

Analysis of derivatized and underivatized theanine enantiomers by high-performance liquid chromatography/atmospheric pressure ionization-mass spectrometry

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Theanine, a naturally occurring non-proteinic amino acid found in tea leaves, has demonstrated wide-ranging physiological activity, from lowering blood pressure to enhancing the anti-tumor activity of chemotherapeutic drugs. The chiral nature of theanine suggests that enantiospecificity plays a significant role in its various pharmacological functions. Using the Chirobiotic T (teicoplanin) chiral stationary phase, native and derivatized theanine enantiomers were separated and detected via high-performance liquid chromatography (HPLC) coupled to atmospheric pressure ionization mass spectrometry (API-MS). With the use of flow rates compatible with each ionization source, native theanine standards achieved excellent sensitivity and detection limits (10 ng/mL) for both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). Optimum sensitivity and detection limits for derivatized theanine standards were achieved using ESI-MS. The enantiomeric composition of six commercially available L-theanine samples was evaluated using the high-flow APCI-MS method and confirmed with photodiode array detection. Five of the six products contained significant amounts of D-theanine. Only one product, SunTheanine[®], appeared to contain only the L-theanine enantiomer. Copyright © 2004 John Wiley & Sons, Ltd.

Tea is the most popular beverage consumed worldwide. The tea plant is grown in over 30 different countries and is available in many different varieties and flavors. There are three main types of tea: green, Oolong, and black tea. Green teas are subject to minimal oxidation, whereas Oolong and black teas are allowed to partially and extensively oxidize, respectively.¹ The taste of all types of teas can be attributed to the presence of many amino acids, in particular 5-N-ethylglutamine (found only in the free amino acid form), also known as theanine. Theanine was first identified by Sakato to be one of the major chemical constituents of green tea leaves.² Later, Cartwright *et al.* demonstrated the presence of theanine in other forms of teas as well.³ Ekborg-Ott *et al.* found a variety of black tea to have the highest concentration of theanine of the 17 teas tested.⁴ Theanine reportedly makes up approximately 1–2% of the dry weight of tea.^{5,6} The only other known natural source of theanine is the mushroom *Xerocomus badius*.⁷

Recently, there has been an increased interest in the pharmacological effects of theanine. A number of papers

have documented the inhibition of peroxidation of low-density lipoproteins (LDL) by tea extracts.^{8,9} Active oxygen species, which are known to cause significant damage to cells, are thought to be taken up by theanine and other tea components. In addition, theanine is thought to play a major role in preventing neuronal death.^{9–11} Kakuda reported that neuronal death induced by glutamic acid was suppressed with the introduction of theanine.^{9,11} These findings prove extremely significant for the treatment and/or prevention of ischemia and reperfusion injury (stroke).

Theanine has also been shown to have an effect on blood pressure and hypertension in rats. In a controlled study, Yokogoshi reported dose-dependent reduction of blood pressure in hypertensive rats during theanine administration, but no change when the related amino acids glutamine and glutamate were introduced.^{12,13} The mechanism by which theanine decreases blood pressure is not well understood. It is known, however, that levels of various neurotransmitters, such as serotonin and dopamine, can affect blood pressure and even heart rate.¹³ Some theories suggest that theanine actually alters the levels of these neurotransmitters in the brain.^{12–16} A number of studies support this theory by measuring neurotransmitter levels in rats after theanine exposure.^{12,14–16} It was found that levels of serotonin and 5-hydroxyindoleacetic acid decreased significantly,¹⁴

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while those of tryptophan¹⁴ and dopamine increased upon administration of theanine.^{15,16} Neurotransmitters such as these are known to have various physiological functions. Specific regulation of these levels could possibly be used in the treatment of neurological diseases, such as Parkinson's and schizophrenia.

A recent study using electroencephalography (EEG) also showed the inhibiting affect of theanine on caffeine stimulation.¹⁷ When mice were given equal molar amounts of caffeine and theanine, the stimulatory affects of caffeine were significantly reduced. In fact, at 10:1 ratios of theanine to caffeine, stimulation was found to be completely quenched.¹⁷ Theanine has also been found to boost immunity^{18,19} as well as improve the anti-tumor activity of various chemotherapeutic drugs,^{20–28} and reduce tumor growth^{29,30} and metastasis.³¹

Clearly, the physiological effects of theanine are varied and significant. Theanine, like most amino acids, is chiral. Therefore, it is quite possible that the pharmacological effects of one enantiomer over another may vary significantly. A few papers have reported the quantitative HPLC analysis of theanine in tea extracts³² as well as in rat serum, tissue, and urine.^{33,34} These methods all suffer from the inability to separate theanine into its D- and L-forms. This was remedied by a method developed by Ekborg-Ott *et al.*⁴ In this procedure, theanine samples from tea extract were derivatized with 9-fluorenylmethyloxycarbonyl glycol chloride (FMOC-Gly-Cl) reagent, and its enantiomers separated using a γ -cyclodextrin (γ -CD, Cyclobond II 2000) column with fluorescence detection. It was found that all teas, regardless of the manufacturing process, contained L-theanine and smaller percentages of D-theanine. The racemization and hydrolysis of theanine in aqueous solution were also evaluated.⁴ Increasingly, chiral stationary phases based on macrocyclic antibiotics have been preferred for the enantiomeric separation for both native and derivatized amino acids.^{35–38} Neither the enantiomeric separation of theanine on the macrocyclic antibiotic chiral selectors, nor the more sensitive MS detection of theanine, has been reported to our knowledge.

Recently, several papers have reported use of HPLC and the macrocyclic antibiotic chiral selectors coupled with MS detection for the enantiomeric analysis of various chiral compounds, including amino acids.^{39–44} MS detection was found to be quite sensitive for analyzing the underivatized amino acid enantiomers. However, derivatization is often necessary to extract target amino acids from biological samples, such as blood and urine.^{45–50} The present study demonstrates the efficacy of MS detection for the analysis of underivatized and derivatized theanine samples. The enantiomeric composition of commercially available theanine samples was also evaluated using this MS method.

EXPERIMENTAL

Chemicals and reagents

Racemic theanine, D-theanine, and L-theanine standards were prepared in-house using a previously reported procedure.⁴ HPLC-grade methanol (MeOH) and ultra-pure HPLC-grade water were acquired from Fisher (Fair Lawn,

NJ, USA) and Alfa Aesar (Ward Hill, MA, USA), respectively. Ammonium trifluoroacetate (NH₄TFA) reagent was purchased from Aldrich (Milwaukee, WI, USA). Formic acid was obtained from J. T. Baker (Phillipsburg, NJ, USA). All underivatized theanine samples were dissolved in water and diluted to 20 μ g/mL for injection.

Commercially available L-theanine samples were obtained from Amax Nutra Source (City of Industry, CA, USA), Honson Industries, Ltd. (Markham, ON, Canada), HWBT (Zheda Technology & Trading Company, Hangzhou, China), Shengma Bio & Chem Co. (Shanghai Waygain Import and Export Co., Ltd., Shanghai, China), Tans (Zhejiang Zhongjin Environmental Protection Corp., Hangzhou, Zhejiang, China), and SunTheanine[®] (Taiyo International, Inc., Edina, MN, USA).

Derivatization of theanine

Solutions of 9-fluorenylmethyloxycarbonyl chloride (FMOC) and 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl), 2.0 mg/mL, both purchased from Aldrich, were prepared in HPLC-grade acetonitrile (Fisher). Derivatization procedures were similar to those previously reported.^{51,52} A 0.1 M borate buffer solution, pH 7.8, prepared using ACS-grade borate (Fisher), was used to formulate a 2.0 mg/mL stock solution of racemic theanine. For each derivatization reagent, 490 μ L of borate buffer, 490 μ L of acetonitrile, 10 μ L of theanine stock solution, and 10 μ L of the appropriate derivatization solution, were combined. The mixtures were vortexed for a few seconds and then allowed to react for at least 10 min before analysis. Solution concentrations were approximately 20 μ g/mL.

AccQ-Fluor Reagent Kit[™], containing 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent powder, acetonitrile (AccQ-Fluor reagent diluent), and borate buffer, was purchased from Waters (Milford, MA, USA). The use of this derivatization method for enantioselective amino acid analysis was first reported by Pawlowski.⁵³ The AQC reagent powder was reconstituted using 1 mL of the reagent diluent. The reagent solution was vortexed for 10 s and then placed on a heating block at 55°C for 10 min. 10 μ L of the theanine stock solution, 70 μ L of AccQ-Fluor borate buffer, and 20 μ L of the reagent solution were added to a sample vial. The mixture was vortexed for 10 s, and once again placed on a heating

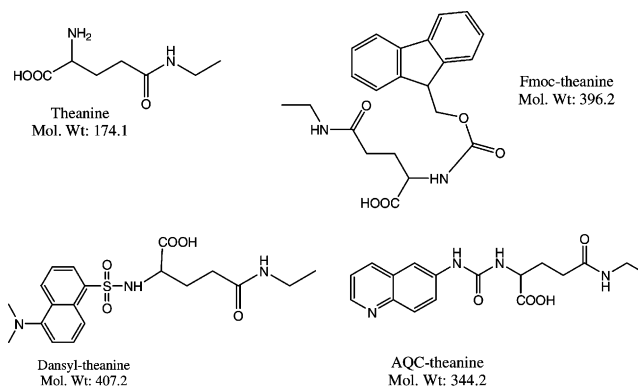


Figure 1. Structures and molecular masses of theanine, FMOC-theanine, dansyl-theanine, and AQC-theanine.

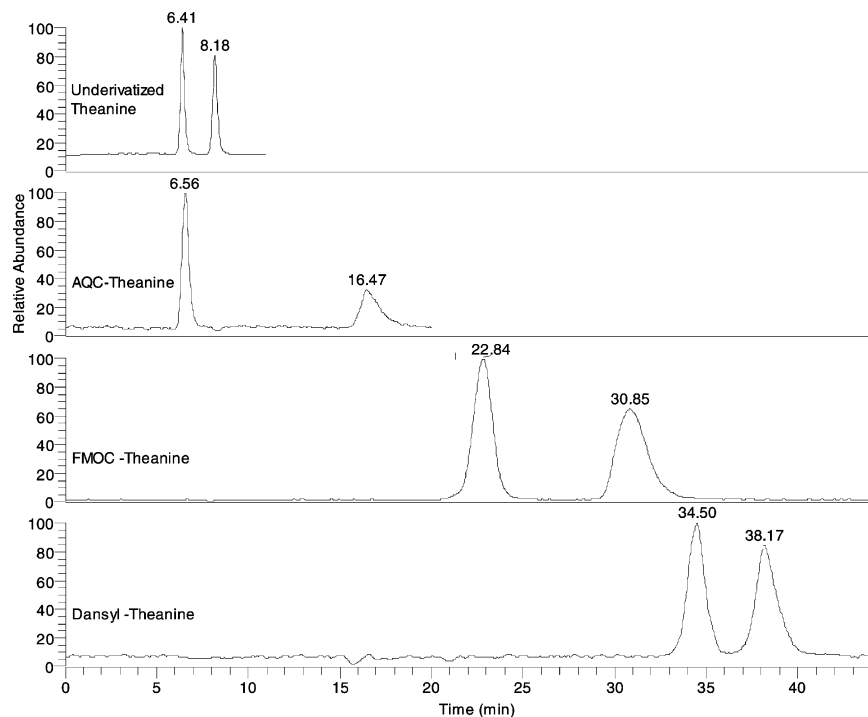


Figure 2. Optimized enantiomeric separation of theanine, AQC-theanine, Fmoc-theanine, and dansyl-theanine. (a) LC/APCI-MS, SIM: m/z 175, mobile phase conditions: 80:20 (1.0% NH_4TFA in MeOH/0.1% formic acid in H_2O) 0.8 mL/min. (b) LC/ESI-MS, SIM: m/z 345, mobile phase conditions: 30:70 (1.0% NH_4TFA in MeOH/100% MeOH) 0.8 mL/min. (c) LC/ESI-MS, SIM: m/z 397, mobile phase conditions: 30:70 (1.0% NH_4TFA in MeOH/100% H_2O) 0.4 mL/min. (d) LC/ESI-MS, SIM: m/z 408, mobile phase conditions: 35:65 (1.0% NH_4TFA in MeOH/100% H_2O) 0.4 mL/min.

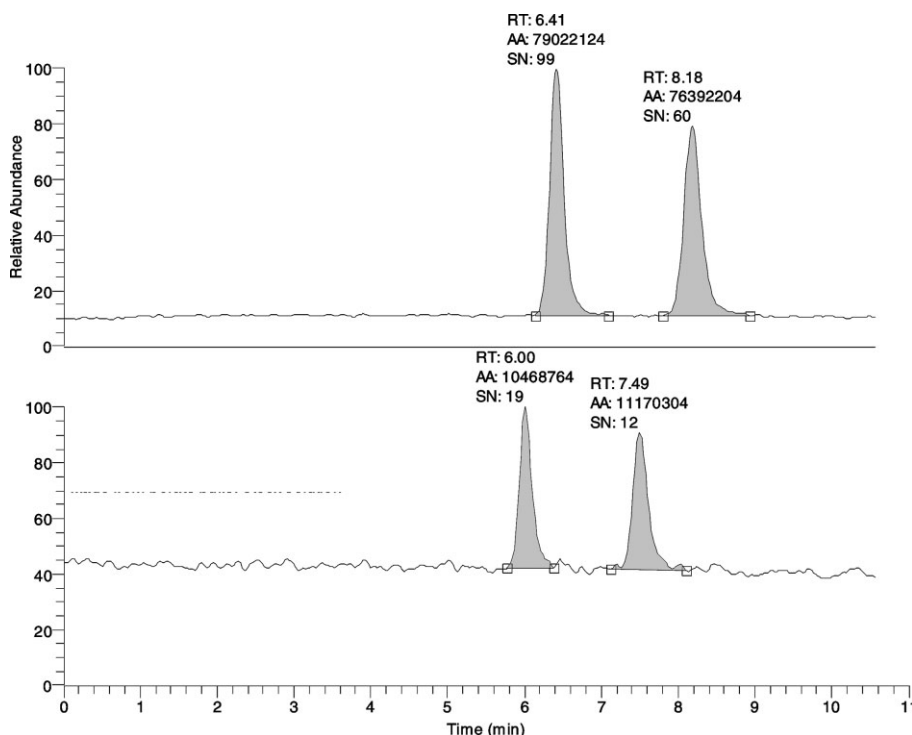


Figure 3. Comparison of theanine standard solution and theanine stock solution. RT: retention time, AA: peak area count, SN: signal-to-noise ratio. Separation conditions for both: LC/APCI-MS, SIM: m/z 175, mobile phase conditions: 80:20 (1.0% NH_4TFA in MeOH/0.1% formic acid in H_2O) 0.8 mL/min. (a) 20 $\mu\text{g/mL}$ racemic theanine standard dissolved in 100% water and (b) 20 $\mu\text{g/mL}$ racemic theanine stock solution dissolved in borate buffer used in derivatization procedure.

Table 1. Sensitivity and detection limits of underivatized and derivatized theanine

Compound	SIM (<i>m/z</i>)	Method	Linearity	<i>r</i> ²	LOD
Theanine	175	80:20 (A:B) 0.4 ml/min ESI	$y = 34818x + 2E+07$	0.9922	10 ng/mL
AQC-theanine	345	30:70 (A:100% MeOH) 0.8 ml/min ESI	$y = 6192.3x - 3E+06$	0.9998	500 ng/mL
FMOC-theanine	397	30:70 (A:100% H ₂ O) 0.4 ml/min ESI	$y = 1256.5x + 2E+06$	0.9889	500 ng/mL
Dansyl-theanine	408	35:65 (A:100% H ₂ O) 0.4 ml/min ESI	$y = 345.03x + 242094$	0.9923	1 µg/mL

A: 1.0 % NH₄TFA in MeOH, B: 0.1 % formic acid in H₂O, LOD: limit of detection.

block at 55°C for 10 min. The solution was then diluted to 20 µg/mL.

Instrumentation

Experiments were performed using a Thermo Finnigan (San Jose, CA, USA) Surveyor LC system with a photodiode array detector (PDA) coupled to a Thermo Finnigan LCQ Advantage API ion-trap mass spectrometer with ESI and APCI ion sources. The mass spectrometer was operated in positive ion mode using single ion monitoring (SIM) detection. SIM was chosen over SRM (single reaction monitoring) as the background ('chemical noise') levels for SIM were not a limiting parameter. Nitrogen (Praxair, Danbury, CT, USA) was used as both the sheath and auxiliary gas. Ultra-high purity helium (Linweld, Lincoln, NE, USA) was used as the damping gas in the ion trap.

ESI conditions

Sheath and auxiliary gases were 50 and 40 (arbitrary units), respectively. MS parameters were optimized to the following: source voltage 4.50 V, capillary voltage 10.0 V, tube lens offset 0.0 V, and capillary temp 270°C.

APCI conditions

Sheath and auxiliary gases were 80 and 20 (arbitrary units), respectively. MS parameters were optimized to the following: APCI vaporizer temp 400.0°C, corona discharge current 5.00 µA, tube lens offset 30.0 V, and capillary temperature 200°C.

All separations were performed at room temperature on a 250 × 4.6 mm Chirobiotic T (teicoplanin) chiral column from Astec (Whippany, NJ, USA). Solvent systems used were either reversed-phase (methanol/water with additives) or polar-organic mode (methanol with additives) for the derivatized and underivatized theanine samples, respectively.⁴⁰ Mobile phase flow rates were 0.4 or 0.8 mL/min.

RESULTS AND DISCUSSION

Enantiomeric separation of underivatized and derivatized theanine standards

Racemic theanine standards were derivatized using FMOC, dansyl, and AQC reagents as per the procedures described previously. Figure 1 shows the structures and molecular masses of native theanine, FMOC-theanine, dansyl-theanine, and AQC-theanine derivatives. Using the Chirobiotic T stationary phase, underivatized theanine was separated using an APCI-MS compatible reversed-phase method (*vide infra*). A baseline separation of the enantiomers was obtained in less

than 9 min. SIM detection was used to monitor the [M+H]⁺ ion for theanine at *m/z* 175.0.

This method was also evaluated for the separation of the derivatized theanine standards. However, different mobile phase compositions were needed to achieve the optimum separation conditions for each of the derivatives. FMOC- and dansyl-theanine were best separated in the reversed-phase mode using 1.0% NH₄TFA in MeOH/100% water at 30:70 and 35:65 ratios, respectively. The elimination of 0.1% formic acid from the previous method seemed to improve the separation. SIM detection at *m/z* 397 and 408 was used for FMOC- and dansyl-theanine, respectively. AQC-theanine,

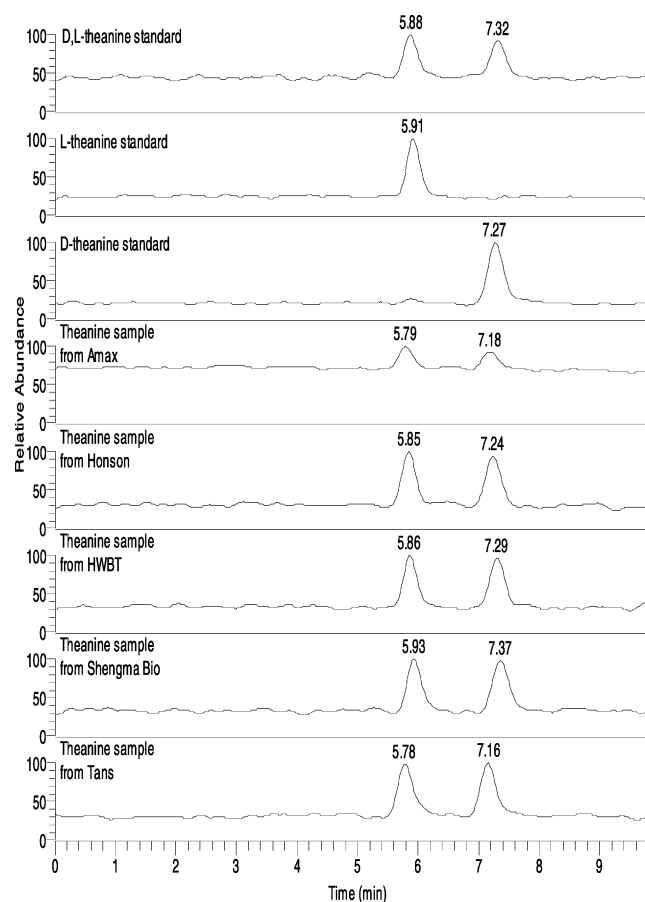


Figure 4. Enantiomeric composition of commercially available theanine samples compared with theanine standards. Retention times are slightly shifted from previously run standards due to day-to-day ambient temperature fluctuations. Separation conditions for all standards and samples: LC/APCI-MS, SIM: *m/z* 175, mobile phase conditions: 80:20 (1.0% NH₄TFA in MeOH/0.1% formic acid in H₂O) 0.8 mL/min.

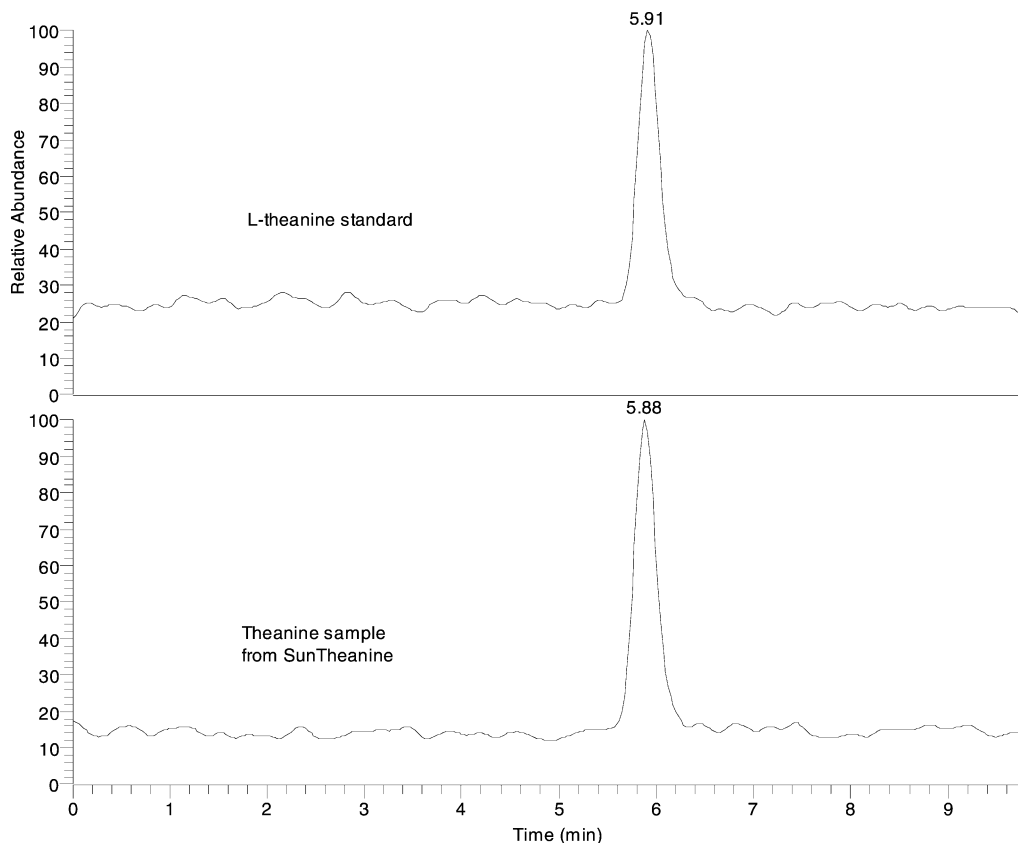


Figure 5. Enantiomeric composition of SunTheanine[®] as compared with L-theanine standard. Separation conditions for both: LC/APCI-MS, SIM: m/z 175, mobile phase conditions: 80:20 (1.0% NH_4TFA in $\text{MeOH}/0.1\%$ formic acid in H_2O) 0.8 mL/min.

however, achieved the best separation using polar-organic mode (containing no water). SIM detection of AQC-theanine at m/z 345 was used. Figure 2 shows the baseline separations for the native and derivatized theanine standards.

It was found that the APCI sensitivity of the derivatized theanine standards was lower than that for the underivatized theanine standard (data not shown). For comparison, the MS signal for the theanine standard dissolved in water was compared with the signal for the theanine stock solution used to make the derivatives (dissolved in borate buffer). Figure 3 shows the peak area counts (AA) for both enantiomers of each sample. The peak areas of the theanine stock solution are far less than those for the theanine standard dissolved in water, as are the signal-to-noise ratios. It is therefore possible that the borate buffer used in the derivatization procedure affected the sensitivity of the APCI-MS method for Fmoc-, dansyl-, and AQC-theanine. As a result, ESI was evaluated for both the derivatized and underivatized theanine standards.

Using flow rates compatible with ESI, the sensitivity and detection limits for underivatized and derivatized theanine standards were assessed for ESI-MS detection. The ESI sensitivity of the underivatized theanine was improved by lowering the flow rate from 0.8 to 0.4 mL/min, whereas the reduction of flow rate for the AQC-theanine method had no effect on the sensitivity. This observation is likely due to fact that the mobile phase of the AQC method contains no water. It is generally understood that, although water easily supports the formation of ions, its surface tension and

solvation energy make desorption more difficult. Table 1 shows the sensitivity and the detection limits for all the theanine standards. The underivatized theanine had at least an order of magnitude better sensitivity than all the theanine derivatives, with a limit of detection of 10 ng/mL. (Sensitivity as defined by IUPAC is the slope of the dose-response curve.⁵⁴) These results are consistent with those of Hemmermeister and co-workers, who found that native amino acids actually had better sensitivity than their dansyl chloride derivatized counterparts for ESI-MS detection.⁴⁸ Of the derivatives, AQC-theanine had the best sensitivity and detection limit for ESI-MS detection, once again most likely due to the use of polar-organic LC mode.

Enantiomeric composition of commercially available L-theanine samples

As the pharmacological activity of theanine continues to be of interest to researchers, a variety of companies have begun marketing theanine as a nutraceutical and/or food/beverage additive. When extracted from tea leaves, theanine is predominately found in the L-form, as are most naturally occurring amino acids.⁴ However, when synthesized, L-theanine may not be the only enantiomer formed. Using the HPLC/APCI-MS method, the enantiomeric composition of commercially available theanine samples was evaluated. All products were marketed as L-theanine. Figure 4 illustrates the results for five of the six theanine products tested. All five samples show significant amounts of D-theanine present. In fact, all

of the products appear to be racemic, within experimental error.

Figure 5 shows the results for the SunTheanine[®] product as compared with the L-theanine standard. This was the only commercially available product tested which showed no substantial amount of D-theanine. These results were confirmed using data obtained from the photodiode array detector on the LC system.

CONCLUSIONS

The HPLC/API-MS system provides excellent sensitivity and detection limits for the analysis of underivatized theanine. Using lower flow rates, the sensitivity of ESI was comparable to that of APCI at the higher flow rates for the native theanine. The sensitivity of derivatized theanine standards was improved with ESI-MS detection, but overall proved less sensitive than the underivatized theanine.

Using the high-flow APCI-MS method, the enantiomeric composition of six commercially available L-theanine products was evaluated and confirmed with PDA detection. Five of the six products appeared to be racemic. Only the SunTheanine[®] sample was the pure L-theanine enantiomer.

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