EVALUATION OF TEA FUNCTIONALITY: DETERMINATION OF L-THEANINE CONTENT IN GREEN AND BLACK TEAS BY LIQUID CHROMATOGRAPHY

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ABSTRACT

L-theanine (γ-glutamyl-L-ethylamide) is a free amino acid classified as functional ingredient because it has shown relevant pharmacological effects such as improving the brain ability to concentrate, learn and memorize. L-theanine is found almost exclusively in tea plants, and its final concentration in commercial tea products mainly depends on the type of raw materials and productive process. Thus, tea infusions could only be considered as functional beverage with respect to L-theanine if an adequate number of tea cups posses the necessary content to produce the desire effect. The objective of this work was to evaluate the functionality of teas marketed in Chile regarding its L-theanine content. A high performance liquid chromatographic method with ultraviolet detection (HPLC/UV) was optimized and validated to quantify L-theanine content in tea infusions. Chromatographic separation was carried out using a mixture of water and phosphoric acid (99.9: 0.1% v/v) as mobile phase and C_{18} Purospher Hibar-STAR (250 mm x 4.6 mm, 5 µm) column. Detection was performed at 210 nm. The L-theanine content in teas marketed in Chile ranged from 4.21 to 24.83 (2.21 to 12.42 mg g^{-1}), with a mean value of 10.06 mg in 200 mL (5.03 mg g^{-1}). Considering the effective dose reported, only one brand (black tea) presented an adequate L-theanine content to produce the desired effect (two serving cups). Therefore, it is possible to conclude that most teas marketed in Chile do not have an adequate L-theanine content to be considered as functional beverage regarding this amino acid.

Keywords: Optimization, L-theanine, chromatography, tea, Chile.

INTRODUCTION

Tea infusions produced with leaves of *Camellia sinensis* or *Thea sinensis* leaves [15,14,17,1] have become in one of the most consumed beverages in the world. Depending on the type of productive processes and harvest time is possible to obtain green (non-fermented), black (fermented), red (oolong, semi-fermented) and white teas (bud, non-fermented) [15,2]. Several beneficial health effects have been ascribed to tea, e.g. antioxidant [6], anti-inflammatory [6], anticarcinogenic [3] and antihypertensive activities [17,4]. These effects have been attributed to compounds such as alkaloids (caffeine, theobromine), polyphenols (catechins), flavonoids and free amino acids (L-theanine) [3,3]. L-theanine (γ-glutamyl-L-ethylamide) is a unique free amino acid, found only in tea plants (*Camellia sinensis*) and bay bolete mushroom (*Xerocomus badius)* [3,5]. In tea plants, L-theanine biosynthesis occurs in roots (principal source of soluble nitrogen) via theanine synthase using glutamic acid and ethylamine as precursors. After that, it is concentrated in leaves reaching about 1-2% w/w of dry leaf weight [4], counting for 50% of total amino acids [6]. Pharmacological activity of L-theanine has been studied by numerous research groups, which described that L-theanine posses an important capacity to improve the brain ability to concentrate, learn and memorize . This action has been associated with two simultaneous effects: increasing levels of alertness and relaxation without sedation [2]. Additionally, there have been described other beneficial effects such as reduction of blood pressure in hypertensive rats [4,5,4]; glutamate antagonist activity, which improves antitumor effect of substances on neoplastic tissues [2]; attenuation of doxorubicin-induced adverse reactions related to oxidative damage [2], and increase dopamine [9], serotonin [6] and γ-aminobutyric acid (GABA) levels [1]. The combination of L-theanine with caffeine can produce an improvement on the performance of demanding cognitive task, with the benefit that L-theanine unlike coffee does not produce hyperactivity states, even is capable of inhibiting the stimulatory effects of caffeine [4]. Currently, tea is the most consumed hot beverage in Chile with 9.500 tons per year, placing Chileans as the major tea drinker in Latin America. Consumption is estimated in 700 grams per year, equivalent to 350 tea bags per person per year. Worldwide, Chile is the sixth largest tea consumer with sales near to US$ 90 millions per year. Considering this consumption level and its beneficial effects, the objective of this work was to evaluate the functionality of teas marketed in Chile regarding its L-theanine content. Several analytical methods have been developed to determine L-theanine content, i.e. high performance liquid chromatography (HPLC) with fluorescence (FLD) [15,4] and ultraviolet (UV) [2,5], including diode array detectors (DAD) [17,19]; HPLC/electrospray ionization mass spectrometry (ESI-MS) [3,5], high performance thin-layer chromatography (HPTLC)/UV [14] and capillary electrophoresis (CE)/UV [1] Due to the lack of important chromophores in L-theanine molecule, some authors considered the use of derivatization reagents to enhance the detection capability, e.g. *o*-phthaladelhyde (OPA) [3,4,19], 9-fluorenylmethyloxycarbonyl chloride (FMOC) [3], 5-dimethylamino-1-naphthalenesulfonyl-chloride (Dns-Cl) [6,3], phenylisothiocyanate PTIC [19], and 6-aminoquinolyl-N-hidroxisuccinimidyl carbamate (AQC) [3]. However, derivatization processes are commonly labor-intensive and time-consuming, requiring several reagents. Some times these reactions also may form unstable derivates and artifacts. Others studies reported the use solid phase extraction (SPE) after derivatization [1,2], but this is also a time-consuming approach. Taking in account this scenario, a simple HPLC/UV method without derivation was chosen for a selective L-theanine determination. The method was optimized using an experimental design and validated following the International Conference on Harmonization (ICH) guidelines.

EXPERIMENTAL

Materials and methods

Reagents and chemicals

L-theanine (≥98%) and polyvinylpolypyrrolidone (PVPP) was purchased from Sigma (St. Louis, MO, USA). Ortho-phosphoric acid 85% was obtained from Merck (Darmstadt, Germany). Ultra-pure water (18 MΩ cm^{-1}) was produced using a Simplicity system from Millipore (Bedford, MA, USA). L-theanine stock solution was prepared in ultra-pure water for a given concentration of 1.8 g L^{-1}. By dilution with ultra-pure water all working solutions were prepared. These solutions stored refrigerated and protected from light were stable for at least five days. In order to evaluate the functionality of teas marketed in Chile with respect to their L-theanine content, nine tea brands were purchased, which represent 80% of the market and almost 100% of the brands available in supermarkets. For each brand two different lots were bought in different periods of the year, analyzing each sample (n=18) in triplicate (n=3).

Sample preparation

In order to evaluate the real L-theanine content available for common consumer, tea infusions were prepared according to the usual method. Tea bags (ca. 2 g) were extracted with 200 mL boiled water during 5 min in a 500 mL Erlenmeyer flask without shake or movement. Thereafter bags were removed without squeeze, allowing cooling down for 20 min. Ten mL of tea infusion were treated with 0.5 g of PVPP (for polyphenols removal) in a KS 125 basic shaker from IKA (Staufen, Germany) at 175 rpm for 15 min. This solution was filtered through a 13 mm PVDF syringe filter (0.45 µm pore size) prior to injection. All sample solutions were protected from light and used immediately.

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Chromatographic conditions

Chromatographic analysis was carried out using a Merck Hitachi (Tokyo, Japan) HPLC system consisted of a L-7100 pump, L-2400 UV detector and a Rheodyne (Cotati, CA, USA) 7125NS injector with 20 µL loop. Externally, the column temperature was controlled with a Merck Hitachi column oven Model 655A-52. Data were acquired and analyzed by means of Varian Star 4.0 software (Walnut Creek, CA, USA). Isocratic separation was performed at 37ºC on a Merck C_{18} Purospher Hibar-STAR (250 mm x 4.6 mm, 5 µm) column using a mobile phase composed of water and phosphoric acid (99.9 : 0.1% v/v) at a flow rate of 0.3 mL min^{-1}. Under these conditions L-theanine was detected at 210 nm, showing a retention time of 13 min.

Statistical analysis

Data were analyzed using descriptive statistics (mean, standard deviation (SD) and relative standard deviation (RSD)). Calibrations were established by applying a linear regression model. Calibrations comparison was carried out using F-test. The results of L-theanine with the proposed method were compared with an already established high performance thin layer chromatography method using Mann-Whitney non-parametric test. L-theanine content comparison was done using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test (pairs comparison). All statistical tests were performed at two-tails with a significance level (α) of 0.05. All above statistical analysis were carried out with GraphPad Prism 5.0 from GraphPad Software (San Diego, CA, USA). Central composite design was prepared and analyzed by means of Statgraphics Centurion XV version 1.15.1902 software (Rockville, MD, USA).

RESULTS AND DISCUSSION

Initial conditions

As described before, several methods have been established for L-theanine determination. The most common ones are based in HPLC/UV and HPLC/FLD. In order to establish a simple, fast and reliable method, derivatization and/or SPE treatment were not considered. Due to chemical nature and molecular characteristics, L-theanine showed the highest absorbance at 210 nm, wavelength on which several compounds present signals. Tea samples chromatograms showed several interfering compounds near to L-theanine peak, to overcome this problem different mobile phases and chromatographic columns were assayed without success. Columns such as Waters (Milford, MA, USA)-Symmetry (C_{18}, 250 mm x 3.0 mm, 5 µm and C_{18}, 150 mm x 4.6 mm, 5 µm); Waters-YMC Pro (C_{18}, 150 mm x 4.6 mm, 3 µm); Phenomenex (Torrance, CA, USA) Luna C_{18} HST (100 mm x 3.0 mm, 2.5 µm) and Waters-YMC-ODS A (C_{18}, 150 mm x 4.6 mm, 5 µm), showed lower separation capacity than the Merck-Hibar-Purospher STAR (C_{18}, 250 mm x 4.6 mm, 5 µm). Regarding mobile phases, organic modifiers like methanol, acetonitrile and ethanol were also assayed without good results. Finally, the combination of water and phosphoric acid as mobile phase and Hibar-Purospher column showed an adequate resolution between interfering and L-theanine peaks (R=2.8). Considering that green and black teas are respectively rich in polyphenols (30% dry weight basis) and oxidized phenolic compounds (25% dry weight basis), and both present an important level of pigments (2% dry weight basis), it was necessary the use of PVPP as sample treatment. The use of this cross-linked molecule, capable of precipitate polyphenols and related molecules, produced chromatogram with lower background.

Optimization of chromatographic conditions

Optimization was performed using central composite design (CCD) with two central points. The variables peak height and resolution were defined as critical for L-theanine evaluation, the first one looking for a lower detection limit and the second one to obtain clear separation from matrix interference peaks. From the factors that possibly affect the responses or variables, three were chosen: flow rate, percentage of acid in the mobile phase and column temperature. Taking into account the preliminary chromatographic assays, a range for each factor was established: flow rate (0.3-0.6 mL min^{-1}), percentage of acid in the mobile phase (0.02-0.10% v/v) and column temperature (25-40ºC). The experimental design model considered 16 runs (data not shown), which were performed duplicate (n=2) in randomized order to minimize the effects of uncontrolled factors. After carry out all experimental runs it was clearly observed that resolution was the most critical variable, thus, resolution response was used to calculate the optimal chromatographic conditions. Resolution data fitted a second-order model with a cubic experimental domain (data not shown). The optimal chromatographic conditions calculated were as follow: flow rate of 0.3 mL min^{-1}, 0.1% v/v of phosphoric acid in the mobile phase and a column temperature of 37ºC. Using these conditions it was possible to obtain a well-resolve L-theanine peak, allowing a reliable quantification. Through analysis of variance (ANOVA), with a significance level (α) of 0.05, it was evaluated which factors significantly affect the variable resolution. As it can be observed in Pareto chart (Figure 1) only acid percentage affected significantly the variable resolution (P<0.05).

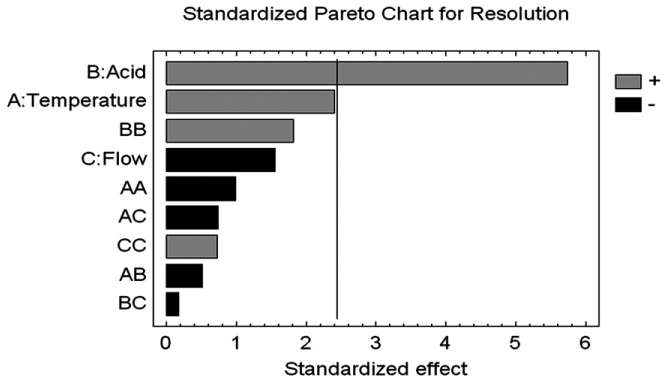

Figure 1: Standardized Pareto chart showing the influence of column temperature (A), acid percentage (B) and flow rate (C) over the response (resolution). Vertical line defines 95% of confidence.

Validation

The optimized method was validated following the ICH recommendations . Calibration curves with and without tea matrix were established in order to evaluate a possible matrix effect. Since slopes were statistically different (F=598.4, P<0.0001) calibration with matrix were used for sample quantification. L-theanine calibration curve was established with five levels in triplicate from 25 to 125 mg L^{-1} (Table 1).

Table 1. Linear function of L-theanine calibration curve.

Parameters	Results
Concentration range (mg L^{-1})	25 – 125
Regression equation	$y = 0.3424 \pm 0.0094\ x + 5.060 \pm 0.7839$
Correlation coefficient (r)	0.9951
Determination coefficient (R^2)	0.9902
S_{yx}	1.295
Confidence interval	a: 0.3329 a 0.3519 b: 4.2761 a 5.8439

Method precision was evaluated through repeatability and intermediate precision. Repeatability was studied injecting in sextuplicate (n=6) a tea sample spiked with 50 mg L^{-1} of L-theanine showing a RSD of 4.5 %. Intermediate precision was determined measuring in triplicate a spiked tea sample (50 mg L^{-1} L-theanine) during three different days (n=3), showing a RSD of 1.3 %. Since PVPP addition was the single sample preparation step, method accuracy was not evaluated through recovery, instead, it was determined comparing the results obtained with the proposed method with those calculated by an already published method that uses a different chromatographic system, in this case, HPTLC [14]. Before comparison, the possible loss of L-theanine due to the interaction/retention with PVPP was discarded (data not shown). For HPTLC determination samples were treated in the same manner than for HPLC determination. Application of samples and standard solutions over 20 cm x 10 cm silica gel 60 HPTLC plates was performed by means of Automatic TLC Sampler 4 (ATS 4) from CAMAG (Muttenz, Switzerland). The settings for 19 tracks per plate were as follows: band length 5.0 mm, track distance 10.0 mm, band velocity 120 nL s^{-1} and first application x axis and y axis at 10.0 mm. Standards from 25 to 125 ng / band were applied at both sides of each plate. Sample application volumes were variables (1-2 µL) depending on L-theanine content in tea infusions. Chromatography was performed in a 20 cm x 10 cm twin trough chamber (CAMAG) up to a migration distance of 50 mm using a mixture of 1-butanol : acetone : acetic acid : water (7:7:2:4 v/v) as mobile phase. After development (chromatographic separation) plates were dried under a stream of warm air for 2 min. L-theanine was detected after a post-chromatographic derivatization with ninhydrin methanolic solution (2 mg mL^{-1}) using a TLC Immersion Device (CAMAG) with the following settings: immersion speed 3.5 cm/s and immersion time 1s; thereafter plates

were heated on a TLC plate heater (CAMAG) for 2 min at 100ºC. Scanning (detection) was performed using a TLC Scanner 3 (CAMAG) in visible absorption mode at 520 nm with a slit dimension of 4.0 x 0.2 mm and a scanning speed of 20 mm s^{-1}. Photo-documentation was done under illumination (reflectance) in visible range by means of DigiStore 2 documentation system (CAMAG). All instruments were controlled via WinCats software 1.4.2 Planar Chromatography Manager (CAMAG). Three samples of different tea brands were evaluated by HPTLC/Vis and with the proposed HPLC/UV method. According to the results of Mann-Whitney test, no statistically significant difference was found between the results obtained with both methods [(P=0.10), (P=0.40) and (P=0.10)]. Thus, it was concluded that the proposed method provides comparable results. Regarding detection capacity, detection and quantification limits were calculated using signal-to-noise ratios (S/N) of 3 and 10, respectively. Considering an injection volume of 20 µL, the detection and quantification limits were 0.3 and 1.1 mg L^{-1} (32 and 108 mg kg^{-1}). Robustness was evaluated simultaneously with optimization showing that the proposed chromatographic method is not robust for the factor acid percentage in the mobile phase (P<0.05). Therefore the mobile phase should be carefully prepared just before its use. As describe before different C_{18} columns were also tested achieving satisfactory results only with Merck-Hibar-Purospher STAR column (Different solvent lots and manufacturers did not show any effect on the chromatographic parameters.

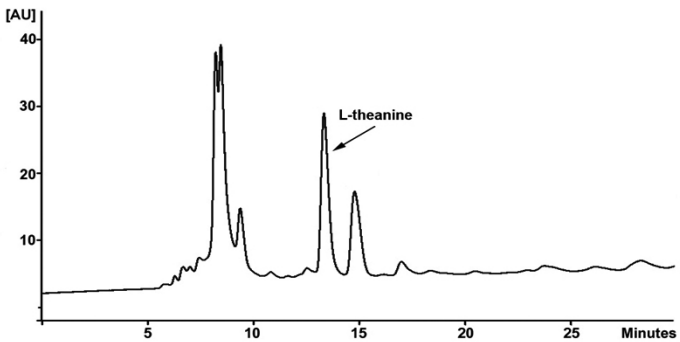

Figure 2: Black tea sample chromatogram obtained with the optimized and validated method showing a well-defined L-theanine peak (8.94 mg /200 mL).

Sample analysis

Since the research objective was the evaluation of tea functionality regarding L-theanine content, only the most representative tea brands were analyzed (80% of the Chilean market). From nine tea brands, 18 samples (14 black and 4 green teas) were analyzed in triplicate (Table 2). Extraction method was similar to the one reported by Keenan et al. [2], which considers 5 min extraction with hot water. No squeezing was performed since it is very difficult to standardize the time and the force applied. The latter may cause an underestimation of L-theanine concentration near to 3 mg/200mL [2]. Teas marketed in Chile showed a L-theanine content from 4.21 to 24.83 mg per cup of tea (considering 200 mL as standard cup volume) with mean values of 11.50 ± 2.91 and 9.65 ± 7.09 mg per cup of green (*n*=2) and black (*n*=7) tea, respectively (Table 2). These values are similar to those reported by Hilal and Engelhardt [2] (10–20 mg/200 mL) and Keenan et al [2] (8-24 mg/200 mL).

Table 2. L-theanine content in tea brands commercialized in Chile (*n*=18).

Tea brand[a]	Varieties of tea	L-theanine content	
		(mg /200 mL)[b]	(mg/g)[c]
LYL	Black	24.83 ± 1.58	12.42 ± 0.79
VL	Green	9.45 ± 1.21	4.72 ± 0.61
CCA	Black	4.31 ± 0.47	2.15 ± 0.23
EC	Black	8.94 ± 0.58	4.47 ± 0.29
CER	Black	10.44 ± 0.30	5.22 ± 0.15
SLO	Black	8.40 ± 1.34	4.20 ± 0.67
SMC	Black	6.39 ± 0.30	3.20 ± 0.15
SR	Black	4.21 ± 0.36	2.21 ± 0.18
SVS	Green	13.56 ± 0.30	6.78 ± 0.15

[a]: Two different lots of each brand were evaluated in triplicate, [b]: Standard cup volume. [c]: tea grams (each bag weight ca. 2 g)

A significant difference in L-theanine content was observed between brands and tea varieties (P<0.0001), which was delimited applying Bonferroni test as following described; the highest theanine concentration was observed in LYL black tea (24.83 ± 1.58 mg in 200 mL) followed by SVS green tea (13.56 ± 0.30 mg in 200 mL), both statistically higher than the rest of tea brands and different among them (P<0.05). No significant difference (P>0.05) was found between VL, EC, CER, and SLO tea brands. Two black tea brands (CCA and SR) presented the lower L-theanine concentration without significant difference among them (P>0.05). Coincidentally, these two brands are the cheapest one with lower particle size (dust). Both green tea brands, SVS and VL, showed significant difference on L-theanine content (P<0.05) with higher values than those reported by Alcazar et al. and Keenan et al. [15,2]. Regarding tea varieties, although green tea samples showed statistically higher (P<0.05) or similar (P>0.05) L-theanine values than almost all black teas, the highest L-theanine content (P<0.05) was observed in one black tea brand (Figure 3). Several authors have reported that green and white teas have higher L-theanine concentration than black and oolong teas. This lower content is attributed to fermentation steps carried out during production process. Contrarily, this work reports that the higher L-theanine content was found in one black tea brand, similar results were published by Keenan, et al [2]. The results obtained for LYL samples with the proposed method are in complete agreement with the values stated in the packet (18 - 22 mg/200 mL), giving some sort of confidence in the analytical procedure. Regarding effective dose, L-theanine produces a feeling of relaxation without sedation with doses from 50 to 200 mg . This effect is achieved after 30 minutes of administered. In view of samples results only one tea brand (LYL) can be considered a functional beverage with regard to L-theanine, because with two consecutive cups of tea is possible to accomplish the effective dose. For the rest of the brands marketed in Chile is necessary to consume of at least 3.7 cups of tea. Comparatively, this study found in black teas higher L-theanine concentration (4.82 ± 0.35 mg g^{-1}) than those reported by Wang *et al*. (1.13 mg g^{-1}) and Zhu *et al.* (1.13 mg g^{-1}) [6,3] but similar to the ones reported by Alcazar *et al*. (0.49 to 4.12 mg g^{-1}) [15]. LYL tea samples showed the highest concentration (12.42 ± 0.79 mg g^{-1}), which is similar to the value found by Keenan *et al.* (12.20 mg g^{-1}) [2] and Zhu *et al.* (9.8 mg g^{-1}) [3]. The average concentration of L-theanine in green tea was 5.75 ± 0.38 mg g^{-1}, which is similar to the values reported by Bedner *et al.* (2 to 5 mg g^{-1}) [5] and higher than those found by Wang *et al.* (3.06 mg g^{-1}), Alcazar *et al*. (1.62 to 3.37 mg g^{-1}) and Keenan *et al.* (3.95 mg g^{-1})[15,6,2]. Considering these results, and regardless its origin (China, Sri Lanka, Brazil, etc.), it is possible to conclude that tea infusions cannot be considered a functional beverage with respect to L-theanine content.

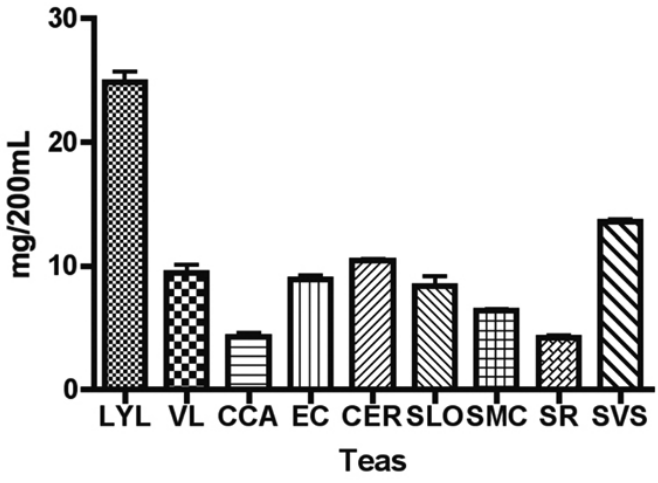

Figure 3: L-theanine content in tea brands marketed in Chile. Each bar indicates the mean ± standard error of the mean.

CONCLUSIONS

According to the validation results, the proposed method is a good alternative for L-theanine determination in teas, showing precise, accurate and reliable results. Sample results indicated that even when all tea brands showed similar L-theanine content than those reported by other authors, the concentration per cup is lower than the effective dose. Only with one brand is

possible to achieve the effective dose after the intake of two consecutively cups (400 mL). To the best of our knowledge this is the first time that L-theanine content is determined in teas marketed in Chile as well as its determination using an optimized and validated HPLC method.

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