Validation of a Densitometric Method for the Determination of Theanine in Tea Extracts Using CP Atlas Software

Dezső Csupor*, Klára Boros, Attila Hunyadi, Katalin Veres, and **Judit Hohmann**

Key Words

***Camellia sinensis* L.**
Theanine
Densitometry
Validation
TLC
CP Atlas

Summary

L-Theanine is a non-protein amino acid that occurs in the leaves of the tea plant *(Camellia sinensis)* and possesses several pharmacologic effects, and therefore it is widely applied in the food industry. Considering the chemical characteristics of the molecule (high polarity, lack of chromophore group), conventional HPLC-based methods are not optimal for the quantification of the compound. However, for TLC chromatographic separation of theanine in tea extracts, there are reliable methods available and TLC analysis allows derivatization for better detection of the compound. Here we report for the first time the development and validation of an eligible densitometric method based on the analysis of digital photographs of TLC plates without the need of densitometer and using a software available free of charge for the quick and reliable determination of theanine in tea extracts.

1 Introduction

L-Theanine (γ-glutamylethylamide, **Figure 1**), a unique amino acid was first discovered in tea leaves in 1949 and has been detected in only three species of the *Camellia* genus (*C. sinensis, C. japonica,* and C. *sasanquaand*) and in one mushroom *(Xerocomus badius).* The only remarkable dietary source of this compound is the tea. According to the literature data, the dried leaves of tea shrub (*Camellia sinensis* L.) contain 1-2% theanine as free amino acid [1].

In the literature, the extent of fermentation has been found to be determinant of the concentration of L-theanine, with more theanine contained in unfermented green teas and less in fermented black teas [2]. 3-4 cups of green tea are expected to contain 60–160 mg of theanine. L-theanine has been linked to the feelings of relaxation reported by those who drink green tea. Similar effects have been observed after the consumption of 100–200 mg theanine by healthy humans. Experimental studies have also shown that L-theanine appears to negate some of the effects of caffeine. L-Theanine facilitates the generation of alpha waves in the brain which are believed to be associated with a relaxed yet alert mental state. This effect has been confirmed in clinical studies as well [3]. Additionally, this compound is also linked with further health benefits including the prevention of certain cancers and cardiovascular disease, the promotion of weight loss and enhanced performance of the immune system. Thus, there has been a significant rise in the demand for theanine [4]. Several food products contain theanine deriving from natural source or chemically or biologically synthesized.

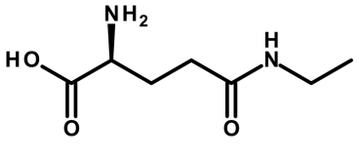

Figure 1

The chemical formula of L-theanine.

Despite the very intensive investigation of the pharmacological characteristics of theanine and the widespread food industrial application of the compound, only a limited number of scientific articles have been published on the quantitative analysis of theanine. The most frequently applied methods are RP-HPLC, TLC and capillary electrophoresis. The main disadvantage of the methods based on HPLC–UV or DAD analysis [5, 6] clearly comes from detection problems and consequential limitations for quantitative determination. UV detection and the identification by PDA is biased by the low UV absorption and uncharacteristic UV spectrum of the compound. Moreover, due to its chemical characteristics, theanine is eluted on the reverse phase with low retention times even with solvents containing very high ratios of water. The chromatographic separation and/or detection of theanine can be improved after derivatization with *o*-phtalaldehyde [7, 9], dimethylaminoazobenzene sulfonyl chloride [10]. Fluorescence detection provides a higher sensitivity [7, 9, 11]. Separation and quantitation of native and derivatized theanine enantiomers can be carried out with HPLC–APCI-MS using a chiral stationary phase [12]. The combination of capillary electrophoresis and light-emitting diode-induced

D. Csupor, K. Boros, A. Hunyadi, K. Veres and J. Hohmann, University of Szeged, Faculty of Pharmacy, Department of Pharmacognosy, Eötvös u. 6, Szeged, Hungary.
E-mail: csupor.dezso@pharmacognosy.hu

fluorescence detection after derivatization with naphthalene-2,3-dicarboxaldehyde has been demonstrated to be a reliable method for the quantitative analysis of amino acids in tea extract [13].

Our experiments aimed at the development of a simple and reliable densitometric method for the quantification of theanine in tea extracts. Interestingly, there are only few articles available on the TLC densitometric quantification of theanine [14, 15]. The chromatographic separation method applied by us was based on a HPTLC method from the literature [14]. For quantitative analysis, digital photographs of plates were analyzed with a freely available software. This method allows the quick and reliable determination of theanine in tea extracts without the need of sophisticated instrumentation.

2 Experimental

2.1 Chemicals, Standard, and Sample Solutions

L-Theanine (T6576, >99%) was purchased from Sigma-Aldrich (Steinheim, Germany), green tea powdered extract (285138) was obtained from Martin Bauer Group (Vestenbergsgreuth, Germany). All solvents used for extraction and chromatography were of analytical grade from Merck. Ninhydrin reagent was prepared by dissolving 2 g of the compound in 1 L of methanol.

Stock standard solution (1.4 mg mL^{-1}) of theanine was prepared in methanol and stored at 4°C. 0.5–2.5 mL of the stock standard solution were transferred to 10.0 mL volumetric flasks and diluted with methanol to obtain final concentrations of theanine of 0.14–1.4 mg mL^{-1}, respectively. Sample solution was prepared by dissolving green tea extract in methanol in a concentration of 3.9 mg mL^{-1}.

2.2 Chromatographic Analysis

TLC was performed on 20 cm × 20 cm glass-backed plates coated with 0.25 mm layers of silica gel $60F_{254}$ (Merck, Darmstadt, Germany). 10 µL of sample and standard solutions were applied to the layers by hand as 6 mm-diameter spots, 15 mm from the bottom of the plate, by use of a TLC syringe (Hamilton, Bonaduz, Switzerland). Calibration was carried out on each plate in the course of quantification of theanine in the sample solution. The plates were developed in *n*-butanol–aceton–acetic acid–water (7:7:2:4, *v/v*) as mobile phase in a horizontal chamber previously saturated with mobile phase vapor for 15 min. Plates were developed to a distance of 17 cm, dried at room temperature and dipped into ninhydrin reagent for 3 s. After heating at 105°C for 5–15 min, the plates were photographed using a Fujifilm digital camera (Finepix J110w, Fujifilm, Japan) in automatic mode. The green channels of the photographs were analyzed by CP Atlas 2.0 software (available free of charge at www.lazarsoftware.com, developed by István Lázár, Debrecen, Hungary) using the *dark on light* option.

2.3 Validation of the Method

2.3.1 Linearity

Standard solutions of theanine (10 µL, equivalent to 1.40–14.0 µg per spot) were applied to assess linearity in the analyzed concentration range. The equation of the calibration curve, and the correlation coefficient was calculated by plotting peak area under curve (AUC) values against amounts of theanine.

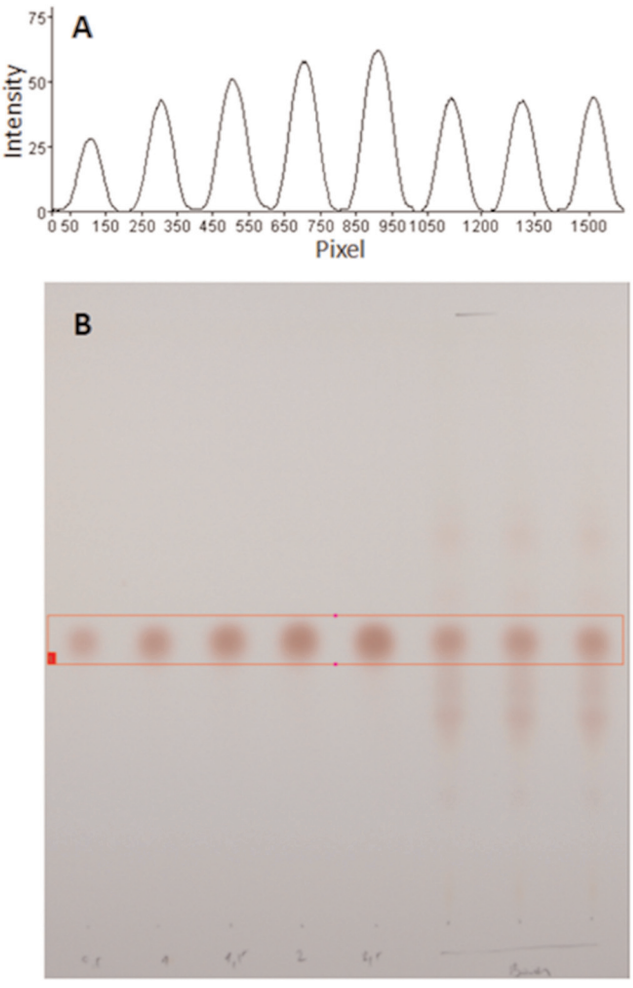

Figure 2

TLC chromatogram (A) and densitogram (B) of the lane of theanine in the tea extract (spots 6–8) and the standard solutions (spots 1–5) applied for quantification.

2.3.2 Accuracy

The accuracy of the method was assessed by recovery studies using the standard addition method, by determination of recovery at two levels, after addition of 50% and 150% theanine to the sample.

2.3.3 Precision

Intermediate precision was assessed by establishing the effects of random events on the precision of the analytical method. Precision was studied by analyzing three spots of sample solution at three concentration levels (intra-day precision) and by analyzing three spots of sample solution per plate on three consecutive days (inter-day precision) and calculating RSD%. Intermediate precision was also assessed by experiments performed by three different analysts (standard solution, one concentration level). Instrument precision was checked by photographing theanine spots from standard solutions at three concentration levels three times and calculating RSD%.

Table 1

Effects of photographic conditions on the quantification of theanine in sample solutions.

Working distance (cm)	Resolution (megapixels)	Zoom	Relative quantity of theanine
25	10	–	100%
50	10	–	96.15%
50	10	2×	99.68%
25	5	–	94.54%
25	2	–	91.81%

Table 2

Method validation data for quantification of theanine by densitometry.

Recovery [%]	
– addition of 50% theanine	95.71
– addition of 150% theanine	102.5
Inter-day precision [RSD%]	0.18
Intra-day precision [RSD%]	
– 2.8 µg theanine/spot	0.42
– 5.6 µg theanine/spot	0.44
– 11.2 µg theanine/spot	0.68
Precision by three different analysts [RSD%]	0.43
Instrument precision [RSD%]	
– 1.4 µg theanine/spot	2.89
– 4.2 µg theanine/spot	2.61
– 7.0 µg theanine/spot	3.13
Repeatability [RSD%]	0.42
Linear range	1.4–14 µg per spot
Typical linear regression equation	$y = 391.88x + 1170.8$
Correlation coefficient	>0.96

Repeatability was tested by analyzing the theanine spot after application and chromatography of a standard solution and calculating RSD%.

2.3.4 Robustness

To assess the robustness of the method, the effects of duration of heating and photographic conditions (resolution, distance, zoom) were studied. Plates containing spots ($n = 3$) of standard solution and spots of sample solutions for quantification were heated for 5, 10, and 15 min at 105°C, respectively. Plates with the same spots were heated for 5 min, and photographs were taken in an artificially lit dark chamber from different distances (25 and 50 cm), with different resolutions (2, 5, and 10 megapixels), without or with 2× zoom.

3 Results

The chromatographic method applied by us provided good separation of theanine from other components of the tea extract.

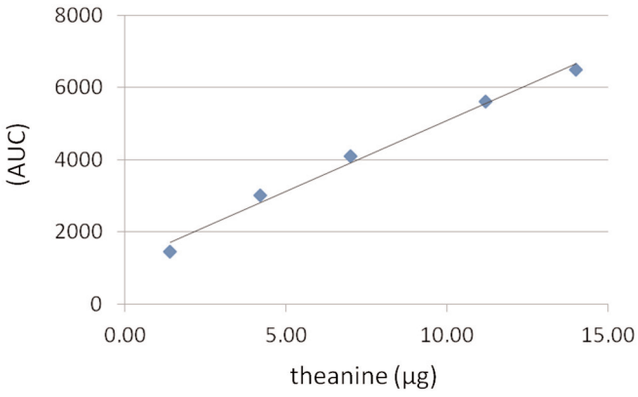

Figure 3

Typical calibration curve for theanine ($y = 391.88x + 1170.8$; $R^2 = 0.989$).

After derivatization with ninhydrin and heating, theanine was detected as reddish spots with $R_F = 0.35$. Neutral compounds of the extract and other unidentified amino acids (which were present in smaller quantities) were separated sufficiently to provide reliable quantification of theanine **(Figure 2).**

The CP Atlas 2.0 software allows the examination of different channels (red, green, blue, greyscale) of color photographs. Our experiments revealed, that in case of theanine, the analysis of the green channels provides maximal detection (highest AUC value) compared to the blue (94.14%), greyscale (74.80%), and red (30.92%) channels. Therefore, for further analysis, the green channel was selected.

Since our method is based on the analysis of digital photographs, the effect of photographic conditions on the results had to be taken into account. Therefore, we studied how the working distance, the resolution of the picture, and the application of zoom influences the quantitative results in the course of quantification of theanine in sample solutions. These experiments revealed that the resolution of the photograph has a major influence; however, working distance and the application of zoom have lesser impact on the AUC values **(Table 1).** For further analysis, photographs were taken from 25 cm distance without zoom with 10 megapixel resolution.

The validation procedure confirmed the reliability of the method **(Table 2).** A linear regression coefficient $R^2 > 0.96$ **(Figure 3)** supported the linearity in the analyzed concentration range. High recovery, high precision, and repeatability indicate the reliability and reproducibility of the method. The RSD% values of the instrument precision reflect that lighting conditions (even the flashing of the neon light) strongly influence the AUC values and stress the necessity to carry out calibration on each plate.

4 Conclusion

The method described here for the determination of theanine is simple, precise, and accurate and can be used for quantification of the compound in tea extracts. TLC combined with densitometry can be regarded as viable alternative to more sophisticated analytical methods. The method developed and validated by us offers the possibility for analysis of theanine without the need of expensive instrumentation.

Acknowledgments

This work was supported by the New Hungary Development Plan projects TÁMOP-4.2.1/B-09/1/KONV-2010-0005 and TÁMOP-4.2.2/B-10/1-2010-0012. The authors are grateful to Attila Horváth (Avidin Ltd.) for his help in the determination of precision by different analysts.

References

[1] *L. Juneja, D. Chu, T. Okubo, Y. Nagato, H. Yokogoshi*, Trends Food Sci. Technol. **10** (1999) 199–204.

[2] *E.K. Keenan, M.D.A. Finnie, P.S. Jones, P.J. Rogers, C.M. Priestley*, Food Chem. **125** (2011) 588–594.

[3] *R. Cooper, D. Morre, D. Morre*, J. Altern. Complement. Med. **11** (2005) 521–528.

[4] *Q.V. Vuong, M.C. Bowyer, P.D. Roach*, J. Sci. Food Agric. **91** (2011) 1931–1939.

[5] *L. Peng, X. Song, X. Shi, J. Li, C. Ye*, J. Food Compost. Anal. **21** (2008) 559–563.

[6] *Y. Wang, X. Yang, K. Li, C. Li, L. Li, J. Li, H. Huang, Y. He, C. Ye, X. Song*, Int. J. Food Sci. Technol. **45** (2010) 1263–1269.

[7] *A. Alcazar, O. Ballesteros, J.M. Jurado, F. Pablos, M.J. Martin, J.L. Vilches, A. Navalon*, J. Agric. Food Chem. **55** (2007) 5960–5965.

[8] *L. Wang, R. Xu, B. Hu, W. Li, Y. Sun, Y. Tu, X. Zeng*, Food Chem. **123** (2010) 1259–1266.

[9] *Y. Ying, J. Ho, Z. Chen, J. Wang*, J. Liq. Chromatogr. Rel. Technol. **28** (2005) 727–737.

[10] *K. Syu, C. Lin, H. Huang, J. Lin*, J. Agric. Food Chem. **56** (2008) 7637–7643.

[11] *F. Tan, C. Tan, A. Zhao, M. Li*, J. Agric. Food Chem. **59** (2011) 10839–10847.

[12] *M. Desai, D. Armstrong-K*, Rapid Commun. Mass Spectrom. **18** (2004) 251–256.

[13] *M. Hsieh, S. Chen*, Talanta **73** (2007) 326–331.

[14] *E. Reich, A. Schibli, V. Widmer, R. Jorns, E. Wolfram, A. DeBatt*, J. Liq. Chromatogr. Rel. Technol. **29** (2006) 2141–2151.

[15] *W. Feldheim, P. Yongvanit, P.H. Cummings*, J. Sci. Food Agric. **37** (1986) 527–534.

Ms received: January 5, 2012
Accepted: July 16, 2012