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TECHNICAL NOTE

An LC-ESI/MS method for determining theanine in green tea dietary supplements

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Abstract Theanine is the major amino acid present in *Camellia sinensis* or green tea. A method for determining theanine in its native state using liquid chromatography with positive-mode electrospray ionization mass spectrometric detection was developed. Quantitation of theanine was achieved using theanine-[$^2H_5$] as an internal standard. This approach was utilized on different green tea matrix materials that are commonly used as dietary supplements including powdered plant leaves, a powdered plant leaf extract, and an oral dosage form that contains green tea. The theanine response was linear over several orders of magnitude, and excellent measurement precision was obtained for all three materials using the developed method.

Keywords Theanine · Green tea · *Camellia sinensis* · Liquid chromatography · Mass spectrometry · Standard Reference Material

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Introduction

Dietary supplements containing green tea consist at least partially of dried leaves or extracts of the plant *Camellia sinensis*. Green tea dietary supplements have gained popularity because of the antioxidant properties of many constituents, most notably the catechins. Green tea also contains amino acids, which are responsible for the savory taste of tea infusions. Theanine (Fig. 1) is the major amino acid component in green tea, and it also has potential health benefits such as reducing anxiety and lowering blood pressure. Like most naturally occurring amino acids, L- and D-enantiomers of theanine exist. In green tea, the most predominant form is L-theanine, and limited measurements indicate that only 2% of the total theanine exists as D-theanine [1]. To date, the biological activity of D-theanine has not been investigated, and quantitative evaluations of this enantiomer are hindered by lack of a commercially available reference standard.

Chromatographic methods with diode array ultraviolet absorbance (UV) detection and fluorimetric detection are common approaches for determining theanine in green tea. While theanine has been measured in tea in its native state using both liquid chromatography (LC) [2] and capillary electrophoresis [3] with UV detection, these approaches are limited by the lack of retention and separation from matrix components and the poor absorbance of theanine, which lacks a sufficient chromophore. To address these issues, quantitation of theanine is often performed by a precolumn derivatization procedure using reagents such as *o*-phthaladelhyde (OPA) and β-mercaptoethanol [4–6]. Derivatization increases both the on-column retention and absorbance of theanine for UV detection; enhanced sensitivity and selec-

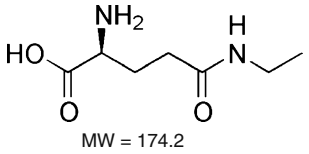

Fig. 1 Structure of theanine

tivity can be obtained when used with fluorimetric detection [5, 7]. However, the derivatization reactions of theanine are time-dependent, and the derivatives can be unstable [4]. Reliable quantitation can only be obtained when freshly prepared derivatives are analyzed and when the time from preparation of the derivative until the chromatographic analysis is well-controlled for both calibrants and samples [8]. This complicates the sample preparation, increases the experimental time, and prevents batch-processing of samples prior to analysis.

To improve the detection sensitivity and selectivity without a need for derivatization, mass spectrometry (MS) methods for determining theanine have been developed. Theanine exhibits favorable ionization behavior and has been determined in green tea using thermospray [9], atmospheric pressure chemical ionization [10], and electrospray ionization (ESI) [6, 10] mass spectrometric methods. Many of the reported MS methods are either qualitative or rely on external calibration for quantitation of theanine. These MS methods are potentially limited because external calibration might not correct for the matrix or ionization suppression effects that exist when tea extracts are analyzed. To correct for these effects, we used labeled theanine, theanine-[$^2H_5$], as an internal standard (IS) for quantitation with ESI detection. Additionally, theanine exhibits instability in aqueous solution where it slowly racemizes to the D-enantiomer, and it is subject to hydrolysis at basic pH [1]. Theanine (as well as many other components like catechins) has been frequently determined in tea by preparation of an aqueous infusion at high temperatures, simulating the widely consumed beverage. To avoid potential instability caused by using these conditions, we have utilized ultrasonic solvent extraction with a buffered solution that contains water and methanol.

The National Institute of Standards and Technology (NIST) is working in collaboration with the National Institutes of Health, Office of Dietary Supplements and the Food and Drug Administration, Center for Food Safety and Applied Nutrition, and Center for Drug Evaluation and Research to develop dietary supplement Standard Reference Materials (SRMs). NIST has developed three green tea SRMs that represent different matrices commonly used for dietary supplements: SRM 3254 *Camellia sinensis* Leaves, SRM 3255 *Camellia sinensis* Extract, and SRM 3256 Green Tea-Containing Solid Oral Dosage Form. Theanine was determined in each of these materials using the new LC-ESI/MS method.

Experimental[1]

Reagents and chemicals

L-Theanine was obtained from Sigma (St. Louis, MO, USA) and isotopically labeled L-theanine-[$^2H_5$] was synthesized by Isotec (Miamisburg, OH, USA). Monobasic and dibasic sodium phosphate were obtained from Sigma. Trifluoroacetic acid was obtained from Fluka (Buchs, Switzerland). Water and methanol were HPLC-grade. The purity of the L-theanine reference standard was determined at NIST using LC with both UV absorbance and evaporative light-scattering detection.

Green tea SRMs

The green tea SRMs were obtained from the Standard Reference Materials Program (NIST, Gaithersburg, MD). SRM 3254 *Camellia sinensis* Leaves consists of ground and sieved green tea leaves, and SRM 3255 *Camellia sinensis* Extract consists of a dried powder from a solvent extract of green tea leaves. SRM 3256, Green Tea-Containing Solid Oral Dosage Form, consists of four commercial green tea dietary supplements (tablets and caplets) that were individually ground and sieved, then blended together. The three SRMs were packaged in nitrogen-flushed 4 mil polyethylene bags, which were then placed in nitrogen-flushed aluminized plastic bags containing two silica gel packets. The individual SRM packets were kept in cardboard boxes in the packaging order (for homogeneity assessments) and were irradiated (absorbed dose 7.9 to 9.5 kGy) to prevent mold growth.

Instrumental

An Agilent Technologies (Palo Alto, CA, USA) 1100 series liquid chromatograph with an SL series mass spectrometric detector and an ESI source was used to measure theanine and theanine-[$^2H_5$]. The system also contained a variable wavelength UV detector, which was set to monitor at 280 nm but was not used for quantitation. An Advanced Chromatography Technologies ACE C18 column that was 15 cm×3.0 mm with 3 μm particles was obtained from Mac

[1] Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Mod (Chadds Ford, PA) and was used to separate theanine from the other matrix components. The mobile phase consisted of two solvents where A was 0.05% (volume fraction) trifluoroacetic acid (TFA) in water and B was methanol. Although theanine is a polar compound that elutes with 100% solvent A, gradient conditions were employed to prevent on-column accumulation of other green tea matrix components such as catechins and caffeine. A linear gradient from 100% A to 72% A was employed from the time of injection to 26 min, and then held at 72% A for 7 min before returning to 100% A. The mobile phase was delivered at a flow rate of 0.4 mL/min, and a post-run time of 10 min was incorporated into the method to allow the column to equilibrate to the initial conditions of the gradient. MS detection was achieved using ESI in positive polarity. Both theanine and theanine-[$^2H_5$] form predominantly $[M + H]^+$ ions, where M is the nominal molecular mass. Selected-ion monitoring (SIM) was used for quantitation at *m*/*z* 175 and *m*/*z* 180 for theanine and theanine-[$^2H_5$], respectively. ESI/MS detection conditions included: fragmentor, 80 V; drying gas flow, 9 L/min; drying gas temperature, 350 °C; nebulizer pressure, 0.276 MPa (40 psi); capillary voltage, 3,000 V.

Calibration

A solution of 60% 25 mmol/L aqueous phosphate buffer, pH 7 and 40% methanol (volume fractions) was used for all extractions and solution preparation. The buffer and methanol were incorporated to increase the stability of theanine [1]. A solution of theanine-[$^2H_5$] was prepared to be 232 μg/g in the above solvent and was used as the internal standard solution. Eight independent solutions of theanine were individually prepared in the solvent, four to mimic levels expected in SRM 3255 (approximately 100 μg/g) and four to mimic levels expected in SRM 3254 and SRM 3256 (approximately 35 μg/g). Eight working calibration solutions were prepared by weighing and mixing 1 mL each of a theanine solution and the internal standard solution. An additional 1 mL of solvent not containing any analytes was added to achieve 3 mL total solution to match the concentrations expected in the green tea sample extracts. All solutions were stored at 4 °C when not in use.

Sample preparation

Six sample packets for each SRM were randomly selected across the packaging (box) order, with two test portions selected from each packet for the determination of theanine (n=12 for each SRM). The following approximate test portion sizes were used for each of the SRMs: SRM 3254 and SRM 3255, 100 mg; SRM 3256, 50 mg. To prepare samples, the appropriate amount of SRM was accurately weighed into a 15-mL plastic tube with a screw cap. A weighed amount of the internal standard solution (1 mL or about 930 mg) was added to the tube, and an additional 2 mL of the extraction solvent (not containing the IS) was also added to the tube to achieve a total volume of 3 mL as used for the calibrants. The tubes with SRM 3254 and SRM 3256 samples were vortex-mixed for 10 s then ultrasonically extracted for 2 h, but samples of SRM 3255 fully dissolved in the solvent and required only brief sonication (≈5 min). Following sonication, the tubes were vortex-mixed for 10 s. The tubes were then centrifuged at 628 rad/s (6,000 rpm) for 10 min, and the supernatant was transferred to a 3-mL plastic syringe with a 25 mm, 0.45 μm pore size, cellulose acetate filter with a GD/X prefilter. The samples were filtered directly into LC vials for analysis.

Linearity of detector response

An individual stock solution of theanine was accurately prepared using the internal standard solution as a diluent to have a nominal concentration of 180 ng/μL. Three subsequent serial dilutions of the solution were prepared using the internal standard solution as a diluent to be 18, 1.8, and 0.09 ng/μL. These solutions were evaluated with the LC method, and the peak height ratio was plotted against the mass ratio of theanine (relative to the internal standard). The amount of theanine injected on-column for this analysis ranged from 0.18 to 357 ng. Regression analysis was performed to evaluate the linearity of the theanine response.

Results and discussion

For reliable determination in its native, underivatized state, theanine must be retained by the analytical column. To improve retention of amino acids on reversed-phase columns, acidic mobile phases are used to suppress ionization of the carboxyl group and protonate the amino group. Additionally, acid modifiers are used to generate ion pairs with the protonated amino group of peptides and proteins [11]. Because the acidic modifier must also be volatile when MS detection is employed, TFA and acetic acid were investigated for the analysis of theanine with the ACE C18 column. Theanine eluted near the solvent front when a mobile phase of 2% acetic acid in water, pH 2.6, was used presumably due to the formation of a hydrophilic ion pair. Theanine exhibited notable retention when a mobile phase of 0.05% TFA in water, pH 2.3, was used presumably because of the formation of a hydrophobic ion pair (observable in Fig. 2). This behavior has been reported

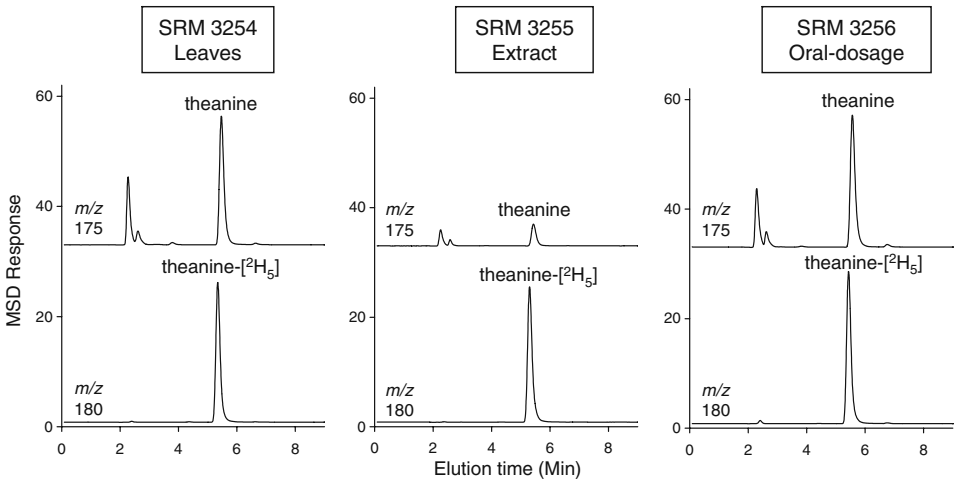

Fig. 2 LC-ESI/MS chromatograms of theanine and theanine-[$^2H_5$] in samples of SRM 3254, SRM 3255, and SRM 3256

for proteins and peptides in reversed-phase separations [11, 12]. TFA is also known to suppress the ionization of peptides during ESI/MS detection [13]. The peak area for theanine was 70% less with TFA than with acetic acid as the modifier, but TFA was selected for the method because of its favorable effect on the retention of theanine. The expected similarity in ionization behavior of theanine and theanine-[$^2H_5$] will minimize the potential effects of ion suppression on quantitation.

Representative chromatograms for samples of all green tea materials are presented in Fig. 2. The chromatograms have very similar peak profiles for all three matrices, differing only in the absolute peak intensities. The chromatograms exhibit negligible baseline noise and have only few extra peaks from matrix constituents, even though the ions being monitored are in the low *m*/*z* range. Theanine also exhibits good signal-to-noise as well as nice peak shape with negligible tailing on the ACE C18 column, which is highly base-deactivated. The chromatograms demonstrate the selectivity and sensitivity of the LC-ESI/MS method for analysis of theanine in green tea matrices. However, some separation of the theanine-[$^2H_5$] and theanine is observable in the chromatograms, and the peaks were estimated to overlap 24%. Incomplete correction for matrix effects and ionization suppression can occur when the analyte and its labeled analogue are baseline resolved [14], but the magnitude of the effect for partial separation is not as well known. To determine if there were effects on the quantitation caused by this partial separation, SRM samples were analyzed on an older ACE C18 column that provided slightly less retention and therefore better co-elution of the theanine-[$^2H_5$] and theanine, with about 55% peak overlap. Even though the samples were analyzed on different days, the average mass fraction of theanine in each SRM (for n= 12 samples) was statistically indistinguishable on both columns. These results suggest that either partial co-elution of theanine and theanine-[$^2H_5$] is sufficient to correct for matrix or ionization suppression effects or that these are not major factors for the quantitation of theanine in the three green tea matrix SRMs. The UV detector response revealed no significant matrix peaks at the elution times of theanine and theanine-[$^2H_5$] (data not shown), which provides some evidence for the latter.

The response linearity was evaluated for theanine with the new method using the calibrants described in the "linearity of detector response" portion of the "Experimental" section. The calibrants for this investigation spanned a few orders of magnitude and covered the concentration region used for the quantitation of the SRM 3254, SRM 3255, and SRM 3256 samples. The plot of peak height ratio versus nanograms theanine injected on-column was found to be linear for calibration over the range of 3.6 to 357 ng. The slope of the line was 0.0019 (±0.00001), and the intercept was not detectably different from zero, 0.001 (± 0.002). The lowest amount of theanine injected on-column, 1.8 ng, produced a detectable signal but exhibited non-linear behavior with respect to the higher levels. This prevented the determination of a formal limit of quantitation (LOQ), but the linearity investigation reveals it is ≤3.6 ng. Most importantly, calibration of theanine in the green tea SRMs was performed within the linear region of the response. Even if the LOQ is equal to 3.6 ng, this LC-

Table 1 Mass fraction of theanine in green tea dietary supplement standard reference materials

Green tea material	SRM	Average (mg/g)	SD (mg/g)	RSD (%)
Leaves	3254	2.03	0.04	2.1
Extract	3255	0.33	0.005	1.4
Oral dosage form	3256	4.16	0.11	2.6

ESI/MS method is still at least 100 times more sensitive than a recently reported method that used OPA derivatization, and has similar sensitivity to the reported phenylisothiocyanate derivatization approach [4]. Also, since theanine is abundant in green tea matrices, no attempts were made to optimize the ESI parameters to achieve the maximum signal-to-noise response. Better sensitivity could be achieved if the ionization were optimized, or if a less suppressive acidic modifier was identified that still provides retention of theanine.

For the quantitative evaluation of theanine in green tea, two test portions from each of six packets from randomly selected boxes of SRM 3254, SRM 3255, and SRM 3256 were prepared and analyzed, resulting in 12 independent measurements (n=12) of the composition of each SRM. The data from the four independently prepared calibrants for theanine were evaluated using linear regression to obtain a response factor that was used for quantitation of theanine. Duplicate LC analyses were performed for each SRM sample and each calibrant solution. The mass fractions of theanine were corrected for the purity of the reference standard and are presented in Table 1. Good repeatability was obtained for the measurements, with relative standard deviations (RSD) <3% for all SRMs. A concentration of 2.028 mg/g theanine (on average) was measured for the green tea leaves, which is consistent with a recent investigation that found a range of 2 to 5 mg/g in commercial green tea from different sources [4]. The extract material contains the lowest level of theanine (≈0.3 mg/g). The process used to prepare the extract dietary supplement material was designed to enhance the catechin content, and it is appears that theanine is lost during the extraction. The oral dosage form contains the highest level of theanine but is in the range found for commercial tea leaves [4].

Conclusions

A simple LC-MS method has been developed for the determination of theanine in green tea dietary supplement materials. Theanine was measured in its native state without derivatization, which is often required for measurement with UV or fluorimetric detection approaches. The reported LC-ESI/MS approach for the analysis of green tea materials may have applications for the determination of theanine in other dietary supplement preparations.

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