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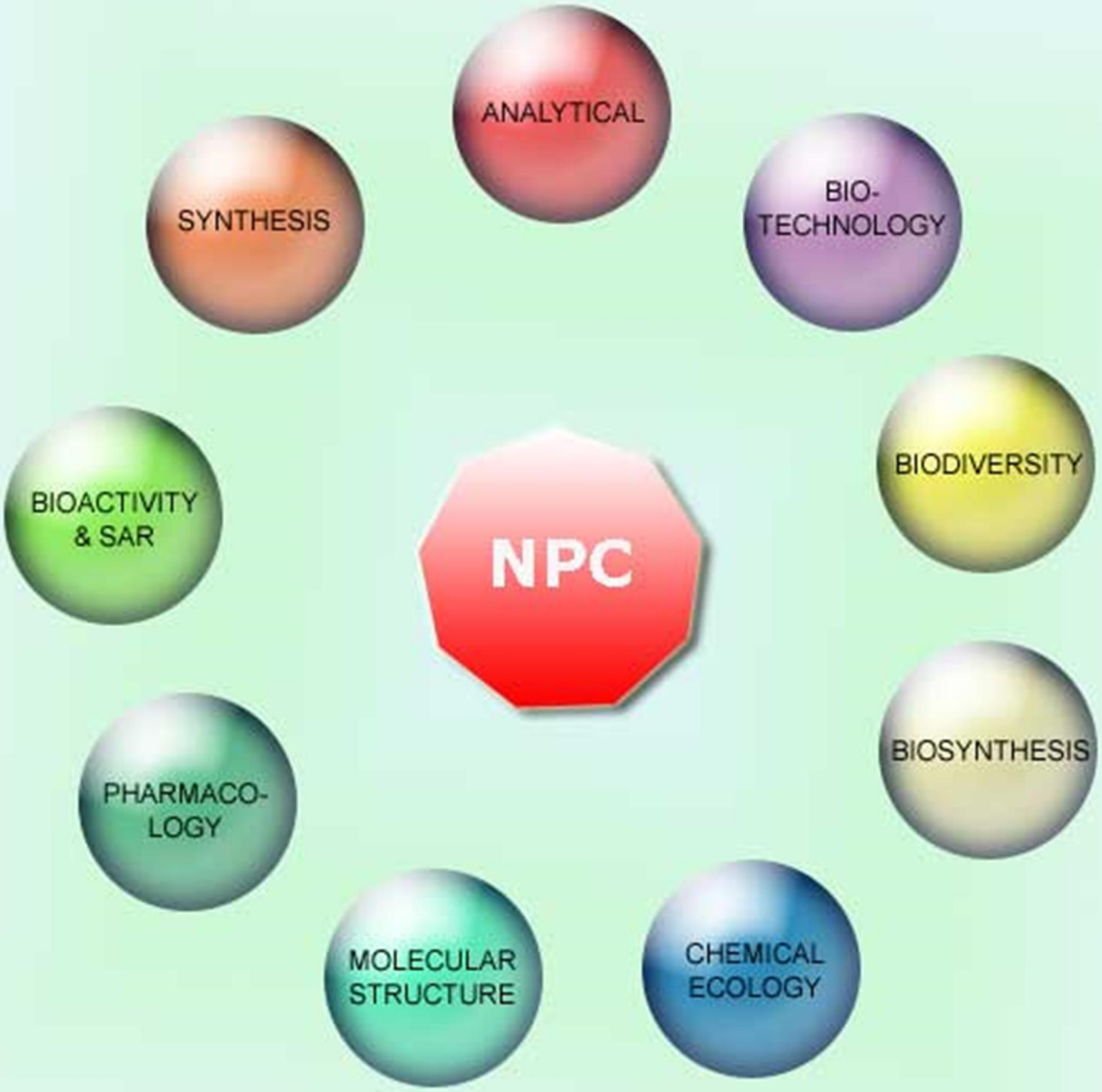

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Quantification of γ-Aminobutyric Acid in Sri Lankan Tea by Means of Ultra Performance Tandem Mass Spectrometry

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γ-Aminobutyric acid (GABA), an important bioactive component of tea, acts as a major inhibitory neurotransmitter and is considered to influence other physiological processes in human as well as *in planta*. In the hereby presented study, the content of this valuable metabolite was investigated in two novel types of Ceylon Tea, explicitly "Silver Tips" and "White Tea", originating from minimally processed buds of the unique cultivar, "TRI 2043". The samples were subjected to hot water infusion, equivalent to the traditional beverage preparation procedure, and analyzed by means of hydrophilic interaction ultra performance liquid chromatography coupled to tandem mass spectrometry (HILIC LC-MS/MS). The registered GABA levels were compared with those obtained for the classic "Black Tea" and "Green Tea" samples from Sri Lanka. A high variation of GABA content was observed among the different tea types, especially in the case of "Silver Tips" and "White Tea", indicating the crucial influence of the manufacturing procedure (processing extent) on the final abundance of the bioactive component of interest. Furthermore, "White Tea" samples boasted the highest GABA concentration reported for this type of tea so far, reaching up to 50% of that characteristic of the high-priced "GABA Tea". Therefore, "White Tea" and "Silver Tips" were proved to be high quality teas with amounts of γ-aminobutyric acid comparable with those described for similar types before. To our knowledge, this is the first report on HILIC LC-MS/MS application for the quantification of GABA and for in-depth characterization of teas from Sri Lanka.

Keywords: Sri Lanka Tea, White Tea, GABA, HILIC LC-MS/MS, Theanine degradation.

Tea is one of the most popular beverages worldwide, consumed in amounts second only to water and comparable with those of coffee. Sri Lanka is the third largest producer of *Camellia sinensis* L., grown mainly in tropical and subtropical regions. Depending on the distinct post-harvest treatment of leaves, the consumable tea types are classified as "Green Tea" (GT, unfermented), "Black Tea" (BT, fully fermented), "Oolong Tea" (partially fermented), and "PuErh Tea" (post fermented) [1-3]. Beside the well-known "Ceylon Black Tea", highly priced special teas, such as "Silver Tips" (ST) and "White Tea" (WT), are nowadays produced through unique and exclusive processing of apical buds, and buds together with the first leaf, respectively. In Sri Lanka, these teas are being manufactured solely from a special cultivar, "TRI 2043", bred and improved by the Tea Research Institute. Due to the presence of bioactive compounds, such as polyphenols, vitamins, carbohydrates, caffeine and purine alkaloids, and amino acids, consumption of tea is associated with important physiological reactions and potential health benefits [1,4-6]. The latter, especially, depends upon the taste, aroma, and quality of tea [3, 7], with γ-aminobutyric acid and L-theanine representing the most abundant amino acids in tea leaves and tea beverages, respectively [2].

GABA is a ubiquitous, non-proteinaceous amino acid that has been linked to stress, signaling, and storage processes in plants [8]. It is abundant in all protein foods, while its richest sources are fish and wheat bran [9]. It is derived from L-glutamate, a breakdown product of L-theanine [10,11]; the two reactions involved are catalyzed consecutively by theanine hydrolase (TH, EC 3.5.1.65) and a cytosolic glutamic acid decarboxylase (GAD, EC 4.1.1.15). GABA can be further metabolized by two mitochondrial enzymes, GABA transaminase (GABA-T, EC 2.6.1.19) and succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.24), to succinate semi aldehyde and succinate, respectively [10].

In mammals, GABA is an important neurotransmitter, exhibiting inhibitory activity in the central nervous system and playing a crucial role in neuronal excitability regulation [12,13]. Furthermore, GABA stimulates overall brain activity and has anti-anxiety, antihypertensive, and anti-convulsive effects [14,15]. Several GABA-enriched foods, such as "GABA Tea" [7,16,17], rice germ [18], brown rice [19], dairy products [20], soft drinks, and black raspberry juice [21], were proved beneficial in treatment of sleeplessness, depression, and autonomic disorders [22]. In recent years, teas enriched in GABA by repeated treatment of fresh leaves with anaerobic and aerobic fermentation have become popular among consumers in Taiwan and Japan [16].

Due to the biological and pharmacological importance of GABA, determining its concentration in different classes of processed tea is important for the definition of the quality and potential health effects of the beverage varieties. Confronted with but scarce recent reports on GABA content in BT and other tea types [3,7,23-25], as

well as utter lack of relevant data for the selected teas (ST and WT) from Sri Lanka, an appropriate quantification of the metabolite of interest was performed. In the present study, fresh tea shoots from plants grown in different locations were harvested and subsequently processed according to the appropriate manufacturing method (Suppl. Tab. 1). GT and BT are well-known standard beverages favored in oriental and western countries, respectively, while the production of WT and ST is rather limited. Unlike the former tea types, ST and WT were processed only minimally, with no heat or steam applied during the procedure. Six different samples of GT, BT, and ST, and 10 samples of WT were analyzed in terms of GABA content. All tea types and samples were prepared as water infusions, comparable with those made traditionally by consumers. The initial profiling of GABA content was performed by means of LC-MS, without any derivatization or further extraction, to determine the concentration most relevant to human consumption.

Hydrophilic interaction ultra performance liquid chromatography (HILIC) coupled to mass spectrometry (MS) enables the separation and analysis of amino acids and other polar metabolites, and has been successfully applied for GABA determination in other matrices [26]. The presence of GABA in the hereby investigated tea samples was proven, as based on the mass spectral characteristics (presence of the molecular ion, $[M+H]^+$, at *m/z* 104.1 and the two fragment ions, at *m/z* 69.0 and 87.0), and co-elution with an authentic standard. While the metabolite of interest was detected in all the analyzed samples, the variation of its content was already evident, as the peak area values were significantly divergent (GT, lowest vs. other samples). Analysis of the replicate samples further confirmed the implied contrasts between the investigated tea types, with the lowest GABA amounts found in GT and the highest, in WT (Figure 1). Furthermore, while the concentration values were highly variable in WT and ST samples, this was not the case for the remaining tea varieties.

More specifically, GABA content in GT samples was the lowest, amounting to 13-48 mg/kg (Suppl. Tab. 2). In contrast, a previous report investigating GT from Taiwan, applying similar processing (infusion), but an alternative analytical approach (Beckman amino acid analyzer), recounted higher GABA levels, of 169.4 +/- 84.6 mg/kg [7]. Moreover, GT samples originating from China and Japan were found to contain higher amounts of the amino acid of interest (19-105 and 138-204 mg/kg, respectively), with 2 to 4 times higher maximum concentrations than the ones detected herein [3, 25]. As for the fully fermented tea type, the 6 different BT samples analyzed in this study contained between 81 and 129 mg/kg GABA, which is more than twice the amount found in the Chinese (34-55 mg/kg), but only one fourth of that characteristic of the Japanese (311-415 mg/kg) "Black Tea" [3,25].

The two special Sri Lankan teas, WT and ST, showed not only the highest average GABA content, but also the highest variation in the investigated parameter between the samples: 91-803 and 63-444 mg/kg, respectively. While the average concentration value calculated for the ST samples was only slightly higher than the one recorded for BT, the maximum values obtained for individual samples exceeded their BT equivalents by 4 to 8 times. Comparing the aforementioned values with those reported for “GABA Tea” from Taiwan [7] resulted in the conclusion that the maximum concentration detected herein constituted but 50% of the average value representative of the high-priced beverage (1809+/-514 mg/kg). However, GABA levels of only around 200 mg/kg were described for a commercially available “GABA Tea” [3]. Thus, the hereby obtained WT data represent the highest GABA content reported so far in this tea category (e.g., Chinese WT, max. 457+/-77 mg/kg [25]).

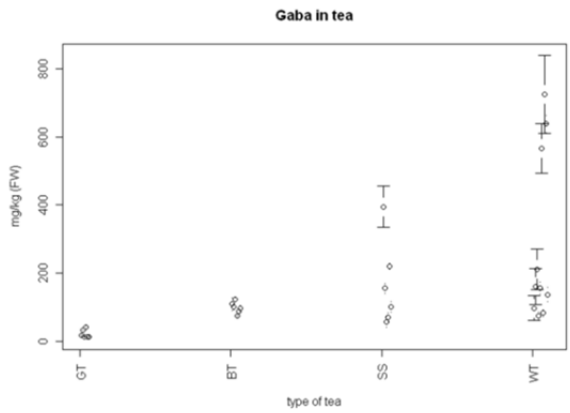

Figure 1: Quantities of GABA in different tea samples in mg/kg of fresh weight. Each point represents the average of 2-4 replicates of each tea sample analyzed; the error bars depict the standard deviation.

The wide variation thus observed led to the assumption that, not only the origin of the crude material but, to an even higher extent, the mode of its processing, influence the final content of GABA in tea. This is in accordance with a previous report investigating amino acid levels (including GABA) in four different tea types from Taiwan [3]. However, the authors used longer infusion times (implying higher extraction rates), probably failing to correspond to the realistic tea preparation procedure, as performed by the consumer.

While the final step of BT processing involves drying with hot air, GT manufacture entails the use of steam or pan firing [26]. In contrast, the Sri Lankan ST and WT were produced with minimal processing, which could account for the high amounts of GABA detected in both teas. In addition, the slow (14 days) air drying process, being part of the ST and WT manufacturing procedure, might prove a contributing factor. As the increase in free amino acid and caffeine levels due to the activity of appropriate hydrolytic enzymes during the withering stage has been well documented for BT [27-29], it could be similarly proposed that the boost in GABA content in the investigated Sri Lankan tea types was an outcome of the enzymatic breakdown of free L-theanine found in leaves to L-glutamic acid and ethylamine, and subsequently to GABA [16,17, 30], catalyzed by TH and GAD. Previous reports, stating that commercial “GABA Tea” usually contained less L-theanine but more GABA, as compared with GT [3], further support the aforementioned claim.

To our knowledge, this is an unprecedented report on the bioactive γ-aminobutyric acid concentration in teas of Sri Lankan origin, as determined by LC/MS-MS. We observed high variability of GABA content, not only between the different tea classes but also between individual samples of the same class, indicating the influence of the sample source and the post-harvest treatment on the investigated parameter. The sample variation was most significant in the case of WT, with some boasting around 50% of the GABA content reported for the high-priced “GABA Teas”. Further studies on the origins of the observed variability could help determine optimal conditions for the improvement of GABA abundance in Sri Lankan tea.

In addition, we hereby established the feasibility of HILIC LC-MS/MS as an alternative technique for successful identification and quantification of GABA in tea extracts, avoiding the use of chromophoric labeling reagents, such as the commonly used dabsyl chloride [3]. Further studies on the activity of enzymes involved in GABA metabolism, together with the identification of L-theanine

and L-glutamic acid during the processing of Sri Lankan ST and WT, are under way and will shed more light on the possibilities of efficient augmentation of the valuable amino acid levels in the introduced novel types of speciality teas.

Experimental

Plant material: Six independently collected "Black Tea" (BT), "Green Tea" (GT), and "Silver Tips" (ST) as well as 10 "White Tea" (WT) samples of Sri Lankan origin, processed according to the general rules immediately after harvest, were selected for the study (29; see Suppl. Tab. 1).

Reagents: Standard GABA and L-norvaline, as well as the solvents (acetonitrile, formic acid, and ammonia of LC-MS grade) were purchased from Sigma-Aldrich (St. Louis, MO). MilliQ water was used for chromatography and sample preparation.

Sample preparation and analysis: One g of each tea sample was mixed with 50 mL of water at 95°C. L-norvaline (25.4 µg) was added to the mixture (internal standard). After 6 min, the samples were quickly cooled in an ice bath, filtered through filter paper into 50 mL volumetric flasks, and the volume adjusted to the mark with MilliQ water. Each sample was re-filtered through a 0.2 µm PTFE membrane and diluted 4-fold with acetonitrile. Two to 4 repetitions per sample were performed. Standard curves were plotted using GABA concentrations of 0.05, 0.10, 0.15, 0.20, 0.25, 0.50, 1.00, 1.51, 2.01, 4.02, and 6.28 µg/mL, each dilution containing the same amount of internal standard.

Analysis of moisture content: Moisture content of all tea samples was determined by the ISO 1573:1980 method [31]. The data were used to express the results on a dry weight basis.

Chromatography and mass spectrometry: Chromatographic conditions were adapted from methods developed in house for the analysis of amino acids and other polar metabolites [32]. In detail, the analysis was performed on an Applied Biosystems ABSciex 5500 triplequad mass spectrometer via an electrospray interface (ESI), controlled by Analyst Software, Version 1.5.1. The mass spectrometer was coupled to a 1290 Agilent UPLC, equipped with an ACQUITY UPLC 1.7 µm, 2.1 × 150 mm BEH amide column (Waters) at 60°C. The samples were eluted in a gradient, starting from 90% A (95% acetonitrile, 5% water, 0.1% formic acid, 0.1% ammonium hydroxide) and increasing linearly over 8 min to 25% B (40% acetonitrile, 60% water, 0.1% formic acid, 0.15 % ammonium hydroxide), followed by 2 min washing with 90% B and reconditioning of the column for 6 min under initial conditions, with a 0.4 mL/min flow rate. The injection volume was 1 µL and the samples were kept at 4ºC throughout the analysis. GABA and L-norvaline were detected by collecting MS/MS spectra in positive ESI mode. The following parameters were applied: dwell time, 100 ms; curtain gas, 20 psi; collision gas, 9 psi; ion spray voltage, 5000 V; source temperature, 400°C; ion source gas 1 and 2, 55 and 60 psi, respectively. The compound parameters set for GABA analysis were: collision cell exit potential, 8; declustering potential, 160; collision energy, 22; entrance potential, 10. For L-norvaline, the corresponding values where: 12, 60, 16, and 10, respectively. Two transitions were monitored for GABA, the positively charged molecular ion, *m/z* 104.1, and 2 fragment ions, *m/z* 69.0 and 87.0, but only the first transition was used for quantification. For the internal standard, L-norvaline, only one transition was monitored (*m/z* 118.1/72.1).

Supplementary data: Suppl. Table 1: Sample list. Suppl. Table 2: GABA concentration in the investigated tea samples. BT, "Black Tea"; GT, "Green Tea"; ST, "Silver Tips"; WT, "White Tea".

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