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Determination of Theanine in Commercial Tea by Liquid Chromatography with Fluorescence and Diode Array Ultraviolet Detection

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Two liquid chromatographic methods that involve precolumn derivatization with *o*-phthaladehyde (OPA) and phenylisothiocyanate (PITC) with fluorescence and diode array UV detection for the determination of theanine have been developed. The chromatographic separations were achieved by reverse-phase high-performance liquid chromatography using octadecyl columns and gradient elution. The methods were applied to evaluate the theanine content of commercial tea leaves. The coefficient of variation of the peak area repeatability for within day ($n = 8$) and between day ($n = 8$ over 10 days) was lower than 3% for both of the methods. The estimated limit of detection (LOD) and limit of quantitation (LOQ) for the OPA method was 0.12 and 0.35 μg theanine, respectively. The PITC method was 500-fold more sensitive with LOD and LOQ values of 0.25 and 0.75 ng, respectively. The theanine content of the commercial tea samples varied from 2−5 mg/g leaf. The overall % recoveries for these methods ranged from 93−99.3. The sensitivity and simplicity of the method render them suitable for use in quality control laboratories.

KEYWORDS: Theanine; black tea; green tea; precolumn derivatization; *o*-phthalaldehyde; phenyl isothiocyanate; reverse phase high-performance liquid chromatography

INTRODUCTION

Tea, made from the tender shoots (flushes) of *Camellia sinensis* (L.), is the most popular and widely consumed beverage the world over aside from water because of its refreshing, attractive aroma, taste, and potential health benefits. The three major categories of tea are (*1*) unfermented or green tea (*2*), semifermented oolong tea, and (*3*) fully fermented black or puerh tea. The fermentation, an oxidation process, produces theaflavins and thearubigins, two families of polyphenols. Black tea is consumed worldwide while green and oolong teas are consumed mainly in Asia and North Africa. Tea has many physiological and pharmacological attributes by a large number of secondary metabolites such as amino acids, polyphenols, caffeine, purine alkaloids, vitamins, and carbohydrates (*1*−*4*).

Green tea infusion has four characteristic taste notes: bitterness, astringency, sweetness, and umami (*2*, *5*, *6*). The brothy, sweet umami is due to amino acids, especially theanine. Twenty-six amino acids comprising 3−4% of the dry matter of tea infusion contribute to this taste and tea quality (*7*). The total content of amino acids in green tea is the highest as compared to the other teas. The predominant amino acid is theanine and represents as much as 50% of the total amino acids in black tea and 1−2% of the dry weight of green tea (*8*, *9*). Theanine, also known as glutamic acid γ-ethyl amide or 5-N-ethyl glutamine, exists only in the free form (nonprotein) and has been discovered as a constituent of green tea. The only other reported natural source of theanine is the mushroom, *Xerocomus badius* (*10*). Theanine is synthesized in the root of the plant and concentrates in the leaves, where sunlight converts theanine to polyphenol. Theanine not only gives the characteristic flavor and delicate taste but also produces a noticeable relaxation effect in human beings (*4*).

Theanine has been known to play an important role in determining the quality and characteristics of green tea (*2*). In black tea, however, the importance of theanine as a flavor component is less since there could be a breakdown of theanine after withering and processing (*11*). The quality of tea continues to be evaluated based on a professional tea taster's judgment. However, considering the wide and varied positive health attributes of both black and green tea, there is a growing concern to assess tea quality based on some form of measurement. A number of methods to assess free amino acids, sugars, chlorogenic acid, purine alkaloids, and catechins have been reported. A routine analytical method for direct microscale determination of theanine that can be adapted by analytical quality control laboratories is not available. Using reverse-phase high-

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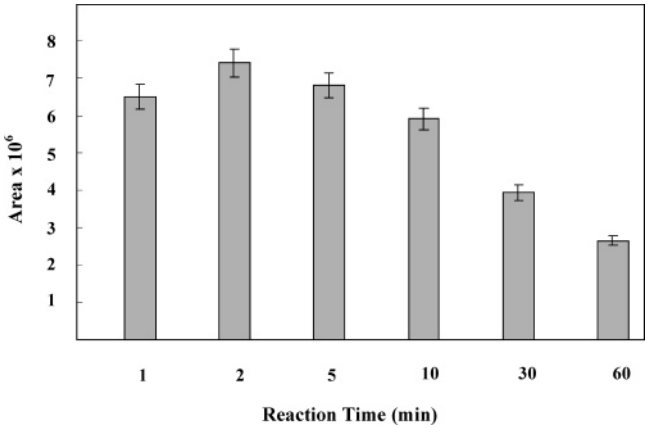

Figure 1. Effect of time on the peak area response for OPA derivatization of theanine.

performance liquid chromatography (RP-HPLC) coupled with a photodiode array detector and electrospray ionization mass spectrometry (ESI-MS), a fingerprint of multiple components of tea was established (*12*). Ekborg et al. (*13*) separated the enantiomers of theanine after 9-fluorenylmethoxycarbonyl glycine chloride (FMOC-Gly-Cl) derivatization. This method involved a laborious and elaborate sample clean up procedure. Using a chirobiotic T (teicoplanin) chiral stationary phase, native and derivatized theanine isomers were separated by HPLC and coupled with atmospheric pressure ionization mass spectrometry (*14*). ^{H}NMR has been effectively used to simultaneously analyze catechins, amino acids, phenolics, fatty acids, and sugars in a green tea extract (*15*).

A microchip-based electrophoresis for short time analysis of amino acids after derivatization with 4-fluoro-7-nitro-2,1,3-benzoxatriazole was developed to analyze theanine in Japanese green tea (*16*). This method required complete removal of polyphenols and a microfabricated hard plastic and poly(methylmethacrylate) chip. Micellar electrokinetic chromatography with photodiode array detection at 194 nm could simultaneously separate several components of tea (*3*). Using a complex ternary gradient elution, Ding and Mou (*17*) separated theanine from glutamine and free sugars by anion exchange chromatography coupled with integrated pulsed amperometic detection. More recently, Ying et al. (*18*) have used a specialized microbore-PTH column coupled with a complex quaternary isocratic solvent and fluorescence detection to determine theanine in different grades of tea. All of the methods known and reported to date require complex sample preparations, which include derivatization, cleanup procedures, special columns not routinely used in quality control labs, and/or sophisticated instrumentation. This limits the use of these methods routinely for the evaluation of theanine, an important quality parameter of green tea.

We report here a relatively instantaneous precolumn derivatization using *o*-phthaladelhyde (OPA), commonly used in the quantification of amino acids. The separation of theanine from the other free amino acids of tea infusions was achieved on a commonly used reverse phase (C_{18}) column using gradient elution and detection at 340 nm. The phenyl isothiocyanate (PITC) precolumn derivatization procedure and RP-HPLC reported by Bindlingmeyer et al. (*19*) for protein hydrolysates was also used to quantitate theanine. The application of the

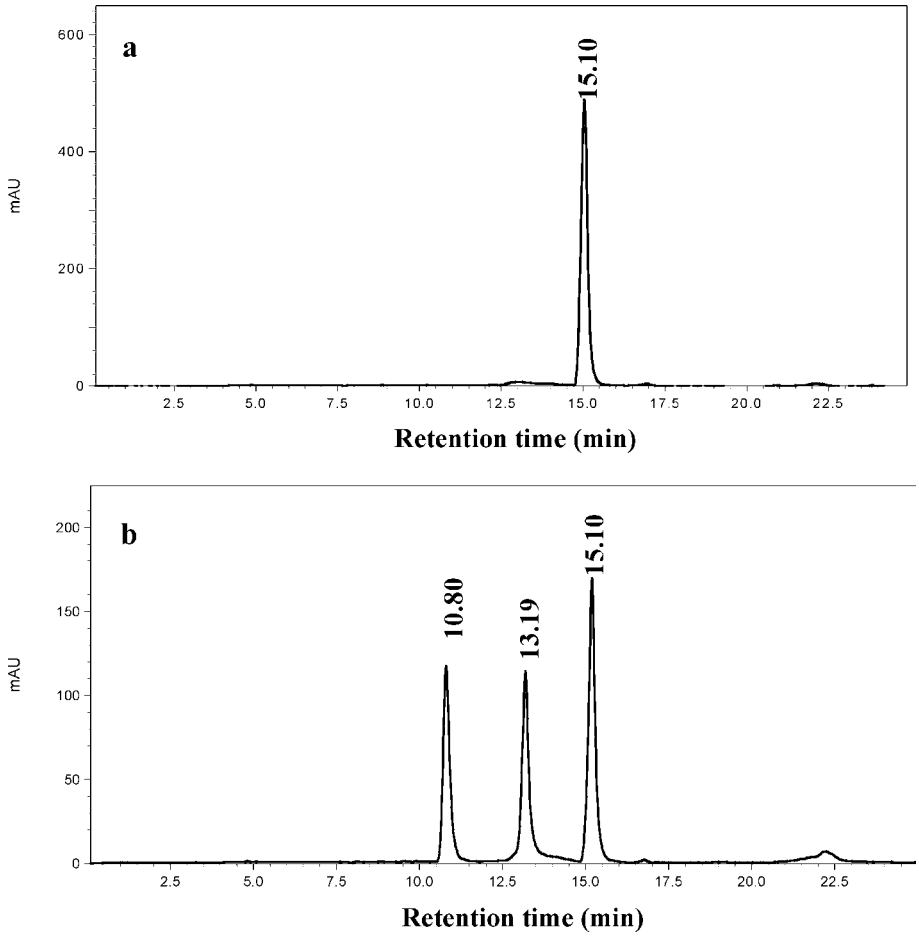

Figure 2. Typical RP-HPLC chromatogram of the OPA derivatized (**a**) theanine and (**b**) a standard mixture of glutamic acid, glutamine, and theanine. Detection was by UV at 340 nm.

methods to commercially available tea is described. The limit of detection (LOD) for theanine by the OPA method was 0.685 nmol, and the LOD by the PITC method was 1.4 pmol.

MATERIALS AND METHODS

Chemical and Reagents. OPA, β-mercaptoethanol, triethylamine (TEA), PITC, and L-theanine were obtained from Sigma-Aldrich Co. (St. Louis, MO). Acetonitrile and methanol were HPLC grade from Spectrochem Pvt. Ltd. (Mumbai, India). Boric acid was from Amresco (Solon, OH), and sodium acetate was from Qualigens (Mumbai, India). All solutions were prepared with water purified with a Milli-Q system (Millipore, Bedford, MA).

Tea Samples. In this study, commercial tea, green tea, tea bags, and dust tea samples were purchased from the local super market.

Tea Infusion Preparation. Ten milliliters of hot water (90 °C) was added to 0.5 g of tea leaves in a beaker and then infused for 20 min. The infusion was filtered through a tea strainer, the filtrate was collected, and the volume was measured. The tea infusion was used for further analysis. Three replicates of the infusions were prepared for each tea sample.

Precolumn Derivatization with OPA. Derivatization reagent was prepared fresh everyday. Five milligrams of OPA dissolved in 0.05 mL of methanol was added to 0.45 mL of 0.4 M sodium borate buffer, pH 10.5, followed by 0.025 mL of β-mercaptoethanol. A 10 μL aliquot of the tea infusion was mixed with an equal volume of OPA reagent and incubated at 25 ± 2 °C for exactly 2 min prior to HPLC analysis. Standard solutions containing 2.5 mM each of theanine, glutamine, and glutamic acid were derivatized as described above. RP-HPLC analysis was performed on a Shimadzu model LC-10ATVP HPLC system (Shimadzu, Japan) equipped with a Rheodyne injector with a 20 μL loop, LC 10 separation module, and SPD-M10AVP PDA detector set to monitor the derivatized amino acids at 340 nm. A reverse-phase Jupiter 5 μm C_{18} 300A°, (250 mm × 4.6 i.d. mm) column was used. The concentration of the optimized mobile phase A was 0.14 M CH_3-COONa containing 0.05% (v/v) TEM adjusted to pH 6.8 with glacial acetic acid and methanol (90:10). Mobile phase B was 60:40 acetonitrile in water. The mobile phase was filtered through a 0.22 μm membrane filter and degassed prior to use. The optimized binary gradient traversing from 0−75% B in 15 min followed by a 5 min wash in 100% B was operated at a flow rate of 1.0 mL/min.

Precolumn Derivatization with PITC. The derivatization of standard theanine and the tea infusion and RP-HPLC was carried out as described by Bidlingmeyer et.al (*20*). The PTC amino acids were separated on a Waters Associate Pico-Tag column and detected at 254 nm using a Waters HPLC system, equipped with a 1525 binary pump and Waters 2996 photodiode array detector.

HPLC Method Validation. Validation tests were performed for accuracy, precision, linearity range, and LOD. The accuracy of the method was assessed by recovery experiments. A known quantity of standard theanine at 50% of the determined level was added to the commercial tea leaf extracts. The recovery was calculated by comparing the theanine measured to that added. About 93−99 ± 0.5% recoveries were obtained by calculating the mean concentration of five replicates each. The precision of the method was evaluated within day ($n = 8$) and between days ($n = 8$ for 10 days) for both the retention time and the peak area of the standard theanine. The precision of the method was expressed by relative standard deviation (RSD) or coefficient of variation (CV). The linearity of the method was evaluated by injecting 20 μL of derivatized standard theanine at 0.047−25 nmol levels. Each standard was analyzed in triplicate.

Linearity and LOD. Calibration curves were constructed over eight different concentrations, three injections were made at each level, and the peak area was plotted against the corresponding concentration. Linear regression analysis was used to generate the standard curves. The LOD and limit of quantitation (LOQ) were calculated based on the standard deviation of the peak area response and slope of the linear calibration curves. LOD and LOQ were expressed as 3.3 d/S and LOQ $= 10\ d/S$ where $d =$ the standard deviation of the y-intercept of the regression line and $S =$ the average slope of the regression lines.

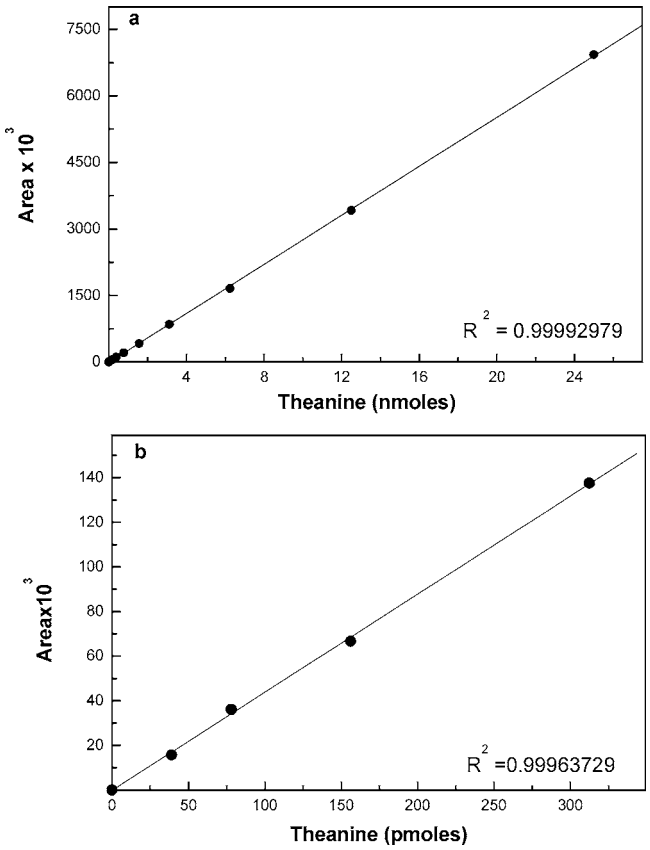

Figure 3. Calibration curve for measuring theanine by (**a**) OPA and (**b**) PITC precolumn derivatization and UV detection.

Data Analysis. For all of the measurements, a minimum of 3−5 replicates were taken for data analysis. Using the software Origin 4.1, all of the values were averaged and mean values were reported. The standard deviation for five replicate data was also tabulated.

RESULTS AND DISCUSSION

The quality of tea is assessed through its appearance, flavor, and aroma. The factors that determine the quality differ from black tea to green tea. Black tea quality is characterized by the content of catechins, theaflavins, thearubigins, and caffeine (*21*, *22*). In contrast, green tea quality is characterized by its amino acids in addition to caffeine and catechin (*15*, *23*). Sweetness of green tea is attributed to amino acids whereas theanine content characterizes the umami (brothy) taste (*21*). Theanine has gained widespread importance, more because of the health-related properties demonstrated in vivo (*4*). The theanine content in tea varies depending on the degree of fermentation and subsequent processing of tea. Therefore, to quantitate theanine, it is essential to develop a reliable and sensitive method that can be universally adapted in quality control laboratories. Recently, we developed a method using OPA precolumn derivatization followed by RP-HPLC to analyze the neurotoxic nonprotein amino acid β-*N*-oxalyl-L-α,β-diaminopropionic (β-ODAP) acid present in the seeds of *Lathyrus sativus* (*24*). The simplicity and reliability of the method prompted us to extend this method to the determination of theanine, which is also an unusual nonprotein amino acid. The same chromatographic conditions were applied to investigate the retention and chromatographic efficiency for the separation of theanine from the other amino acids present in tea infusions. The amount of OPA selected for the derivatization was based on earlier reports (*24*). The maximum of reaction between OPA and theanine measured

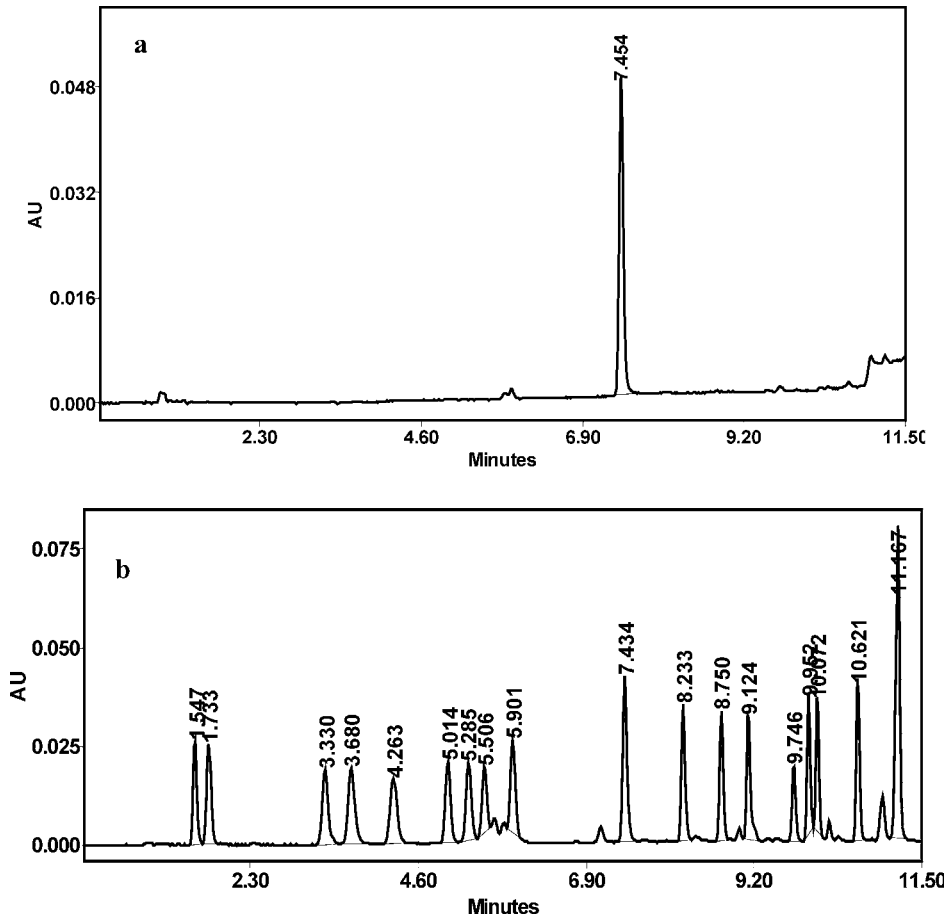

Figure 4. Typical RP-HPLC chromatogram of PITC derivatized (**a**) theanine and (**b**) a mixture of Pierce H standard protein hydrolysate and theanine. Order of elution and retention time: 1, Asp, 1.547; 2, Glu, 1.733; 3, Ser, 3.330; 4, Gly, 3.680; 5, His, 4.263; 6, Arg, 5.014; 7, Thr, 5.285; 8, Ala, 5.506; 9, Pro, 5.901; 10, theanine, 7.434; 11, Tyr, 8.233; 12, Val, 8.750; 13, Met, 9.124; 14, Cys, 9.746; 15, Ile, 9.952; 16, Leu, 10.072; 17, Phe, 10.621; and 18, Lys, 11.167.

as a response of A_{340} was achieved at 2 min at 25 ± 2 °C, following a measurable decrease that was observed (**Figure 1**). The derivatization time was fixed at 2 min for all further studies. Employing the same gradient as that reported for OPA-derivatized β-ODAP, derivatized theanine was chromatographically resolved (*24*). The retention time for theanine was 15.10 ± 0.08 min (**Figure 2a**). Besides theanine, there exist structurally similar amino acids such as glutamic acid and glutamine in tea infusions that would generally interfere with the chromatographic separation. To investigate the veracity of the separation, the elution profile of a mixture of glutamic acid, glutamine, and theanine was evaluated. Under the given chromatographic conditions, standard theanine is well-resolved from glutamic acid and glutamine (**Figure 2b**). The retention times are 10.80 and 13.19 min for glutamic acid and glutamine, respectively, as compared to 15.10 min for theanine. The separation of theanine and glutamine, the major amino acids in Japanese green tea, was insufficient using microchip electrophoresis and fluorescent detection (*16*). The use of a ternary gradient eluent consisting of water and 0.25 M NaOH and 1.0 M sodium acetate together with anion exchange chromatography resulted in the coelution of theanine and glutamine (*17*). The method described in this investigation is relatively instantaneous, and the OPA reagent was prepared fresh each day prior to analysis. However, the method of Ying et al. (*18*) requires overnight standing for a similar OPA reagent and the isocratic elution of OPA-derivatized theanine followed by fluorescence detection shows chemical interference and a progressive decay of the fluorescence with time.

The calibration curve for theanine was linear over the concentration range from subnanomole to 25 nmols with a coefficient of linearity near unity ($R^2 = 0.9995$) and the intercept not significantly different from zero (**Figure 3a**). The LOD for this method was 0.12 μg, and the LOQ was 0.35 μg. The reproducibility of the retention times ($n = 8$ injections) within the day was 0.55% CV, and between days ($n = 10$ over 10 days), it was 2.2% CV. The peak area repeatability intra- and interday (10 days) obtained for $n = 8$ injections was 1.56 and 2.78% CV, respectively.

Precolumn derivatization with PITC followed by RP-HPLC is a reproducible, reliable, accurate, and rapid method with a detection limit of 1 pmol developed for amino acids analysis of protein hydrolysates (*19*, *20*) and has been applied to several hundred proteins and peptides. We have expanded the capability of this method to the analysis of theanine in tea. The chromatographic separation of theanine alongside the Pierce H standard protein hydrolysate is represented in **Figure 4**. Theanine is well-resolved from all of the other amino acids of the standard mixture. PTC-theanine elutes between proline and tyrosine (**Figure 4b**). The response studied over the standard range of 2−312.5 pmol was linear with a regression coefficient close to unity ($R^2 = 0.999$). The detection limit for theanine was 1.4 pmol (0.25 ng), whereas the LOQ was 4.3 pmol (0.75 ng). The detection limit for standard amino acids of protein hydrolysates reported is 1 pmol (*20*). The reproducibility of the derivatization procedure and chromatographic analysis was 0.6% RSD at the 312.5 pmol level. The repeatability and precision of the same day and between day retention times ($n = 5$) was

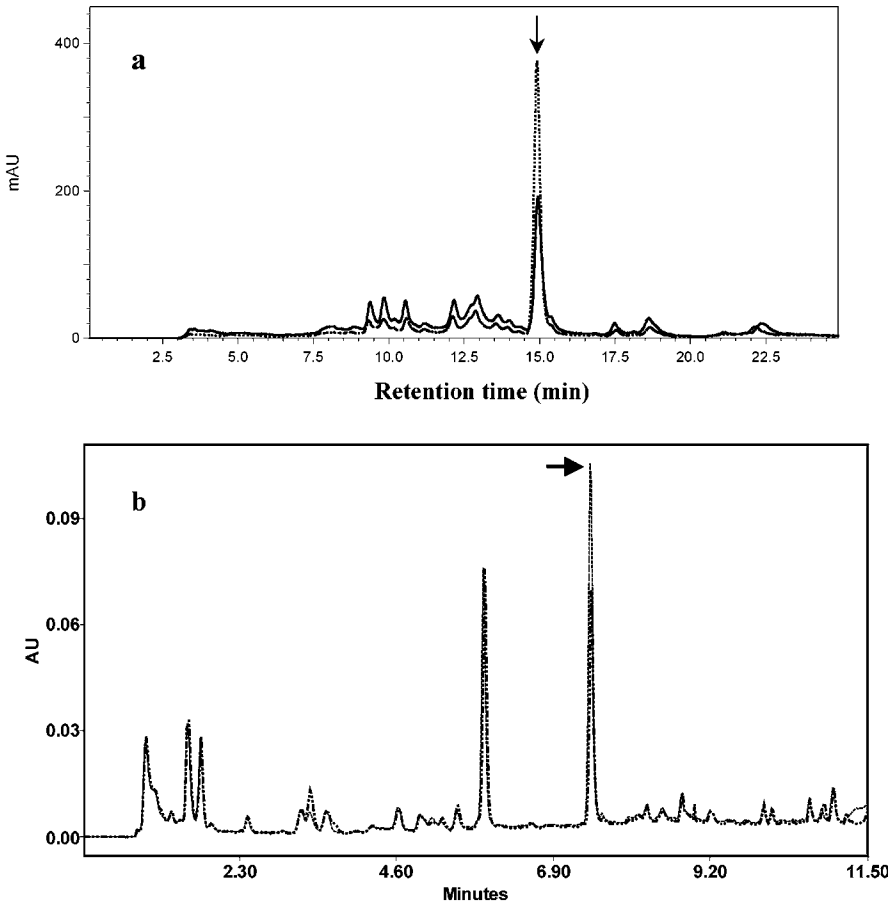

Figure 5. RP-HPLC chromatograms relevant to a commercial tea infusion (—) and the same sample spiked with a known quantity of theanine (- - -). (**a**) Derivatized with OPA and detection at 340 nm. The arrow indicates theanine. (**b**) Derivatized with PITC and detection at 254 nm.

0.08% RSD. The reproducibility for the amino acid analysis of purified proteins that includes hydrolysis, PITC derivatization, and chromatography is reported to be 4% RSD (*20*). The reproducibility for theanine using this method is higher probably due to it being a nonprotein amino acid like norleucine that is commonly used as an internal standard. This method is very selective for theanine, as there is no interference from other amino acids. Therefore, the ambiguities in the identification of theanine in tea infusions are minimal. The reproducible results together with the high sensitivity for theanine detection render this method suitable for the analysis of free amino acid content of tea infusions.

The veracity and practicability of these two methods were evaluated by analyzing tea and green tea extracts. A representative chromatogram of commercial tea samples is presented in **Figure 5**. It can be observed that in addition to theanine, other amino acids are present, however, at trace levels with the exception of proline. The theanine contents of various commercial tea and green tea infusions are listed in **Table 1**. It is observed that the theanine content varies from 2 to 5 mg/g leaf. These differences reflect the natural abundance of theanine in different types of tea. The different theanine levels of several grades of tea were attributed to the fermentation process (*18*). Flowery orange pekoe considered to be one among the finest grades of dark tea that produces strong dark brew has a very low theanine content (*18*). The theanine content of green tea has been shown to decline with late plucking and among leaf parts; the bud contains higher contents (*3*).

The recovery test for theanine in the commercial teas was performed by spiking the tea infusion with standard theanine at 50% of the determined level (**Figure 5**). The average recoveries ranged from 93 to 99.3%. The recovery using the PITC method was higher than the OPA method. Spiking with theanine at 50−100% of the determined level, the recovery reported was 92−103.8% using anion exchange chromatography and integrated pulsed amperometric detection (*17*). Mean recoveries vary from level to level of spiking. At a high level of spiking, the recovery was 93.5−104.8%, whereas at the low level it was 89.3−104.2% (*12*). Therefore, caution must be exercised when recovery data are evaluated.

Table 1. Representative Theanine Content of Tea Infusions[a]

commercial tea samples	theanine (mg/gm leaf) OPA method	theanine (mg/gm leaf) PITC method
1	2.84 ± 0.10	2.74 ± 0.04
2	5.31 ± 0.13	4.90 ± 0.12
3	5.23 ± 0.26	4.55 ± 0.21
4	3.39 ± 0.13	3.00 ± 0.12
5	4.00 ± 0.09	4.16 ± 0.20
6	3.10 ± 0.07	3.07 ± 0.05
7	3.25 ± 0.07	3.52 ± 0.21
8	2.56 ± 0.07	2.46 ± 0.12
9	4.52 ± 0.03	5.43 ± 0.24

[a] Key: 1–4, black tea; 5 and 6, green tea; and 7–9, tea bags.

The theanine content measured using both the PITC method and the OPA method correlates well (**Table 1**). These results coupled with the sensitivity and reproducibility should serve as a quantitative method for theanine measurements.

In conclusion, in this investigation, the theanine content of tea was analyzed using liquid chromatography and diode array

UV detection. High sensitivity, reproducibility, and selectivity were achieved with both the OPA and PITC precolumn derivatization and the UV detection at 340 and 254 nm, respectively. The method involves simple sample preparation with efficient chromatographic separations for selective measurement of theanine. The methods can be easily adapted in quality control analytical laboratories as they involve the use of RP-HPLC columns and detectors that are routinely used for HPLC, the most versatile analytical technique. This method also fulfills the requirements as a robust and simple technique to evaluate the quality of tea based on amino acid profiling.

ABBREVIATIONS USED

CV, coefficient of variation; ESI-MS, electrospray ionization−mass spectrometry; FMOC, 9-fluorenylmethoxycarbonyl; LOD, limit of detection; LOQ, limit of quantitation; OPA, *o*-phthalaldehyde; β-ODAP, β-*N*-oxalyl-L-α,β-diaminopropionic; PITC, phenyl isothiocyanate; RP-HPLC, reverse-phase high-performance liquid chromatography; RSD, relative standard deviation.

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