Chapter

GABA-enriched Oolong Tea: Reducing Stress in a Student Cohort May Involve More than Just GABA

Tina Hinton, Kong M. Li, Vincent Viengkhou, Sin Yoo Kam, Sandra Kindaro, Herbert F. Jelinek, Slade Matthews and Graham A.R. Johnston

Abstract

We have previously shown that the consumption of GABA-enriched oolong tea is effective in reducing stress in a student cohort. However, key constituent content has not been previously investigated, especially as applied to a standard cup of tea. Further, it has not been substantiated whether it is the suggested GABA content or other constituents that lead to these observed changes in stress behaviour. Using reverse-phase HPLC, we determined the actual content of four chemicals known to influence stress in 200 mL cups of regular or GABA-enriched oolong tea brewed to manufacturer's instructions. We found eight times as much γ-aminobutyric acid (GABA) and 1.5 times as much caffeine in GABA-enriched oolong tea as in regular oolong tea. In contrast, there was 10 times less epigallocatechin gallate (EGCG), and half as much theanine in the GABA-enriched tea. Thus, there are changes in multiple constituents in GABA-enriched oolong tea that may contribute to the biological effects we observed in students consuming these teas.

Keywords: GABA, theanine, epigallocatechin gallate, caffeine, tea, stress, HPLC

1. Introduction

We have previously shown that consumption of GABA-enriched oolong tea is effective in reducing stress and improving cardiac rhythm in a university student cohort [1]. As constituents other than GABA are known to influence stress, we have quantified three other key chemicals in oolong tea known to influence stress: the methylxanthine caffeine, the flavonoid epigallocatechin gallate (EGCG) and the amino acid theanine in addition to GABA. The chemical structures of these constituents are shown in **Figure 1**. The aim of the current study was to quantify, using HPLC, the concentrations of these constituents in 200 mL cups of regular oolong and GABA-enriched oolong tea as prepared according to manufacturer's specifications and consumed by the students.

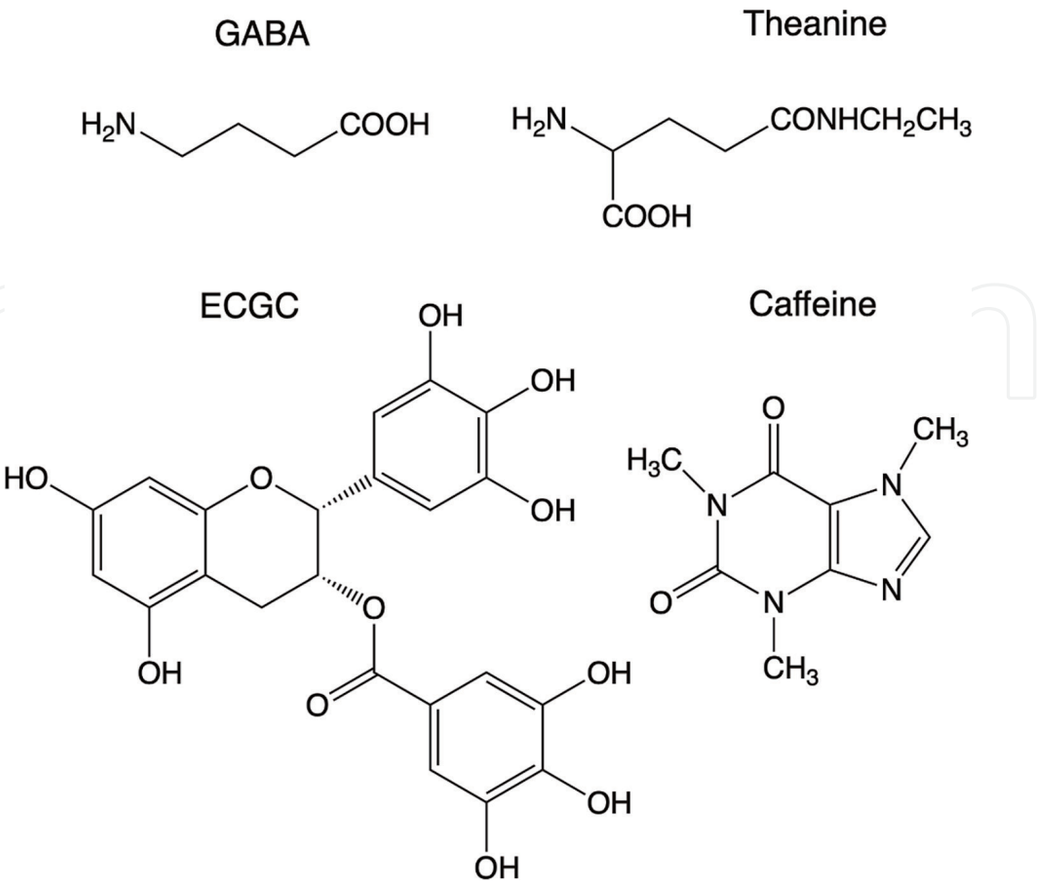

Figure 1.
Chemical structures of four neuroactive constituents found in regular oolong and GABA-enriched oolong teas.

GABA is the major inhibitory neurotransmitter in the brain [2] and plays a pivotal role in stress as well as modulating autonomic and cardiovascular function centrally and peripherally. GABA-enriched foods and GABA in single oral administration have been shown to reduce stress as measured through heart rate variability, heart rate, and other stress-related biomarkers and psychological tests. Hence, GABA content in tea has been enhanced to produce an improved therapeutically useful beverage for consumption [3]. GABA-enriched tea is obtained by incubation of tea under anaerobic conditions, or cycling under aerobic and anaerobic conditions, which has been shown to accumulate GABA to produce a tea with concentrations of GABA >150 mg/100 g tea [4–9].

The possible roles for other oolong tea constituents of oolong tea, such as EGCG, theanine and caffeine, may then also relate to their interaction with GABA. There are several known mechanisms of action to explain the effects of caffeine in oolong tea in addition to its stimulant effect [10]. The most prominent is that it is a potent adenosine receptor antagonist, and it has also been shown to potentiate GABA release via its effect on A_1 adenosine receptors [11].

EGCG may mediate some of its stress-reducing effects via interaction with the GABAergic system [12–15]. EGCG is readily incorporated into the brain following intragastric administration in mice [14], and has shown stress-reducing, anxiolytic and sedative properties in a number of animal models [12, 13, 15]. EGCG has also been found to be a biphasic modulator of $GABA_A$ receptors, at low doses enhancing diazepam action at recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes [16].

Although the action of theanine is not well understood [17, 18], it has been proposed to contribute to the relaxation experienced following tea consumption. For example, an oral 200 mg dose of theanine was also shown to increase α-brain waves in the occipital and parietal cortices of human subjects, indicative of a relaxation effect [17]. While one study showed it to increase GABA release [19], theanine does not appear to impact behaviours mediated by the GABAergic system in animal models [19–21]. Instead, theanine may mediate its effects through modulation of other transmitter systems including dopamine [18, 22] and glycine [22].

The aim of the current study was to quantify, using HPLC, the concentrations of GABA, EGCG, caffeine and theanine in 200 mL cups of regular oolong and GABA-enriched oolong tea as prepared according to manufacturer's specifications and consumed by the students.

2. Materials and methods

2.1 HPLC sample preparation and analysis

Golden Wulong (regular Oolong tea) and Organic GABA Body and Mind tea (GABA-enriched Oolong tea) both originated from Taiwan and were purchased from www.teas.com.au. Standard cups of each oolong tea were prepared and analysed to quantify both the differences in active constituent content between the teas and also the consistency of GABA, theanine, caffeine and EGCG extraction between separate brews of the same tea. Teas were prepared in accordance with manufacturer's instructions for consumption, by the addition of 5 g tea to 200 mL of 90°C deionised Milli-Q water (dH_2O) and infused for 10 min.

Samples (20 mg) of the authentic standard compounds, GABA (RBI; Natick, MA, U.S.A.), theanine (Tocris; Ballwin, MO, U.S.A.), caffeine (Sigma, St. Louis, MO, U.S.A.) and EGCG (Sigma, St. Louis, MO, U.S.A.), were weighed accurately and added to a 100 mL volumetric flask before being dissolved in 12.5% acetonitrile, yielding final stock solutions of 200 μg/mL. Standard solutions in the range of 5.0–200 μg/mL were made by diluting the stock solution with 12.5% acetonitrile directly before each run. The standard solutions were filtered through 0.45 μm, 13 mm diameter HPLC nylon syringe filters (Grace Davison Discovery Science; Deerfield, IL, U.S.A.). Analysis was via reverse-phase HPLC using a Varian ProStar 210 solvent delivery system coupled to a Varian autosampler model 410 with cooling tray set at 4°C, Degassit degasser (Varian Inc.; Walnut Creek, CA, U.S.A.). Data were collected and analysed using Varian Star Chromatography Workstation, Interactive Graphic, System Control and Method Builder version 6.30 (Varian Inc.).

2.2 GABA and theanine measurements

GABA and theanine were determined using pre-column derivatisation of standards and tea samples at the same time before each run for detection of amino acids. Immediately prior to derivatisation, 50 μL tea samples were mixed with 50 μL 0.2 M borate buffer (pH 8.5). To derivatise the amino acids, 100 μL 15.5 mM 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) was added and allowed to react for 1 min before addition of 60 μL cleavage reagent (0.5 M hydroxylamine hydrochloride: 1.7 M sodium hydroxide: water) to remove excess FMOC-Cl which can interfere with amino acid separation. The reaction was stopped after three minutes with 100 μL quenching

reagent (1:4 glacial acetic acid: acetonitrile). Each sample was then diluted 1:3 in water and filtered through a 0.45 µm HPLC nylon syringe filter (13 mm diameter). All samples were stored at 4 °C and analysed within 24 hours.

For amino acid detection, a Shimadzu RF-10A$_{XL}$ spectrofluorometric detector (excitation 263 nm; emission 313 nm) with a Varian Microsorb-MV 100 C18, 5 µm, 250 × 4.6 mm column maintained at room temperature were used.

2.3 Caffeine and EGCG measurements

For caffeine and EGCG quantification, samples were centrifuged at 2,500 g for 10 mins, a 1:2.5 dilution was made using 12.5% acetonitrile, and then samples were filtered through 0.45 µm, 13 mm diameter HPLC nylon syringe filters. All samples were stored at 4°C for no greater than 24 hours prior to performing HPLC. For caffeine and EGCG measurement, a Kinetex 5u XB-C18 (5 µm, 150 × 4.6 mm) column maintained at room temperature was used. Detection was via analysis at UV wavelength 254 nm.

2.4 Gradient elution

All chromatographic experiments were performed using gradient elution. For GABA and theanine, optimised mobile phase A consisted of 2 M ammonium phosphate buffer (pH 6.7), methanol and water (0.75:15:84.25). Mobile Phase B was acetonitrile and water (90:10). For caffeine and EGCG, the composition of the optimised mobile phase A was water/acetonitrile/formic acid (94.7/4.3/1) and mobile phase B was water/acetonitrile/formic acid (49.5/49.5/1). Each mobile phase was filtered through a 0.45 µm Teflon membrane filter under vacuum prior to use. The flow rate was 1.0 mL/min.

The gradient elution profile for GABA and theanine was 0 min 12% B, 5 min 30% B, 20 min 34% B and 22–27 min 99% B. The gradient elution profile for caffeine and EGCG was 0 min 1% B, 10 min 35% B, 13.3 min 90% B, 19.5 min 90% B, 21 min 1% B and 26 min 1% B. The injection volume was 5 µL and the detection wavelength was set at 254 nm. GABA, theanine, caffeine and EGCG were identified by comparison of the retention time of peaks produced by the tea sample against that of the respective GABA, theanine, caffeine and EGCG standards.

2.5 Method validation

Calibration curves were constructed from duplicate analyses of GABA and theanine standards at 1.2, 2.4, 4.8, 7.2, 9.6 and 12.0 µM, and triplicate analyses of caffeine and EGCG standards at 5, 50, 100, 160 and 200 µM. To evaluate the intraday and interday precision, 96 µM GABA and theanine standards were analysed in duplicate of two consecutive days, 50 µM caffeine and 160 µM EGCG standards were analysed in triplicate on three consecutive days, and coefficients of variation (CV) for the retention time and peak area were calculated. Accuracy was assessed using a recovery test of spiked samples with 50 µM of each of GABA, theanine, caffeine and EGCG. Recoveries were calculated comparing the obtained amounts with those added using the formula: recovery (%) = [(concentration found – endogenous concentration) / 50] × 100% (Zhao *et al*., 2011). Validation of GABA, theanine, caffeine and EGCG peak identification in the samples was conducted by comparing the retention time of the peak of increased size in the spiked to the unspiked samples.

3. Results

3.1 HPLC method validation

Mean retention times of the standards were: GABA 14.05 min; theanine 13.15 min; caffeine 8.65 min; and EGCG 11.32 min. Consistent retention times were also observed for both regular oolong tea (GABA 14.08 min; theanine 13.19 min; caffeine 8.46 min; and EGCG 11.21 min) and GABA-enriched oolong tea (GABA 14.01 min; theanine 13.11 min; caffeine 8.39 min; and EGCG 11.13 min). The calibration curves for quantified constituents obtained from the duplicate and triplicate standards exhibited linear correlation coefficients (r^2) of 0.991 for GABA, 0.996 for theanine, 0.992 for caffeine, and 0.998 for EGCG. Intraday CVs of peak area and retention time were GABA 1.61%; theanine 0.01%; caffeine 1.03%; and EGCG 0.06%. Interday CVs were GABA 5.41%; theanine 0.01%; caffeine 2.45%; and EGCG 0.01%. The spiked samples demonstrated mean recoveries of 100.33 ± 28.40% (range: 87–134%) for GABA and theanine, and 104.64 ± 19.07% for caffeine and EGCG.

3.2 GABA, theanine, caffeine and EGCG content in a cup of tea

Two separate brews of each tea were analysed in duplicate for GABA and theanine, and three separate brews were analysed in triplicate for caffeine and EGCG in order to determine both the difference in constituent content between the teas and also the consistency of constituent content between separate brews of the same tea. Given the greater degree of variability encountered with the measurement of the caffeine and EGCG content in different brews, triplicate measurements were undertaken. The relative amounts of GABA, theanine, caffeine and EGCG and differences in constituent content between the two types of tea are shown in **Table 1** expressed as mg per 200 mL of brewed tea from 5 g of dry tea leaf. Good reproducibility in the extraction of constituents was demonstrated with our preparation protocol. Both teas showed consistent quantified constituent content between separate brews of the same tea.

Representative reverse-phase HPLC chromatograms are shown in **Figures 2** and **3**.

Constituent (mg/100 g)	**Regular tea**				**GABA-enriched tea**			
	Brew 1	**Brew 2**	**Brew 3**	**Average**	**Brew 1**	**Brew 2**	**Brew 3**	**Average**
GABA	5.00	5.00		5.00	40.4	40.0		40.2
Theanine	163	166		165	82.8	82.0		82.4
Caffeine	300	252	250	267	457	368	383	402
EGCG	377	355	338	356	1.80	3.60	2.40	2.60

Data are expressed in mg per 100 g of dry tea leaf.

Table 1.
Amount of GABA, theanine, caffeine and EGCG in separate brews of oolong tea.

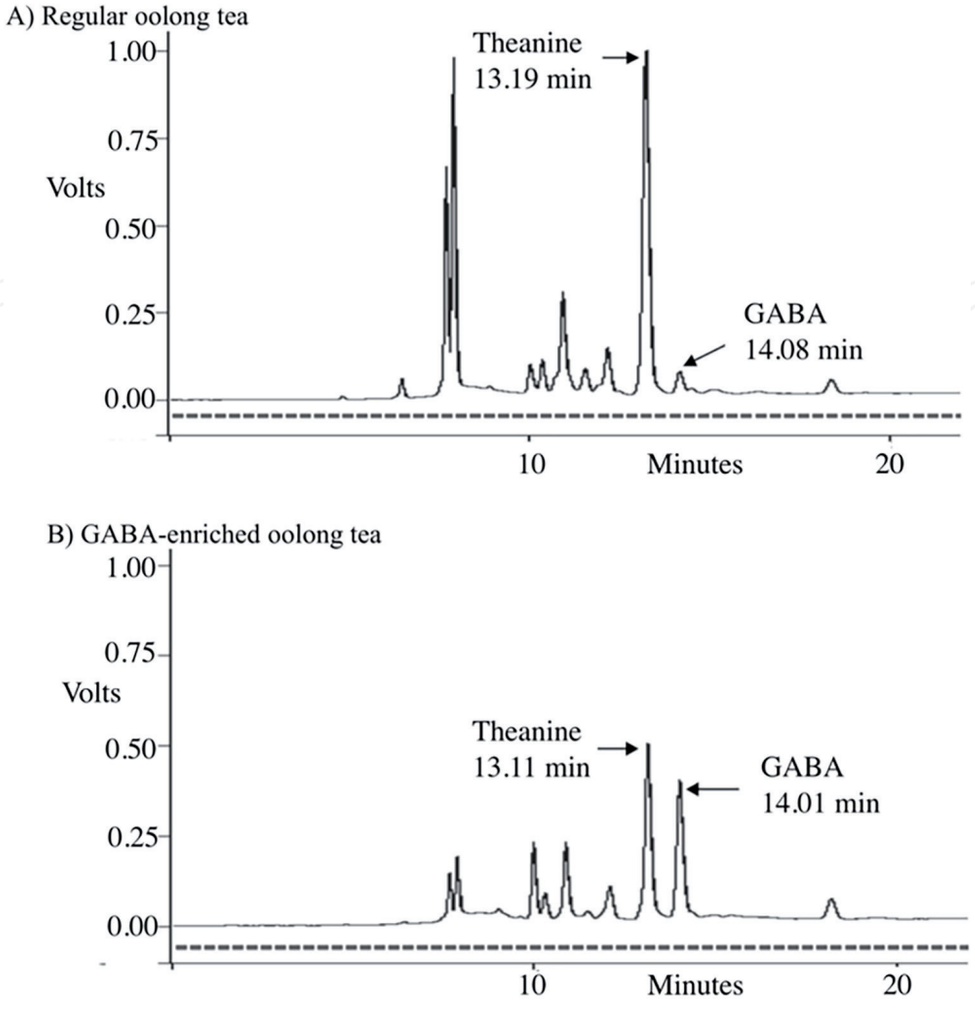

Figure 2.
Representative reverse-phase HPLC chromatograms of GABA and theanine in (A) regular oolong, and (B) GABA-enriched oolong teas.

4. Discussion

The advantage of our study is that four active constituents of tea were measured using HPLC in one tea type (oolong) and changes relative to GABA-enrichment were documented. Further, our method of sample preparation was appropriate for determining constituent content in a cup of tea prepared according to usual consumption practices and in our studies of effects on a student cohort. In future, testing these constituents across a range of commercially available products would be valuable for a wider comparison of different tea types. Choice of oolong tea for the current study was based on mid-range fermentation. Analysis of constituents of GABA-enriched green or black teas will likely yield differing results.

Previous studies using HPLC have determined GABA to be present to varying extents in commercially prepared GABA-enriched teas, including 1560 mg/100 g [9]; 19 mg/100 g [6] and 272 mg/100 g [4]. The type of tea according to the degree of oxidation (e.g., oolong, black) was not specified in these studies. On the other hand, GABA concentration in freshly prepared GABA tea has been measured at between 30 and 700 mg/100 g (average 275 mg/100 g) using HPLC, with concentrations dependent on the method of preparation (aerobic vs anaerobic cycling, or both), and plant

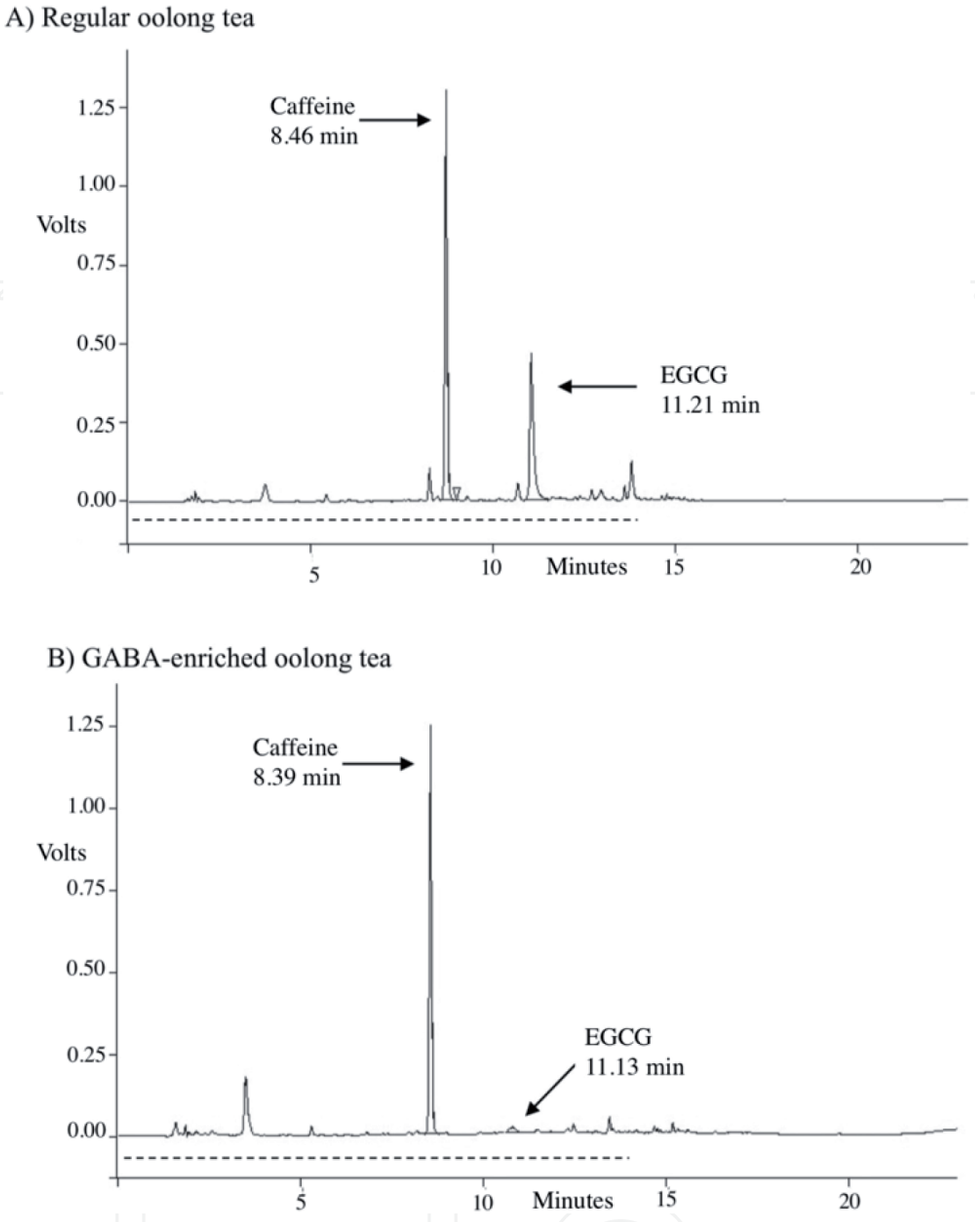

Figure 3.
Representative reverse-phase HPLC chromatograms of caffeine and EGCG in (A) regular oolong, and (B) GABA-enriched oolong teas.

part used (leaves, buds or stem) [5, 7, 8, 23, 24]. These reported results compare with our finding of 40.2 mg GABA/100 g GABA-enriched oolong tea.

In non-GABA enriched (i.e., regular) teas, GABA quantities have been measured using HPLC, Zhao et al. [9] found the concentration of GABA in teas differed according to tea type. Relative concentrations from greatest to least were white (50.5 mg/100 g) > black (31.1–41.5 mg/100 g) > green (13.8–33.9 mg/100 g) > oolong (14.8–20.7 mg/100 g). On the other hand, Syu and colleagues [6] found the GABA content of different teas to be green (19.6–105 mg/100 g) equal to oolong (10–101 mg/100 g) > black (34–55 mg/100 g). In contrast to our findings of 5 mg GABA/100 g regular oolong tea, others have shown, on average, higher GABA concentrations in commercially prepared regular oolong teas.

The differences between GABA content determined in our study and findings from previous investigations may be explained by the fact that our teas were prepared

according to manufacturer's instructions using simple aqueous extraction, as in making a cup of tea, without repeated extractions using different solvents and drying down to increase yield. In this way, we provide a realistic estimation of the concentration of GABA that one may encounter in a commonly prepared cup of tea.

Like GABA, theanine content measured across a range of commercial tea types is found to vary in concentration, and even within the different tea types, there is significant variation [6, 25, 26]. For example, commercially prepared white teas measured between 53 and 3337 mg theanine/100 g w/w [25] while oolong teas ranged from 85 to 282 mg/100 g [25], averaging 101 mg/100 g [6]. Our findings of 163.2 mg/100 g of theanine in a cup of regular oolong tea are in keeping with these previous studies. One study that prepared tea for analysis similar to the method used in the present investigation (brewing for a specified period of time in 200 mL water) found a standard (200 mL) cup of black tea contained 24.2 mg L-theanine, white tea contained 11.5 mg, while a cup of green tea contained the least theanine at 7.9 mg [27]. These values are lower than those that we report and may be related to the type of tea tested. Oolong tea was not investigated by Keenan and colleagues [27].

Syu and colleagues [6] measured theanine content in GABA-enriched tea and found it occurred in a higher concentration (198 mg/100 g) than regular teas on average. This contrasts with our finding that GABA-enriched tea measured half the theanine concentration (82.4 mg/100 g) of the regular oolong tea. On the other hand, Wang et al., [8] and Tsushida and Murai [7] found that GABA enrichment did not substantially alter theanine concentration compared to non-enriched teas. Moreover, theanine content in black teas was shown to be equivalent to green and oolong teas [25–27], therefore it appears to remain unaffected by oxidation.

Zuo [28] found caffeine concentrations were fairly stable across different commercial teas tested, including green (up to 99 mg/ 100 g), oolong (37–121 mg/100 g) and black (43 mg/100 g) teas. Roughly equivalent amounts of caffeine were also found across different teas [29–31]. Caffeine constituted 3.62% w/w of white tea, ~2.40% w/w of green teas tested (0.77–3.35% w/w), 2.77% w/w of oolong tea, and ~2.90% w/w of black teas (2.41–3.69% w/w) [29–31].

Compared with previous studies, we show higher concentrations of caffeine in the teas we tested: 267 mg/100 g dried tea leaf in regular oolong tea and 402 mg/100 g in GABA-enriched oolong tea. These differences in concentration may be explained by the method of tea sample preparation for HPLC analysis. Zuo [28] extracted tea samples in methanol and HCl then further diluted samples in water, focusing on efficient extraction. Wang and colleagues [8] dried samples, infused them in 80°C water for 20 minutes, while we infused tea samples directly in 90°C water for 10 minutes in accordance with manufacturer's instructions for consumption.

Caffeine content has previously been shown to be relatively unaffected by the cycling fermentation process involved in creating GABA-enriched tea, with no difference between freshly prepared green (3.2 mg/100 g) and GABA-enriched (3.3 mg/100 g) [8] However, we found variation in caffeine content in GABA-enriched compared with regular oolong tea.

Our findings show a lower concentration of EGCG (356 mg/100 g) in regular oolong tea compared to some previous studies, and levels near the lower limit of detection in GABA-enriched oolong tea (2.6 mg/100 g). For example, Tang and colleagues [32] found that EGCG varied between 2070 mg/100 g and 3670 mg/100 g across commercially prepared oolong teas tested. This may be due to sample preparation as well as tea source. Tang et al., [32] extracted samples with 10 mL tetrahydrofuran at 30 °C for 30 minutes, followed by 10 mL methanol:acetic acid:water

(50:3.7:46.3, *v/v/v*) mixture at 30 °C for 30 minutes to obtain all soluble components. While this sample preparation technique may be more rigorous in extraction of different constituents, it does not represent common consumption practices. Thus, our findings provide a more realistic estimate of EGCG concentrations consumed in a cup of tea. The significant reduction in EGCG content in GABA-enriched compared with regular oolong tea observed in our study likely arose through the additional fermentation process required to increase GABA content in the tea.

There are many ways of increasing the levels of GABA in food and beverages. There is a wide range of GABA-enriched fermented food products [33], lactic acid fermented green tea being an example [34]. The GABA content of mulberry leave powder as a potential functional food ingredient has been increased by sodium glutamate immersion, cold shock and anoxia [35]. A different approach is being used in tomatoes, with CRISPR/Cas9 gene editing technology used to selectively increase GABA levels by deleting the autoinhibitory domain of the enzyme that converts glutamate to GABA [36]. GABA levels in such gene-edited tomatoes increased by 11- to 18-fold accompanied by a drastic reduction in glutamate and aspartate levels. Sales of these tomatoes were launched onto the Japanese market in 2021[37]. Such gene editing technology can be applied to annual crops like tomatoes but would be difficult to readily apply to tea.

In conclusion, teas contain a range of bioactive constituents including GABA, theanine, EGCG and caffeine. GABA-enriched oolong tea was shown to have 8 times more GABA than regular oolong tea, although the quantity may not be sufficient alone to account for the stress-reducing effects of this GABA-enriched tea. It is very likely that additional constituents measured here contribute to the purported relaxant effects of GABA-enriched tea, possibly via interaction with the GABAergic system. The differences in GABA, theanine, caffeine and EGCG content between GABA-enriched and regular oolong teas demonstrated here may arise through the additional fermentation process required to enrich GABA content in the tea. In particular, the increase in the amount of caffeine may be significant.

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