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Analysis of Theanine in Tea (Camellia sinensis) Dietary Ingredients and Supplements by High-Performance Liquid Chromatography with Post-Column Derivatization: Single Laboratory Validation

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- Submitter notes: Tables and chromatograms are grouped at the end of the document.

Analysis of Theanine in Tea (*Camellia sinensis*) Dietary Ingredients and Supplements by High-Performance Liquid Chromatography with Post-Column Derivatization: Single Laboratory Validation.

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Tea has been consumed all over the world throughout human history and its positive effects on mood, cognitive functions and overall health is well recognized. The leaves of the tea plant (*Camellia sinensis*) contain a number of biologically active compounds such as caffeine, polyphenol antioxidants and a unique non-proteinogenic amino acid, Theanine. Theanine content generally accounts for 1-4 % of dry weight of tea leaves and depends on growing conditions, tea variety, grade, and degree of fermentation (1, 2).

Studies have found that Theanine promotes relaxation and alertness, decreases anxiety, may protect from environmental neurotoxins and even enhances the activity of certain anti-tumor medications (1, 3-7). It was also noticed that many health effects of Theanine are more pronounced at higher levels of intake than made possible by drinking brewed tea alone.

Dietary supplements containing green tea have gained popularity as sources of antioxidants, weight loss agents and the means to improve energy level and alertness. Currently, the majority of supplement manufacturers list polyphenols content and the amount of green tea extract but do not specify the amount of Theanine present in the formulation. As awareness of Theanine health benefits grows consumers and manufacturers alike are looking to expand label claims to include Theanine. Since the quality of starting materials as well as manufacturing processes effect amino acid profile of tea-containing products, it is expected that the amount of Theanine varies greatly from supplement to supplement. To support label claims and ensure the integrity of the supplements market, it is important for the industry to have reliable methods for Theanine analysis in dietary ingredients and final products.

In 2015, the AOAC Stakeholder Panel on Dietary Supplements (SPDS) developed and adopted Standard Method Performance Requirements (SMPR®) for the determination of Catechins, Methyl Xanthines, Theaflavins, and Theanine in Tea (*Camellia sinensis*) Dietary Ingredients and Supplements (8). AOAC stakeholder panels composed of representatives from industry, regulatory organizations, contract laboratories, and academic institutions are tasked with determining the need for methods as well as the method evaluation parameters.

Analyzing amino acids in natural products comes with a unique set of challenges. Most amino acids, including Theanine, do not exhibit strong light absorption or fluorescence, making them difficult to detect, especially in complex plant matrices. Reported methods for analyzing Theanine in teas mostly employ chromatographic techniques like HPLC, capillary electrophoresis and micellar electrokinetic capillary chromatography (9-13). Theanine is then detected with or without derivatization using UV or fluorescence detection, amperometric detection or mass-spectrometry (9, 14-17).

Cation-exchange chromatography with post-column Ninhydrin derivatization has long been a trusted technique for amino acids analysis in foods, animal feeds, pharmaceuticals and clinical samples. A selective retention mechanism allows separating of free amino acids from other matrix components, so no extensive sample clean-up is required. And since the derivatization reaction occurs after the compounds are chromatographically separated, there are no matrix effects on reaction rate and signal intensity. This ensures that the same method and detection parameters could be used for analyzing wide variety of complex matrices.

Green tea containing supplements come in variety of forms such as tablets, liquid and dry capsules, tinctures and softgels. They often also contain other active ingredients including vitamins, minerals, oils and other plant extracts. The presented method for Theanine analysis in dietary ingredients and supplements uses simple buffer extraction followed by cation-exchange chromatography, post-column reaction with Ninhydrin reagent and UV/Vis detection. Single Laboratory Validation was completed for a wide range of tea-containing formulations and method performance characteristics were compared with requirements listed in AOAC SMPR® 2015.014.

Experimental

Scope

This method is applicable to the determination of L-Theanine in Tea (Camellia sinensis) Dietary Ingredients and Supplements in the form of powders, liquids, tablets, capsules, softgels and gelcaps.

Principle

Theanine is extracted from the samples with Lithium Citrate buffer pH 2.2 using ultrasonic water bath. L-Norleucine is used as Internal Standard. The extract is filtered and injected on a Lithium cation-exchange HPLC column and Theanine is separated from other free amino acids using Lithium citrate buffers with different pH and concentrations as mobile phases. All amino acids, including L-Theanine, react with Ninhydrin reagent in the post-column derivatization system at 130 ^{o}C and are converted to a colored derivative. Detection is performed at 570 nm using a UV/Vis detector.

Apparatus

(a) *HPLC system.* – Ternary or quaternary LC pump capable of delivering pulse-free flow of 0.1-2 mL/min. Autosampler with injection loop suitable for 10 – 50 uL injection. UV/Vis or DAD detector capable of monitoring signal at 570 nm. (Agilent Technologies 1290 or equivalent)

(b) *Post-column derivatization system.* – Single pump post-column derivatization system equipped with: pulse-free pump capable of delivering flow of 0.3 mL/min, 0.5 mL reaction coil capable of maintaining temperature of 130 +/- 0.5 ^{0}C, column oven controlling temperature between 30 to 75 ^{0}C. (Pinnacle PCX, Pickering Laboratories, Inc. or equivalent).

(c) *Post-column reagent bottles.* – 1 L safety coated glass bottles, pressure resistant up to 10 psi (Pickering Laboratories, Inc.; P/N 3107-0137 or equivalent).

(d) *HPLC columns and Guards.* – Lithium cation-exchange analytical column 4 x 100 mm (Pickering Laboratories, Inc.; P/N 0354100T). Cation-exchange GARD™ (Pickering Laboratories, Inc.; P/N 1700-3102).
(e) *Ultrasonic water bath.* – (Fisher Scientific FS30, or equivalent).
(f) *Centrifuge.* – Capable of accepting 50 mL centrifuge tubes (Thermo IEC Centra CL2, or equivalent).
(g) *Centrifuge tubes.* – Plastic, 50 mL with screw cap (Fisher Scientific).
(h) *Analytical Balance.* – With readability of 0.1 mg, maximum capacity of 120 g (Fisher Scientific Accu-124, or equivalent).
(i) *Pipets.* – Various sizes, adjustable (Eppendorf or equivalent).
(j) *Pipet tips.* – Various sizes.
(k) *Syringe filters.* – Nylon, 0.45 um, 13 mm (Whatman or equivalent).
(l) *Disposable syringes.* – Plastic 1 mL with lure connection (BD Luer-Lok™ or equivalent).

Reagents

(a) *Deionized Water.* – HPLC grade water (Millipore or equivalent).
(b) *LC mobile phases.* – Lithium citrate buffer solutions for cation-exchange separation of amino acids pH 2.8 – pH 13 (Pickering Laboratories, Inc.; P/N Li275, Li750, RG003).
(c) *Post-column derivatization reagent.* – Ninhydrin reagent for amino acids analysis (Pickering Laboratories, Inc. Trione® reagent; P/N T100C or T200).
(d) *Extraction solution.* – Lithium Citrate buffer, pH 2.2 (Pickering Laboratories, Inc.; P/N Li220).
(e) *L-Theanine Reference standard.* - L-Theanine, CAS 3081-61-6, purity ≥98 % (Sigma-Aldrich).
(f) L-Norleucine Reference Standard. – L-Norleucine, CAS 327-57-1, purity ≥98 % (Sigma-Aldrich).
(g) Standard Reference Materials. – Standard Reference Materials SRM 3254 *Camelia sinensis* (Green Tea) Leaves, SRM3255 *Camelia sinensis* (Green Tea) Extract, SRM 3256 Green Tea-Containing Solid Oral Dosage Form (NIST, MD).
(h) Tea (Camellia sinensis) Supplements. - Tea supplements used in this study were purchased from the local health stores. Content information is taken from the product label.
    1. Liquid green tea leaf extract - Prepared in water/grain alcohol USP (35%-45%), 500 mg/mL dry herb equivalent.
    2. Capsules with dry green tea extract – 500 mg of green tea extract per capsule, 50% polyphenols. Capsules also contains magnesium stearate, cellulose and silicone dioxide. Capsules are made of gelatin.
    3. Green tea extract gelcaps – Each gelcap contains 350 mg of green tea extract in glycerin. Gelcap shell is made of vegetable cellulose.
    4. Green tea softgels – Each softgel contains green tea extract, fish oil, black pepper extract, ginger extract, gelatin, soy lecithin, titanium dioxide. Softgel shell is beeswax-based.

5. Green Tea Extract tablets – Each tablet contains 500 mg of green tea extract, calcium phosphate, stearic acid, modified cellulose gum, silica.

Preparation of Standard Solutions

(a) *L-Theanine stock solution (500 ug/mL).* – Accurately weigh 50 mg of L-Theanine into a 100 mL volumetric flask. Bring to volume with *Extraction solution*. Correct final concentration for purity stated in Certificate of Analysis. Store refrigerated for up to 8 weeks.
(b) *Internal Standard (IS) stock solution (500 ug/mL).* – Accurately weigh 50 mg of L-Norleucine into a 100 mL volumetric flask. Bring to volume with *Extraction solution*. Correct final concentration for purity stated in Certificate of Analysis. Store refrigerated for up to 8 weeks.
(c) *L-Theanine intermediate stock solution (50 ug/mL).* – Pipette 2.5 mL of *L-Theanine Stock Solution* into a 25 mL volumetric flask. Bring to volume with *Extraction solution*.
(d) *Mixed working calibration solutions*. – Prepare mixed working calibration solutions by diluting stock solutions of L-Theanine and L-Norleucine with *Extraction solution* according to Table 1. Prepare all working calibration solutions on the day of analysis. Use at least 6 calibration solutions covering the range of concentrations in the samples.

Sample Preparation and Extraction

Choose sample size and volume of extraction solutions based on sample availability, sample type and expected Theanine concentration.

(a) For samples in tablet form. – Finely grind at least 20 tablets and mix the resulting sample thoroughly before taking out the test portion. Accurately weigh 0.1 g – 1 g portion into a 10 mL or 25 mL volumetric flask. To the 25 mL volumetric flask, add 500 uL *Internal Standard stock solution* and 20 mL of *Extraction solution* and mix well. To the 10 mL volumetric flask, add 200 uL Internal Standard stock solution and 8 mL of Extraction solution and mix well. Place the flask into an ultrasonic water bath for 2 hours. Take the flask out of the ultrasonic bath and allow to cool to room temperature. Bring to volume with *Extraction solution,* mix well and transfer to a 50 mL centrifuge tube. Centrifuge for 20 min at 3,800 rpm. Filter the extract through 0.45 um syringe filter into an HPLC autosampler vial to be analyzed.

(b) For samples in powder form. – Mix the sample thoroughly before taking out the test portion. Accurately weigh 0.1 g – 1 g portion into a 10 mL or 25 mL volumetric flask. To the 25 mL volumetric flask, add 500 uL *Internal Standard stock solution* and 20 mL of *Extraction solution* and mix well. To the 10 mL volumetric flask, add 200 uL *Internal Standard stock solution* and 8 mL of *Extraction solution* and mix well. Place the flask into an ultrasonic water bath for 2 hours. Take the flask out of the ultrasonic bath and allow to cool to room temperature. Bring to volume with *Extraction solution*, mix well and transfer to a 50 mL

centrifuge tube. Centrifuge for 20 min at 3,800 rpm. Filter the extract through 0.45 um syringe filter into an HPLC autosampler vial to be analyzed.

(c) *For samples in liquid form.* – Mix the sample thoroughly before taking out the test portion. Accurately weigh 0.1 g – 1 g portion into a 10 mL or 25 mL volumetric flask. To the 25 mL volumetric flask, add 500 uL *Internal Standard stock solution* and 20 mL of *Extraction solution* and mix well. To the 10 mL volumetric flask, add 200 uL *Internal Standard stock solution* and 8 mL of *Extraction solution* and mix well. Place the flask into an ultrasonic water bath for 2 hours. Take the flask out of the ultrasonic bath and allow to cool to room temperature. Bring to volume with *Extraction solution*, mix well and transfer to a 50 mL centrifuge tube. Centrifuge for 20 min at 3,800 rpm. Filter the extract through 0.45 um syringe filter into an HPLC autosampler vial to be analyzed.

(d) *For softgels, gelcaps or encapsulated dry supplements samples.* – Remove the contents of at least 15 capsules and mix the resulting sample thoroughly before taking out the test portion. Accurately weigh 0.1 g – 1 g portion into a 10 mL or 25 mL volumetric flask. To the 25 mL volumetric flask, add 500 uL *Internal Standard stock solution* and 20 mL of *Extraction solution* and mix well. To the 10 mL volumetric flask, add 200 uL *Internal Standard stock solution* and 8 mL of *Extraction solution* and mix well. Place the flask into an ultrasonic water bath for 2 hours. Take the flask out of the ultrasonic bath and allow to cool to room temperature. Bring to volume with *Extraction solution*, mix well and transfer to a 50 mL centrifuge tube. Centrifuge for 20 min at 3,800 rpm. Filter the extract through 0.45 um syringe filter into an HPLC autosampler vial to be analyzed.

(e) *For Standard Reference Materials.* – Follow NIST instructions for using the reference material. Accurately weigh 0.1 g portion into a 10 mL or 25 mL volumetric flask. To the 25 mL volumetric flask, add 500 uL *Internal Standard stock solution* and 20 mL of *Extraction solution* and mix well. To the 10 mL volumetric flask, add 200 uL *Internal Standard stock solution* and 8 mL of *Extraction solution* and mix well. Place the flask into an ultrasonic water bath for 2 hours. Take the flask out of the ultrasonic bath and allow to cool to room temperature. Bring to volume with *Extraction solution*, mix well and transfer to a 50 mL centrifuge tube. Centrifuge for 20 min at 3,800 rpm. Filter the extract through 0.45 um syringe filter into an HPLC autosampler vial to be analyzed.

Safety

Review Safety Data Sheets (SDS) for all reagents and chemicals. Follow manufacturers' manuals and instructions while running HPLC system, post-column derivatization system and other devices.

Post-column Ninhydrin reagent (Trione®) is sensitive to oxidation, so reagent bottles are pressurized with nitrogen at 5 psi. Use only safety coated bottles.

A 100 psi back-pressure regulator is installed on the detector outlet line to prevent liquid boiling in the post-column reactor.

HPLC Conditions

Connect the equipment in the following order: HPLC pump – autosampler – guard column – analytical column – post-column derivatization system – UV/Vis detector. Use a Lithium cation-exchange column and guard. Use Lithium-based buffer solutions as mobile phases. Set HPLC pump flow rate at 0.35 mL/min. Set post-column reagent pump flow rate at 0.3 mL/min. Set column oven temperature at 37 ^{0}C. Set post-column reactor temperature at 130 ^{0}C. Use HPLC pump gradient conditions listed in Table 2. Monitor UV/Vis signal at 570 nm. For detection use reference wavelength of 630 nm if available. Inject 10-50 uL.

Equilibrate the system for at least 30 minutes before starting the analysis. Make sure all temperatures and pressures are stable. Inject at least one reagent blank to equilibrate the system. Inject working calibration solutions, control samples, samples extracts and reagent blank. Run middle range calibration solution to confirm stability of the calibration curve every 10 injections.

Retention time of L-Theanine is 18.5 ± 0.5 minutes. Retention time of L-Norleucine is 35.0 ± 0.5 minutes.

System Suitability

(a) Retention times for L-Theanine in sample extracts and calibration solutions are within 0.5 minutes.
(b) Retention times for L-Norleucine in sample extracts and calibration solutions are within 0.5 minutes.
(c) Correlation Coefficient R^2 for weighted linear regression calibration curve is ≥ 0.9998.
(d) Relative error for back calculated concentration of middle range calibration standard is within ± 4%.

Calculations

Use HPLC data processing software or manual calculations.

Plot calibration standards response ratio ($Area_{L\text{-}Theanine}/Area_{IS}$) versus its corresponding concentration ratio ($Concentration_{L\text{-}Theanine}/Concentration_{IS}$). Obtain weighted linear regression calibration curve.

Calculate L-Theanine concertation (ug/mL) in sample extracts by interpolating from the calibration curve.

Calculate amount of L-Theanine in the sample by using formula:

$$CONCENTRATION\ sample(mg/g) = \frac{CONCENTRATION\ extract\left(\frac{ug}{mL}\right) * VOLUME\ extract(mL)}{MASS\ sample(g) * 1000}$$

Results and Discussion

Validation Study

Single Laboratory Validation study was conducted to compare performance characteristics of this method with AOAC SMPR 2015.014 *Standard Method Performance Requirements* for Determination of Catechins, Methyl Xanthines, Theaflavins, and Theanine in Tea (*Camellia sinensis*) Dietary Ingredients and Supplements (8).

Matrices

Eight matrices were used in validation study: five green tea-containing dietary supplements and three NIST Standard Reference Materials.

The dietary supplements included tablets, dry capsules, liquid formulation, softgels and gelcaps. According to label claims, all dietary supplements contained green tea extract. The liquid formulation contained up to 45% of alcohol; tablets and dry capsules contained calcium and magnesium salts as well as common inactive ingredients. Gelcaps contained glycerin and softgels contained fish oil, caffeine, lecithin, glycerin and several plant extracts. None of the dietary supplements had label claims regarding Theanine content.

NIST Standard Reference Materials included SRM 3254 *Camelia sinensis* (Green Tea) Leaves, SRM3255 *Camelia sinensis* (Green Tea) Extract, SRM 3256 Green Tea-Containing Solid Oral Dosage Form. Only reference (noncertified) mass fraction values for L-Theanine were available from NIST. L-Theanine reference values represented data from a single laboratory using an LC/MS method.

Selectivity

The post-column reaction with Ninhydrin reagent (Trione®) is specific for primary amino groups and allows for selective detection of amino acids in complex matrices. Lithium cation-exchange columns and lithium citrate buffers represent a chromatographic system designed for separating free amino acids. Only free amino acids and a very limited number of organic amines are retained on Lithium cation-exchange column under the analytical conditions used for analysis and so could be detected after reaction with Ninhydrin post-column reagent.

The L-Theanine peak identity was confirmed by comparing the HPLC elution profiles of L-Theanine standard solution with that of the samples using two types of cation-exchange columns and different

sets of buffers as mobile phases. The L-Theanine and L-Norleucine (Internal Standard) peaks are fully resolved from other peaks present on the chromatograms with the resolution $R_S \geq 1.5$.

Chromatograms of dietary supplements analyzed in a course of this study are shown in Fig. 1-7

Precision

Method precision was evaluated using the eight matrices discussed earlier. The chosen samples represented common forms of green tea dietary ingredients and supplements and were found to cover a wide range of L-Theanine concentrations – from 0.04 mg/g to 4 mg/g.

Each matrix was analyzed in triplicate over four days. Working calibration solutions were prepared on each day of the analysis. Repeatability precision was assessed by calculating S_r and RSD_r (%) for same day replicates measured under the same conditions. To determine Intermediate Precision the conditions of analysis were intentionally varied by performing the analysis on different days by two different analysts using different lots of reagents and different calibration curves. In addition, samples SRM3254, SRM3255 and SRM3256 were analyzed using two different HPLC systems. The Grubbs Outlier test for 95% Confidence Interval was applied to the results. No outliers were detected using the Grubbs test. Repeatability and Intermediate Precision data for L-Theanine analysis are presented in Table 3.

The SMPR 2015.014 document approved by AOAC stakeholders' panel sets repeatability and reproducibility requirements for different levels of L-Theanine in the samples with upper limits for RSD_r and RSD_R being 5%-7% and 8%-10% respectively (8). Repeatability Relative Standard Deviation (RSD_r) for this method ranged from 0.76 % to 2.95% and Intermediate Precision (RSD_{iR}) ranged from 1.81% to 5.33%, thereby meeting the method precision requirements for all the studied matrices.

Calculated HorRat values ranged between 0.32 and 0.62 and met the acceptance criteria for within-laboratory precision of 0.3-1.3 (18).

Accuracy

Accuracy was evaluated by analyzing Standard Reference Materials SRM3254, SRM3255 and SRM3256 as well as conducting spike recoveries studies for seven matrices.

Standard Reference Materials were analyzed in triplicate over four days by two different analysts using two different HPLC systems and different lots of reagents and columns. Results are presented in Table 4 and are in close agreement with Reference Values for L-Theanine obtained by NIST laboratory using an LC/MS method.

Spike recoveries studies were completed for a total of seven matrices, including SRM 3254, SRM 3255 and SRM 3256, to cover dietary ingredients such as green tea leaves and pure green tea extract in addition to different supplements formulations. Each matrix was spiked at two levels and samples were

analyzed in duplicate over three days by two different analysts using different lots of reagents. L-Theanine stock solution and L-Theanine intermediate stock solution were used to spike the samples.

Spike concentrations varied from 0.02 mg/g to 3.6 mg/g and for most matrices represented 50% and 100% overspike of the native levels. The overall mean for un-spiked samples determined in the course of the precision study was used for calculating the recoveries. Data for total and marginal recoveries are presented in Table 5 and Table 6.

Total recoveries ranged between 98.8% and 102.1% with maximum relative standard deviation of 3.3% for liquid gelcaps samples. Marginal recoveries ranged between 97.6% and 108.7% with the highest standard deviation of 6.4% again obtained for liquid gelcaps.

Relative Standard Deviations over 6% for marginal recoveries were observed for the lowest level of L-Theanine and for two samples with non-uniform distribution of material: the liquid extract formulation and liquid gelcaps. Liquid green tea extract formulation contained insoluble materials and liquid gelcaps content considerably varied in density, most likely due to partial evaporation.

These findings highlight the challenges of obtaining a uniform and representative sample for analysis of formulations containing natural products.

The SMPR 2015.014 specifies the ranges for acceptable recoveries as 80-110% for 10 to 50 ppm of L-Theanine, 90-107% for 51 to 500 ppm of L-Theanine and 95-105% for L-Theanine concentrations exceeding 501 ppm. These recoveries specifications are more restrictive than the specifications listed in AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (19).

Total recoveries for all spiked levels of L-Theanine in all the studies samples met the specifications outlined in the SMPR 2015.014.

Marginal recoveries of 108.7% obtained for 0.285 mg/g spike of Liquid green tea extract formulation exceeded Standard Method Performance Requirements of 107%. For all other spike levels in all the studied matrices, marginal recoveries met the specifications listed in SMPR 2015.14.

Linearity

Sample analysis throughout the validation study was performed using a 7-point working calibration curve covering range from 1 ug/mL to 40 ug/mL (Table1).

Additionally, the linearity of the method was evaluated by building extended calibration curves consisted of 13 mixed calibration standards ranging from 0.5 ug/mL to 100 ug/mL.

Extended calibration curves were obtained on six separate days by two different analysts using different lots of reagents. Calibration standards for each curve were prepared fresh on the day of analysis. The

Correlation Coefficient R^2 for resulted weighted linear regressions was between 0.99993 – 0.99998. The relative errors of back-calculated concentrations for standard solutions are presented in Table 7.

For all calibration curves, the back-calculated errors for standards at concentrations 1 ug/mL and above were below 5% with most calibrators falling within 2%. For the lowest calibration level of 0.5 ug/mL, back-calculated error exceeded 5%, with two out of six days coming to 6.82% and 14.9%.

Ruggedness Test

The method ruggedness was evaluated using the Youlden ruggedness trial (20). This experimental design allows for assessing the effects of changes in seven factors by performing eight experiments. Each factor can have one of two values and in each experiment the values of four factors are changed. The effect of any specific factor is evaluated by comparing the difference between the averages of two sub-sets of four experiments with $\sqrt{2}$ *SD, where SD is the standard deviation between the replicates done under the same conditions.

Dry green tea extract capsules were used for the ruggedness trial and each experiment was done in duplicate. The experimental design and the results of the ruggedness trial are presented in Tables 8 and 9.

The following seven factors were studied during this trial: different formulations of Ninhydrin post-column reagent, post-column reactor temperature, different lots of extraction solution, HPLC flow rate, sample/extraction solution ratio, extraction time and different analysts.

For five out of seven factors the differences between two subsets of four experiments were below $\sqrt{2}$ x SD, indicating that expected differences in Ninhydrin formulation, extraction solutions, extraction time, HPLC flow rate and in an analyst's way of performing the analysis do not affect the final results.

For factors such as sample/extraction solution ratio and post-column reactor temperature, the calculated differences were slightly above $\sqrt{2}$ *SD of 0.0639 coming to 0.0656 and 0.0658 respectively. Though observed differences are small, the results underline the importance of performing Theanine extraction using sufficient volume of the extraction solution and performing regular calibration of the post-column reactor temperature.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

Ten low-level L-Theanine standards (0.7 ug/mL) were prepared and analyzed as samples using 10 uL injection volume. Up to 50 uL of extract can be injected for analysis if detecting even lower levels of L-Theanine is required.

Limit of Detection (LOD) was calculated as 3*SD and Limit of Quantitation was calculated as 10*SD.

LOD=0.09 ug/mL

LOQ= 0.30 ug/mL

Limits of detection and quantitation for the samples (ug/g) were calculated for 1 g of sample extracted with 10 mL of extraction solution:

LOD=0.91 ug/g

LOQ= 3.05 ug/g

Limits of detection and quantitation met requirements outlined in SMPR 2015.14 for L-Theanine.

Conclusion

The presented method allows for the analysis of Theanine in green tea dietary supplements and ingredients. The method is based on proven methodology for detecting amino acids in native samples and is rugged, sensitive and easy to implement. The easy extraction with no additional clean-up steps is suitable for a wide array of matrices without the need for additional optimization. Results of Single Laboratory Validation show that this method meets the Standard Method Performance Requirements approved by AOAC Stakeholders Panel on Dietary Supplements (SPDS) and therefore will be well suited for laboratories tasked with testing Theanine in green tea-containing samples.

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Table 1. Preparation of mixed working calibration solutions

Volume of L-Theanine stock solution	Volume of L-Norleucine stock solution	Total volume of calibration solution	L-Theanine concentration	L-Norleucine concentration
2.00 mL	0.5 mL	25 mL	40 ug/mL	10 ug/mL
1.250 mL	0.5 mL	25 mL	25 ug/mL	10 ug/mL
0.500 mL	0.5 mL	25 mL	10 ug/mL	10 ug/mL
0.374 mL	0.5 mL	25 mL	7.48 ug/mL	10 ug/mL
0.250 mL	0.5 mL	25 mL	5 ug/mL	10 ug/mL
0.125 mL	0.5 mL	25 mL	2.5 ug/mL	10 ug/mL
0.050 mL	0.5 mL	25 mL	1 ug/mL	10 ug/mL

Table 2. HPLC pump gradient conditions

Time, min	Li275, %	Li750, %	RG003, %
0	100	0	0
12	100	0	0
45	66	34	0
45.1	0	0	100
50	0	0	100
50.1	100	0	0
62	100	0	0

Table 3. Repeatability and Intermediate Precision data for L-Theanine analysis in green tea-containing matrices.[(1)]

Sample	Concentration Level, mg/g	SD_r	RSD_r, %	SD_{iR},	RSD_{iR}, %
Liquid green tea extract formulation	0.575	0.017	2.95	0.022	3.79
Dry green tea extract capsules	3.959	0.058	1.46	0.074	1.88
Green tea liquid gelcaps	0.1897	0.0047	2.47	0.0063	3.31
Green tea softgels	0.1432	0.0042	2.92	0.0044	3.06
Green tea extract tablets	0.0410	0.00096	2.39	0.0022	5.33
SRM 3254	2.051	0.037	1.78	0.059	2.89
SRM 3255	0.3168	0.0053	1.66	0.0068	2.16
SRM 3256	3.949	0.030	0.76	0.071	1.81

[(1)]Number of replicates (3 replicates x 4 days)

Table 4. Analysis of Standard Reference Materials from NIST

Standard Reference Material	Description	Results	RSD_{IR}, % n=12	L-Theanine Mass Fraction Reference Value[(1)]
SRM 3254	*Camelia sinensis* (Green Tea) Leaves	2.051 mg/g	2.89	2.130 ± 0.054 mg/g
SRM 3255	*Camelia sinensis* (Green Tea) Extract	0.3168 mg/g	2.16	0.340 ± 0.008 mg/g
SRM 3256	Green Tea-Containing Solid Oral Dosage Form	3.949 mg/g	1.81	3.7 ± 1.2 mg/g

[(1)]Only reference mass fraction values are available from NIST for L-Theanine. Reference values are noncertified values that are the best estimate of the true values based on available data. Reference values for L-Theanine represent data from a single laboratory using a LC/MS method.

Table 5. Total Recoveries for L-Theanine

Sample	Level in the sample, mg/g	Spike Level 1, mg/g	Rec. %	RSD,%	Spike Level 2, mg/g	Rec., %	RSD, %
Liquid green tea extract formulation	0.575	0.285	102.8	2.1	0.570	100.7	1.8
Green tea liquid gelcaps	0.1897	0.1025	101.9	1.4	0.2050	101.8	3.3
Green tea softgels	0.1432	0.707	99.6	1.3	1.414	99.5	1.3
Green tea extract tablets	0.0410	0.0200	100.1	0.7	0.0401	100.3	3.0
SRM 3254	2.051	1.002	100.3	1.2	2.004	98.8	1.5
SRM 3255	0.3176	0.1515	99.5	1.8	0.3030	101.1	1.8
SRM 3256	3.949	1.804	100.7	0.9	3.607	102.1	1.0

Table 6. Marginal Recoveries for L-Theanine

Sample	Level in the sample, mg/g	Spike Level 1, mg/g	Rec. %	RSD,%	Spike Level 2, mg/g	Rec. %	RSD, %
Liquid green tea extract formulation	0.575	0.285	108.7	6.2	0.570	101.4	3.6
Green tea liquid gelcaps	0.1897	0.1025	105.7	4.2	0.2050	103.6	6.4
Green tea softgels	0.1432	0.707	99.5	1.6	1.414	99.4	1.4
Green tea extract tablets	0.0410	0.0200	100.2	2.3	0.0401	100.7	6.1
SRM 3254	2.051	1.002	101.0	3.6	2.004	97.6	3.0
SRM 3255	0.3176	0.1515	98.4	5.5	0.3030	102.2	3.6
SRM 3256	3.949	1.804	102.1	2.9	3.607	104.4	2.0

Table 7. Relative errors for back-calculated concentrations for calibration standards

Std	L-Theanine concentration	L-Norleucine concentration	Relative back-calculated errors, %					
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	100 ug/mL	10 ug/mL	0.37	0.51	-0.31	0.17	0.69	0.29
2	80 ug/mL	10 ug/mL	0.02	1.21	0.28	-0.11	-0.05	-0.88
3	60 ug/mL	10 ug/mL	0.09	0.26	0.22	0.82	-0.06	0.36
4	50 ug/mL	10 ug/mL	0.14	-0.41	0.88	0.01	-0.55	1.08
5	40 ug/mL	10 ug/mL	-0.78	-1.01	-0.14	-0.73	0.02	0.24
6	25 ug/mL	10 ug/mL	-0.67	-0.73	-0.49	-0.49	0.14	-0.49
7	20 ug/mL	10 ug/mL	-1.35	-2.13	-0.63	0.16	-0.71	-1.45
8	10 ug/mL	10 ug/mL	1.35	-1.99	0.68	-0.69	0.02	-0.25
9	7.48 ug/mL	10 ug/mL	1.05	-1.91	-1.03	-1.36	0.01	-2.03
10	5 ug/mL	10 ug/mL	-0.02	-3.33	-1.65	-0.30	-0.48	1.69
11	2 ug/mL	10 ug/mL	3.74	3.49	-3.55	-1.75	0.82	2.13
12	1 ug/mL	10 ug/mL	4.19	-0.79	1.09	-0.17	-0.33	4.24
13	0.5 ug/mL	10 ug/mL	1.70	6.82	14.9	4.43	0.88	-4.95

Table 8. Ruggedness Trial Experimental Design

Factor	Value 1	Value 2
Formulation of Ninhydrin Reagent	T100, 1-part Ninhydrin Reagent (A)	T200, 2-part Ninhydrin Reagent (a)
HPLC Flow Rate	0.35 mL/min (B)	0.38 mL/min (b)
Extraction Volume	25 mL (C)	10 mL (c)
Analyst	Analyst 1 (D)	Analyst 2 (d)
Extraction time	2h (E)	1.5 h (e)
Extraction Solution Li220	Lot 1 (F)	Lot 2 (f)
Reactor Temperature	130 ^{o}C (G)	125 ^{o}C (g)

Experiment Number	Combination of Factors
1	ABCDEFG
2	ABcDefg
3	AbCdEfg
4	AbcdeFG
5	aBCdeFG
6	aBcdEfG
7	abCDefG
8	abcDEFg

Table 9. Results of the Ruggedness Trial

The Effect of Changing Factors Calculated as Described (20) $\sqrt{2}$ ***SD = 0.0639**	
A-a	0.0467 < $\sqrt{2}$ *SD
B-b	0.0002 < √2 *SD

C-c	-0.0656 > √2 *SD
D-d	0.0431 < $\sqrt{2}$ *SD
E-e	0.0156 < $\sqrt{2}$ *SD
F-f	0.0078 < $\sqrt{2}$ *SD
G-g	-0.0658 > $\sqrt{2}$ *SD

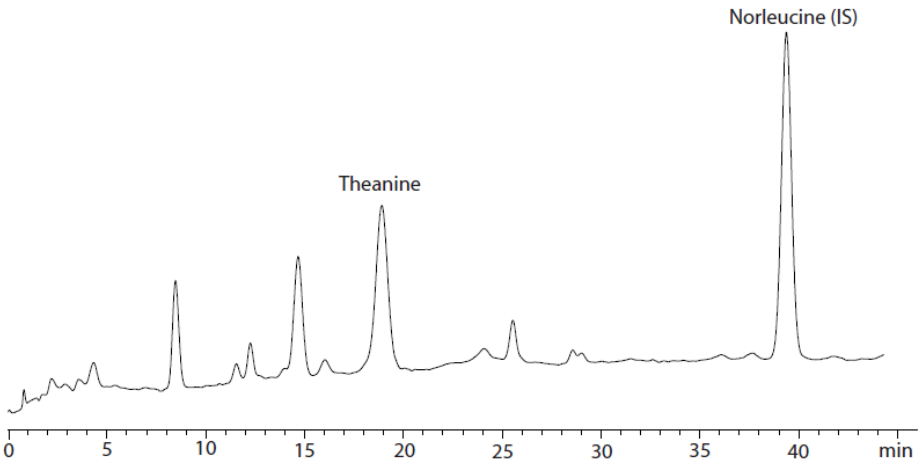

Figure 1. Chromatogram of SRM3254 *Camelia sinensis* (green tea) leaves

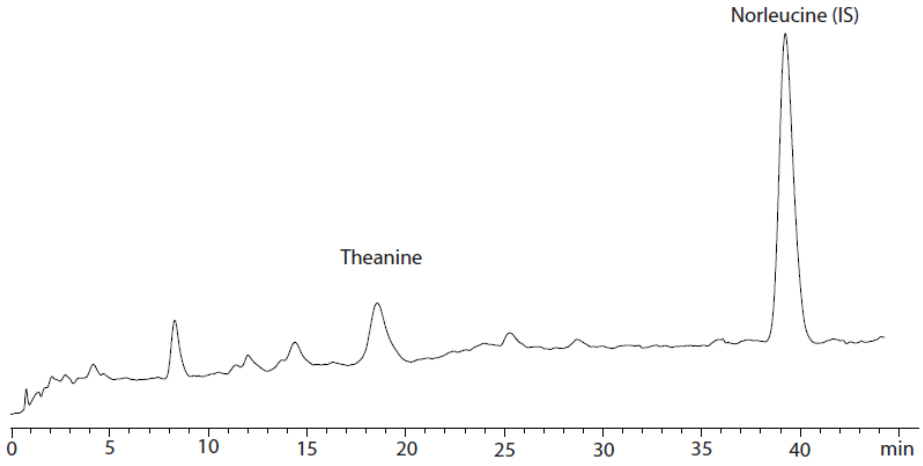

Figure 2. Chromatogram of SRM3255 *Camelia sinensis* (green tea) extract

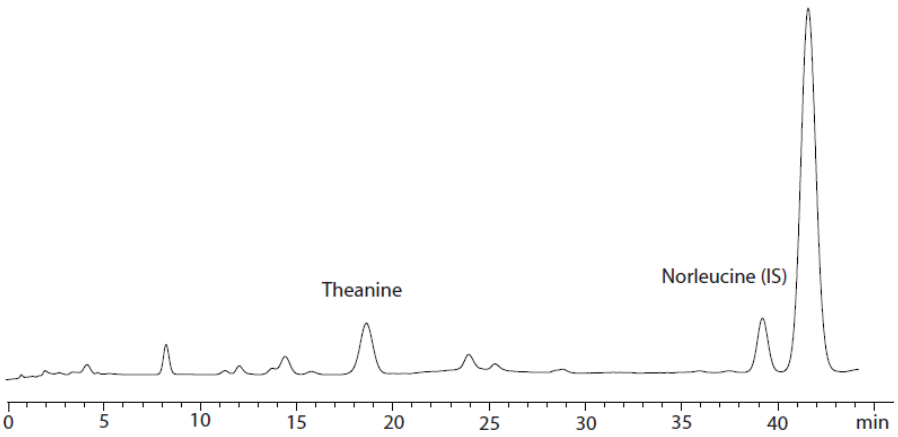

Figure 3. Chromatogram of SRM3256 green tea-containing solid oral dosage form

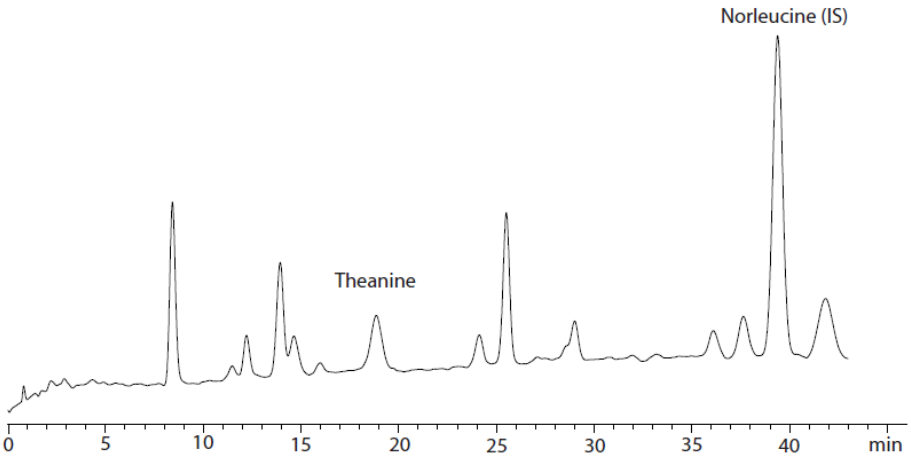

Figure 4. Chromatogram of green tea softgels

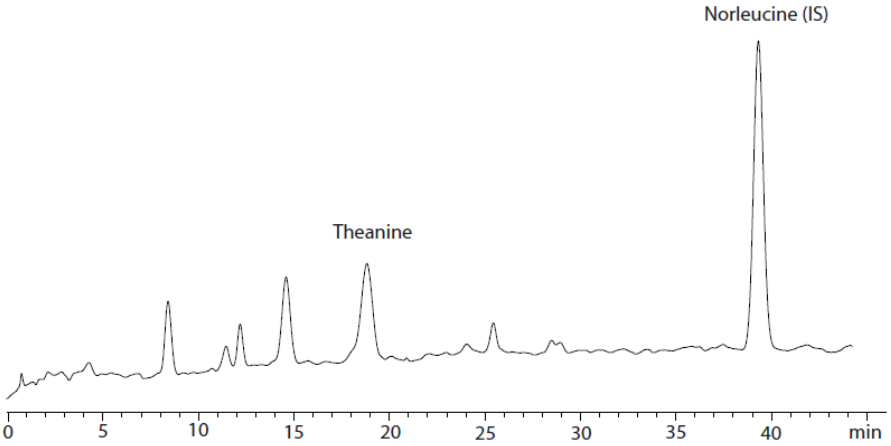

Figure 5. Chromatogram of liquid green tea leaf extract

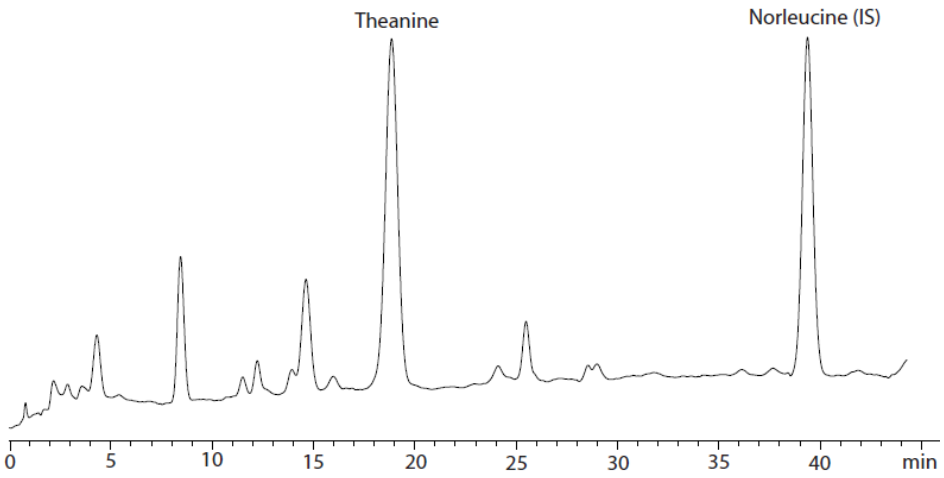

Figure 6. Chromatogram of dry green tea extract capsules

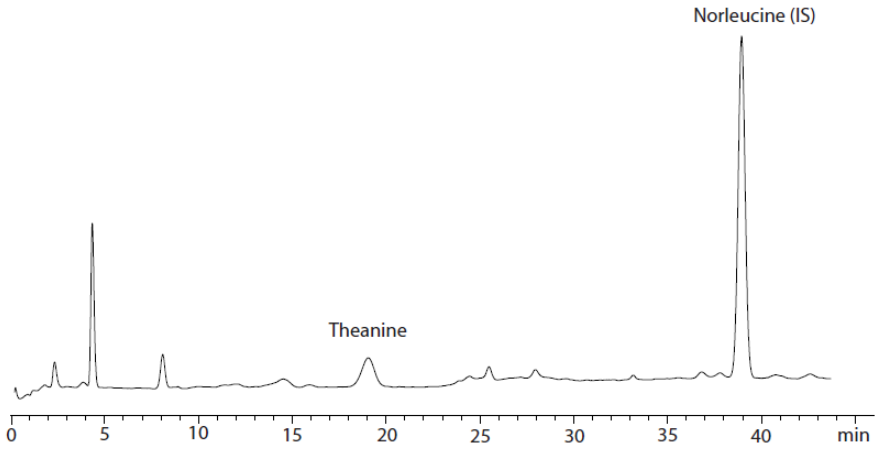

Figure 7. Chromatogram of green tea extract tablets