Central European Journal
of Chemistry
DOI: 10.2478/s11532-006-0014-4
Research article
CEJC 4(2) 2006 216–222

Isotachophoretic determination of theanine

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Received 16 January 2006; accepted 4 March 2006

Abstract: Free amino acids, the key quality components of tea, are contained in higher amounts in higher grade teas. Among free amino acids in tea, the content of theanine, which is the amino acid of the highest amount, shows high correlation to the price of green tea. A capillary isotachophoresis (CITP) method was developed for the analysis of L-theanine in tea and food supplements. The optimized electrolyte system was following: 0.01 mol/l HCl + 0.02 mol/l TRIS + 0.05 % HEC (leading electrolyte), 0.01 mol/ L-valine + barium hydroxide to pH 10 (terminating electrolyte). Good separation of L-theanine from other components of sample was achieved within 20 min. Method characteristics, *i.e.*, linearity (0 – 200 mg/l), accuracy (99 ± 2 %), intra-assay (1.5 %), quantification limit (2 mg/l), and detection limit (0.7 mg/l) were determined. Sufficient sensitivity, low labouriousness (extraction only) and low running cost are important attributes of this method. It was proved that the developed method is suitable for the routine analysis of L-theanine in green tea and food supplements containing green tea extract.
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Keywords: Green tea, theanine, capillary isotachophoresis, food supplements

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Abbreviations

CITP – capillary isotachophoresis,
TRIS – $1,1,1-$tris(hydroxymethyl)-aminomethane,
HEC – hydroxyethylcellulose,
FEP – fluorinated ethylene-propylene copolymer.

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1 Introduction

L-theanine is a derivative of L-glutamic acid and is also known as gamma-ethylamino-L-glutamic acid, gamma-glutamylethylamide, r-glutamylethylamide, L-glutamic acid gamma-ethylamide and N-ethyl-L-glutamine. Theanine is a non-protein amino acid mainly found naturally in the green tea plant *(Camellia sinensis).* It is the predominant amino acid in green tea and makes up to 60 % of the total free amino acids in the plant. The amino acids constitute between 1 % and 2 % of the dry weight of green tea leaves [1, 2]. The occurrence of L-theanine in tea leaves was discovered in 1949 [3]. Its chemical structure is shown in Fig. 1.

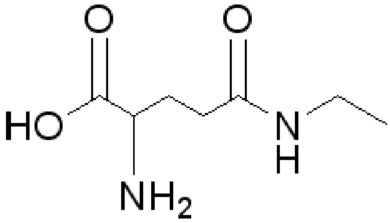


Fig. 1 Structural formula of L-theanine; (4-carbamoyl-2-ethylamino-butyric acid, $C_7H_{14}N_2O_3$, MW 174.2; water soluble white powder).

The studies published in recent years have shown that L-theanine has an important effect in physiology, biochemistry, pharmacology and health care [4, 5]. L-theanine is marketed in Japan as a nutritional supplement for mood modulation. It is considered the main component responsible for the taste of green tea, which in Japanese is called umami. It has been shown that theanine not only gives flavor and taste to green tea but also produces a noticeable relaxation effect in human beings [6].

Analytical methods based on HPLC with pre- or post-column derivatization have been developed to measure free amino acid in green tea [7]. Low stability of amino acid derivatives, high cost of maintenance of post-column derivatization system and reagent interference in pre-column derivatization are typical shortcomings of these methods. Ding *et.al.* [8] published a method based on anion-exchange chromatography with direct pulse amperometric detection of theanine and they succesfully applied it for the determination of L-theanine in green tea. Analysis of theanine was reported by means of capillary electrophoresis and micellar electrokinetic capillary chromatography using UV absorbance detection [9, 10]. Kato *et.al.* developed electrophoresis on a chip with fluorimetric detection for the analysis of amino acids in green tea [11]. L-theanine is derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole prior to electrophoresis. Under optimized conditions the main amino acids of tea including theanine were well separated and detected within 2 min.

The aim of our work was to develop an optimized method for the determination of L-theanine in green tea and selected food supplements. The method is based on isotachophoretic separation of L-theanine in anionic mode with direct conductivity detection.

2 Experimental

2.1 Chemicals and apparatus

Standard of L-theanine (99+%, batch. No. 128H0784), hydrochloric acid (1 N solution), TRIS (99+%), and HEC were purchased from Sigma-Aldrich, Ltd. (Prague, Czech Republic). Barium hydroxide octahydrate (p. a.) was obtained from Lachema (Brno, Czech Republic) and DL-valine (p. a.) was purchased from Reanal (Budapest, Hungary). Deionized water was used for electrolyte and standard solutions preparation. Samples of green tea were bought at local market, food supplement "L-theanine" was purchased from Brainway Inc. (Prague, Czech Republic) and other food supplements were donated from Dr. M. Staněk (Dr. Stanek Ltd., Prague, Czech Republic).

Samples were extracted in ultrasonic bath UC 003 PS1 (power input 300 W, Tesla, Czechoslovakia).

CITP analyses were performed using electrophoretic analyzer EA 101 (Labeco-Villa, Ltd., Slovak Republic) with column coupling (pre-separation FEP capillary 90×0.8 mm; and analytical FEP capillary 90×0.3 mm) equipped with a conductivity detector. The isotachopherograms were evaluated by the help of PC software package (KasComp Ltd., Slovakia) supplied with analyzer. The samples and/or standard solutions were injected by a valve with fixed internal sample loop (30 μl).

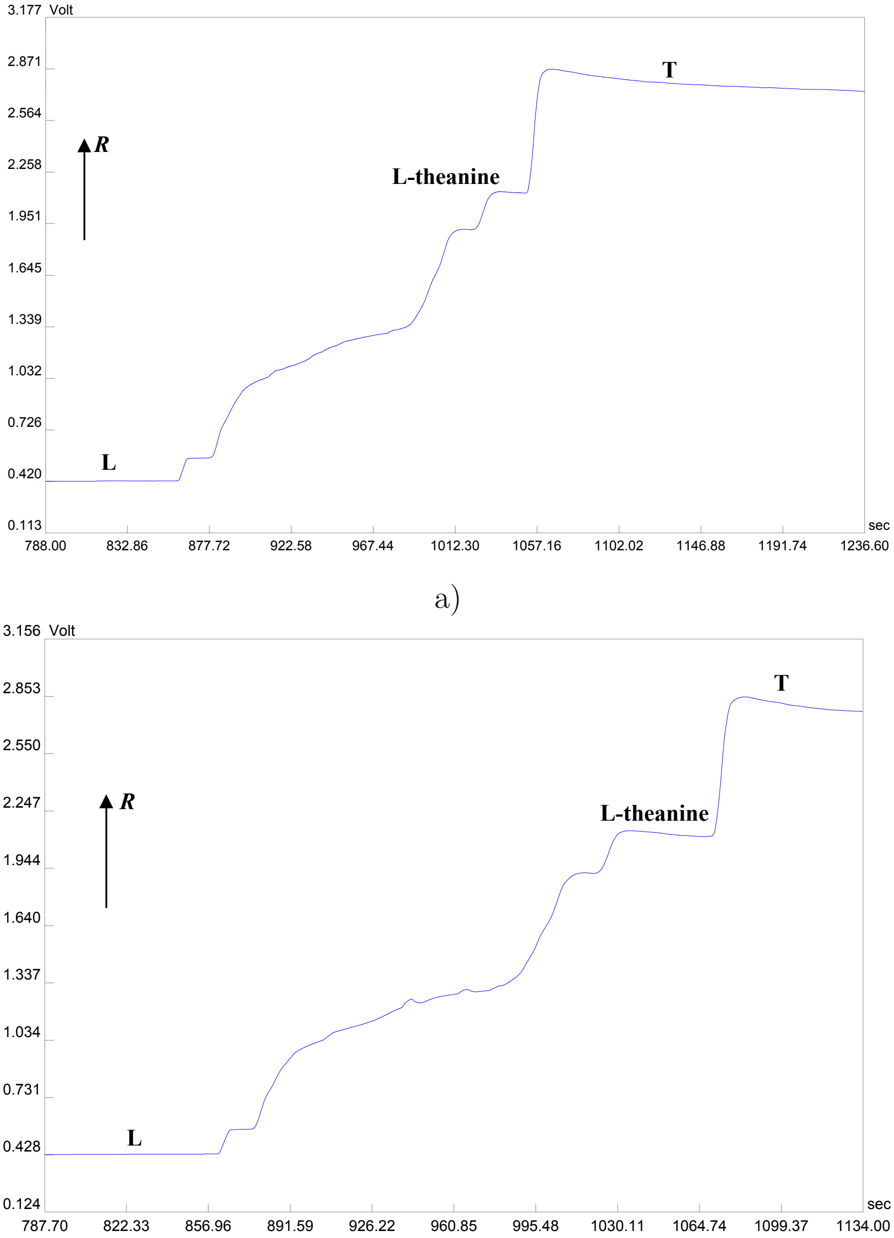
2.2 Condition of analysis, calibration and sample treatment

L-theanine was determined as anion under following conditions. Leading and terminating electrolytes consisted of 0.01 mol/l HCl + 0.02 mM TRIS + 0.05% HEC, pH 8.1, and 0.01 mM DL-valine + barium hydroxide to pH 10, respectively. Driving current applied in pre-separation capillary was 200 μA and in analytical capillary 40 μA that was decreased to 20 μA prior to detection. Total analysis time was 20 minutes. The external standard technique was used. A standard solution of L-theanine in water was injected (in duplicate) at six levels (from 2 up to 200 mg/l) by the use of a sample valve with fixed internal loop (volume 30 μl). The standard solutions were prepared from the stock solution of L-theanine (1 g/l) in demineralized water.

Samples (1–5 g) were placed into 500ml beaker, to which 250 ml of demineralized water was added and boiled for 10 minutes. After cooling to room temperature, samples were made up to 1000 ml with demineralized water. Filtrate (through filter paper) was injected into electrophoretic analyser.

3 Results and discussion

While developing the method, we tested different leading electrolytes within the pH range 7 – 10 (tested counter cations were imidazole, tris, ammediol and ethynolamine). A pH of 8.1 allowed for full separation of theanine from sample matrices. In Fig. 2 electropherograms of green tea extract is shown. From this figure is clear that L-theanine is fully separated from sample matrix.



a)

b)

Note: At the given conditions the separation of enantiomers of theanine (L- and D-form) is not possible.

Fig. 2 Electropherograms of samples; (a) analysis of green tea extract (2 g/l); (b) analysis of green tea extract (2 g/l) spiked with 20 mg of L-theanine/l; trace from the conductimeter of the analytical capillary; R = response of conductimeter, L =leading anion (chloride), T = terminating anion (valine).

We had to optimise conditions of extraction of theanine from tea. Fig. 3 summarizes results of measurement of extraction efficiency of L-theanine from green tea by hot and cold water. It was found that a 10 minute extraction of tea in boiling water gives highest yield of theanine extraction.

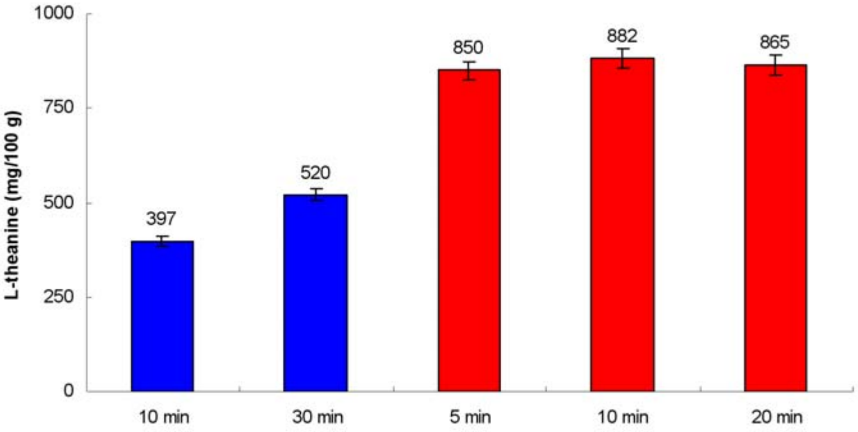


Fig. 3 Extraction efficiency of L-theanine from green tea; influence of temperature and time; extraction by 20 °C water in ultrasonic bath (blue bar); extraction in boiling water (red bar).

The method characteristics, *i.e.*, linear dynamic range, intra-assay, accuracy (recovery) and detection limit are displayed in Table 1. These results clearly show that the method is sufficiently sensitive, precise and reproducible and that is why it is suitable for intended purpose.

Table 1 Method characteristics for L-theanine.

Characteristic	value
Intra-assay[a] (RSD in %, $n = 6$)	1.5
Accuracy (recovery)[b] (RSD in %, $n = 4$)	99 ± 2
Linearity (mg/l)	2 – 200
Slope (mg/l/s)	0.67
Intercept (mg/l)	0.08
Correlation coefficient	0.99998
Detection limit[c] (mg/l)	0.7
Quantification limit[d] (mg/l)	2

[a] - repeated injection of standard solution at concentration level 8 mg/l of L-theanine;
[b] - green tea water extract (2 g/l) spiked with 6 mg/l and 12 mg/l of L-theanine;
[c] - based on minimal detectable zone length 1 s;
[d] - treble of detection limit.

Results of isotachophoretic analysis of samples are summarized in Table 2. Levels of L-theanine found in green tea varied between 0.6 – 0.9 %. These figures correspond to literature data (*vide supra*). Amount of theanine determined in samples of food supplements proportionates to green tea extracts addition. Analysis of sample "L-theanine" revealed this food supplement contains almost 90 % of L-theanine.

Table 2 Results of sample analyses.

sample	L-theanine (mg/100 g)
Green Tea, China Sencha	880
Green Tea, Pickwick with lime-blossom	730
Green Tea, Pickwick with jessamine	690
Green Tea, Teesan with lemon	865
Food supplement[a], L-theanine	86 480
Food supplement[b], AntiOxi Fit	14
Food supplement[c], Revital Ginkgo+Green tea	88

[a] - pure extract from leaves of Camellia sinensis;
[b] - 1.13 g of green tea extract/100 g;
[c] - 0.1 g of green tea extract/tablet (=4 g).

4 Conclusion

The developed isotachophoretic method for the determination of L-theanine is sensitive, accurate, reproducible, and relatively fast. Due to low consumption of water-based diluted electrolytes it is environmental friendly method. Method was applied to analyses of green tea and food supplements containing green tea extract. The obtained results clearly showed that the method is well suited for the intended purpose. Due to different separation principles, CITP is complementary technique to chromatography and can be use as an alternative. Markedly lower running cost of CITP than that of HPLC is the main advantage of isotachophoretic method.

Acknowledgment

Financial support of The Grant Agency of the Czech Republic (grant No 525/03/0351) is greatly acknowledged. Thanks are extended to PharmDr. Martin Staněk from Dr. Stanek Ltd Company for food supplements samples.

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