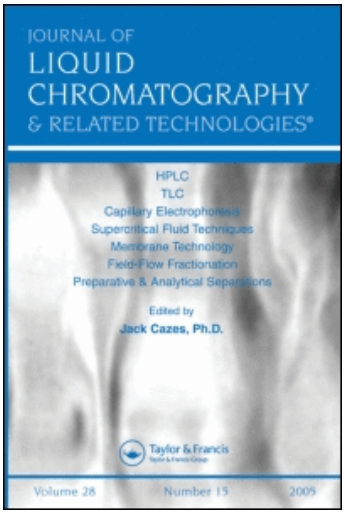

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Analysis of Theanine in Tea Leaves by HPLC with Fluorescence Detection

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Abstract: Theanine, one of the main amino acid components in tea, is known as a precursor of the non-peptide antigen ethylamine, which mediates a memory response leading to secretion of IFN-γ. Tea which contains theanine is alleged to have various therapeutic benefits to man. Different types of tea contain various amounts of theanine. A method for reversed-phase high performance liquid chromatographic separation of theanine with fluorescence detection and its application are described. The method was applied to determining theanine in different types and grades of tea samples, which were extracted in boiling water, followed by filtration through a 0.45 μm filter. Theanine was derivatized with o-phthaldialdehyde (OPA) prior to analysis. Separation of theanine, using an isocratic elution with a mobile phase containing 15 mM sodium acetate, isobutanol, isopropanol, and acetonitrile (75 : 3 : 2.5 : 8, pH 7.1) was achieved in less than ten minutes. The relative fluorescence of derivatized theanine remained steady during analysis. The limit of detection of theanine standard was 33.2 picograms, while the limit of quantitation of theanine in tea extract was about 0.33 ng/mL. The results show that there is a relative quantitative relationship between the degree of fermentation and the level of theanine. The findings indicate that non-fermented green tea and partially fermented yellow tea contained more theanine than dark, black, and woo-loong tea, which all undergo the fermentation.

Keywords: Theanine, HPLC, o-phthaldialdehyde, tea, analysis

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INTRODUCTION

Theanine is one of the major amino acid components in beverage teas. Its chemical structure is shown in Figure 1. Theanine was initially identified as an ninhydrin-reactive substance.[1] Other natural sources of theanine include the mushroom *Xerocomus badius*.[2] Theanine can be as much as >50% of the total amino acids in tea leaves. It is found in the developing shoot tips, where it is used as the major source of nitrogen for alkaloids.[3,4] Theanine is an essential precursor for the biosynthesis of flavonols in tea leaves.[5] Theanine was reported to reduce blood pressure,[6] and to regulate the physiological levels of norepinephriine, serotonin, 5-hydroxyindoleacetic acid, and dopamine.[7–9] More recently, theanine has been shown to regulate human γ and δ T cells that play a role in mediating innate immunity to microbes.[10] Primed peripheral blood γ and δ T cells may mediate a memory response to ethylamine and IFN-γ in response to bacteria. These findings suggest health benefits of tea consumption. Tea can be categorized based on the degree of fermentation. The fermentation process essentially involves the oxidation of the polyphenolic compounds of tea. The fermentation step yields different types and the characteristic taste of tea. The theanine content in tea varies, depending on the degree of fermentation and subsequent processing of tea.

A simple method for the determination of theanine in tea sample has not been reported. Existing methods for quantitation of theanine required tedious solid-phase extraction and pre-concentration protocols.[11] The separation procedures employed novel mobile phase techniques with fluorometric detection.[11–13] More recently, a method for determination of theanine using gradient elution with 9-fluorenylmethoxycarbonylglycine chloride (FMOC-Gly-Fl) as a pre-column derivatizing agent has been reported.[14] These methods vary in complexity.

In order to reliably evaluate the theanine content and its innate immune potential through drinking of tea phages by tea diversity, it is essential to develop a simple and sensitive method for the determination of theanine in tea. The desirability for an efficient separation and quantitation of theanine prompted the development of a HPLC method. The quantitation of theanine in tea is prone to difficulties, such as incomplete extraction or low sensitivity. In addition, other co-extractives from tea leaves may interfere by co-eluting with theanine.

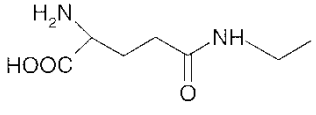

Figure 1. Chemical structure of theanine.

The present paper describes a reversed-phase HPLC method with fluorometric detection for the determination of theanine. The application of the method to tea samples is also described.

EXPERIMENTAL

Chemicals

Standard theanine used in this study was a commercial product purchased from Taiyo International lnc. (Boise, ID). *O*-phthalaldehyde (OPA) was obtained from Sigma (St. Louis, MO, USA). Acetonitrile purchased from BDH Laboratory Supplies (Poole BH151TD, UK) was of HPLC grade. Other chemicals and solvents were of analytical grade.

HPLC System and Condition

The HPLC system consisted of a Hewlett-Packard Series 1100 Liquid Chromatograph. The detection system was a Model FL-1 Dynamax fluorescence detector (RAININ). The integrator was a Model 3395B purchased from Hewlett-Packard. The micro-C_{18} PTH column (2.1 mm × 250, 5 μm) was supplied by Agilent Technologies (Palo Alto, CA).

The mobile phase was composed of 15 mM sodium acetate, isobutanol, isopropanol, and acetonitrile by volume ratio (75/3/2.5/8, pH 7.1). The flow rate of mobile phase was set at 0.2 mL/min. The temperature was maintained at 30°C. The excitation and emission wavelengths of the detector were fixed at 330 and 418 nm, respectively.

Sample Preparation

Two grams of dried woo-loong tea leaves were boiled in water for 10 min. The extraction was repeated thrice. The combined extract was filtered through an inert 0.45 μm filter and was derivatized with OPA prior to injection onto the HPLC. The theanine standard (0.4 mg) was dissolved in 1 mL of double-distilled water. For recovery study, the woo-loong tea samples were spiked with a calibrated amount of theanine (0.2 mg) before extraction. The indigenous theanine was corrected in the recovery calculation.

Derivatization with o-Phthaldialdehyde (OPA)

Preparation of OPA reagent was performed according to the published method.[15] A 27 mg portion of OPA was dissolved in 500 μL of absolute

ethanol, followed by addition of 4.5 mL of 0.4 M sodium borate buffer (pH 9.5) and 25 μL β-mercaptoethanol. The reagent should stand overnight before use. A 50 μL portion of standard or tea extract was mixed with 50 μL of OPA reagent for 4 min. The reaction was stopped by the addition of 100 μL of 0.4 M potassium monobasic phosphate (pH 4.0). An aliquot of 2 μL was injected onto the HPLC for analysis.

RESULTS

The HPLC chromatograms (Fig. 2) showed an efficient separation of theanine standard by isocratic elution with the mobile phase, which contained 15 mM sodium acetate, isobutanol, isopropanol, and acetonitrile (75 : 3 : 2.5 : 8, v/v/v/v, pH 7.1), with fluorescence detection. Confirmation of theanine in tea samples was made by comparison of the UV spectra of the analyte and the authentic standard obtained under the same analytical conditions. The recovery of theanine in a tea sample was shown in Table 1. A simple extraction protocol for theanine from tea sample was developed. The extract was derivatized with OPA prior to injection onto the HPLC. No interfering peak co-eluted with theanine in the tea samples. The utility of the method was demonstrated through the analysis of theanine in tea. A typical chromatogram shows the theanine in Longjing (Dragon Well) tea leaves (Fig. 2). Longjing is a world famous and special tea found in West Lake, Hanzhou, China. The distribution and quality of Longjing tea depends on the geo-chemical characteristics of the growth environment and climate conditions.[16] The method was additionally applied to Gynostemma Pentaphyllum (Jiao gu lan), commercial tea samples (Fig. 2). Jiao gul lan is believed to reinforce overall health and to show anti-fatigue effects.[17] Consumption of aqueous extracts of jiao gu lan has been associated with a marked lowering of cholesterol and triglyceride levels.[18,19] However, details of the active principles in jiao gu lan remain to be elucidated. Table 2 summarizes the determination of theanine in various tea samples. The analysis clearly shows varietal differences in the theanine content of the tea samples.

The relative fluorescence of the derivatized theanine is shown in Fig. 3, while the fluorescence decay of the derivative is shown in Fig. 4. Theanine from the tea sample matched the retention time and spectral characteristics to the authentic theanine standard. The sensitivity of the HPLC method with fluorescence detection is reflected in the limit of detection (LOD) value for theanine (Table 1). The calibration curve was linear over the concentration range of interest from sub-pico mole to 1 μM with a coefficient of linearity close to unity (0.997) for theanine.

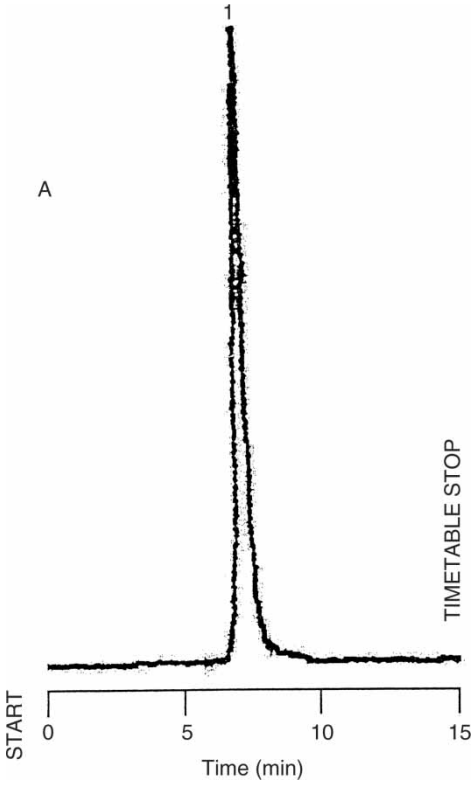

Figure 2. Chromatograms of derivatized theanine. Chromatogram of derivatized theanine standard (0.3 μmole) with OPA solution. The mobile phase contained 15 mM sodium acetate, isobutanol, isopropanol and acetonitrile by volume ratio (75 : 3 : 2.5 : 8). The excitation and emission wavelengths were set at 330 and 418 nm, respectively. Elution profile of derivatized theanine (1) from Longjing tea leaves. Elution profile of derivatized theanine (1) from Jiao Gu Lan tea leaves.

(*continued*)

DISCUSSION

Theanine is the major amino acid component in tea leaves. Its health benefits have been reported in previous studies.[8,9] The analysis of theanine in beverages provides useful information for comparison of theanine level in tea samples (Table 2). The differences in the theanine levels in tea samples reflects the natural abundance of theanine in different types of tea samples. Fermentation steps effect the theanine content of tea. Fermentation involves oxidation in the processing of the commercial tea leaves. Green tea does not undergo the fermentation step. For black tea processing, the green leaves are incubated for about 4 h under conditions of high humidity

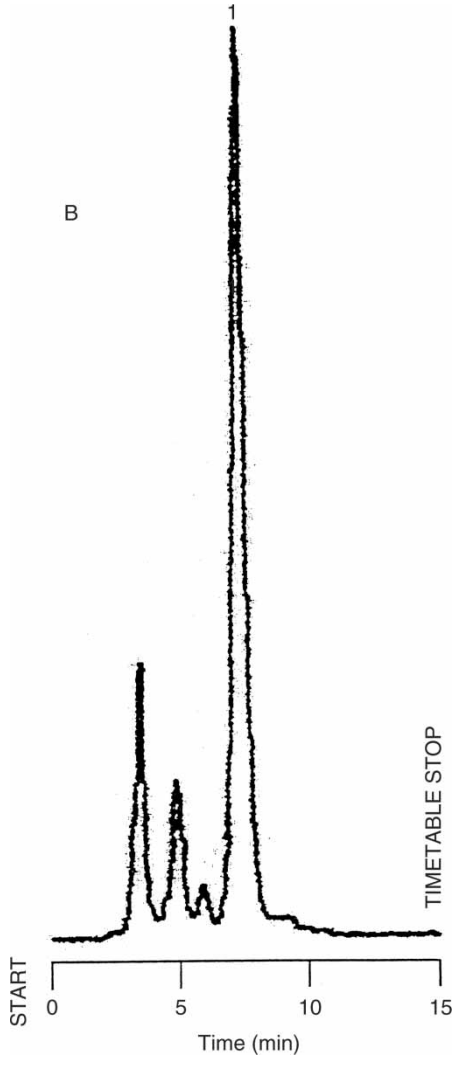

Figure 2. Continued.

and low temperature. The half-green tea preparation is a compromise process between black and green tea. Green tea appears to contain the highest level of theanine while dark tea has the lowest theanine content. Among the tea samples tested, half-green tea and black tea contain theanine at levels that are mid-way between green and dark teas. The difference in theanine levels among different grades of tea seems to be attributed, in part, to the fermentation process. The longer tea leaves are fermented, the less theanine remains in

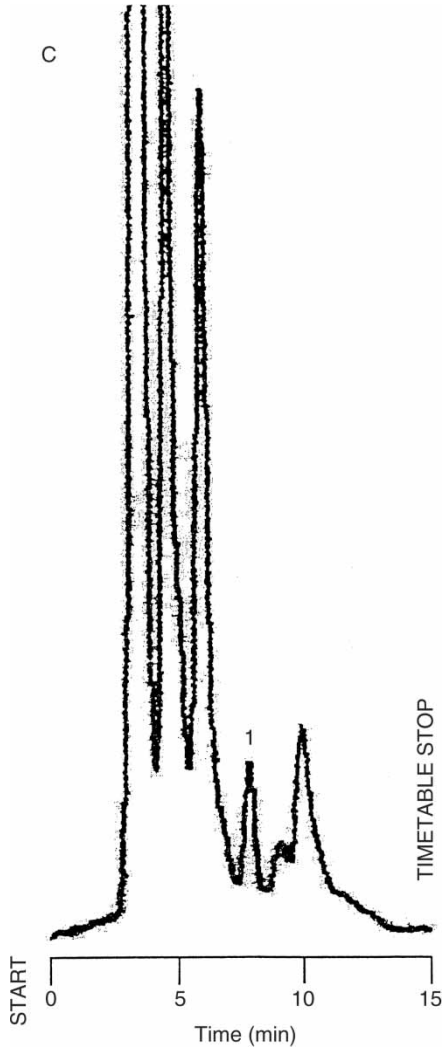

Figure 2. Continued.

the tea sample. The higher concentration of theanine in green tea could be associated with its alleged salubrious effects. An interesting trend relating to the theanine level was found in Table 2. The teas that had the lowest amounts of theanine were always of Flowery Orange Pekoe (FOP) grade. FOP is considered to be one of the finest dark teas with small tea leaves that produce strong brews. The results suggest that there may be a relative quantitative relationship between the grade of tea and the level of theanine. The method of the present study employed simple derivatization of

Table 1. Chromatographic characteristics of the HPLC method

	t_R (min) ± RSD (%)	LOD[a] (Pg)	LOQ[b] (ng/mL)	Recovery (%)[c]
Theanine	7.31 ± 0.2	33.2	0.33	99.6 ± 3.9

Data represent the mean ± RSD from replicate experiments (n = 3).
[a]LOD, Limit of detection at a signal-to-noise ratio of 2.
[b]LOQ, Limit of quantitation at a precision and accuracy of <20%.
[c]Recovery, a calibrated amount of theanine (0.2 mg) was spiked in tea leaves before extraction.

theanine with OPA. The stability and the fluorescence of the derivatized theanine remained steady during the analysis. The derivatization of theanine with OPA is simple. The derivatized theanine is photostable and spectrally distinct. There was hardly any variation of the derivatized theanine during analysis by the HPLC method. The relative fluorescence intensity of the derivatized theanine remained unchanged during analysis. The sensitivity of the method for the determination of theanine in tea samples is about 3-fold higher than the existing detection methods with UV absorbance or other fluorometric measurements.[11–14] The recovery study also showed a

Table 2. Total amount of theanine in various tea samples

Tea samples		Producing area (province)	Theanine in dry tea leaves (%)	Ref.
Green tea	Dragon well (grade one)	Zhejiang	1.75	[16]
	Dragon well (before Tomb-sweeping day)	Zhejiang	2.40	[16]
	Green Spring Snail	Jiangsu	2.61	[1]
	Green Spring Snail	Jiangsu	1.73	[1]
			1.26	[1]
			1.75	[1]
White tea	Longevity Eyebrow	Fujian	1.34	[18]
	Longevity Eyebrow (white peony)	Fujian	0.90	[18]
Yellow tea	Silver Needle	Hunan	2.17	[14]
Woo-loong tea	Iron Guan Yin	Fujian	0.46	[19]
	Big Red Robe	Fujian	0.53	[19]
Black tea	Yun Nan Black tea	Yunnan	1.12	[17]
	Qi Men Black tea	Anhui	0.49	[17]
Dark tea	Yun Nan tea	Yunnan	0.005	[20]
	Pu'er	Yunnan	0.007	[20]

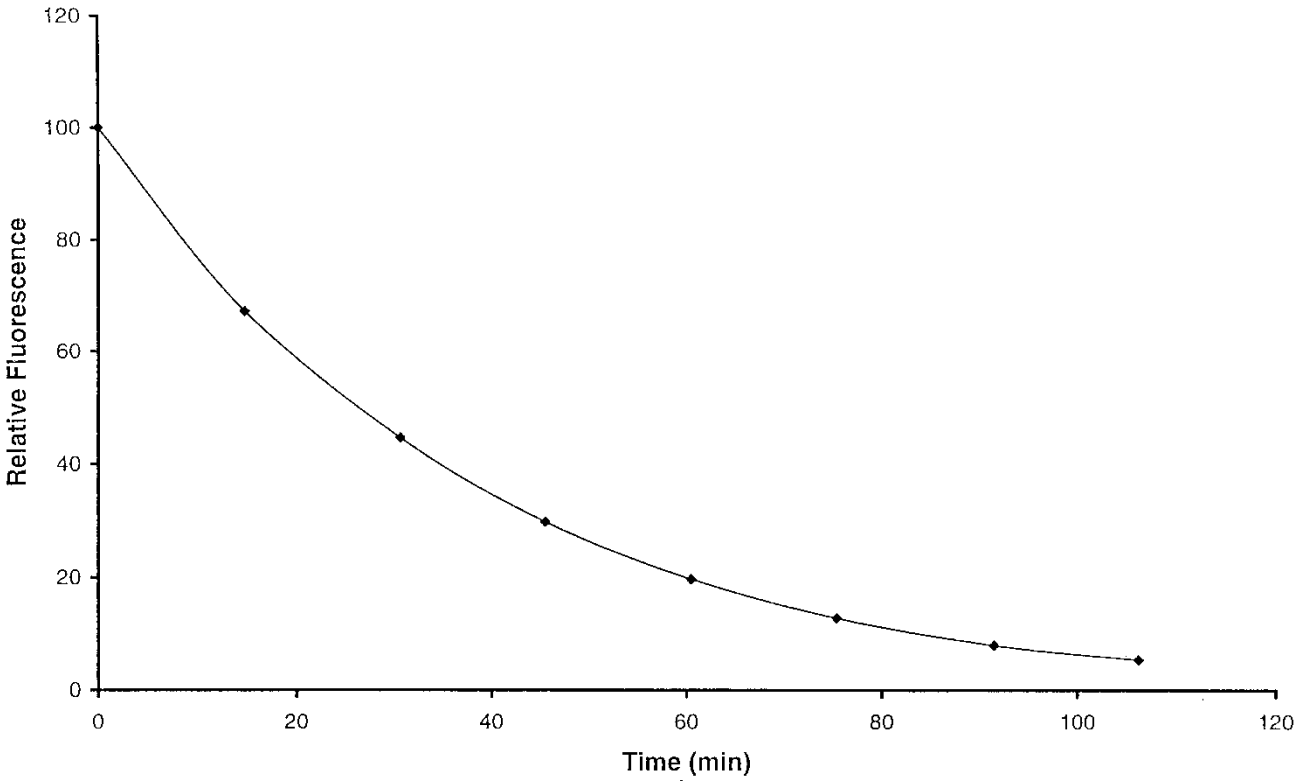

Figure 3. Decay of derivatized theanine fluorescence with time.

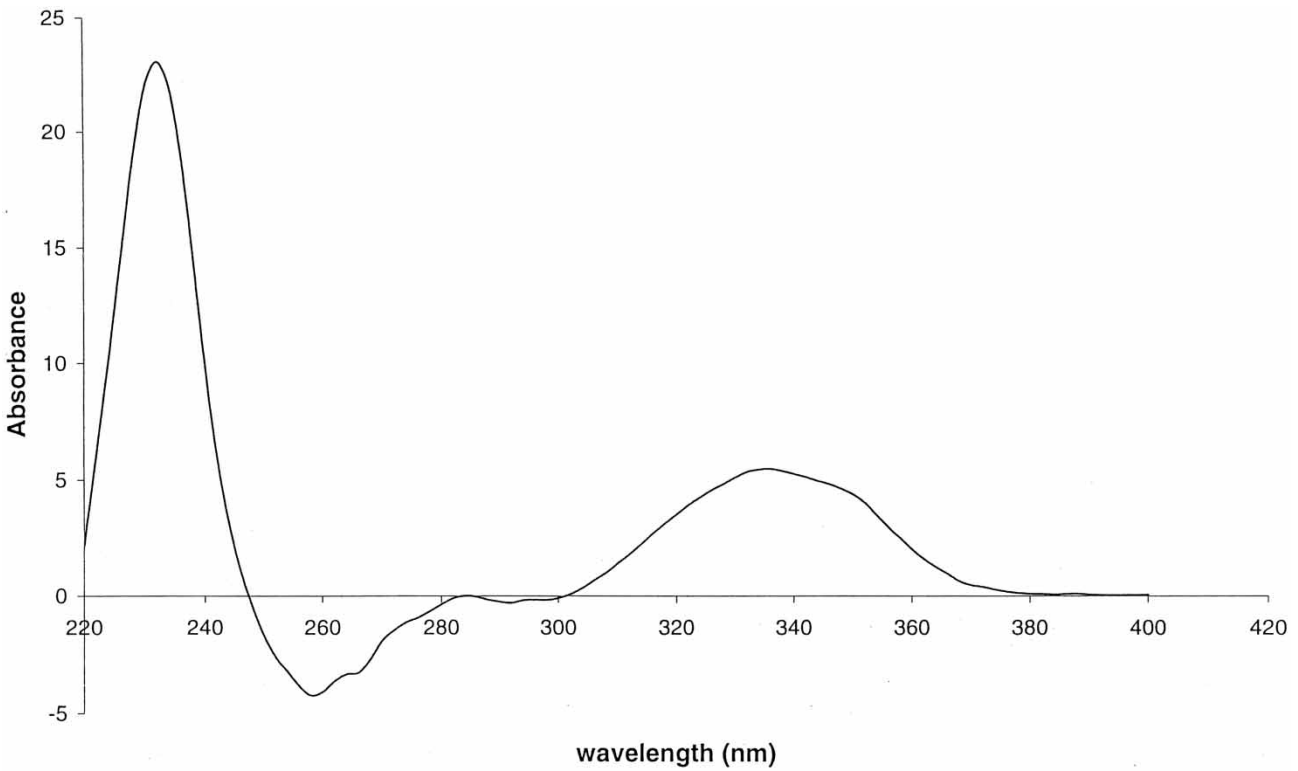

Figure 4. Scanning spectrum of derivatized theanine (0.3 μM) with OPA.

quantitative extraction of theanine in tea samples (Table 1). The simple extraction procedure, coupled with the sensitive detection method, provide an efficient method for the analysis of theanine in different types of tea samples. The linearity of the calibration of theanine is close to unity (>0.99) over a dynamic range of concentration of sub- micromolar level (0.5 μM). The present method is selective and reduced chemical interferences in tea samples during analysis. It can minimize ambiguities in identification of theanine in tea samples due to spectral distinct fluorescent peak and lack of interfering peaks.

ACKNOWLEDGMENTS

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