through a 0.45 μm nylon filter membrane.

2.3. Treatment of tea infusion by solid-phase extraction

The SPE cartridges were conditioned by rinsing with 30 ml of methanol and 10 ml of distilled water. The tea infusion (1.0 ml) was loaded onto the conditioned cartridge, and the retained compounds were eluted with 5.0 ml of 10% ethanol. The resulted elute was collected and evaporated to dryness in a rotary vacuum evaporator (Heidolph, Germany). Then, the residue was dissolved in 1.0 ml distilled water and filtered through a 0.45 μm nylon filter membrane before its precolumn derivatisation with OPA.

2.4. Precolumn derivatisation with OPA

The derivatisation with OPA was carried out according to the method reported with some modifications (Alcazar et al., 2007; Thippeswamy et al., 2006). Briefly, a 70 μl aliquot of tea infusion or standard amino acid solution was mixed with 10 μl of OPA solution and incubated at 25 ± 1 °C for exactly 2 min. Then, the reaction mixture was immediately used for HPLC analysis. The derivatisation solution was freshly prepared everyday as following: five milligrammes of OPA dissolved in 0.05 ml of methanol was added to 0.45 ml of 0.4 M boric acid/borate buffer (pH 9.5), followed by addition of 25 μl of β-mercaptoethanol.

2.5. HPLC-DAD analysis

The determination of amino acids were performed by using an Agilent 1100 series HPLC system (Agilent Technologies, CA, USA), consisting of a model G1379A degasser, a model G1311A quatpump, a model G1316A column oven, and a model G1315B DAD. An Agilent ChemStation was used for instrument control and data acquisition. The separation was completed on a Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm, 5 μm, Agilent). The temperature of column oven was set at 40 °C. The mobile phase consisted of methanol/acetonitrile/water (45/45/10, A) and phosphate buffer (pH 7.5, B). Elution was performed with a linear gradient as shown in Table 1. The flow rate was 1.0 ml/min. The DAD was set at 338 nm to monitor the derivatised amino acids. The injection volume was 20 μl.

2.6. Validation of HPLC-DAD method

HPLC-DAD validation tests were performed for accuracy, selectivity, linearity range, limit of detection (LOD), and limit of quantification (LOQ). The accuracy of the HPLC-DAD method was assessed by recovery experiments. Known amounts of standards of amino acids were added to tea infusion. The recovery was calculated by comparing the amount measured to that added. The selectivity criterion for an assay method is that the analyte peaks will have a chromatographic baseline with a suitable resolution from all of the other sample components. Calibration curves were constructed over five different concentrations. Each standard was analyzed in triplicate. LOD and LOQ were calculated as three and 10 times, respectively, the ratio between the standard deviation of the *y*-intercept of the regression line and the average slope of the regression lines.

Table 1
Scheme of elution gradient for HPLC-DAD analysis.

Time (min)	Solvent A (%)	Solvent B (%)
0	10	90
10	18	82
15	24	76
21	41	59
21.5	41	59
22	42.2	57.8
23	42.5	57.5
25	58	42
27	59	41
30	60	40