Optimization of L-theanine and caffeine relative extraction/infusion from *Camellia sinensis* (tea)

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Abstract

Tea (*Camellia sinensis*), the traditional and popular drink, contains several bioactive compounds. The tea type and the preparation practices both affect the compositions of these compounds. The relaxant and anxiolytic L-theanine is an important bioactive compound unique to tea in the plant kingdom. The well-known stimulant, caffeine, is another bioactive compound of tea. The balance of concentrations of these two compounds highly determines the health effects of the tea beverage. To obtain a relaxing beverage, the L-theanine level should be higher than the level of caffeine. Several factors affect this balance such as the time, temperature, pH and solvent of extraction and any drying procedures subsequently used. The stability of these compounds is important and determines the functionality and shelf life of the tea-based products.

This research aimed to find the optimized conditions for preparing tea extracts with higher L-theanine and lower caffeine contents. Three types of tea (white, green and black tea) were extracted in water at different temperatures (~10-11, 50 and 90-100°C) and for different durations (5, 30 and 60 min) followed by simultaneous HPLC analysis of L-theanine and caffeine. At ~50 and 90-100°C, on average 16.49 and 19.76 mg/mL L-theanine and 1.34 and 1.66 mg/mL caffeine were extracted from white tea, 7.71 and 1.26 mg/mL L-theanine and 0.75 and 1.17 mg/mL caffeine extracted from black tea and 1.38 and 2.12 mg/mL L-theanine and 0.43 and 0.75 mg/mL caffeine extracted from green tea, respectively. At ~10-11°C, on average more L-theanine and caffeine were extracted from black (1.07 and 0.37 mg/mL respectively) and green tea (1.41 and 0.35 mg/mL respectively) than from white tea (0.37 and 0.08 mg/mL respectively). Overall, on average more L-theanine and caffeine were extracted from white tea, followed by black and green teas.

Hence, white tea was further extracted in room temperature water, resulting in less L-theanine (0.39±0.0005 mg/mL) and more caffeine (0.56±0.009 mg/mL) extraction compared to when infused at ~10-11°C (0.51±0.03 mg/mL L-theanine and 0.34±0.01 mg/mL caffeine).

The DPPH radical scavenging activity of tea preparations at <10°C revealed that black tea possesses the highest DPPH activity (~85%) followed by white (~76%) and green tea (~71%).

Then, tea leaves were pretreated in methanol to reduce caffeine content, through a preliminary selective extraction, before L-theanine extraction. This pre-treatment increased L-theanine by ~18% and decreased caffeine by ~19% in the water infusions subsequently prepared.

Applying two drying techniques on aqueous white tea infusions revealed that freeze-drying best retained L-theanine and caffeine levels; in contrast, spray-drying resulted in ~30% and ~45% reduction in L-theanine and caffeine levels respectively.

Ultrasound-assisted extraction (UAE) was studied on white tea in cold water and the process significantly increased the efficiency of the low-temperature extraction.

Moreover, reducing the pH of the solution did not affect L-theanine and caffeine levels. Adding milk (10% V/V) to the tea infusion reduced the caffeine content of tea preparations by ~17%. However, L-theanine was reduced only when a higher concentration of milk (50% V/V) was added.

Storage of dried and liquid white tea infusions for 60 days showed that drying can restore the DPPH activity. During the storage, the DPPH activity in freeze-dried tea was between ~86% and 93%; in spray-dried tea, it was between ~85% and 91%; and in liquid tea, it reduced from ~75% to 15%. During the storage, the L-theanine and caffeine levels in the freeze-dried samples

were relatively stable whereas in the spray-dried tea samples, L-theanine decreased by ~10%. In the liquid tea samples, L-theanine and caffeine decreased by ~32% and ~22% respectively.

In conclusion, the research results show that natural tea-based preparations can be produced with higher L-theanine and lower caffeine levels. Such preparations possess relatively high antioxidant activities which are stable for at least two months following freeze-drying and spray-drying suggesting their potential use as functional ingredients.

Résumé

Le thé (*Camellia sinensis*), est une boisson traditionnelle et populaire qui contient de nombreux composés bioactifs. Le type de thé et la méthode de préparation ont un effet sur les concentrations de ces composés. Le composé relaxant et anxiolytique L-théanine est important et particulier parce que principalement retrouvé dans le thé. Le très connu ingrédient stimulant, la caféine, est un autre composé important du thé. La relative concentration de ces deux composés influence les effets sur la santé de la consommation de thé. Afin d'assurer un effet relaxant, la concentration en L-théanine devrait être supérieure à la concentration en caféine. Plusieurs facteurs influencent les concentrations présentes tel que le temps d'infusion, la température, le pH, le solvent d'extraction, et les méthodes de séchage subséquentes. Il est important de maintenir la stabilité des composés bioactifs afin d'assurer la fonctionnalité et la durabilité des produits du thé.

Cette recherche a visé à déterminer les conditions optimales de préparation du thé assurant une plus haute concentration de L-théanine par rapport à la caféine. Trois types de thé (blanc, vert et noir) ont été infusés avec de l'eau à des températures différentes (~10-11, 50 et 90-100°C) et des durées d'infusion différentes (5, 30 et 60 min) et les concentrations de L-théanine et de caféine ont été analysées conjointement par HPLC. À ~50 et 90-100°C, des concentrations entre 16.49 et 19.76 mg/mL de L-théanine et entre 1.34 et 1.66 mg/mL de caféine ont été extraites du thé blanc, entre 7.71 et 1.26 mg/mL de L-théanine et entre 0.75 et 1.17 mg/mL de caféine ont été extraites du thé noir et entre 1.38 et 2.12 mg/mL de L-théanine et entre 0.43 et 0.75 mg/mL de cafféine extraites du thé vert, respectivement. À ~10-11°C, des concentrations plus élevées de L-théanine et de caféine ont été extraites du thé noir (1.07 et 0.37 mg/mL respectivement) et du thé vert (1.41 et 0.35 mg/mL respectivement) par rapport au thé blanc (0.37 et 0.08 mg/mL respectivement). En

général, plus de L-théanine et de caféine ont été extraits du thé blanc, suivi du thé noir et du thé vert.

De ce fait, le thé blanc a été étudié davantage en infusion à température pièce, résultant en une diminution de L-théanine (0.39±0.0005 mg/mL) et une augmentation de la caféine (0.56±0.009 mg/mL) lorsque comparé à une infusion à ~10-11°C (0.51±0.03 mg/mL L-théanine et 0.34±0.01 mg/mL caféine).

L'analyse de l'activité des radicaux par épreuve DPPH des infusions de thé à <10°C a révélé que le thé noir possédait une plus grande activité antioxydante (~85%) suivi du thé blanc (~76%) et du thé vert (~71%).

Par la suite les feuilles de thé ont été prétraitée par infusion au méthanol afin de réduire la concentration de caféine, par extraction sélective, précédant l'extraction de la L-théanine. Ce pré-traitement a augmenté la concentration en L-théanine d'environ 18% et a diminué la concentration de caféine d'environ 19% dans l'eau de l'infusion subséquente.

L'étude de deux méthodes de séchage des infusions aqueuses de thé blanc a révélé que la lyophilisation était meilleure pour la rétention des concentrations de L-théanine et de caféine; par contre le séchage par atomisation a mené à une réduction d'environ 30% de L-théanine et de 45% de la caféine.

Une extraction aux ultrasons (UAE) a été étudiée lors de l'infusion à froid du thé blanc et le traitement aux ultrasons s'est avéré améliorer l'efficacité de l'extraction à basse température.

De plus, la réduction du pH de l'infusion s'est avérée ne pas avoir d'effet particulier sur les concentrations en L-théanine et en caféine. L'ajout de lait (10% V/V) à l'infusion de thé a de

son côté réduit la concentration de caféine de 17% dans l'infusion alors que la réduction de la concentration de L-théanine a été plus importante avec un plus important ajout de lait (50% V/V).

L'entreposage des infusions de thé blanc liquides ou séchées a été étudié pendant 60 jours, et a démontré que le séchage permettait de maintenir l'activité antioxydante. Lors de l'entreposage, l'activité DPPH des échantillons lyophilisés était entre 86 et 93% alors que pour les échantillons atomisés l'activité DPPH était entre 85 et 91%. L'activité DPPH des échantillons liquides a diminué entre 75 et 15% au cours des 60 jours. Lors de l'entreposage les échantillons lyophilisés ont maintenu les concentrations de L-théanine and caféine les plus stables.

En conclusion, les résultats de recherche encouragent la préparation de produits à base d'infusion de thé ayant été produits avec une concentration supérieure en L-théanine et inférieure en caféine pour en assurer les effets anxiolytiques. De plus ces infusions possèdent une bonne et stable activité antioxydante, et ce pour un minimum de deux mois suivant la lyophilisation ou l'atomisation de l'infusion, pouvant en faire un intéressant ingrédient fonctionnel.

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Contribution to Original Knowledge

In this work, after reviewing the literature available on anti-anxiety plants and the active components that contribute to their anxiolytic effects, tea (*Camellia sinensis*) was chosen for further studies on the optimization of extraction/infusion conditions and stability of its relaxant component L-theanine. Because of the antagonistic effects of caffeine on L-theanine and the co-extraction of these two major tea components in tea extracts, it was decided to work on studying the tea preparation conditions, resembling those of tea consumers, to minimize caffeine content in tea preparations. Therefore, the levels of these active compounds were first measured and compared in black, green and white tea samples. Then different approaches were tested to either minimize caffeine or enhance L-theanine, as the main relaxing compound of tea. Hence, the impacts of extraction parameters, drying methods and storage of tea-based preparations on these compounds were investigated.

The data presented in this dissertation have provided valuable insights into the effects of the tea type, extraction temperature, extraction time, solvent, pH, ultrasound application and addition of milk on L-theanine and caffeine levels in the extracts. Also, this work provides information on the association of active components present in black, green and white tea extracts with their antioxidant status as estimated by the DPPH radical scavenging activity. Data on the effects of different drying techniques and storage on L-theanine and caffeine levels, DPPH radical scavenging activities and moisture content of dried tea infusions are important contributions to knowledge about natural tea-based drinks and extracts as consumables with high levels of L-theanine. Additionally, a fast and efficient HPLC technique was developed to simultaneously and quantitatively determine both the L-theanine and caffeine levels in the samples.

Contribution of Authors

Mina Allameh, Ph.D. candidate in the Department of Bioresource Engineering at McGill University, designed and performed experiments, data analysis and preparation of the Ph.D. dissertation and related publications. Prof. Valérie Orsat, Professor in the Department of Bioresource Engineering, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada, supervised this project. She assisted in directing, planning and executing this work, provided funding and scientific guidance, and edited and reviewed the dissertation and related publications. So far, the following manuscripts have been published:

Review article:

1. Allameh, M., & Orsat, V. (2023). Herbal Anxiolytics: Sources and Their Preparation Methods. Food Reviews International, 39(8), 4992–5020. https://doi.org/10.1080/87559129.2022.2043895

Research articles:

2. Allameh, M., & Orsat, V. (2023). Optimization of extraction conditions for the maximum recovery of L-theanine from tea leaves: Comparison of black, green, and white tea. JSFA Reports, 3(12), 655–662. https://doi.org/10.1002/jsf2.175
3. Allameh, M., & Orsat, V. (2024). Effects of time, ultrasonic treatment and pH during extraction on l-theanine and caffeine yields from white tea leaves. Future Foods, 9, 100304. https://doi.org/10.1016/j.fufo.2024.100304
4. Allameh, M., & Orsat, V. (2024). Freeze drying and spray drying for the retention of the active components, L-theanine and caffeine, and antioxidant activity of tea-based ingredients.

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1. Allameh, M., & Orsat, V. Improvement of extraction and drying techniques for increasing L-theanine yield in white tea infusion. Submitted to Journal of Medicinal Food.
2. Allameh, M., & Orsat, V. The correlation of L-theanine and caffeine levels with the antioxidant activity of different tea infusions and changes in L-theanine and caffeine contents of white tea infusions supplemented with fresh milk. Submitted to Measurement: Food.

Conference presentations:

1. Presented poster: Optimization of extraction conditions to increase theanine and decrease caffeine contents in extracts prepared from three types of tea; Mina Allameh and Valérie Orsat. Canadian Society for Bioengineering/ La Société Canadienne de Génie Agroalimentaire et de Bioingénierie (CSBE/SCGAB), Charlottetown, PEI, Canada (July 24-27, 2022).
2. Presented poster: Effects of time and ultrasonic treatment during extraction on L-theanine and caffeine yields from white tea leaves; Mina Allameh and Valérie Orsat. Green Food Tech, Montreal, QC., Canada (May 18-19, 2023).

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List of Abbreviations

ΔT: temperature change

°C: degree Celsius

Å: angstrom

ANOVA: analysis of variance

$A\beta_{1-42}$: amyloid beta

BBB: blood-brain barrier

C: (+)-catechin

CG: (−)-catechin gallate

CNS: central nervous system

CO_2: carbon dioxide

c_p: specific heat capacity

CTC: cut, tear, curl; or crush, tear, curl

DIS: dewatering and impregnation soaking

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EAE: enzyme-assisted extraction

EC: (−)-epicatechin

ECG: (−)-epicatechin gallate

EGC: (−)-epigallocatechin

EGCG: (−)-epigallocatechin gallate

FAO: Food and Agriculture Organization of the United Nations

FD: freeze-drying

g: gram

GABA: gamma-aminobutyric acid; γ-aminobutyric acid

GC: (−)-gallocatechin

GCG: (−)-gallocatechin gallate

GHz: gigahertz

HD: hydro-distillation

HPA: hypothalamic–pituitary–adrenal

HPLC: high-performance liquid chromatography

hr: hours

J: joule

kHz: kilohertz

L: litre

m: total mass

MAE: microwave-assisted extraction

MAOIs: monoamine oxidase inhibitors

MD: microwave drying

mg: milligram

MHz: megahertz

min: minute

mL: millilitre

mm: millimeter

mM: millimolar

mol: mole

nm: nanometer

OD: osmotic drying

PEF: pulsed electric field-assisted extraction

pH: potential of hydrogen

PI: isoelectric point

PPO: polyphenol oxidase

PVPP: polyvinylpolypyrrolidone

Q: total energy absorbed by the sample

ROS: reactive oxygen species

RPM: revolutions per minute

RT: retention time

RTD: ready-to-drink

s: second

SC-CO_2: supercritical CO_2

SD: standard deviation

SE: Soxhlet extraction

SFE: supercritical fluid extraction

SNRIs: serotonin and norepinephrine reuptake inhibitors

SSRIs: selective serotonin reuptake inhibitors

TCAs: tricyclic antidepressants

TF_1: theaflavin

TF_2A: theaflavin-3-gallate

TF_2B: theaflavin-3’-gallate

TF_3: theaflavin-3,3’-digallate

TFs: theaflavins

TRs: thearubigins

UAE: ultrasound-assisted extraction

US: ultrasound

USD: the United States dollar

UV: ultraviolet

V: volume

VD: vacuum drying

VWD: variable wavelength detector

W: weight

μm: micrometer

Chapter 1: Introduction

1.1. Background

Both tea and coffee contain caffeine as a stimulating agent (Francis, 2000), however, contrary to coffee, considered as a stimulating beverage, tea is considered as a relaxing beverage. The compound responsible for the relaxing effect of tea has been recognized to be L-theanine (Giles et al., 2017; Juneja et al., 1999; Rogers et al., 2008).

Some people do not tolerate caffeine and its stimulating effects on the body (Ramalakshmi & Raghavan, 1999). In addition, the caffeine present in tea, at equal or higher doses may suppress the anti-stress effects of L-theanine (Unno et al., 2016; Unno et al., 2013). L-theanine in the presence of caffeine, has been shown to successfully impart its anti-stress effect when its level is higher than that of caffeine (Unno et al., 2013).

L-theanine and caffeine have known counteracting effects, and to benefit from the relaxing effects of L-theanine, either L-theanine should be isolated or caffeine should be removed or at least kept to a minimal concentration. Isolation of the naturally present L-theanine from tea has been proven to be costly, time-consuming, complicated and inefficient (Mu et al., 2015). The production rate of L-theanine using a 732 cationic resin column followed by preparative HPLC was low and only 2.53 g purified L-theanine could be obtained in 24 hr (Y. Zhang et al., 2004). A long and tedious procedure to isolate L-theanine from tea solution using a cation exchange resin was reported, requiring the regeneration of the resin after each adsorption-desorption cycle (Ye et al., 2011). The chemical synthesis of L-theanine, offering the potential for large-scale availability, is not considered adequately acceptable to the majority of consumers (Mu et al., 2015). The

decaffeination of tea is problematic as the process involves using organic solvents and causes the presence of trace amounts of organic solvents in the final product which can have negative health effects for the consumers. Also, the solvents used for decaffeination are sometimes non-selective and can cause the loss of other valuable compounds present in the tea (Varnam & Sutherland, 1994). Additionally, the decaffeination process may negatively affect the taste and aroma of the final product (Ramalakshmi & Raghavan, 1999). Known as the most commonly used decaffeination method, liquid CO_2 supercritical fluid extraction removes other compounds along with caffeine which negatively affects the taste and aroma of the decaffeinated product (Lean et al., 2011). Other processes reported, including the use of resin and charcoal or microbial treatment are considered costly (Baldi et al., 2020). Therefore, a cost-effective and environmentally friendly alternative is to reduce the caffeine concentration, in proportion to L-theanine, to benefit from L-theanine's health effects without being inhibited by a more modest amount of caffeine remaining. This may be achievable by accurate modulation of the extraction and drying procedures used in the preparation and processing of tea.

1.2. Rationale

Tea contains both L-theanine and caffeine and these compounds are extracted simultaneously in the water used for the infusion of tea. Therefore, to suppress the inhibitory effect of caffeine on the relaxing effect of L-theanine, the balance of the relative concentrations of these two compounds is important. This study attempted to obtain an appropriate balance between L-theanine and caffeine by modifying different factors namely, the type of tea, extraction time, extraction temperature, extraction solvent, the use of ultrasounds and the use of additives (i.e. lemon juice and milk). The impacts of drying and storage on these compounds and the

functionality, stability and antioxidant activity of the tea samples were assessed. The rationale for each factor examined is mentioned below.

1.2.1. Tea Type

Due to the abundance of tea cultivars, varieties and grades, the composition and relative amounts of bioactive compounds are highly different in various tea types such as black, green and white tea. The level of these compounds initially depends on many factors such as cultivation conditions, amount of sunshine, plant breed and geographical region (Unno et al., 2016). The compositions of amino acids that exist in different tea varieties have been successfully used to differentiate and classify different types of tea (Alcázar et al., 2007). Alcázar et al. (2007) observed that non-fermented teas such as white and green tea, contained higher amounts of free amino acids. Alcázar et al. (2007) therefore suggested a correlation between the level of tea fermentation and the amount of free amino acids in the tea. Higher amounts of L-theanine were also observed in white and green teas compared to black, oolong and pu-erh teas (Alcázar et al., 2007).

1.2.2. Extraction Time

Other factors responsible for the differences in the amounts of active compounds in tea are linked to the tea preparation practices. Bioactive compounds in tea have different leaching rates and amino acids have been shown to infuse faster than caffeine in cold water (Fukushiyama et al., 1999). Also, it was shown that in 80°C water, most of L-theanine was extracted from tea during the first 5 min of the extraction and, following the first 5 min, only a small increase in the extraction of L-theanine was observed (Keenan et al., 2011). In addition, Perva-Uzunalić et al. (2006) observed that after aqueous extraction of green tea at 80°C or 95°C starting temperatures, the extraction of caffeine plateaued after 2.5 min; however, at a 60°C starting temperature, the

extraction of caffeine reached a constant maximum after 15 min of extraction (Perva-Uzunalić et al., 2006), indicating that at a lower temperature, the rate of caffeine extraction is significantly slower. These results indicate that L-theanine may have a faster or slower extraction rate than caffeine depending on the temperature of the extraction.

1.2.3. Extraction Temperature

It has been shown that the extraction temperature influences the composition and concentrations of the bioactive compounds found in tea infusions (Monobe, 2018). The caffeine in tea is easily extracted in hot and boiling water; while it is less efficiently extracted in cold water. In contrast, L-theanine is more easily extracted in cold water. Infusion of tea in cold water (0.5°C) yielded less than 20% of the amount of caffeine extracted with hot water (80°C); whereas about 80% of the amount of L-theanine extracted with hot water (80°C) was extracted in cold water (0.5°C) (Monobe, 2018). Fukushiyama et al. (1999) showed that when infused in cold water (7 to 8 °C), tea amino acids were extracted faster than caffeine (Fukushiyama et al., 1999). S.-D. Lin et al. (2008) showed that caffeine levels extracted from green tea with cold water (4°C for 24 hr) and hot water (90°C for 20 min) were different and the extraction of caffeine from green tea was lower with cold water compared to hot water (S.-D. Lin et al., 2008). It was also shown that significantly more caffeine is dissolved in water at 100°C compared to 75°C and 50°C extraction temperatures (H. Liang et al., 2007). At room temperature, the maximum water solubility of caffeine is only 2%, while its solubility in boiling water reaches 70% (Ramalakshmi & Raghavan, 1999). While, at 0°C, 1 g of L-theanine can be dissolved in as little as 2.6 mL water (Ho et al., 2009). These results confirm that at different temperatures, caffeine and L-theanine are infused/extracted at different rates. The solubility of caffeine in water is widely affected by the temperature of the water (Ramalakshmi & Raghavan, 1999), whereas, a temperature change seems to have a smaller effect

on the solubility of L-theanine. This was confirmed by the study of Keenan et al. (2011), which showed that equal amounts of L-theanine are extracted from tea with either hot or cold water (80°C and 12°C) (Keenan et al., 2011). In addition, it was shown that spraying a hot water shower at 95°C on freshly plucked tea leaves for 180s or 280s could preferentially extract caffeine and significantly reduce the caffeine level in the tea leaves to approximately 1/4 to 1/5 of the caffeine level in untreated tea leaves (i.e. not sprayed with hot water); while this 180s and 280s hot-water treatment only slightly reduced the L-theanine levels to 93% and 83% respectively, compared to tea leaves not sprayed with hot water (Unno et al., 2016).

1.2.4. Extraction Solvent

The molecular affinity between the solvent and the bioactive compounds, defined by their polarity, determines the efficiency of the extraction solvent (Azmir et al., 2013; Jun, 2009). L-theanine is considered a very water-soluble amino acid, which is however claimed to be insoluble in many organic solvents such as ether, ethanol, methanol and chloroform (Ho et al., 2009; Vuong et al., 2011; Williams et al., 2016). Compared to caffeine, L-theanine, with its zwitterionic structure is relatively more soluble in water and less soluble in organic solvents, including methanol (Vuong et al., 2011). Caffeine is only moderately water-soluble (Ramalakshmi & Raghavan, 1999), while it has been shown to be more soluble in methanol. Methanol (pure or in aqueous solutions at 50% V/V) was shown to be more efficient in the extraction of caffeine from green tea leaves than either pure water or pure ethanol (Jun, 2009). The differences in the solubility of these compounds in different solvents can be used for their selective extraction and isolation.

1.2.5. Ultrasound-Assisted Extraction

Ultrasound-assisted extraction (UAE) is known to increase the extraction efficiency of bioactive compounds. It has been suggested that UAE can increase the extraction efficiency of compounds at lower temperatures which could be favourable for the preferential extraction of L-theanine over caffeine from tea leaves (T. Xia et al., 2006). Additionally, T. Xia et al. (2006) showed that UAE may have a heightened selective effect as it had an inhibitory effect on the extraction of some compounds, namely protein and pectin from tea (T. Xia et al., 2006).

1.2.6. Additives

Around the world, tea beverage is prepared in different ways. Adding milk or lemon juice is customary in many countries (Astill et al., 2001; Venditti et al., 2010; Weisburger, 1997). It has been reported by S.-H. Kim et al. (1999) that the total amino acid content of green tea aqueous solutions did not change at pH values between 4 and 7. However, the caffeine content of the tea solutions increased as the pH increased from 4 to 7 (S.-H. Kim et al., 1999). In another report, Vuong et al. (2013) observed no change in L-theanine and caffeine levels extracted from green tea at pH values between 1 and 9 (Vuong et al., 2013). Keenan et al. (2011) adjusted the pH level of brewing water to 3, 7 or 9 before adding tea and observed no substantial difference in the amount of L-theanine extracted from tea after brewing under these pH values (Keenan et al., 2011).

Adding a high amount of milk to tea (50 mL milk per 200 mL of tea infusion) has been reported to substantially reduce the level of L-theanine. However, lower amounts of milk (5 and 12 mL per 200 mL of tea infusion) did not greatly affect the L-theanine level (Keenan et al., 2011). Additionally, it has been reported that the addition of milk to the green tea brew, significantly reduced the caffeine level in the resulting tea-milk preparations (Ferruzzi & Green, 2006).

These reports indicate that the method of preparation of a tea beverage may affect the levels of L-theanine and caffeine in the beverage. Additives such as milk and lemon juice may be beneficial in reducing the caffeine level or increasing the L-theanine level in tea preparations.

1.2.7. Drying Techniques

Food products are dried for a variety of reasons such as preservation and maintaining the high quality and functionality of the product (Akpinar et al., 2003; Lewicki, 2006; Phisut, 2012; Sagar & Suresh Kumar, 2010), reducing the volume and weight of the product and consequently the costs of packaging, handling, transport and storage (Akpinar et al., 2003; Dev & Raghavan, 2012; Lewicki, 2006; Orikasa et al., 2014). Freeze drying and spray drying are two of the well-established drying methods preferred for quality food products. Freeze-drying (lyophilization) is known to preserve the original structure and shape of the product (Ratti, 2001) and the quality of the heat-sensitive materials (Parikh, 2015). Freeze-drying is recognized as the best drying technique that can preserve the high quality of foods and food ingredients (Ratti, 2024). Spray drying on the other hand is also very commonly used (Jafari & Rashidinejad, 2021) for drying fluids, and is a relatively low-cost technique (J. Shi, 2016). The prospect of using these drying techniques for preparing powdered nutraceuticals and functional food ingredients is beneficial in reducing the water activity, moisture content and bulk density, while increasing the shelf life of a variety of food and food ingredients with desirable quality (Kalkan et al., 2017; Manickavasagan et al., 2015). The application of these drying techniques for producing stable and functional tea-based products with high L-theanine and low caffeine levels needs to be assessed.

1.2.8. Antioxidant Activity

In the human body, an imbalance between oxidants and antioxidants results in oxidative stress and excessive free radical production which can be detrimental to health (Rahal et al., 2014; Salim, 2017). Tea has been shown to possess strong antioxidant properties (Yen & Chen, 1995), mainly attributed to its polyphenols (Dufresne & Farnworth, 2001; C. S. Yang et al., 2002). Rusak et al. (2008) reported a significant linear correlation between the total polyphenol content and the antioxidant capacity of white and green tea extracts (Rusak et al., 2008). Similarly, Seeram et al. (2006) found a significant correlation between antioxidant activity and the total polyphenol content of green tea dietary supplements (Seeram et al., 2006). However, Song et al. (2012) reported that major tea catechins, i.e. (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC) and (−)-epicatechin gallate (ECG), only account for about 20% of the antioxidant activity of tea leaf extracts (Song et al., 2012). While, Joo et al. (2012) observed that the free radical scavenging activities of different tea extracts were higher than the standard phenolic compounds, (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG), at the same concentration with the total phenolic content equivalent (Joo et al., 2012). Therefore, it has been speculated that other factors may contribute to the antioxidant properties of tea (Yen & Chen, 1995), especially due to the possible low bioavailability of tea polyphenols (C. S. Yang et al., 2002). Joo et al. (2012) observed that in addition to the phenolic compounds present, the amino acid content enhanced the free radical-scavenging activities of tea extracts (Joo et al., 2012). Deng et al. (2016) demonstrated the antioxidative activity of L-theanine *in vivo*. They observed that the antioxidative activity of L-theanine is through the regulation of non-enzymatic pathways (Deng et al., 2016). Jo et al. (2011) reported that L-theanine significantly and dose-dependently reduced the generation of intracellular reactive oxygen species induced by amyloid beta ($A\beta_{1-42}$) in human

neuroblastoma cells. They also observed that pre-treatment of neuronal cells with L-theanine helped in reducing lipid and protein oxidative damage (Jo et al., 2011). Zeng et al. (2020) demonstrated the antioxidant activity of L-theanine using a rat model. They reported that L-theanine can improve the activities of antioxidant enzymes (Zeng et al., 2020). Nagai et al. (2018) showed the antioxidant activity of L-theanine by suppressing oxidative stress in rats (Nagai et al., 2018). G. Li et al. (2012) observed that L-theanine could prevent oxidative stress in the liver caused by ethanol *in vivo* and *in vitro*, by significantly decreasing the production of reactive oxygen species (ROS), inhibiting lipid peroxidation and enhancing the activities of antioxidant enzymes (G. Li et al., 2012). On the other hand, caffeine has been shown to also possess some antioxidant activities. Devasagayam et al. (1996) showed that 1 mM caffeine has strong antioxidant activities significantly higher than that of ascorbic acid (Devasagayam et al., 1996). Similarly, X. Shi et al. (1991) demonstrated that caffeine is an efficient scavenger of hydroxyl radicals (X. Shi et al., 1991). Moreover, other bioactive compounds in tea have been shown to have strong antioxidant activity. Leung et al. (2001) showed that the antioxidant activity of black tea theaflavins and green tea catechins was higher than that of ascorbic acid (Leung et al., 2001).

1.2.9. Storage

Increased demand for healthy, minimally processed and long-lasting food products has led to intensive research into different ways of increasing the storage life of food products while maintaining their inherent qualities (Cohen & Levin, 2011). Food products naturally undergo chemical changes during storage causing the loss of their nutrients and detrimental changes in their properties. Processing and storage conditions may exacerbate these quality changes and affect the storage life (Alam, 2021). Food products are often dried to increase their storage life and for the products to be preserved from deteriorating factors such as microorganisms, insects and oxidative

and enzymatic reactions (Mujumdar, 2021; Orsat et al., 2006). The impact of storage on the stability and functionality of tea-based products in terms of their bioactive compounds, antioxidant activities and moisture change is essential to investigate.

1.3. Hypotheses

According to the background data from scholarly sources and the rationales discussed, the following hypotheses are put forth and evaluated in this thesis.

1. Tea, with proven health benefits, can be utilized as an anti-anxiety and antioxidant functional food ingredient with favourable stability and shelf-life when the caffeine content is reduced to comparatively lower levels than the L-theanine content.
2. Adjusting the extraction conditions such as tea type, extraction time and temperature, extraction solvent and pH, applying ultrasound and addition of milk can alter L-theanine and caffeine levels and their relative compositions in the final product.
3. Different types of tea, manufactured with different processes, contain different levels and compositions of L-theanine and caffeine.
4. According to the data provided in the literature, non-fermented white and green tea may have higher levels of L-theanine compared to black tea.
5. In cold water, amino acids have a faster infusion rate compared to caffeine, and therefore L-theanine is easily extracted in low-temperature water, while the extraction of caffeine is more efficient in hot water. Tea infused for shorter times at lower temperatures thus contains more L-theanine than caffeine.
6. Pre-treatment of tea in methanol preferentially extracts the caffeine content, leaving the L-theanine unextracted from the tea leaves. This principle can be used for the preliminary

removal of caffeine. After the pre-treatment of tea with methanol, the methanol can be separated from the tea leaves for subsequent further extraction. Infusion of this pre-treated tea in water yields greater concentrations of L-theanine and lower concentrations of caffeine than the infusion of methanol-untreated tea leaves. The separated methanolic filtrate, on the other hand, will contain more caffeine than L-theanine which can also find an application for caffeine extraction.

7. Applying ultrasound can enhance extraction efficiency at lower temperatures. Given that L-theanine is the main ingredient of a tea infusion obtained at low temperatures, the extraction efficiency of this compound can be preferentially increased using ultrasound at low temperatures.
8. Based on the data available in the literature about the effect of pH on the extraction of L-theanine and caffeine, the common practice of adding lemon juice to a tea infusion would not affect the L-theanine level of tea infusions however, adding lemon juice to the tea infusion, and thus reducing the pH, may reduce the caffeine levels.
9. Adding small amounts of milk to tea infusions will not have a significant effect on the L-theanine levels; however, higher amounts of milk may reduce the level of L-theanine in the tea infusions. Also, adding milk will dose-dependently reduce the caffeine level in tea infusions.
10. Freeze-drying the tea infusions can yield a concentrated, high-quality product which can be used as the quality standard for comparison of spray-dried and liquid/undried tea infusions.
11. Infusions of different types of tea prepared in cold water have good levels of antioxidant activity which may be correlated with their L-theanine and/or caffeine levels.

12. Storage of dried and undried tea infusions will provide information about their stability and functionality in terms of their L-theanine and caffeine contents, antioxidant activity and moisture uptake in the case of the dried products during storage.
13. Drying the tea infusions increases the stability of L-theanine and caffeine in the final products during storage.
14. Drying the tea infusions also increases the stability of the antioxidant activities of the final products during storage.
15. Selecting the appropriate preparation process can help modify the L-theanine-to-caffeine ratio, by either minimizing the elution of caffeine or increasing the extraction rate of L-theanine more effectively than that of caffeine.
16. By selecting the appropriate preparation process, a low-caffeine natural tea-based functional food ingredient can be developed with several potential applications, with desirable quality and stability.

1.4. Objectives

The general purpose of this research study is to understand the behaviour of L-theanine and caffeine during the extraction of tea and find a way to adjust their proportion in a tea infusion and ultimately gain from the beneficial physiological effects (anti-anxiety) of L-theanine and minimize the over-stimulating effects of caffeine. The specific objectives of the present study are to:

1. find out which tea type contains more L-theanine and/or less caffeine and which has higher antioxidant activities;

2. know how much L-theanine and caffeine is extracted from tea at different temperatures and after different infusion durations;
3. understand what combination of tea type, extraction time and temperature is optimal in terms of a high L-theanine-to-caffeine ratio;
4. ascertain the feasibility of reducing caffeine and increasing the L-theanine-to-caffeine ratio by pre-treatment of tea leaves in methanol;
5. determine which drying technique can better retain the functionality of tea infusions in terms of their L-theanine and caffeine levels;
6. find out whether or not applying ultrasound-assisted extraction (UAE) can improve the cold-water extraction of L-theanine while limiting the caffeine extraction from tea (i.e. improving the L-theanine-to-caffeine ratio);
7. know whether or not adding milk or lemon juice affects the levels of L-theanine and caffeine and their proportion in tea infusions;
8. determine the stability of the bioactive molecules, L-theanine and caffeine, in dried and undried tea infusions and the stability of antioxidant activities of dried and undried tea infusions during storage;
9. develop extraction procedures and HPLC analyses to simultaneously determine L-theanine and caffeine levels in tea extracts;
10. develop a low-caffeine or L-theanine-rich tea extract as a valuable anti-anxiety food ingredient that also has antioxidant activities, stable L-theanine levels, and acceptable shelf life and can be introduced in a variety of food formulations.

Chapter 2: Literature Review

2.1. Functional Foods and Bioactive Compounds

The Food and Agriculture Organization of the United Nations (FAO) defines functional food as "a foodstuff that provides a health benefit beyond basic nutrition, demonstrating specific health or medical benefits, including the prevention and treatment of disease" (Zaid et al., 2001). Health Canada defines a functional food as being "similar in appearance to" or maybe "a conventional food" that "is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (Health Canada, 1998).

Bioactive compounds can be defined as compounds/molecules present in foods that when consumed, affect the body (Fernandes et al., 2019).

Owing to their several biological effects, L-theanine and caffeine are considered as bioactive compounds of tea; and tea with demonstrated physiological benefits is considered a functional food. Sometimes, bioactive compounds need to be processed and preserved. Different extraction and drying techniques have been developed for this purpose.

2.2. Extraction Techniques

Techniques for the extraction of bioactive compounds have been divided into two categories: conventional techniques and non-conventional techniques. Some of the conventional extraction techniques include Soxhlet extraction (SE), hydro-distillation (HD) and maceration. Some of the non-conventional extraction techniques include ultrasound-assisted extraction (UAE),

microwave-assisted extraction (MAE), pulsed electric field-assisted extraction (PEF), supercritical fluid extraction (SFE) and enzyme-assisted extraction (EAE) (Azmir et al., 2013; Subramanian & Anandharamakrishnan, 2023).

2.2.1. Soxhlet Extraction (SE)

The Soxhlet extraction (SE) technique involves a specific apparatus in which the solvent is heated until it reaches its boiling point where the solvent vapour travels up a distillation arm, and floods into the thimble housing the plant material. An attached condenser ensures that any solvent vapour is condensed and falls back onto the plant materials in the thimble resulting in the extraction of the bioactive compounds. The bioactive compounds are then separated from the solvent, traditionally by using a rotary evaporator. The procedure used in this technique is time-consuming and may cause the loss of heat-sensitive compounds (Harbourne et al., 2013; Jha & Sit, 2022; Radrigán et al., 2017; Subramanian & Anandharamakrishnan, 2023).

2.2.2. Hydro-Distillation (HD)

In the hydro-distillation (HD) technique, the plant material is placed in contact with boiling water (called water distillation), steam (called direct steam distillation) or a mixture of the two (called water and steam distillation), resulting in the extraction of bioactive compounds in hot water and steam. The steam is then cooled and condensed and the bioactive compounds are separated. The main physicochemical processes involved in this technique are hydro-diffusion, hydrolysis and decomposition by heat. This technique is not suitable for heat-sensitive compounds due to the higher temperatures used (Aramrueang et al., 2019; Azmir et al., 2013; Radrigán et al., 2017).

2.2.3. Maceration

Maceration is a traditional and simple technique which involves grinding and soaking the plant materials in a solvent, called “menstruum”, for a long period of time (ranging from a few hours to a few days) and usually at lower temperatures which results in the rupture of the cell walls and release of the bioactive compounds into the solvent. This extraction procedure is followed by filtering out the solvent and pressing the solid residue, called “marc”. This technique is time-consuming with low efficiency. Also, the procedure used in this technique suffers from the potential growth of microorganisms leading to spoilage or fermentation (Azmir et al., 2013; Jha & Sit, 2022; Radrigán et al., 2017; Subramanian & Anandharamakrishnan, 2023).

2.2.4. Ultrasound-Assisted Extraction (UAE)

UAE is an environmentally friendly extraction technique (Egüés et al., 2021), which uses acoustic cavitation to pose mechanical and thermal effects on cell walls leading to their disruption and subsequent extraction of bioactive compounds (Subramanian & Anandharamakrishnan, 2023). Ultrasounds are sound waves with frequencies above the range of human hearing (16 kHz). When ultrasound (US) waves are passed through a medium, they produce alternating compression and expansion cycles. While compression cycles cause positive pressure, the expansion cycles cause negative pressure which can overcome the tensile strength of the media and produce cavities. The cavities are formed by the expansion of the gas trapped in the crevices of solid particles present in the medium which leads to the release of small bubbles. These bubbles (cavities) absorb the compression and expansion energy and keep a cycle of growing and contracting. The cavities keep growing until they implode which leads to the generation of shock waves that dissipate leading to a significant rise in the temperature of their surrounding liquid. The temperature rise usually does

not increase the temperature of the whole medium because it occurs in very small localized spots. In the presence of solid surfaces in the liquid, the pressure caused by ultrasounds is distorted leading to the generation of a liquid jet with a high speed. This jet along with the shock waves caused by the implosion of cavities, erodes the solids' surfaces. This leads to the breakdown of the cell walls, consequently facilitating the penetration of the solvent into the plant material and improving the mass transfer (Carciochi et al., 2017; Chemat et al., 2011; Suslick, 1989).

2.2.5. Microwave-Assisted Extraction (MAE)

Microwaves are electromagnetic waves with frequencies between 300 MHz to 300 GHz. Similar to other electromagnetic waves, microwaves consist of an electric field and a magnetic field (C.-H. Chan et al., 2011). The electric fields of the microwaves induce the movement of polar molecules which creates heat (Letellier & Budzinski, 1999; Radrigán et al., 2017). The main mechanisms of heating by MAE are ionic conduction and dipole rotation. During the ionic conduction, the ions migrate causing resistance of the solution which results in friction and heat dissipation in the solution. During the dipole rotation, the dipoles are realigned which also results in heat dissipation (Sparr Eskilsson & Björklund, 2000). When heat is generated inside cells, their moisture content evaporates which poses internal pressure to the cell walls and disrupts them enough until the cell walls rupture leading to an enhanced extraction (Mandal et al., 2007). Due to the heat levels generated in this technique, MAE is usually not suitable for the extraction of heat-sensitive compounds (Routray & Orsat, 2012).

2.2.6. Pulsed Electric Field-Assisted Extraction (PEF)

In the PEF-assisted extraction technique, the plant/food material is placed between two electrodes and exposed to a pulsed voltage field. The electric power passes through the cell

membrane and increases the potential difference across the cell membrane which disrupts the cell membrane and increases its permeability by electroporation, also known as electro-permeabilization. This facilitates the transfer of cell molecules to the solvent. The PEF-assisted extraction is considered to be a costly process (Radrigán et al., 2017; Subramanian & Anandharamakrishnan, 2023; Vorobiev & Lebovka, 2010; Wijngaard et al., 2013).

2.2.7. Supercritical Fluid Extraction (SFE)

In this technique, the solvent should be at the supercritical region (high temperature and pressure combination). The supercritical region of a solvent is when it is above its critical temperature and critical pressure. The critical temperature is the highest temperature above which a gas can no longer be converted into its liquid form by increasing the pressure while the critical pressure is the highest pressure above which a liquid can no longer be converted into gas by increasing the temperature (Wijngaard et al., 2013). This technique, therefore, uses supercritical solvents, which have low viscosity and high diffusivity. The density of a supercritical fluid is similar to that of a liquid, its viscosity is similar to that of a gas and its diffusivity is between gas and liquid (Herrero et al., 2006). In this technique, the chemical compounds present in the plant material are solubilized in the supercritical solvent followed by the removal of the solvent by reducing the pressure or increasing the temperature (da Silva et al., 2016). Various solvents can be used; however, CO_2 is most often utilized because it is environmentally safe, cheap, food-grade, available at high purity, and has relatively low critical temperature and pressure, making it useful for heat-labile compounds and simplifying the processing conditions. When CO_2 is used, this technique is called supercritical CO_2 extraction (SC-CO_2) (Wijngaard et al., 2013). In summary, for the SFE technique, the gas utilized is compressed until it is liquefied. Then the liquid and the plant matrix are mixed and after the extraction is carried out the supercritical fluid containing the

extracted molecules is moved to a separation chamber. In the separation chamber, the temperature of the fluid is increased or its pressure is decreased to reduce its solvating power and subsequently separate the extracted bioactive compounds (Ingle et al., 2017; Radrigán et al., 2017). The equipment used for this technique is considered to be more costly than conventional extraction techniques (Subramanian & Anandharamakrishnan, 2023).

2.2.8. Enzyme-Assisted Extraction (EAE)

The EAE technique involves hydrolysis of the plant cell wall using specific exogenous enzymes that have the capacity to degrade the cell wall, to facilitate cell disruption and thus extract the compounds that are otherwise inaccessible. Such enzymes include cellulase, α-amylase, and pectinase (Kleekayai et al., 2023; Radrigán et al., 2017; Wijngaard et al., 2013). The EAE technique for the extraction of bioactive compounds from plants is considered to be relatively costly, with unpredictable and low efficiency, and difficult to scale up (Puri et al., 2012).

In summary, most of the well-known extraction methods are based on applying high temperatures (e.g. Soxhlet extraction, hydro-distillation and microwave-assisted extraction), while others are either time-consuming, low-efficiency, costly or difficult to scale up (e.g. maceration, pulsed electric field-assisted extraction, supercritical fluid extraction and enzyme-assisted extraction). Therefore, it is important to choose/develop an extraction technique that is highly efficient at lower temperatures, in order to obtain an extraction output with higher yield and quality.

According to Monobe (2018), cold water is less efficient than hot water in the extraction of caffeine from tea while it is almost equally efficient as hot water in the extraction of L-theanine (Monobe, 2018). This was further confirmed by Keenan et al. (2011) by showing that the extraction

of L-theanine from tea was not affected by the water temperature (hot or cold) (Keenan et al., 2011). However, L-theanine has been shown to thermally degrade at 180°C (Yamanishi et al., 1989). Therefore, to reduce the extraction of caffeine from tea without negatively affecting the extraction of L-theanine, it is important to apply low temperatures during extraction.

Accordingly, for the present research, a conventional and a non-conventional extraction technique was studied for the extraction of L-theanine from tea. The conventional technique applied in this research work uses water as the solvent and resembles the domestic tea preparation practice. Ultrasound-assisted extraction (UAE) was selected as the non-conventional extraction technique because it can operate at low temperatures which is beneficial in enhancing the product's quality and decreasing the extraction of caffeine.

2.3. Drying Techniques

Traditionally, food products have been dried to be preserved (Akpinar et al., 2003; Lewicki, 2006). Besides increasing the shelf life, drying is useful for reducing the volume and weight of food and agricultural products and consequently the cost and difficulty of packaging, handling, storage and transport (Akpinar et al., 2003; Dev & Raghavan, 2012; Gaukel et al., 2017; Lewicki, 2006; Orikasa et al., 2014; Orsat et al., 2006). Drying is the process of removing or reducing the moisture content of food and agricultural products to reduce their water activity and consequently reduce the enzymatic and microbial activities, inactivate insects which may be present in such products, and reduce resulting quality loss (Gaukel et al., 2017; Phisut, 2012; Sagar & Suresh Kumar, 2010). Several drying techniques exist including microwave drying (MD), osmotic drying (OD), vacuum drying (VD), freeze-drying (FD) and spray drying. Dryers are categorized into two groups: direct dryers and vacuum dryers. Direct dryers (or convective dryers) use hot air, for

convective heat and mass transfer purposes, and near atmospheric pressure. Whereas vacuum dryers, including freeze dryers and vacuum dryers, use reduced pressure to help draw the moisture out of the samples (Parikh, 2015).

2.3.1. Microwave Drying (MD)

Microwaves (in the electromagnetic spectrum), are of frequencies between 300 MHz to 300 GHz (Orsat et al., 2017). In this drying technique, microwaves interact with materials as a function of their dielectric properties where the microwave energy is absorbed by the material and the energy is dissipated as heat through mechanisms including ionic conduction and dipolar rotation (Schiffmann, 1995). By ionic conduction, the electric field moves ions in the opposite direction to their polarity which causes their collision with unionized molecules resulting in the transfer of energy and heating (Orsat et al., 2006; Schiffmann, 1995). By dipolar rotation, the alternating electric field causes the dipolar molecules (such as water) to rotate into alignment and random orientation which converts the electrical energy to thermal energy pushing the moisture out of the samples (Schiffmann, 1995; Sutar & Prasad, 2008) during drying coupled with forced-air or vacuum.

2.3.2. Osmotic Drying (OD)

This technique is also known as osmotic dehydration, or dewatering and impregnation soaking process (DIS). In this drying technique, food products are immersed in highly concentrated aqueous solutions (sugar or salt solutions). This results in the transfer of water out of the product and into the solution (Raoult-Wack, 1994). This technique is suitable as a pre-treatment for other drying methods since the dehydration level is only intermediate (Dev & Raghavan, 2012).

2.3.3. Vacuum Drying (VD)

In this technique, reduced pressure is used which makes it possible to apply lower drying temperatures (Parikh, 2015). Under vacuum, air and water vapour in the product are expanded at lower temperatures which increases the surface area, heat and mass transfer (Jaya & Das, 2003) and vapour escape, with better retention of the thermo-sensitive quality parameters (Dev & Raghavan, 2012).

2.3.4. Freeze-Drying (FD)

Freeze-drying, also known as lyophilization, is a drying technique involving freezing the moisture content of the product, and subsequent sublimation of the frozen moisture under reduced pressure directly from the solid state into the gas state (Dev & Raghavan, 2012; Gaukel et al., 2017; Ngamwonglumlert & Devahastin, 2018; Parikh, 2015). This technique is a form of vacuum drying (Gaukel et al., 2017) with sublimation. Due to the low temperature used for moisture removal, along with the mass transfer occurring from solid to gas forms, freeze drying produces final products of higher quality (Dev & Raghavan, 2012; Ratti, 2001), and is suitable for drying heat-sensitive materials (Parikh, 2015). This method notably can preserve the original structure and shape of the product (Ratti, 2001). However, this technique is considered to be time-consuming, costly and highly energy-intensive (Ngamwonglumlert & Devahastin, 2018; Ratti, 2001, 2024). The freeze-drying procedure involves three processes: 1- freezing the product, 2- sublimation of ice into vapour, also called primary drying and 3- desorption of the bound water, also called secondary drying (Ratti, 2024).

2.3.5. Spray Drying

This technique involves atomizing a liquid in contact with a hot gas (usually air) to evaporate the moisture and convert the liquid to a solid powder (Gharsallaoui et al., 2007; Mujumdar, 1995; Roustapour et al., 2009). This method is also widely used for encapsulation and microencapsulation of food products by adding wall materials such as starch derivatives and food gums to protect the bioactive compounds from degradation (Murugesan & Orsat, 2012). This technique may be suitable for preserving heat-sensitive materials due to the rapid evaporation which keeps the temperature of the drying material relatively low. Also, it can be used for large-scale production which has been largely commercially adopted (Mujumdar, 1995). Microencapsulation by spray drying is considered cost-effective and simple and widely used in food ingredients and bioproducts drying (Murugesan & Orsat, 2012). However, some materials such as fruit and vegetable juices tend to produce a sticky output which makes their spray-drying difficult with lower yields due to sticking to the drying chamber (Krishnaiah et al., 2014). Additionally, this technique may not be suitable for exceptionally heat-sensitive materials such as biopharmaceuticals which easily degrade (Krishnaiah et al., 2014). Spray drying follows four phases: 1- feed atomization which maximizes the heat transferring surface, 2- hot gas and droplet contact during atomization, 3- drying of feed (droplets) by evaporation, and 4- separation of the dry products in a collection chamber (Gharsallaoui et al., 2007; Murugesan & Orsat, 2012).

Spray-drying and freeze-drying techniques are efficient drying techniques commonly used for processing functional foods with high quality. Functional foods have the potential to promote human health by affecting the body's biological responses. They have health-promoting effects on diseases including gastrointestinal diseases, cancers, cardiometabolic syndrome, neurodegenerative disorders and mental health disorders and can improve healthy aging (Aguiar

et al., 2019; Dutta et al., 2020). Therefore, in the present research, spray-drying and freeze-drying techniques are used to dry tea extracts and preserve their L-theanine contents as the functional anti-anxiety component of tea.

2.4. Functional Foods for Alleviating Mental Health Disorders

Anxiety disorders are the most common mental health disorders in the world followed by depressive disorders (World Health Organization, 2022, 2023). Similar to other health conditions, the state of mental health disorders can be affected by food and dietary intake (Musa, 2022).

Anxiety is defined as the anticipation of a threat rather than the response to a real threat. Anxiety disorders typically last for at least six months and affect women twice as much as men (American Psychiatric Association, 2013). Some of the different types of anxiety disorders as defined by the American Psychiatric Association are separation anxiety disorder (persistent fear of separation from or harm to attachment figures), selective mutism (constant refusal to speak in social situations where the person is expected to speak), specific phobia (persistent fear or avoidance of specific objects or situations such as animals, natural environment, blood and flying), social anxiety disorder (persistent fear or avoidance of social situations in which the person may be scrutinized), panic disorder (recurrent and unexpected panic attacks and persistent concern about having more panic attacks), agoraphobia (persistent fear or avoidance of situations such as using public transport, being in open or enclosed spaces, being in a crowd or standing in line or leaving home alone) and generalized anxiety disorder (persistent and excessive worry about different issues including work, school, health and finances, accompanied by physical symptoms including restlessness, irritability, muscle tension and sleep disturbance) (American Psychiatric Association, 2013).

Anxiety disorders are usually treated with pharmacological agents such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), benzodiazepines, selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs) (Constable et al., 2022; Garakani et al., 2020). These medications can cause drowsiness, memory impairment, impaired concentration, gastrointestinal problems, sleep disturbances, sexual dysfunction, fatigue, weight gain and ocular side effects. They may also be associated with the risk of dependence, tolerance or abuse (Anagha et al., 2021; Constable et al., 2022; Garakani et al., 2020). Therefore, naturally sourced bioactive compounds with demonstrated anti-anxiety effects can be good alternatives or adjuvants to ameliorate mental health conditions.

2.4.1. Anti-anxiety Phytochemicals and Their Sources

There are several natural compounds with anxiolytic effects. Some of these compounds and their natural sources are introduced in Table 1 (Allameh & Orsat, 2023; Juneja et al., 1999; Muszyńska et al., 2020).

Table 1- Some of the naturally sourced anxiolytics and their sources

Anxiolytic compound	Natural source
Saponins	• American ginseng (*Panax quinquefolius*) • Fenugreek (*Trigonella foenum-graecum*)
Quercetin	• Cardamom (*Elettaria cardamomum*) • Coriander (*Coriandrum sativum*)

	• Ginkgo (*Ginkgo biloba*) • Common hop (*Humulus lupulus*) • Indian cassia (*Cinnamomum tamala*)
Linalool	• Coriander (*Coriandrum sativum*) • True lavender (*Lavandula angustifolia*) • Indian cassia (*Cinnamomum tamala*)
Kaempferol	• Ginkgo (*Ginkgo biloba*) • Indian cassia (*Cinnamomum tamala*)
Rosmarinic acid	• Lemon balm (*Melissa officinalis*) • Rosemary (*Rosmarinus officinalis*)
Chrysin	• True passion flower (*Passiflora incarnata*) • Skullcap (*Scutellaria lateriflora*)
1,8-cineole	• Rosemary (*Rosmarinus officinalis*) • Sage (*Salvia* spp.)
Camphor	• Rosemary (*Rosmarinus officinalis*) • Sage (*Salvia* spp.)

α-pinene	• Rosemary (*Rosmarinus officinalis*) • Sage (*Salvia* spp.)
L-theanine	• Tea (*Camellia sinensis*) • Bay bolete (*Imleria badia*)

2.5. Tea (*Camellia sinensis*)

The present study is focused on tea for its high content of L-theanine. Tea (*Camellia sinensis* (Linnaeus) O. Kuntze) is a perennial crop (Hilal & Engelhardt, 2007; Ho et al., 2009; Nair, 2021) belonging to the genus *Camellia*; family Theaceae. *C. sinensis* is native to Southwestern China and the name "tea" originates from the Chinese "ch'a" (Nair, 2021).

With an annual global production of more than 17 billion USD (FAO, 2022), the tea plant is adaptable to extreme conditions. However, the optimal conditions for its growth are tropical and subtropical climates, 23 to 30 °C temperature, 2500–3000 mm annual rainfall, well-drained soils with pH between 4.5 to 5.5 and shading for diffused light (Ho et al., 2009; Nair, 2021). The largest tea-producing country is China, followed by India (FAO, 2022).

During the last decade, the global per capita consumption of tea increased by 2.5 percent (FAO, 2022). Tea has been shown to have several positive health effects. Tea consumption by humans has been associated with reduced risk of type 2 diabetes mellitus (Y. Chen et al., 2020), lung cancer (Seow et al., 2020), colorectal cancer (Shimizu et al., 2008), cardiovascular diseases

(X. Li et al., 2017; X. Wang et al., 2020), osteoporosis (Huang et al., 2023), anxiety and depression (Bakhriansyah et al., 2022; S.-P. Chan et al., 2018; Pan et al., 2017), reduced body weight (Auvichayapat et al., 2008) and reduced blood pressure (Hodgson et al., 2012) to name a few.

After water, tea is the most consumed drink in the world (Ho et al., 2009) and there are about 3000 different tea types (Nair, 2021). Once harvested, tea leaves are processed not only to reduce their water content but also to generate taste and aroma (L. Zhang et al., 2019). Some of the tea types are described below:

2.5.1. Black Tea

Black tea is fully fermented tea leaves and buds and is manufactured when tea polyphenols are oxidized by atmospheric oxygen which is catalyzed by the tea polyphenol oxidase enzyme (Lunder, 1992; Nair, 2021; Zhen, 2002). This process which is incorrectly known as tea fermentation, is an enzymatic oxidation and polymerization process, in which the polyphenols (monomers) and oxidized polyphenols form dimers or polymers (Long et al., 2023; Nair, 2021). Two important pigment products in black tea are formed as a result of this process: theaflavins (TFs) (which are orange-red) and thearubigins (TRs) (which are dark brown). The mixture of theaflavins and thearubigins in black tea is called oligomeric polyphenols (Nair, 2021). Generally, for the manufacture of black tea, following harvest, fresh tea leaves are withered, rolled, fully fermented at about 25–35°C and more than 95% humidity and fired (dried) to inactivate the enzyme systems and stop the fermentation/oxidation process. Finally, the leaves are graded according to the tea leaves particle size (Ho et al., 2009; Spiller, 1998). There are two major categories of black tea based on the rolling technique: 1- orthodox and 2- CTC (cut/crush, tear, curl). Orthodox black tea is produced when the leaves are cut, squeezed and twisted by machines

called rollers. CTC black tea is produced when the rolling of the leaves is done by cutting them into less than 1 mm particles (Ho et al., 2009; Nair, 2021; Spiller, 1998).

2.5.2. Green Tea

Green tea is a non-fermented tea which is generally produced by immediately fixing (a term used for steaming or heating) freshly harvested tea leaves to rapidly inactivate the polyphenol oxidase, followed by rolling in order to break the leaf cells and release the leaf sap. Finally, the leaves are fired (dried). Due to the inactivation of the polyphenol oxidase, this type of tea does not undergo any fermentation/oxidation, hence, the main pigment in green tea infusions is chlorophyll (Ho et al., 2009; Nair, 2021; Zhen, 2002).

2.5.3. Oolong Tea

Oolong tea is a semi-fermented tea which is mainly produced in China and Taiwan (Nair, 2021; Spiller, 1998). For the production of oolong tea, the harvested tea leaves are first withered in the sunlight for 30 to 60 min. Then, the leaves undergo another withering indoors for 6 to 8 hours while being manually agitated. During this step, which is also called “rotating”, fermentation happens only on the edges of tea leaves. Subsequently, the leaves are pan-fired to inactivate enzymes and stop fermentation (a step called fixing), rolled and dried (Ng et al., 2018; Varnam & Sutherland, 1994; Zhen, 2002).

2.5.4. White Tea

White tea is made from the tea buds still covered with fine white hair and leaves that are not fully opened. White tea is minimally processed hence the structure of the leaf cells remains intact. After harvesting, the tea buds and immature leaves are sun-withered, followed by drying.

During white tea processing, the tea leaves undergo slight oxidation and consequently, a small amount of catechins converts to theaflavins and thearubigins (Damiani et al., 2014; Ho et al., 2009).

2.5.5. Dark Tea

This tea is manufactured by microbial fermentation of the leaves. Briefly, following harvesting, the fresh tea leaves are steamed or heated (a process which is called fixing) and then rolled. Subsequently, they are oxidized by pile fermentation and finally dried. One important type of dark tea is pu-erh tea, which originates from China (Ho et al., 2009; Zhu et al., 2020). There are two processes for manufacturing pu-erh tea: 1- the classical pressing process and 2- the wet-piling process. In both processes, the harvested tea leaves are baked on a hot pan to be blanched, followed by rolling and sun-drying of the rolled leaves to obtain a product called “dried raw tea”. At this stage, the classical pressing process involves storing the “dried raw tea” in a dry place, followed by classifying it, removing foreign objects, weighing it and then steaming it for 40–50 seconds, and subsequently pressing it into different shapes and incubating it for ripening. In the wet-piling process, on the other hand, the “dried raw tea” undergoes fermentation by increasing the water content to 20% or higher, and piling up the tea biomass, followed by air-drying of the fermented tea biomass, classifying, pressing into different shapes and storing (Ho et al., 2009).

2.5.6. Yellow Tea

This type of tea is produced in a manner similar to green tea with an extra processing step. After freshly harvesting the tea leaves, they are fixed (which is the process of steaming or heating the tea leaves) and rolled and before being dried, they undergo a process called “yellowing”, also known as “sealed yellowing”. The “yellowing” process involves piling the tea leaves and auto-

oxidizing them. During the “yellowing” process, the chlorophyll and polyphenols in the tea leaves undergo oxidation due to the presence of moisture and heat (Ho et al., 2009; J. Xu et al., 2018).

2.5.7. GABA Tea

GABA tea, also known as Gabaron tea in Japan, is a newer type of tea which was developed in 1987. This tea is high in γ-aminobutyric acid (GABA). GABA accumulates in plant tissue as a result of different stressors such as extreme temperature and pH, lack of oxygen and water, and physical damage. This principle is used for the accumulation of GABA within the tea leaves during the processing and manufacture of GABA tea. Different procedures can be used for the manufacture of different types of GABA tea. But generally, anaerobic treatment is used in all procedures (Baldi et al., 2020; Ho et al., 2009).

2.6. Bioactive Compounds in Tea

The different manufacturing processes applied for producing each type of tea, result in different compositions of many different bioactive compounds of these teas (Butt & Sultan, 2009). Tea leaves contain over 4000 important bioactive compounds including purine alkaloids, amino acids and polyphenols (Koch, 2021). Purine alkaloids in tea include caffeine, theobromine and theophylline (Ho et al., 2009). The most abundant amino acid in tea is L-theanine (Alcázar et al., 2007; Horanni & Engelhardt, 2013; Jiang et al., 2019). Polyphenols present in tea include catechins, theaflavins, and thearubigins (Ho et al., 2009). Tea catechins (flavan-3-ols) include (+)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), (−)-gallocatechin gallate (GCG) and (−)-epigallocatechin gallate (EGCG) (Baldi et al., 2020; Dalluge & Nelson, 2000). Theaflavins and thearubigins are two important pigments of black tea. Theaflavins are reddish-orange pigments

and thearubigins are dark brown (Ho et al., 2009; Nair, 2021). Theaflavins (TFs) include theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3'-gallate (TF_2B), and theaflavin-3,3'-digallate (TF_3) and are produced by oxidation and polymerization of their parent catechins during the fermentation/oxidation process (Koch, 2021; Sang et al., 2011; Takemoto & Takemoto, 2018). Thearubigins however, are believed to be a group of poorly identified heterogeneous compounds (Koch, 2021; Long et al., 2023). Since theaflavins and thearubigins are formed during the fermentation/oxidation process by the conversion of catechins, initiated by polyphenol oxidase (PPO), unfermented green tea does not contain these pigment compounds while semi-fermented oolong tea contains a mixture of theaflavins and thearubigins as well as catechins (Ho et al., 2009).

L-theanine and caffeine, two of the most important bioactive compounds in tea, have opposite effects on mood. While caffeine is a stimulating compound, L-theanine causes relaxation. Since these compounds are co-extracted during tea infusion, the possibility of preferential extraction of L-theanine over caffeine, for imparting relaxation effects, needs to be investigated.

2.7. L-Theanine

L-theanine, L-γ-glutamylethylamide (5-*N*-ethyl glutamine) (molecular formula: $C_7H_{14}N_2O_3$) is a rare non-protein amino acid, almost exclusive to *Camellia sinensis* (Ho et al., 2009; L. Wang et al., 2022). L-theanine is the most abundant amino acid in tea and constitutes 30% to more than 50% of the total amino acids in *C. sinensis* (Alcázar et al., 2007; Horanni & Engelhardt, 2013). L-theanine is very water-soluble with an umami taste (Ho et al., 2009; Vuong et al., 2011; Williams et al., 2016).

Drinking tea is associated with a feeling of relaxation attributed to its L-theanine (Juneja et al., 1999; L. Wang et al., 2022). L-theanine is considered a safe compound (Juneja et al., 1999)

and does not accumulate in the body (Ho et al., 2009). After ingestion, L-theanine is absorbed through the intestinal brush-border membrane and distributed to different body organs (L. Wang et al., 2022). L-theanine is absorbed into the brain through the blood-brain barrier (BBB) (Yokogoshi et al., 1998). Figure 1 shows the chemical structure of L-theanine.

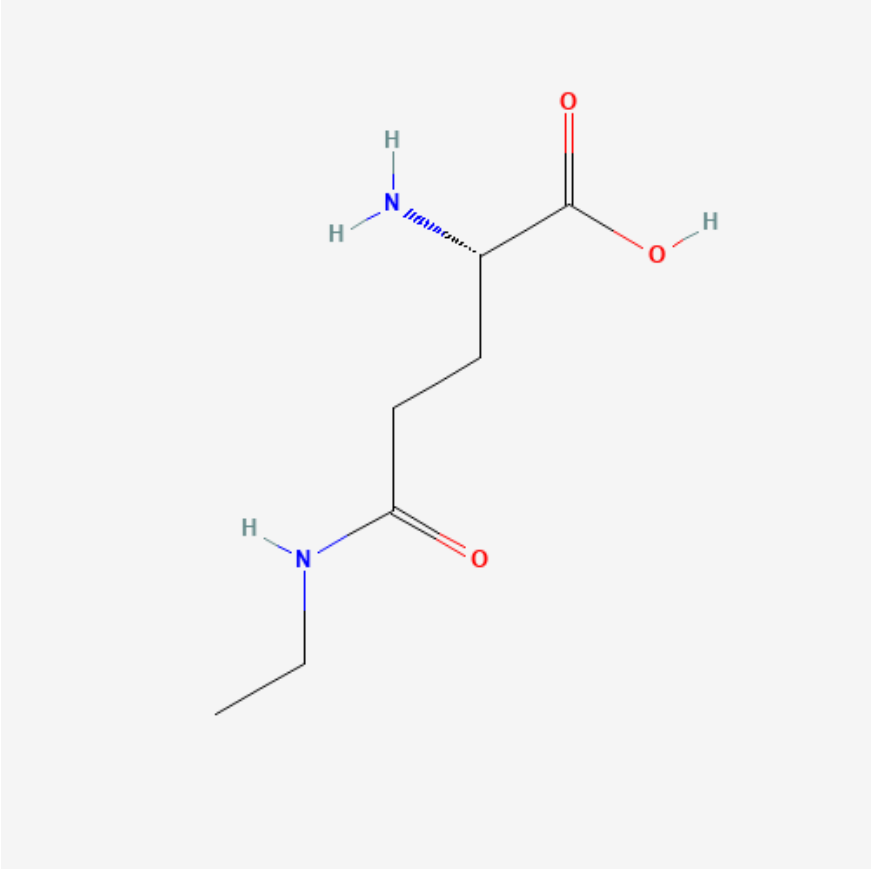


Figure 1- Chemical structure of L-theanine.

Reproduced from a figure from the National Center for Biotechnology Information, PubChem (National Center for Biotechnology Information, 2004b).

2.8. Caffeine

Caffeine, 1,3,7-trimethylxanthine ($C_8H_{10}N_4O_2$), one of the most popular drugs in the world and a central nervous system (CNS) stimulant is found in more than 60 plants, including tea, coffee, cacao, guarana, kola and mate (Carvalho et al., 2012; Fiani et al., 2021; Francis, 2000; Ho et al., 2009; Spiller, 1998). Caffeine easily dissolves in boiling water (Spiller, 1998) and the over-consumption of caffeine can be dangerous, while the consumption of about 10 g of caffeine by humans can be fatal (Ramalakshmi & Raghavan, 1999). Figure 2 presents the chemical structure of caffeine.

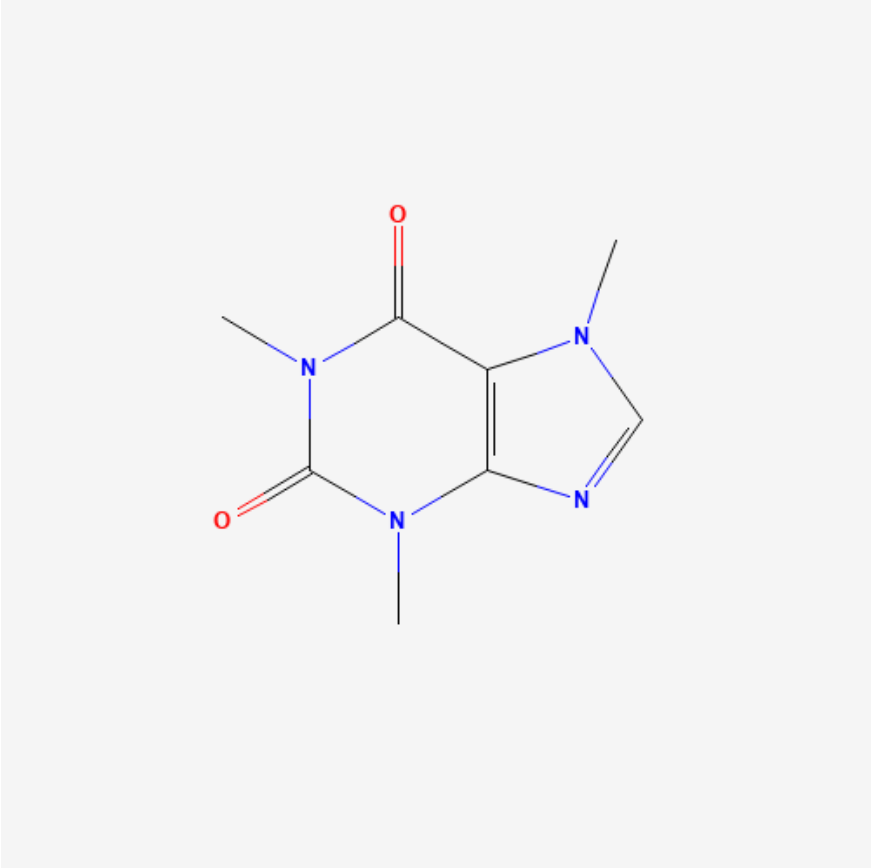


Figure 2- Chemical structure of caffeine.

Reproduced from a figure from the National Center for Biotechnology Information, PubChem (National Center for Biotechnology Information, 2004a).

Table 2 presents some of the reported properties of L-theanine and caffeine (Carvalho et al., 2012; Ho et al., 2009; H. Liang et al., 2007; Mu et al., 2015; Vuong et al., 2011).

Table 2- Physical properties of L-theanine and caffeine

Physical property	L-theanine	Caffeine
Molecular formula	$C_7H_{14}N_2O_3$	$C_8H_{10}N_4O_2$
Molecular mass	174.2 g mol^{-1}	194.19 g mol^{-1}
Water solubility	At 0°C: 385 g L^{-1} At 100°C: 556 g L^{-1}	At 20°C: 21.7 g L^{-1} At 100°C: 666 g L^{-1}

2.9. Biological Effects of L-Theanine and Caffeine and Their Interactions

L-theanine and caffeine each have health effects when consumed by humans. L-theanine induces relaxation indicated by the generation of alpha brain waves (Juneja et al., 1999; Kobayashi et al., 1998). It is an anxiolytic and antidepressant and can improve sleep (Hidese et al., 2017; Hidese et al., 2019; Shamabadi et al., 2023). Animal studies showed that L-theanine had antidepressant effects by increasing the levels of monoaminergic neurotransmitters such as serotonin and dopamine in limbic–cortical–striatal–pallidal–thalamic-circuit related brain regions and suppressing the HPA axis alterations during chronic stress (Shen et al., 2019; Unno et al.,

2013). Accordingly, modulation of the HPA axis activity by L-theanine was implicated in its anti-stress effects (Unno et al., 2013). Other animal studies showed that L-theanine could reduce blood pressure (Yokogoshi et al., 1995) and prevent ischemic neuronal death (Kakuda, Yanase, et al., 2000). Other *in vivo*, *in vitro* and *ex vivo* studies showed the protective effects of L-theanine against cancers (Q. Liu et al., 2009; J. Ma et al., 2022), its antioxidative and anti-inflammatory effects (Nagai et al., 2018; C.-C. Yang et al., 2023; Zeng et al., 2020), regulation of immune functions (A. Liu et al., 2021), and facilitation of neurogenesis in the developing hippocampus (Takeda et al., 2011).

Caffeine, on the other hand, has been shown to reduce the risk of pre-diabetes and to modify insulin levels and insulin resistance in humans (Mirmiran et al., 2018). In addition, caffeine consumption in humans has been associated with a lower risk of Parkinson's disease (Palacios et al., 2012) and Alzheimer's disease (Maia & De Mendonça, 2002). However, in humans, caffeine consumption has been shown to increase blood pressure (Hartley et al., 2004), and maternal caffeine intake was associated with adverse birth outcomes including lower birth weight (L.-W. Chen et al., 2018) and preterm birth (Okubo et al., 2015).

When consumed by humans, caffeine was shown to increase systolic and diastolic blood pressure, whereas L-theanine antagonized this increase (Rogers et al., 2008). However, when consumed together by humans, the combination of L-theanine and caffeine could improve attention and memory (Einöther et al., 2010; Owen et al., 2008). In contrast, their effects on excitation and stimulation of the central nervous system in rats were antagonistic (Kakuda, Nozawa, et al., 2000). In mice, ingestion of L-theanine reduced stress. However, this anti-stress effect of L-theanine was suppressed by caffeine at equal or higher doses (Unno et al., 2016; Unno

et al., 2013). It has therefore been suggested that the anti-stress effects of L-theanine in tea, can be enjoyed when the amount of caffeine is lowered (Unno et al., 2016).

Chapter 3: Methodology

After a thorough review of the literature and gathering a comprehensive list of anxiolytic plants and their bioactive compounds (Allameh & Orsat, 2023), tea (*Camellia sinensis*) was chosen for further experiments exploring tea as a potential functional food ingredient for its active anxiolytic component, L-theanine.

3.1. Materials

High-quality tea samples were purchased from the local market. The tea samples, research materials, chemicals and equipment used are listed below:

- White tea (bai hao yin zhen; from Fujian province, China)
- Black tea (lapsang souchong; from Fujian province, China)
- Green tea (from Hubei province, China)
- L-theanine standard (≥ 98%; HPLC; Sigma® Life Science; China)
- Caffeine standard (Sigma reference standard; Sigma-Aldrich; USA)
- Polyvinylpolypyrrolidone (PVPP) (Fluka® Analytical; Sigma-Aldrich; Germany)
- Polyvinylpolypyrrolidone (PVPP) (Supelco®; Sigma-Aldrich; China)
- DPPH (2,2-diphenyl-1-picrylhydrazyl) (Aldrich® Chemistry; Sigma Aldrich)
- Maltodextrin (dextrose equivalent 4.0-7.0; Aldrich® Chemistry; Sigma Aldrich)
- Gum acacia (Importers Service Corporation; USA)
- Filter paper #2 (Whatman™)
- Filter paper P8 (Fisherbrand®; Fisher Scientific)
- Filter paper #4 (Whatman®)

- Syringe filter 0.45 μm (Whatman™ Puradisc™)
- Screw thread amber glass vials with rubber-lined caps (Fisherbrand®; Fisher Scientific; USA)
- Labconco FreeZone® 2.5 litre benchtop freeze dryer system
- Büchi B-290 mini spray dryer
- Probe sonicator (450 Sonifier, Analog Cell Disruptor; Branson Ultrasonics Corporation; USA)
- UV/Visible spectrophotometer (Ultrospec 2100 pro)
- Agilent 1100 Series HPLC system (Agilent Technologies, Inc.) with variable wavelength detector (VWD) (wavelength = 210 nm)
- Discovery® C18 column (25 cm × 4.6 mm, 5 μm) for HPLC
- Phenomenex® column-Gemini 5μm C18 110Å (150 × 4.60 mm, 5 μm) for HPLC

3.2. Effect of Time, Temperature and Tea Type on L-Theanine and Caffeine Extraction

3.2.1. Extraction of Different Teas at Different Temperatures for Different Durations

Three tea types were selected (white tea, black tea and green tea) and were ground using a laboratory mortar and pestle and sieved using a #35 or 500 μm sieve. One gram of each ground and sieved tea was used for extraction and analysis.

For extraction, HPLC-grade water at different temperatures was used as the solvent. The temperature of HPLC-grade water was adjusted to about 10-11°C, 50°C, or 90-100°C. For that purpose, the HPLC-grade water was heated on a hotplate to reach about 50°C or 90-100°C; or placed in a refrigerator to reach about 10-11°C. Then, 20 mL of the water at each temperature was added to 1 g of the white, black, and green powdered tea samples. The mixtures (1 g tea/20 mL water) were kept at constant temperature (~ 10-11°C, 50°C, or 90-100°C) for 5, 30, and 60 min extraction times. For that, the mixtures were either placed on the hotplate at about 50°C or 90-100°C for 5, 30 or 60 min while stirring at 200 RPM; or were placed in the refrigerator (~ 4°C) for 5, 30 or 60 min without stirring. Figure 3 summarizes the procedure used for this experiment.

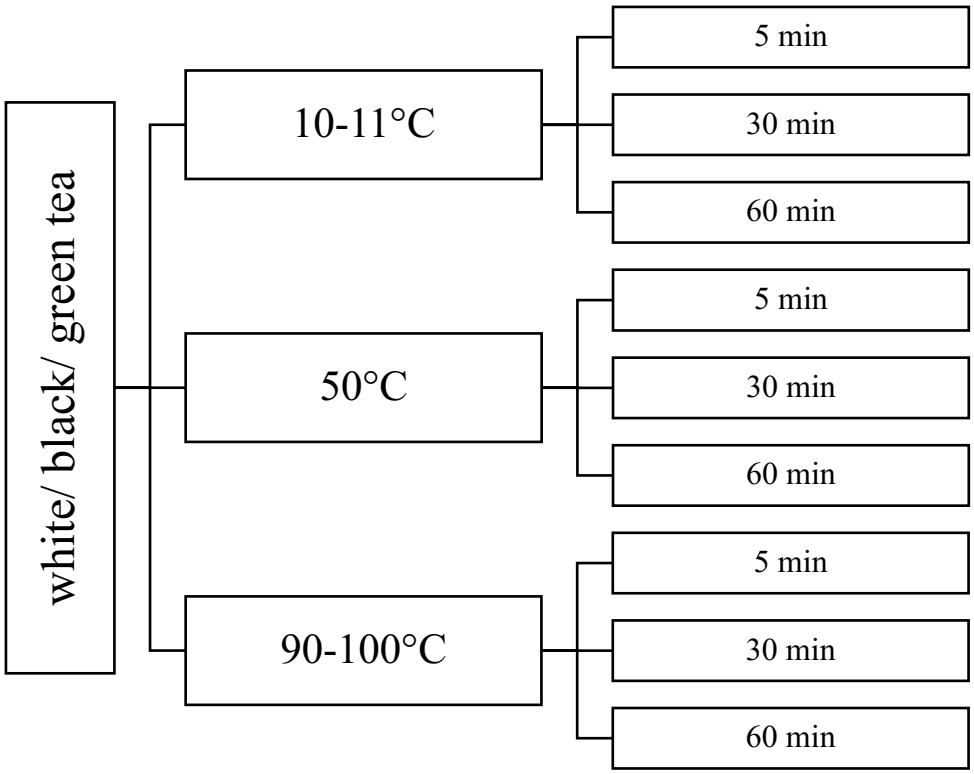


Figure 3- Testing of the effect of temperature and time on the extraction of L-theanine and caffeine from white, black and green teas.

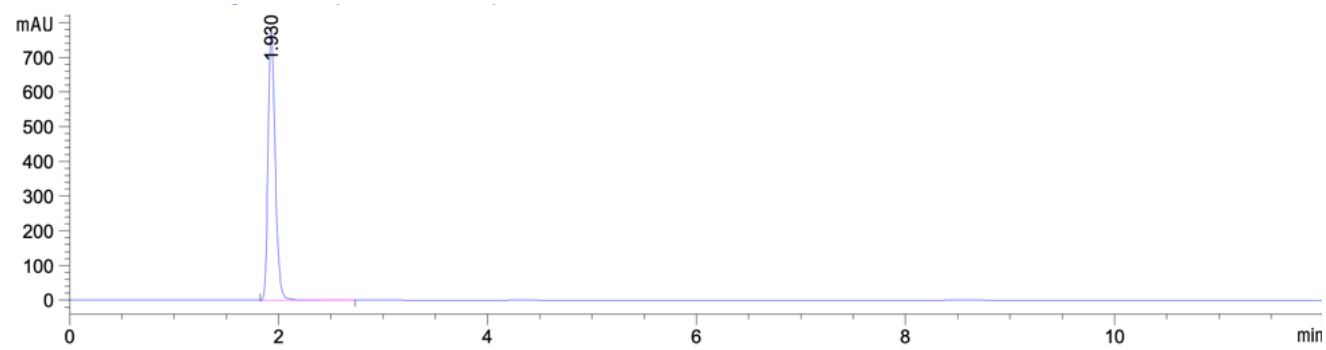
When the extraction time (5, 30 or 60 min) reached completion, each tea and water mixture was filtered using filter paper #2 (Whatman™), to separate the tea biomass. Then, polyvinylpolypyrrolidone (PVPP; Fluka® Analytical; Sigma-Aldrich; Germany) was added to each sample at the ratio of 1 g PVPP/20 mL tea, to eliminate the polyphenols, and each sample was stirred at 180 RPM and room temperature for 15 min (method adopted from Henríquez-Aedo et al. (2013) and Unno et al. (2016)). Finally, each tea sample was transferred into HPLC sample vials by filtering through 0.45 μm syringe filters (Whatman™). The samples were protected from light and kept in the freezer at -18°C until they were analyzed by HPLC in triplicate.

3.2.2. Determination of L-Theanine and Caffeine Levels in Tea Extracts Using HPLC

For the simultaneous HPLC analyses of L-theanine and caffeine in the samples, first, separate standard curves for L-theanine and caffeine were prepared. Different concentrations of authentic standard L-theanine (≥ 98%; HPLC; Sigma® Life Science; China) and caffeine (Sigma reference standard; Sigma-Aldrich; USA) were prepared and separated by HPLC (Agilent 1100 Series), using the modified procedure described by Agilent Technologies, Inc. (Naegele, 2016). The peak areas for standard L-theanine and caffeine solutions obtained by HPLC were plotted against the concentrations of the standards, to prepare separate standard curves for L-theanine and caffeine. Then, the trendline equation of each standard curve, the retention time (RT) of each standard obtained by HPLC and the peak areas of the samples obtained by HPLC were used to calculate the concentrations (mg/mL) of L-theanine and caffeine in each tea sample.

For the HPLC analyses, HPLC Agilent 1100 Series, Discovery® C18 column (25 cm × 4.6 mm, 5 μm), and variable wavelength detector (VWD) (wavelength = 210 nm) were used. The mobile phase was methanol with HPLC-grade water (25:75% V/V). The column temperature was set at 25°C and the flow rate was 1 mL/min. The run time was 12 min and the post-run time was 3 min. All the analyses were performed in triplicate. Figure 4 shows representative HPLC peaks of both the standards, L-theanine and caffeine.

a



b

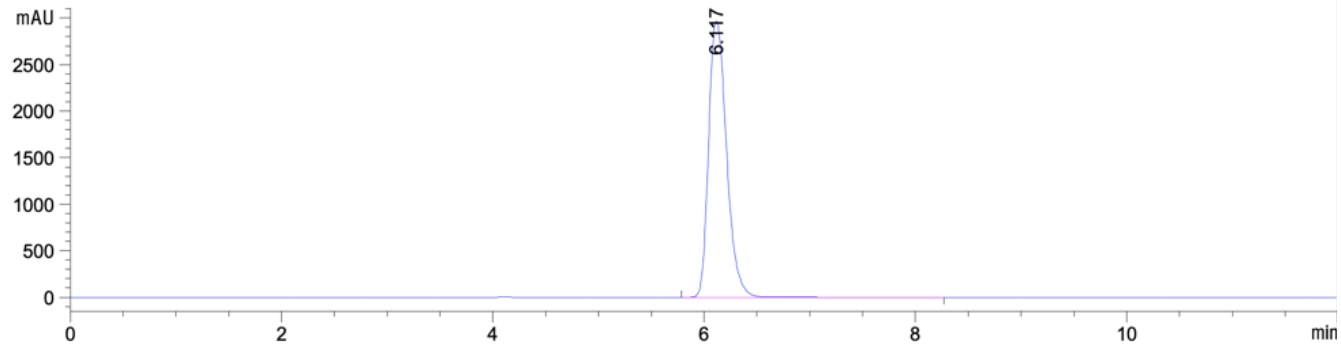


Figure 4- Standard peaks for a) L-theanine and b) caffeine obtained by HPLC.

The retention time (RT) for the L-theanine standard was 1.9 min and for the caffeine standard, it was 6.1 min.

3.3. Effect of Room Temperature on L-Theanine and Caffeine Extraction from Tea

3.3.1. Extraction Procedure of White Tea at Room Temperature

In the previous section, white, black and green teas were extracted at about 10-11°C, 50°C and 90-100°C for 5 min, 30 min and 60 min. From the results obtained, extraction of white tea at ~10-11°C for 5 min was chosen as the optimum condition, since under these conditions, the lowest level of caffeine was extracted and the highest L-theanine-to-caffeine ratio was obtained. Therefore, the 5 min extraction of white tea at ~10-11°C was selected for further experiments. In this section, this optimum extraction condition is compared with extraction at room temperature.

Exactly 20 mL cold HPLC-grade water (~10-11°C) was added to exactly 1 g of the ground and sieved white tea leaves (bai hao yin zhen, sieved through a #35 or 500 μm sieve). In parallel, exactly 20 mL HPLC-grade water at room temperature (~21°C) was added to another 1 g of the ground and sieved white tea.

The sample prepared with ~10-11°C water was placed in the refrigerator (at ~ 4°C) for 5 min, while the sample prepared with ~21°C water was left at room temperature for 5 min extraction time. After the 5 min extraction, each of the two samples was vacuum filtered using #4 Whatman® filter paper. Then, PVPP (Supelco®; Sigma-Aldrich; China) was added to each sample at the ratio of 1 g PVPP/20 mL tea, and the samples were stirred on a magnetic stirrer for 15 min at room temperature (method adopted from Henríquez-Aedo et al. (2013) and Unno et al. (2016)). Subsequently, the samples prepared at both temperatures were transferred into HPLC vials using 0.45 μm syringe filters (Whatman™) and analyzed in triplicate by HPLC.

3.3.2. HPLC Analysis of L-Theanine and Caffeine in White Tea Samples Infused at Room Temperature

The modified procedure described by Agilent Technologies, Inc. (Naegele, 2016) was used for the simultaneous HPLC analysis of L-theanine and caffeine in the tea-infused samples. Separate standard curves were prepared for L-theanine and caffeine by injecting different concentrations of the standard L-theanine (≥ 98%; HPLC; Sigma® Life Science) and caffeine (Sigma reference standard; Sigma-Aldrich) into the HPLC system and determining the peak areas for the standard L-theanine and caffeine solutions. The trendline equation of the standard curves, the retention times (RT) of the standards and the peak areas obtained by HPLC analysis of each sample were used to calculate L-theanine and caffeine levels (mg/mL) in each sample.

All the analyses were performed in triplicate with HPLC Agilent 1100 series, Phenomenex® column-Gemini 5µm C18 110Å (150 × 4.60 mm, 5 µm), variable wavelength detector (VWD) (wavelength = 210 nm) and methanol and HPLC-grade water (25:75% V/V) as the mobile phase. The column temperature was set at 25°C, the flow rate was set to 1 mL/min, with a 12 min run time and a 3 min post-run time.

3.4. DPPH Radical Scavenging Activity in the Extracts of White, Green and Black Teas

3.4.1. Extraction Procedure of White, Green and Black Teas

First, 40 mL cold HPLC-grade water (< 10°C) was added to 2 g of ground and sieved (#35 or 500 µm sieve) white tea (bai hao yin zhen), green tea and black tea (lapsang souchong) (with a ratio of tea to water: 1 g/20 mL). Each tea and water mixture was placed in a refrigerator (~ 4°C) for 5 min and after the 5 min extraction they were vacuum filtered using filter paper #4 Whatman®. Then, PVPP (Supelco®; Sigma-Aldrich) was added to each tea extract sample to remove their polyphenols (1 g/20 mL) (Henríquez-Aedo et al., 2013; Unno et al., 2016). The tea extract samples were then stirred on a magnetic stirrer for 15 min at room temperature and then vacuum filtered using filter paper #4 Whatman®. Three aliquots of each tea sample were transferred into HPLC amber vials by filtering through 0.45 µm syringe filters (Whatman™) and analyzed by HPLC in triplicate to determine their L-theanine and caffeine levels. The remaining portion of each tea infusion sample was used for the DPPH radical scavenging assay.

3.4.2. DPPH Radical Scavenging Assay on the Extracts of White Tea, Green Tea and Black Tea

The DPPH radical scavenging activity of each tea extract was assessed using the DPPH radical scavenging assay. For completing the DPPH radical scavenging assay, 0.004% (W/V %) DPPH solution in methanol was prepared by dissolving 0.0008 g DPPH (2,2-diphenyl-1-picrylhydrazyl) (Aldrich® Chemistry; Sigma Aldrich) in methanol and making the volume up to 20 mL with methanol. Then, 1 mL of this freshly prepared 0.004% DPPH stock solution was added

to 3 mL of each aqueous tea extract (white, green or black tea). After shaking, the samples and the DPPH stock solution were incubated in darkness at room temperature for 30 min. After the 30 min incubation, the absorbance of each sample was recorded in triplicate at 515 nm using a UV/Visible spectrophotometer (Ultrospec 2100 pro) following established methods. The control was the DPPH methanolic solution without any tea extract (Afroz Bakht et al., 2019; Espín et al., 2000; Islam et al., 2021; Jayabalan et al., 2008; Mimica-Dukic et al., 2004). The DPPH radical scavenging activity (%) was determined using the following equation (Eq.1) (Jayabalan et al., 2008):

DPPH radical scavenging activity (%) = [(control absorbance - sample absorbance)/control absorbance] × 100

Eq.1

Ascorbic acid was used as the positive control (Islam et al., 2021). For that, 0.1 g ascorbic acid was dissolved in methanol and made up to 10 mL to make a 10 mg/mL solution and then serially diluted. Then, 1 mL of the DPPH stock solution was added to 3 mL of each ascorbic acid dilution. After shaking, the ascorbic acid samples were incubated in darkness and at room temperature for 30 min. After 30 min the absorbance for each dilution was recorded at 515 nm in triplicate by a UV/Visible spectrophotometer (Ultrospec 2100 pro).

3.4.3. HPLC Analysis of the Tea Samples for Their L-Theanine and Caffeine Levels

For the concurrent determination of L-theanine and caffeine in the samples using HPLC analysis, first separate standard curves for standard L-theanine and caffeine were prepared using a range of concentrations of authentic standard L-theanine (≥ 98%; HPLC; Sigma® Life Science) and caffeine (Sigma reference standard; Sigma-Aldrich). The modified procedure described by

Agilent Technologies, Inc. (Naegele, 2016) was used to calculate L-theanine and caffeine levels (mg/mL) in the samples, using the trendline equation of the standard curves, the peak areas of the samples obtained by HPLC and the retention times of the standards.

All the samples were analyzed in triplicate. HPLC Agilent 1100 series, Phenomenex® column-Gemini 5µm C18 110Å (150 × 4.60 mm, 5 µm) and variable wavelength detector (VWD) (wavelength = 210 nm) were used. The mobile phase was methanol and HPLC-grade water (25:75% V/V). The column temperature was set at 25°C. The flow rate was set to 1 mL/min. The run time was selected as 12 min and the post-run time was set to 3 min.

3.5. Effect of Methanol Pre-treatment on L-Theanine and Caffeine Extraction from Tea

An extraction procedure was developed, with the aim of removing the caffeine content from tea leaves in a pre-treatment. This procedure involved an initial extraction of tea leaves in methanol aiming to preferentially remove the caffeine content, followed by the subsequent extraction of the methanol-pre-treated tea (tea biomass) in water to then preferentially extract the L-theanine content.

3.5.1. Methanol Pre-treatment of Tea Leaves

White tea leaves (bai hao yin zhen) were ground and sieved (#35 sieve or 500 μm). Then, 20 mL cold methanol (< 10°C) was added to 1 g of the tea powder. This mixture (1 g tea/20 mL methanol) was placed in a refrigerator (~ 4°C) for 15 min extraction time. After extraction, the mixture was filtered using filter paper P8 (Fisherbrand®) to remove and separate methanol from the tea leaves (tea biomass).

3.5.2. Extraction of Methanol-Treated Tea Leaves in Water

The tea biomass (i.e. tea leaves once pre-treated/extracted with methanol) was mixed with 20 mL HPLC-grade water and placed in the refrigerator for 15 min (the second step of the extraction).

Another 1 g of the ground and sieved white tea was mixed with 20 mL cold HPLC-grade water (< 10°C) and placed in the refrigerator (~ 4°C) for 15 min to serve as the control.

After the 15 min extraction time, the sample and the control were filtered using filter paper P8 (Fisherbrand®), followed by adding PVPP from Supelco® Sigma-Aldrich (1 g/20 mL) (Henríquez-Aedo et al., 2013; Unno et al., 2016). The sample and the control were stirred on a magnetic stirrer at 180 RPM at room temperature for 15 min. Then, they were transferred into HPLC vials using 0.45 µm syringe filters (Whatman™) and analyzed in triplicates using HPLC.

The methanolic fraction separated and obtained from the first extraction was also analyzed by HPLC for comparison. This fraction was transferred into HPLC vials using 0.45 µm Whatman™ syringe filters. The procedure sequence followed is shown in Figure 5.

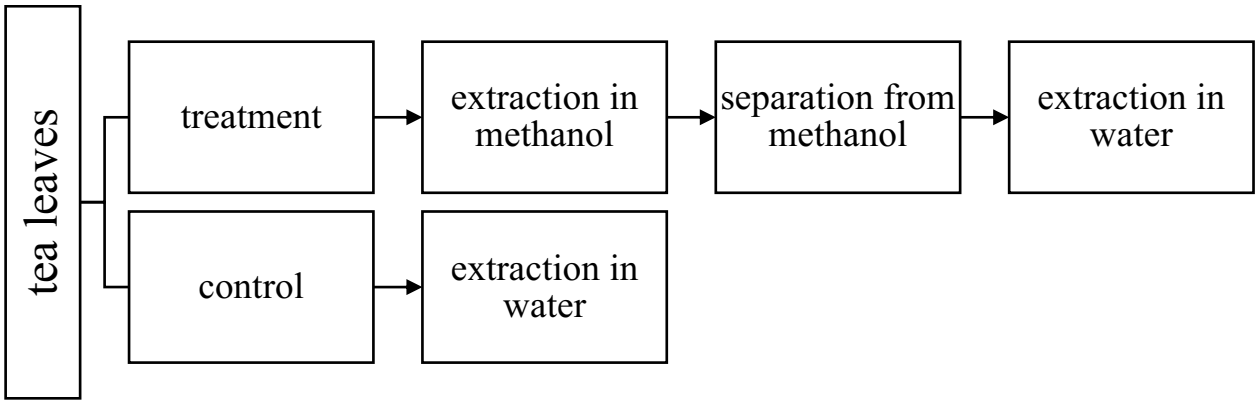


Figure 5- White tea pre-treatment with methanol followed by water extraction and comparison with the control.

3.5.3. HPLC Analysis of L-Theanine and Caffeine in Aqueous Infusion of Methanol-Treated White Tea Leaves

The modified procedure described by Agilent Technologies, Inc. (Naegele, 2016) was used to simultaneously analyze L-theanine and caffeine in the samples using HPLC. First, separate standard curves were prepared for the standards by injecting different concentrations of the

authentic standard L-theanine (≥ 98%; HPLC; Sigma® Life Science) and caffeine (Sigma reference standard; Sigma-Aldrich) into the HPLC system, determining the peak areas for the standards and plotting the peak areas of the standards against their concentrations. Then, L-theanine and caffeine levels (mg/mL) were calculated in each sample using the trendline equation of the standard curves, the retention times (RT) of the standards and the peak areas of the samples obtained by HPLC.

All the analyses were performed in triplicate using the HPLC Agilent 1100 series. Phenomenex® column-Gemini 5µm C18 110Å (150 × 4.60 mm, 5 µm) and variable wavelength detector (VWD) (wavelength = 210 nm) were used. Methanol with HPLC-grade water (25:75% V/V) was used as the mobile phase. The column temperature was set at 25°C and the flow rate was set to 1 mL/min. The run time was 12 min and the post-run time was 3 min.

3.6. Effect of Drying on L-Theanine and Caffeine Contents of Tea Infusions

3.6.1. Tea Extraction Procedure

The effects of spray drying and freeze drying of tea infusions on their L-theanine and caffeine levels were analyzed. First, white tea infusion/extract was prepared by mixing exactly 10g of the ground and sieved white tea (bai hao yin zhen, sieved through a #35 or 500 μm sieve) with 200 mL of ~10 to 11°C HPLC-grade water (1/20 g/mL) and leaving the tea and water mixture in the refrigerator (~ 4°C) to infuse for 5 min. This mixture was then filtered to remove the tea leaves (P8 Fisherbrand® filter paper) followed by adding 1 g/20 mL PVPP (Fluka® Analytical, Sigma-Aldrich; Germany) to remove the polyphenols (Henríquez-Aedo et al., 2013; Unno et al., 2016) and stirring on a magnetic stirrer at 180 RPM and room temperature for 15 min. A part of the tea extract was transferred into HPLC amber vials in triplicate, by passing through 0.45 μm syringe filters (Whatman™) to be used as the control. The rest of the tea samples were passed through filter paper, protected from light and stored in a freezer (-18°C) before being dried.

3.6.2. Freeze-Drying and Spray-Drying Tea Infusions

90 mL of the pre-frozen tea extract was freeze-dried at about -50°C for 24 hr in a Labconco FreeZone® 2.5 litre benchtop freeze dryer system.

50 mL of the thawed tea sample was stirred with 2% (W/V) maltodextrin and 2% (W/V) gum acacia, thoroughly mixed and spray-dried with a Büchi B-290 mini spray dryer. The inlet temperature used for spray drying was set at 180°C, the aspiration rate was 100%, the feed temperature was at room temperature and the outlet temperature was about 103°C.

After drying, the tea powders were reconstituted in water to be analyzed by HPLC. For that, 0.08 g of the freeze-dried and spray-dried tea extracts were each dissolved in 1.6 mL HPLC-grade water (ratio: 1 g/20 mL) and vortexed. Each reconstituted tea sample was then separately transferred into HPLC amber vials by passing through 0.45 μm syringe filters (Whatman™) for HPLC analyses.

3.6.3. HPLC Analysis of L-Theanine and Caffeine in Spray-Dried and Freeze-Dried White Tea Infusions

Using the modified procedure described by Agilent Technologies, Inc. (Naegele, 2016), the L-theanine and caffeine levels were simultaneously determined in the tea samples by HPLC. After injecting different concentrations of the authentic standard L-theanine (≥ 98%; HPLC; Sigma® Life Science) and caffeine (Sigma reference standard; Sigma-Aldrich) into the HPLC system, determining the peak areas for standard L-theanine and caffeine solutions and plotting the standard concentrations against their peak areas, separate standard curves were prepared for L-theanine and caffeine. L-theanine and caffeine levels (mg/mL) in each sample were calculated using the trendline equation of each standard curve, the retention times (RT) of the standards and the peak areas of the samples obtained by HPLC.

All the analyses were performed in triplicate. HPLC Agilent 1100 series, Phenomenex® column-Gemini 5μm C18 110Å (150 × 4.60 mm, 5 μm) and variable wavelength detector (VWD) (wavelength = 210 nm) were used. The mobile phase was methanol and HPLC-grade water (25:75% V/V) and the column temperature was set at 25°C. The flow rate was 1 mL/min. A 12 min run time and a 3 min post-run time were selected.

3.7. Effect of Ultrasound (US)-Assisted Extraction on L-Theanine and Caffeine Levels in Tea Infusions

3.7.1. US-Assisted Extraction of White Tea

Firstly, 1 g/20 mL tea and water mixtures were prepared using ~10-11°C HPLC-grade water and ground and sieved (#35 or 500 μm sieve) bai hao yin zhen white tea sample. The water and tea mixtures were placed in the refrigerator (~ 4°C) for either 5 or 20 min without stirring to prepare the control samples.

For the preparation of the US-treated samples, the initial temperature of the solvent (HPLC-grade water) was lowered to ~8°C instead of ~10-11°C to compensate for the temperature rise during sonication, since sonication increased the temperature by ~2°C. Therefore, 1 g/20 mL tea and water mixtures were prepared using ~8°C HPLC-grade water and ground and sieved (#35 or 500 μm sieve) white tea sample (bai hao yin zhen). These mixtures were treated with a probe sonicator (450 Sonifier, Analog Cell Disruptor; Branson Ultrasonics Corporation; USA), at 20 kHz electrical energy for 5 or 20 min. The sonicator was set to pulsed operation mode at 0.1 seconds in order to minimize the temperature increase in the solution. This transmitted the ultrasonic vibrations to the solution at one pulse per second for 0.1 of each second. Also, the amplitude of the ultrasonic vibrations and the ultrasonic intensity were controlled and adjusted by setting the Output Control at 1. A solid stepped disruptor horn with a 3/8 inch (9.5 mm) diameter and a horn amplitude (tip movement) of 36 μm (0.0014 inches) was used (Branson Ultrasonics Corporation, 1998). The total energy absorbed by the sample was calculated to be about 8.7 J/mL using the formula Q/V where V is the sample volume and Q is calculated by $Q = m \times c_p \times \Delta T$. In this

equation, m is the total mass of the sample, c_p is the specific heat capacity, and ΔT is the temperature change (Natolino & Celotti, 2022).

After the treatment time had elapsed (5 min or 20 min), both the US-treated and the control samples were first filtered through P8 Fisherbrand® filter papers to remove the tea leaves. Then, PVPP (Fluka® Analytical, Sigma-Aldrich) was added to the US-treated and the control samples at a PVPP to tea infusion ratio of 1 g/20 mL to remove the polyphenols in the samples (Henríquez-Aedo et al., 2013; Unno et al., 2016). Subsequently, the US-treated and the control samples were stirred on a magnetic stirrer for 15 min at 180 RPM and room temperature and then, they were transferred into HPLC vials using 0.45 μm syringe filters (Whatman™). The samples were protected from light and stored in a freezer (-18°C) before being analyzed by HPLC in triplicate.

3.7.2. HPLC Analysis of L-Theanine and Caffeine in the US-Assisted-Extracted White Tea Samples

The simultaneous HPLC analysis of L-theanine and caffeine in the samples and the controls was conducted after plotting separate standard curves. Separate standard curves for L-theanine and caffeine were plotted by injecting a range of concentrations of authentic standard L-theanine (≥ 98%; HPLC; Sigma® Life Science) and caffeine (Sigma reference standard; Sigma-Aldrich) to the HPLC system (Agilent 1100 Series, Agilent Technologies, Inc.), following the modified procedure described by Agilent Technologies, Inc. (Naegele, 2016). The peak area of each standard solution was plotted against its concentration to obtain the standard curves. The trendline equation of each standard curve, the peak areas of the samples and the retention time (RT) of each standard obtained by HPLC were used for the quantification (mg/mL) of L-theanine and caffeine in the tea samples and the controls.

For the HPLC analysis, Phenomenex® column-Gemini 5µm C18 110Å (150 × 4.60 mm, 5 µm), and variable wavelength detector (VWD) (wavelength = 210 nm) were used. The mobile phase was methanol and HPLC grade water (25:75% V/V) and the column temperature was 25°C. The flow rate was 1 mL/min. The run time and the post-run time were 12 min and 3 min respectively. All analyses were performed in triplicate.

3.8. Effects of Common Additives on the Levels of L-Theanine and Caffeine in Tea Infusions

3.8.1. Effect of Adding Lemon Juice During Infusion on L-Theanine and Caffeine Levels of Tea Infusions

Firstly, HPLC-grade water at ~10-11°C was added to the ground and sieved (#35 or 500 µm sieve) white tea (bai hao yin zhen) (1 g tea/20 mL water). Then, 5% V/V freshly squeezed lemon juice was added to the tea and water mixture. The mixture was then placed in the refrigerator (~ 4°C) for 5 min. A similar procedure, but without adding lemon juice (0% V/V), was conducted to prepare the control sample. The pH and temperature of the control and test samples were recorded and adding lemon juice reduced the pH of the solution from 5.17 to 2.79. At the end of the 5 min extraction, the tea leaves were removed by filtering both the control and treated samples through filter paper #2 (Whatman™). After that, the sample and the control were treated with PVPP (1 g/20 mL) (Fluka® Analytical, Sigma-Aldrich) to eliminate the polyphenols (Henríquez-Aedo et al., 2013; Unno et al., 2016). Then, the sample and the control were magnetically stirred for 15 min at 180 RPM and room temperature and subsequently transferred into HPLC vials using 0.45 µm syringe filters (Whatman™) and stored in a freezer at -18°C until the HPLC analysis could be conducted.

3.8.2. Effect of Adding Milk to Tea Extracts/Infusions on Their L-Theanine and Caffeine Levels

80 mL cold HPLC-grade water (at ~10 to 11°C) was added to 4 g ground and sieved (#35 or 500 µm sieve) leaves of white tea (bai hao yin zhen) to make a 1/20 (g/mL) aqueous tea mixture.

This mixture was left in a refrigerator (~ 4°C) for 5 min. Thereafter, it was filtered (P8 filter paper; Fisherbrand®; Fisher Scientific) and added with PVPP (Supelco® Sigma-Aldrich) (1/20 g/mL) to eliminate the polyphenols, followed by stirring on a magnetic stirrer for 15 min at 180 RPM and room temperature (Henríquez-Aedo et al., 2013; Unno et al., 2016). Then, the tea sample was divided into 4 aliquots. One aliquot was without added milk (0% milk) and served as the control. The control sample (0% milk) was transferred into HPLC amber vials using 0.45 µm syringe filters (Whatman™). The three other aliquots of the tea sample were first filtered (P8 filter paper; Fisherbrand®; Fisher Scientific) to remove PVPP and then, added with 10%, 20% or 50% (V/V) milk (partly skimmed, 2% milk fat). After shaking, the samples were placed in the refrigerator (~ 4°C) for 20 min to equilibrate (method adopted with modifications from Ferruzzi and Green (2006)). After 20 min, the milk-treated tea samples were transferred into HPLC amber vials using 0.45 µm syringe filters (Whatman™). The samples and the control were immediately analyzed by HPLC in triplicate.

3.8.3. HPLC Analysis of L-Theanine and Caffeine in Lemon Juice- and Milk-Treated Tea Samples

L-theanine and caffeine contents of the treated samples and the controls for both experiments described were simultaneously determined by HPLC using the modified procedure described by Agilent Technologies, Inc. (Naegele, 2016). Before the analysis of tea samples, separate standard curves for L-theanine and caffeine were plotted by injecting different concentrations of authentic standard L-theanine (≥ 98%; HPLC; Sigma® Life Science) and caffeine (Sigma reference standard; Sigma-Aldrich) into the HPLC system. Then, the peak area of each standard solution was determined and plotted against its concentration. The trendline equation of

these standard curves along with the retention times of each standard and the peak areas obtained by HPLC separation of each sample were used to calculate L-theanine and caffeine levels (mg/mL) in the samples and the controls.

HPLC Agilent 1100 Series (Agilent Technologies, Inc.) was used and all the samples were analyzed in triplicate. Phenomenex® column-Gemini 5µm C18 110Å (150 × 4.60 mm, 5 µm) and variable wavelength detector (VWD) (wavelength = 210 nm) were used. The mobile phase used was methanol and HPLC-grade water (25:75% V/V). The column temperature was set at 25°C. The flow rate was set to 1 mL/min. The run time was selected as 12 min and the post-run time was set to 3 min.

3.9. Storage of Freeze-Dried, Spray-Dried and Liquid Tea Preparations

Tea infusions were freeze-dried or spray-dried and their quality in terms of antioxidant activity, moisture content, L-theanine and caffeine contents was analyzed during 60 days of storage under extreme temperature conditions (39 ± 1°C).

3.9.1. Tea Extraction Procedure

Tea infusions (tea-to-water ratio = 1/20 g/mL) were prepared by adding cold HPLC-grade water (~10-11°C) to ground and sieved white tea leaves (bai hao yin zhen sieved through a #35 or 500 μm sieve) and placed in the refrigerator (~ 4°C) for 5 min. The tea infusions were subsequently filtered through nylon mesh and then vacuum filtered through filter paper #4 (Whatman®). PVPP (Supelco®; Sigma-Aldrich) was then added to the tea infusions at the ratio of 1 g/20 mL, to eliminate the polyphenols (Henríquez-Aedo et al., 2013; Unno et al., 2016). The tea infusions were stirred on a magnetic stirrer for 15 min at room temperature and subsequently filtered using nylon mesh followed by filter paper #4 (Whatman®) under vacuum, to remove the PVPP. The tea infusion samples were stored in a refrigerator (~ 4°C) before being spray-dried or freeze-dried.

3.9.2. Spray-Drying and Freeze-Drying Tea Infusions

For spray drying, an aliquot of the tea infusion sample was added with 2% (W/V) maltodextrin (dextrose equivalent 4.0-7.0 from Aldrich® Chemistry, Sigma Aldrich) and 2% (W/V) gum acacia (from Importers Service Corporation, USA) and stirred well before being dried using a Büchi B-290 mini spray dryer with compressed air, inlet temperature at 180°C, the aspiration rate at 100% and the pump output of 30%. For freeze-drying, another aliquot of the tea infusion sample was first frozen at -18°C and then dried in a Labconco FreeZone® 2.5 litre

benchtop freeze dryer system for 48 hours at about -50°C. A third aliquot of the tea infusion sample was used without drying to serve as the control for comparison. All the samples were processed in triplicate.

3.9.3. Storage of the Dried and Undried Tea Infusion Samples

The spray-dried, freeze-dried and undried tea infusion samples were stored in screw thread amber glass vials with airtight rubber-lined caps (Fisherbrand®; Fisher Scientific; USA) in triplicate, at 39 ± 1°C for 60 days. The DPPH radical scavenging activity and the L-theanine and caffeine contents of all the spray-dried, freeze-dried and undried tea samples were analyzed every 20 days in triplicate, for a period of 60 days. The moisture content of the freeze-dried and spray-dried tea infusion samples was similarly analyzed in triplicate, at 20-day intervals for 60 days. These analyses were conducted on days 0, 20, 40 and 60 of storage.

3.9.4. DPPH Radical Scavenging Activities of the Dried and Undried Tea Samples During the Storage Period

DPPH radical scavenging assay was performed for the freeze-dried, spray-dried and undried tea infusion samples on days 0, 20, 40 and 60 of storage. For this assay, the dried tea samples were dissolved in HPLC-grade water and vortexed to make 1 mg/mL freeze-dried and spray-dried tea solutions. In addition, 0.004% (W/V %) DPPH solution in methanol was freshly prepared, following the method of Islam et al. (2021), with modifications. For the preparation of the 0.004% DPPH stock solution in methanol, 0.0008 g DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma Aldrich) was dissolved in methanol and the volume was made up to 20 mL with methanol followed by shaking this solution. Then, 1 mL of the DPPH methanolic solution was added to 3

mL of each tea sample (i.e. the aqueous solutions of freeze-dried and spray-dried tea infusions and the undried tea infusions). After shaking, all the samples and the DPPH stock solution were incubated in darkness at room temperature for 30 min. After the 30 min incubation, the absorbance of each sample and the control (consisting of DPPH in methanol solution without any tea sample) was recorded in triplicate at 515 nm using a UV/Visible spectrophotometer (Ultrospec 2100 pro) (Afroz Bakht et al., 2019; Espín et al., 2000; Mimica-Dukic et al., 2004).

Ascorbic acid was used as the positive control (Islam et al., 2021). For that, an ascorbic acid methanolic solution (10 mg/mL) and its serial dilutions were prepared. Following the same procedure used for the test samples, 1 mL of the DPPH solution was added to 3 mL of each dilution, shaken and incubated in darkness at room temperature for 30 min. After 30 min, the absorbance of each dilution of the ascorbic acid solution was recorded in triplicate at 515 nm using a UV/Visible spectrophotometer (Ultrospec 2100 pro).

The following equation (Eq.2) was used to determine the DPPH radical scavenging activity (%) of each tea sample (Jayabalan et al., 2008):

DPPH radical scavenging activity (%) = [(control absorbance - sample absorbance)/control absorbance] × 100

Eq.2

3.9.5. Determination of L-Theanine and Caffeine Contents of the Dried and Undried Tea Samples During Storage

On days 0, 20, 40 and 60 of storage, the L-theanine and caffeine contents of the dried samples, reconstituted in water, and the undried samples were determined using HPLC analysis.

On each analysis day, the spray-dried and freeze-dried tea infusions were reconstituted in water by dissolving 0.24 g of each dried sample in 4.8 mL HPLC-grade water (ratio of 1 g/20 mL) and vortexed. Then, the reconstituted dried tea samples and the undried tea samples were transferred into HPLC amber vials using 0.45 µm syringe filters (Whatman™).

The simultaneous HPLC analysis of L-theanine and caffeine in each tea sample was performed by following the modified procedure described by Agilent Technologies, Inc. (Naegele, 2016). Separate standard curves for L-theanine and caffeine were plotted by injecting different concentrations of authentic standard L-theanine (≥ 98%; HPLC; Sigma® Life Science) and caffeine (Sigma reference standard; Sigma-Aldrich) into the Agilent 1100 series HPLC system, determining their peak areas and plotting the peak areas against concentrations. The trendline equation of each standard curve, the retention times (RT) and the peak areas of the samples obtained by HPLC were used to calculate L-theanine and caffeine levels (mg/mL) in the samples. All the samples were analyzed in triplicate.

The HPLC conditions used were as follows: HPLC Agilent 1100 series; Phenomenex® column-Gemini 5µm C18 110Å (150 × 4.60 mm, 5 µm); variable wavelength detector (VWD) (wavelength = 210 nm); mobile phase: methanol and HPLC-grade water (25:75% V/V); column temperature = 25°C; flow rate = 1 mL/min; run time = 12 min; post-run time = 3 min.

3.9.6. Moisture Contents of the Dried Tea Infusion Samples During Storage

The moisture content of powdered foods is an important factor in determining the quality, stability, preservation and deterioration of the product (Nielsen, 2010; Pomeranz & Meloan, 1994). As powders absorb moisture during storage, the powders agglomerate and become clumpy and

difficult or unpleasant to handle. Therefore, it is advantageous if the produced powder does not take up too much moisture during storage.

The moisture contents of the dried tea infusion samples stored in screw thread amber glass vials with airtight rubber-lined caps were determined in triplicate, on days 0, 20, 40 and 60 of storage following the oven-drying method described by Nielsen (2010) with some modifications, to assess the stability of the dried tea samples during storage and the tendency for the powders to absorb moisture during storage. First, 6 disposable aluminum pans were pre-dried in an oven at 200°C for 24 hr. Then, the pans were weighed after cooling down in a desiccator. About 0.5 g of each dried tea sample (freeze-dried or spray-dried) was added to each pan (in triplicate) and weighed (pan + sample). Then, the pans containing the tea samples were placed in a preheated oven at 70°C for 24 hr (Şahin Nadeem et al., 2011), after which, they were weighed again. After weighing the pans containing the tea samples, they were placed back in the oven at 70°C for another 15 min followed by weighing them again. This was repeated until a constant weight was reached. The moisture content of the spray-dried and freeze-dried tea samples was determined on each analysis day as percentage moisture (W/W) using Eq.3 (Nielsen, 2010).

% moisture = (weight of water in the sample/weight of the sample before drying) × 100

Eq.3

Chapter 4: Research Findings

4.1. Effect of Time, Temperature and Tea Type on L-Theanine and Caffeine Extraction

The HPLC data obtained for L-theanine and caffeine content of each tea preparation in this experiment were used to calculate the concentrations (mg/mL) of these compounds in each tea sample and to calculate the L-theanine-to-caffeine ratio of each tea sample to understand the proportions between these two compounds in the samples. These concentrations are presented in Table 3, as means ± SD of triplicate samples.

Table 3- L-theanine and caffeine levels (mg/mL) extracted from white, black and green teas at different temperatures and after different infusion times

Tea type	Temperature (°C)	Time (min)	L-theanine (mg/mL)	Average L-theanine (mg/mL)	Caffeine (mg/mL)	Average caffeine (mg/mL)
White	10-11	5	0.23 ± 0.05	0.373	0.006±0.009	0.08
		30	0.40 ± 0.12		0.09 ± 0.02	
		60	0.48 ± 0.02		0.13 ± 0.001	
	50	5	15.05 ± 0.71	16.491	1.38 ± 0.21	1.343
		30	16.19 ± 1.06		1.11 ± 0.07	
		60	18.23 ± 0.86		1.53 ± 0.04	

	90-100	5	21.52 ± 2.64	19.765	1.91 ± 0.18	1.66
		30	17.91 ± 0.33		1.50 ± 0.10	
		60	19.85 ± 0.53		1.55 ± 0.10	
Black	10-11	5	1.04 ± 0.16	1.074	0.36 ± 0.04	0.379
		30	1.07 ± 0.05		0.44 ± 0.01	
		60	1.10 ± 0.17		0.33 ± 0.06	
	50	5	11.72 ± 2.50	7.715	1.24 ± 0.02	0.75
		30	9.63 ± 2.74		0.88 ± 0.13	
		60	1.78 ± 0.01		0.12 ± 0.002	
	90-100	5	1.39 ± 0.07	1.261	1.38 ± 0.24	1.179
		30	1.21 ± 0.20		1.25 ± 0.14	
		60	1.17 ± 0.41		0.89 ± 0.19	
Green	10-11	5	2.05 ± 0.55	1.416	0.46 ± 0.04	0.355
		30	1.05 ± 0.10		0.36 ± 0.03	
		60	1.13 ± 0.07		0.23 ± 0.01	
	50	5	0.40 ± 0.006	1.389	0.10±0.0003	0.439

		30	0.91 ± 0.01		0.70 ± 0.008	
		60	2.84 ± 0.15		0.50 ± 0.22	
	90-100	5	2.45 ± 0.42	2.123	0.83 ± 0.18	0.754
		30	1.80 ± 0.63		0.65 ± 0.27	
		60	2.11 ± 0.29		0.77 ± 0.04	

L-theanine and caffeine concentrations (mean ± SD of 3 samples) for all the tea preparations, obtained by HPLC analysis of the samples, are also depicted in Figures 6 and 7 respectively, which clearly show the remarkable effects of tea type, extraction time and temperature on the content and extractability of these bioactive compounds. GraphPad Prism software version 10.1.1 for macOS, was used for the statistical analyses and plotting the graphs.

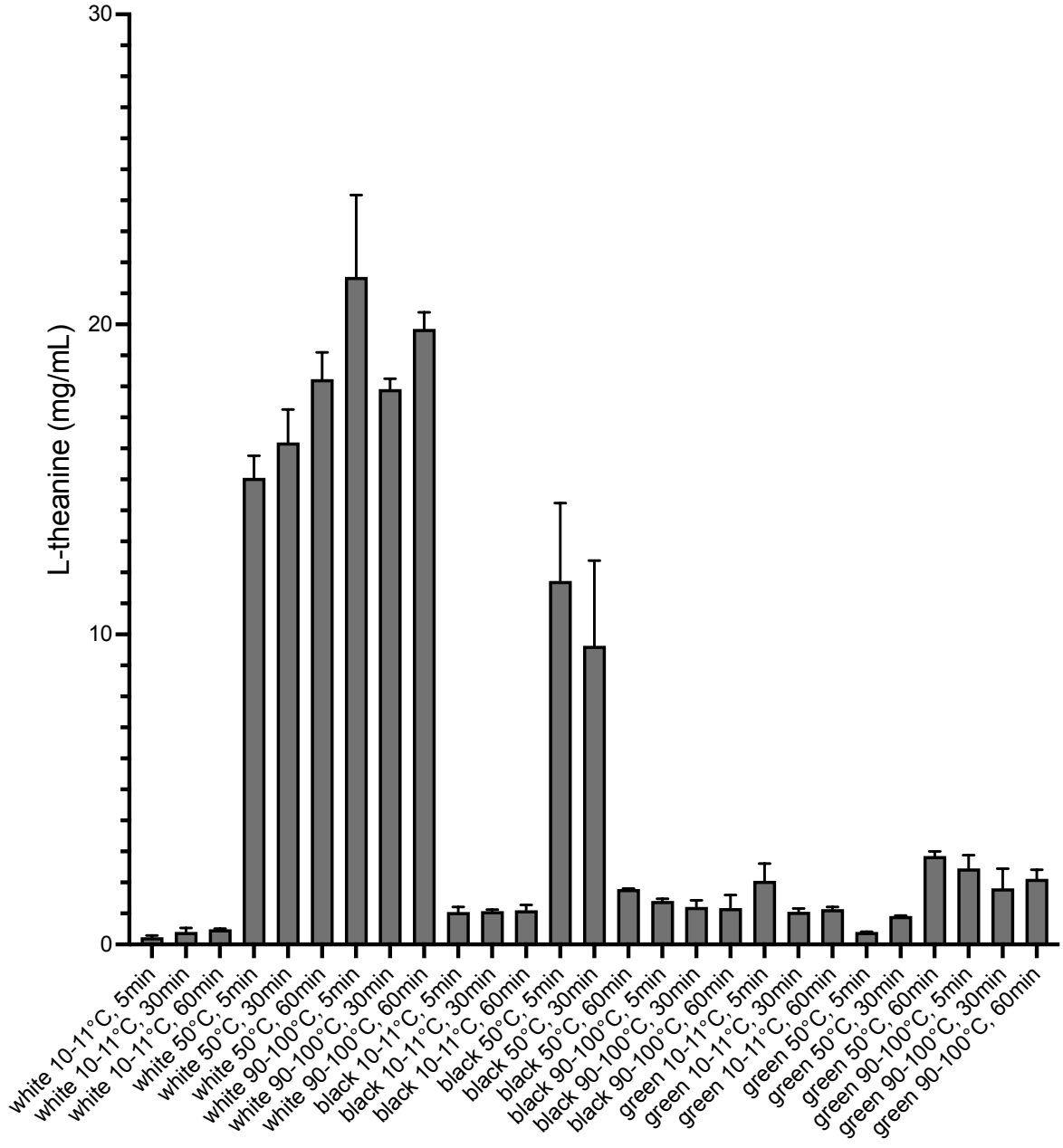


Figure 6- Concentration of L-theanine (mg/mL) in white, black and green tea infusions prepared at different temperatures and for different durations.

Data are means ± SD of samples analyzed in triplicate. GraphPad Prism software version 10.1.1 for macOS, was used for plotting the graph and for the statistical analyses.

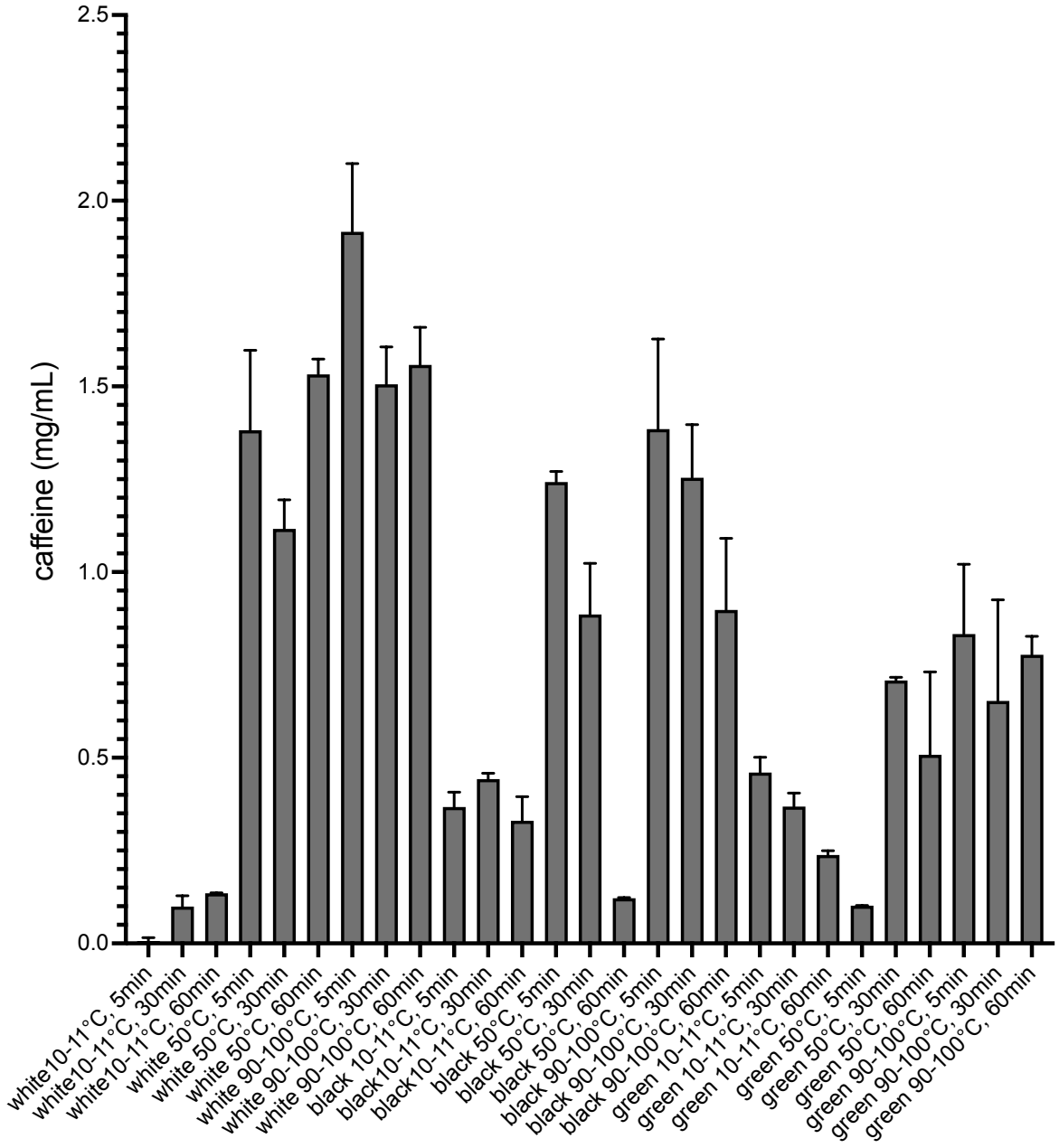


Figure 7- Concentration of caffeine (mg/mL) in white, black and green tea infusions prepared at different temperatures and for different durations.

Data are means ± SD of samples analyzed in triplicate. GraphPad Prism software version 10.1.1 for macOS, was used for plotting the graph and for the statistical analyses.

These data and pairwise comparisons for L-theanine are shown in Figure 8, a to c, in more detail. As shown in Figure 8a, at 10-11°C, significantly ($P < 0.05$) more L-theanine (2.05 ± 0.55 mg/mL) was extracted from green tea after 5 min infusion, compared to black and white tea extracted at 10-11°C for 5 min as well as green tea extracted at 10-11°C for 30 min and 60 min (data are means ± SD of 3 replicates) (Figure 8a).

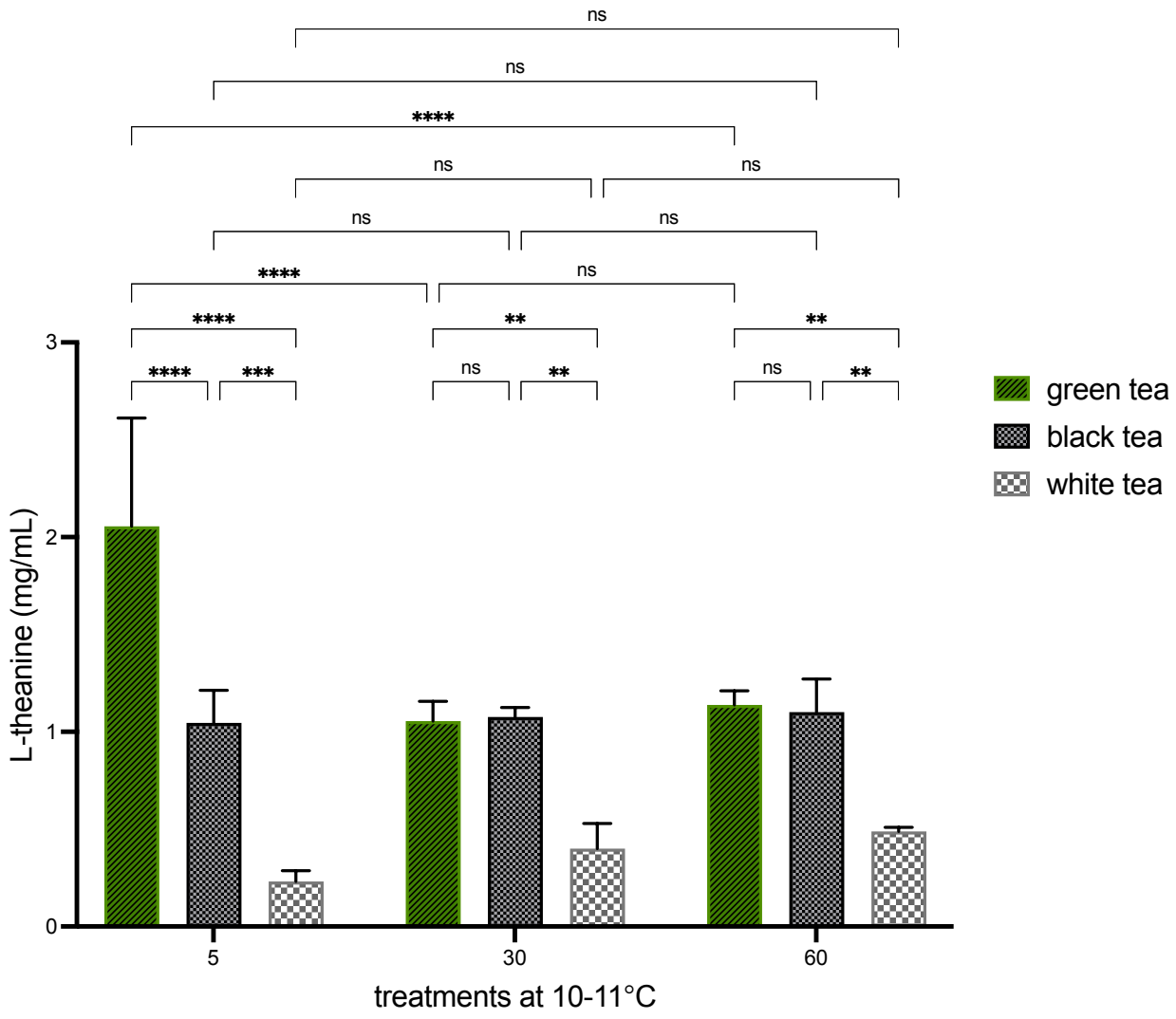
Additionally, as shown in Figure 8a, at 10-11°C significantly ($P < 0.05$) less L-theanine (0.23 ± 0.05 mg/mL) was extracted from white tea infused for 5 min, compared to green and black tea infused for the same duration. At 10-11°C, the infusion of L-theanine from white tea increased time-dependently and longer infusion times yielded more L-theanine, although this increase was not statistically significant ($P \geq 0.05$). Hence, the L-theanine level extracted from white tea at 10-11°C after 5 min infusion, was not significantly different ($P \geq 0.05$) from the L-theanine extracted from white tea at this temperature after 30 min and 60 min (data are means ± SD of 3 replicates) (Figure 8a).

Similarly, at 10-11°C, the level of L-theanine extracted from black tea increased with infusion time ($P \geq 0.05$) (Table 3). Contrarily, at this temperature, significantly ($P < 0.05$) less L-theanine was extracted from green tea when infused for 30 min and 60 min compared to 5 min of infusion (Figure 8a).

When extraction was conducted at 50°C, the extraction of L-theanine from green and white tea increased time-dependently ($P \geq 0.05$). Contrary to green and white tea, L-theanine extraction from black tea at 50°C decreased time-dependently. At this temperature, the highest concentration of L-theanine was extracted from white tea when infused for 60 min (18.23 ± 0.86 mg/mL); and

the lowest concentration of L-theanine at 50°C was extracted from green tea after 5 min infusion (0.4 ± 0.006 mg/mL) (data are means ± SD of 3 replicates) (Figure 8b).

At 90-100°C, the highest level of L-theanine was extracted from white tea after 5 min infusion (21.52 ± 2.64 mg/mL) and the lowest level of L-theanine at this temperature was extracted from black tea after 60 min infusion (1.17 ± 0.41 mg/mL) (data are means ± SD of 3 replicates) (Table 3). At this temperature, the time of infusion had non-significant effects on the L-theanine levels extracted from black and green tea ($P \geq 0.05$). However, the L-theanine levels extracted from white tea infused at 90-100°C for 30 min and 60 min were significantly ($P < 0.05$) lower than when infused for 5 min (Figure 8c).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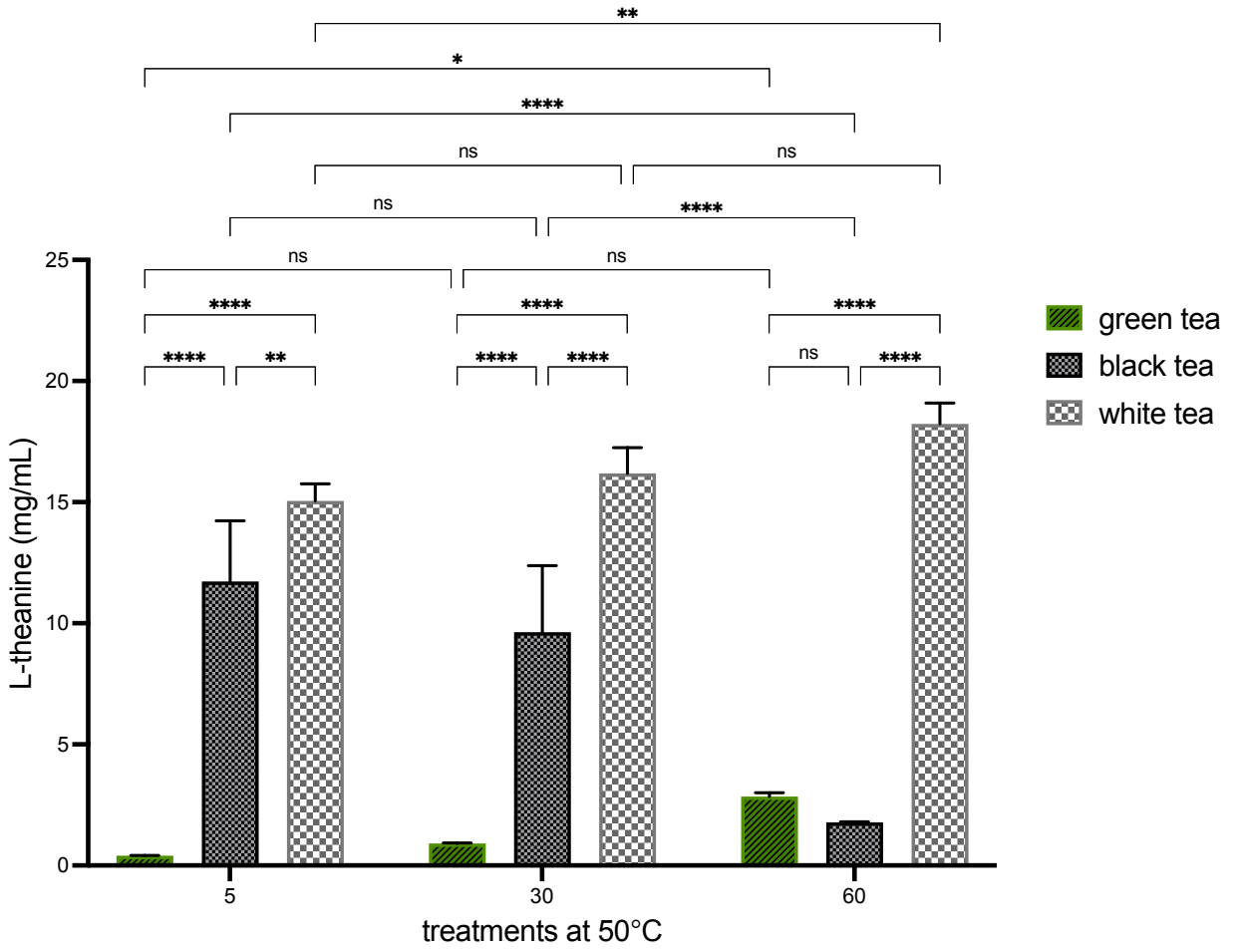
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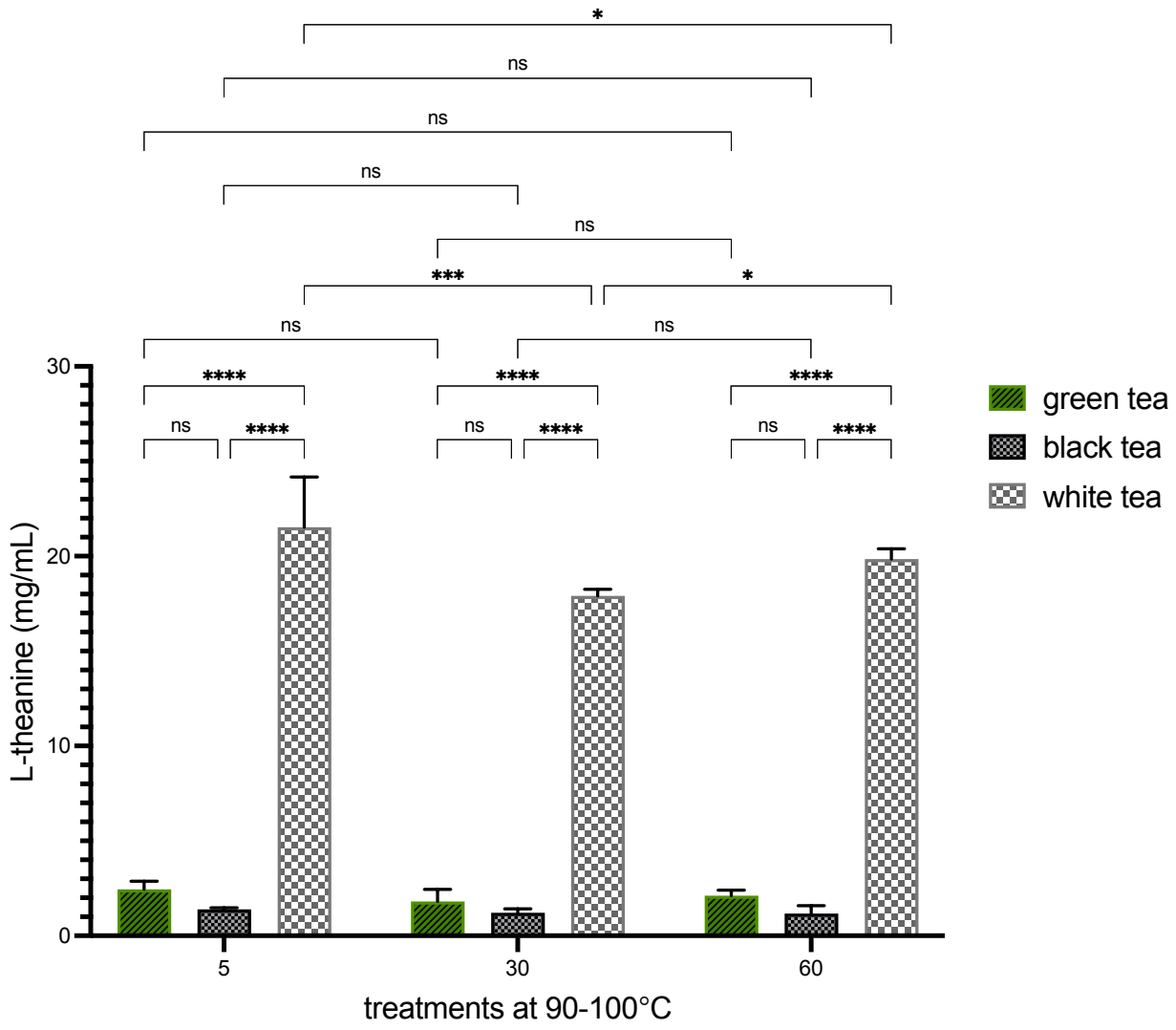


c

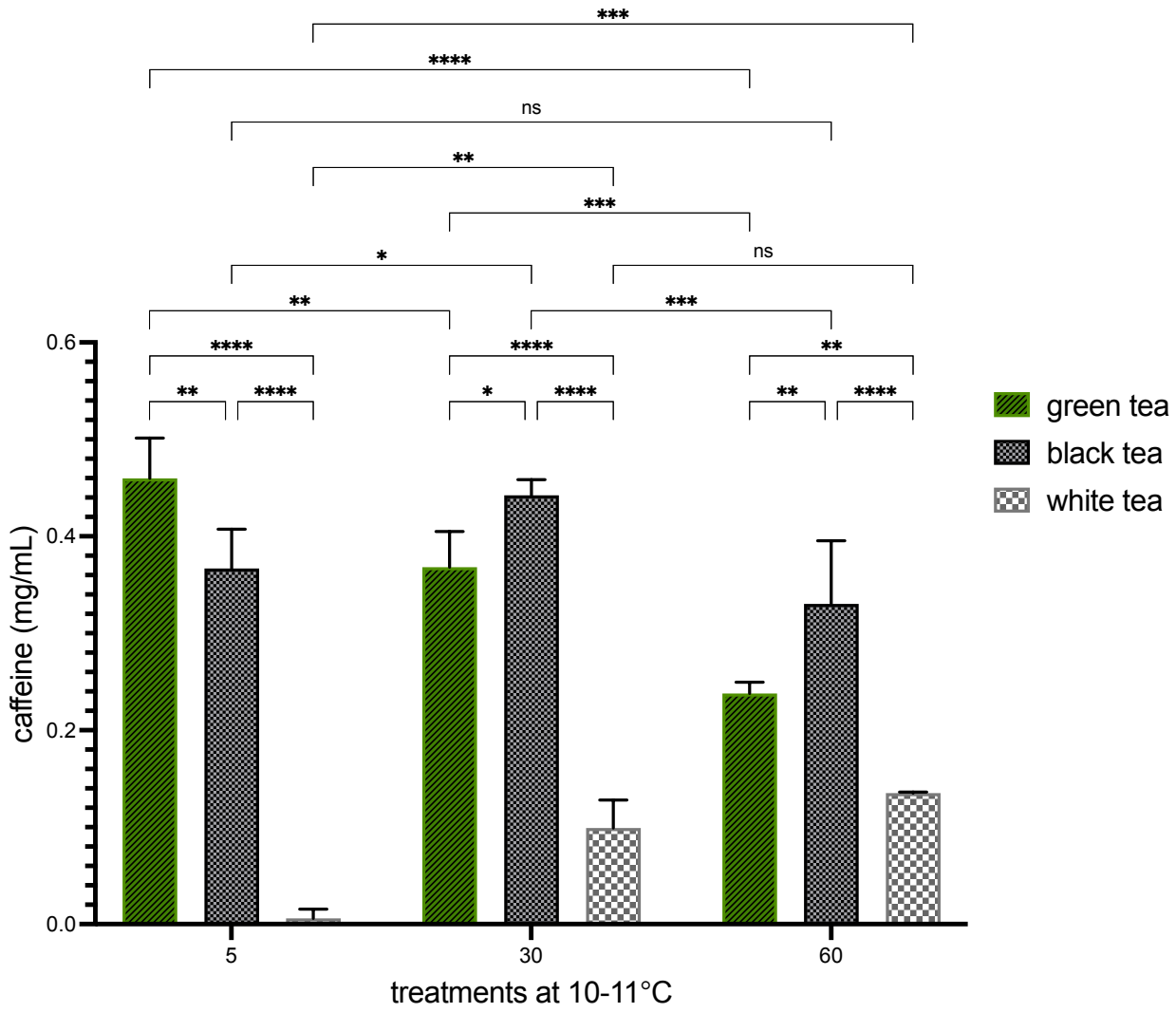
Figure 8- L-theanine levels (mg/mL) extracted from green, black and white tea at about a) 10-11°C, b) 50°C and c) 90-100°C.

Data are means ± SD of 3 samples. Two-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. $P < 0.05$ was considered statistically significant. * indicates P-value 0.01 to 0.05; ** indicates P-value 0.001 to 0.01; *** indicates P-value 0.0001 to 0.001; **** indicates $P < 0.0001$; ^{ns} indicates $P \geq 0.05$.

Similarly, the data and pairwise comparisons for caffeine levels extracted from white, green and black tea under different time and temperature conditions are shown in Figure 9, a to c. In a similar manner to L-theanine, the highest caffeine level at 10-11°C was infused from green tea after 5 min extraction (0.46 ± 0.04 mg/mL) and the lowest caffeine level at this temperature was infused from white tea after 5 min extraction (0.006 ± 0.009 mg/mL) (data are means ± SD of 3 replicates). At this temperature, the extraction of caffeine from green tea decreased while from white tea it increased time-dependently. The caffeine level infused from black tea at 10-11°C after 30 min was significantly ($P < 0.05$) higher than that extracted after both 5 min and 60 min (Figure 9a).

Similar to L-theanine, the highest caffeine level at 50°C was extracted from white tea after 60 min of infusion (1.53 ± 0.04 mg/mL) and the lowest caffeine level at this temperature was extracted from green tea after 5 min of infusion (0.1 ± 0.0003 mg/mL) (data are means ± SD of 3 replicates). At this temperature, the caffeine level infused from green tea was highest after 30 min of infusion/extraction; while by opposition, the level of this compound in white tea infusion was lowest at this temperature after 30 min of infusion. At 50°C the infusion of caffeine from black tea decreased time-dependently and significantly ($P < 0.05$) (Figure 9b).

At 90-100°C, similar to L-theanine extraction, the highest caffeine was infused from white tea after 5 min (1.91 ± 0.18 mg/mL); however, dissimilar to L-theanine, the lowest caffeine at this temperature was infused from green tea after 30 min (0.65 ± 0.27 mg/mL) (data are means ± SD of 3 replicates). Similar to the 50°C extraction, at 90-100°C, the caffeine infusion from black tea decreased time-dependently. However, at 90-100°C, the caffeine infusion from both white and green tea was lowest after 30 min of extraction (Figure 9c).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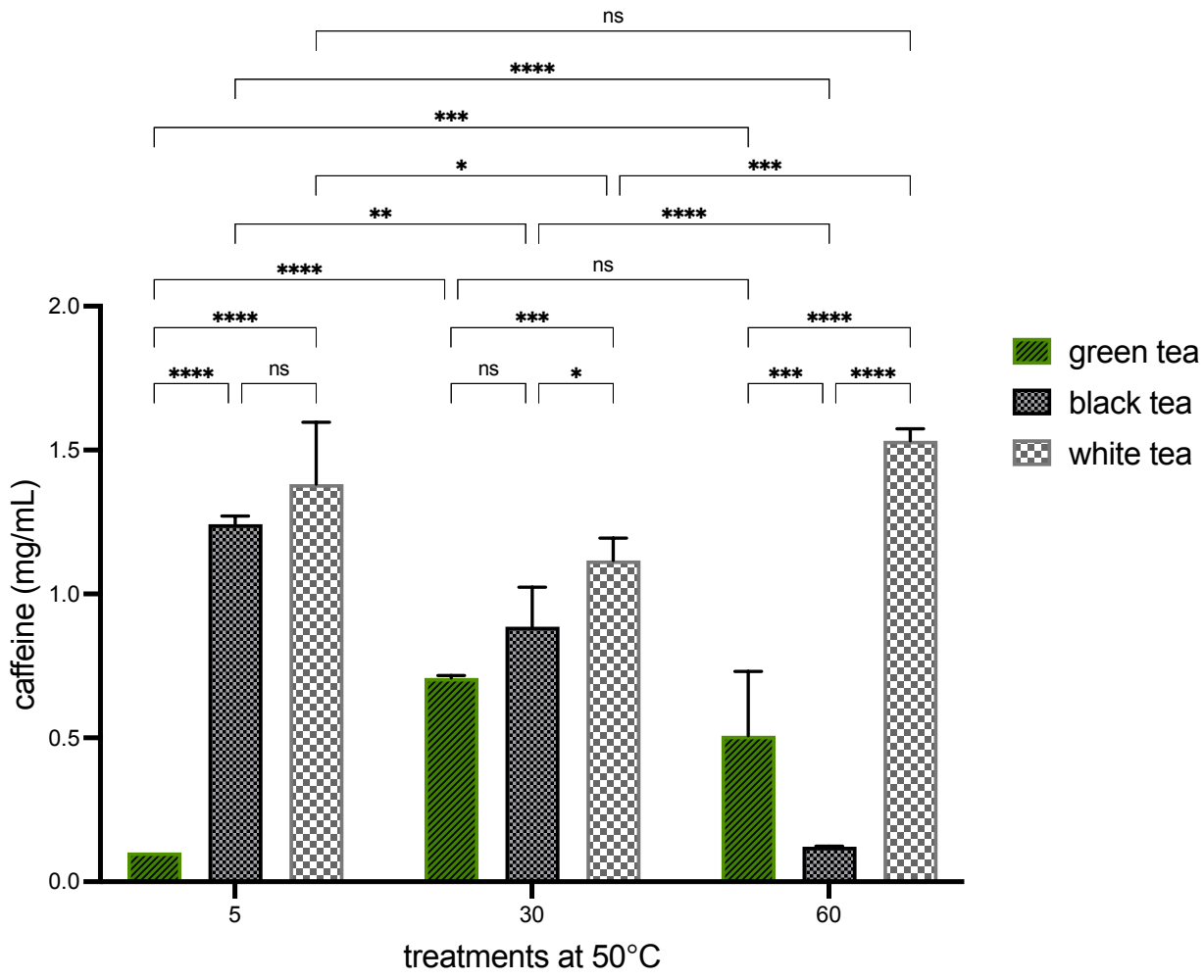
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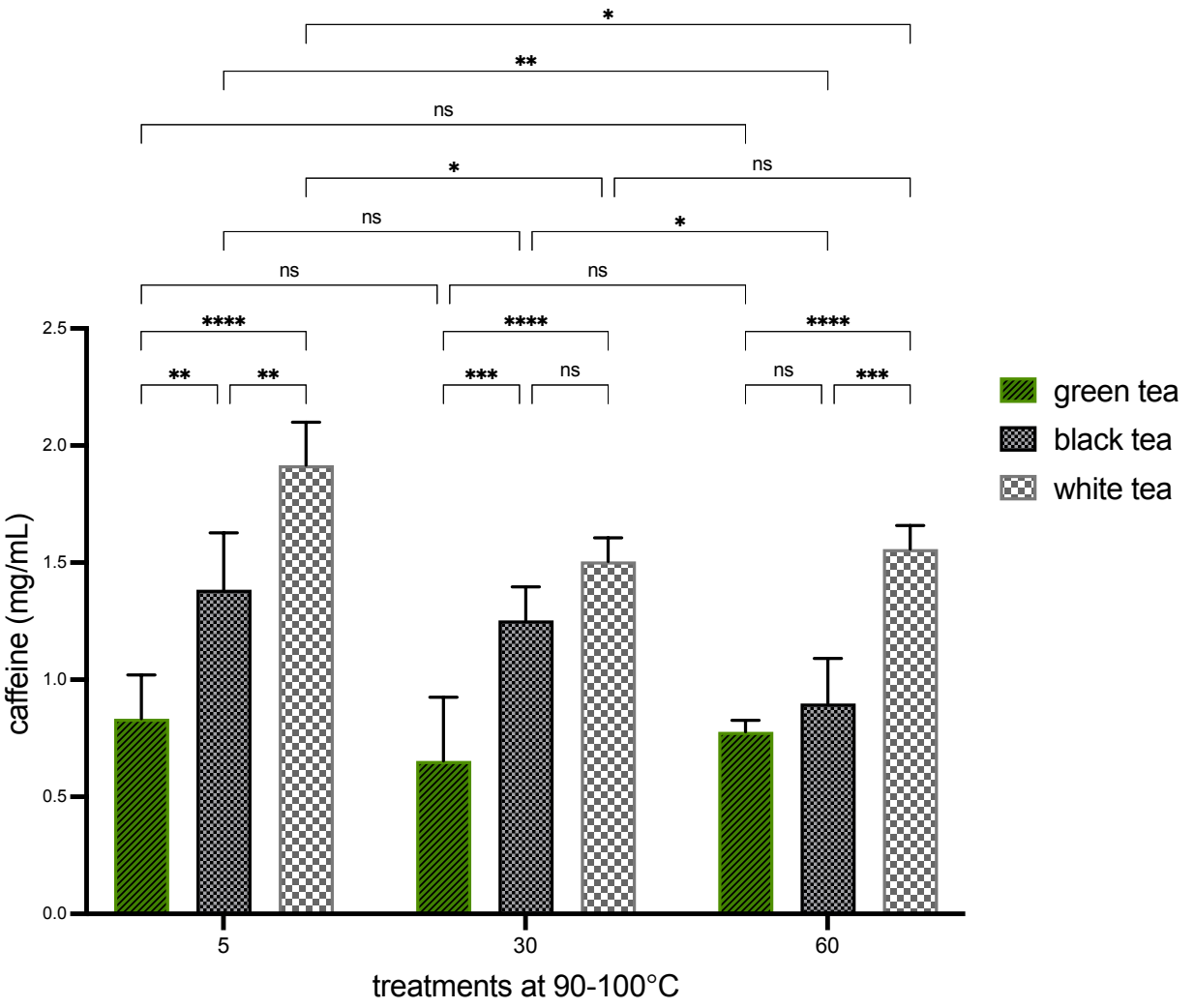


c

Figure 9- Caffeine levels (mg/mL) extracted from green, black and white tea at about a) 10-11°C, b) 50°C and c) 90-100°C.

Data are means ± SD of 3 samples. Two-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. $P < 0.05$ was considered statistically significant. * indicates P-value 0.01 to 0.05; ** indicates P-value 0.001 to 0.01; *** indicates P-value 0.0001 to 0.001; **** indicates $P < 0.0001$; ^{ns} indicates $P \geq 0.05$.

To provide a better view of the correlation of L-theanine and caffeine and their proportion in different tea infusions, the ratios of average L-theanine concentration to average caffeine concentration, obtained by HPLC analysis of each tea preparation, were calculated for all samples. This ratio was highest when white tea was infused at 10-11°C for 5 min (L-theanine : caffeine = 38) and lowest when black tea was infused at 90-100°C for 30 min (L-theanine : caffeine = 0.96) (Figure 10).

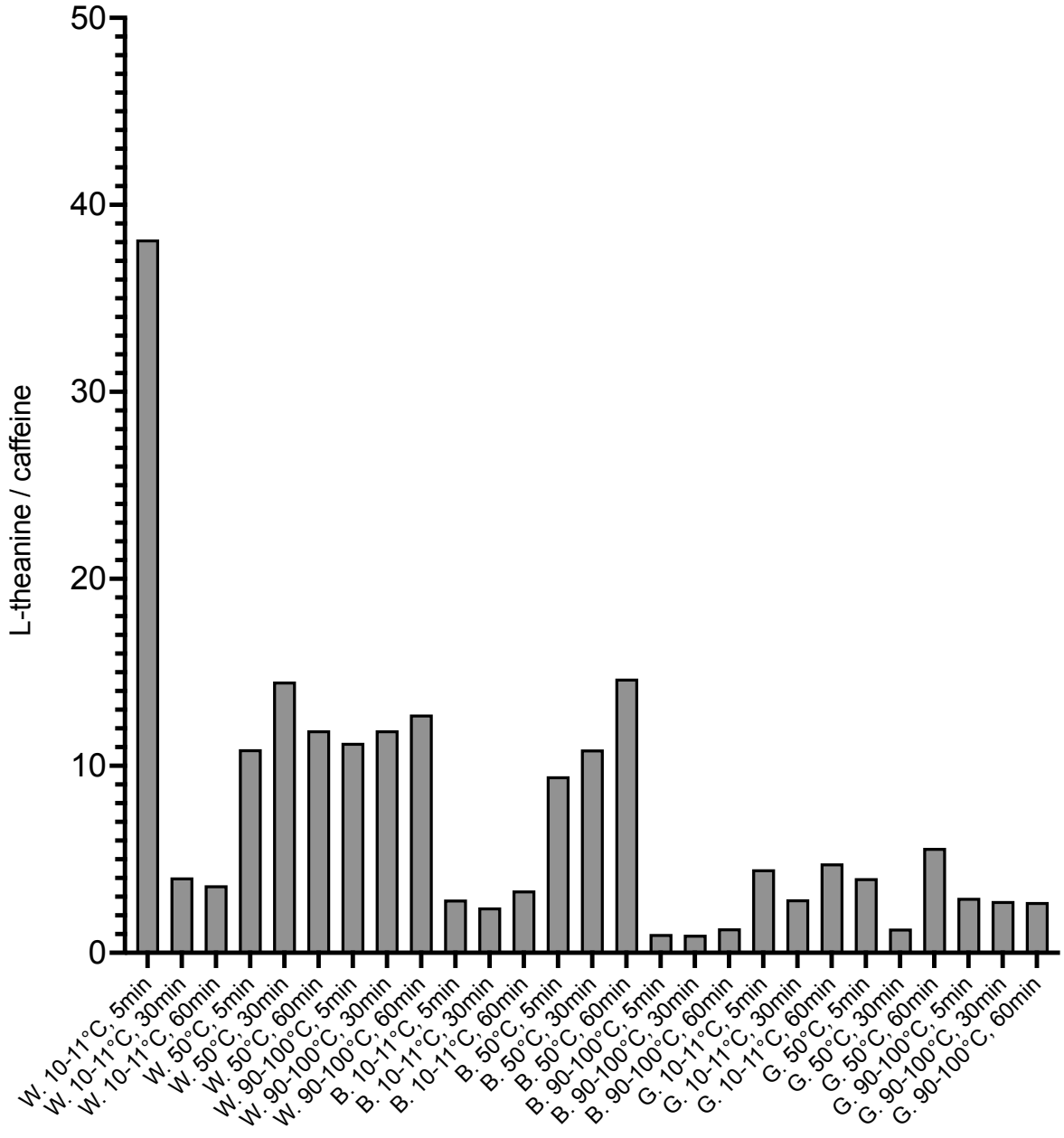


Figure 10- L-theanine-to-caffeine ratios determined from the average concentrations of L-theanine and caffeine obtained by HPLC analysis of white (W.), black (B.) and green (G.) tea, extracted at ~10-11°C, 50°C and 90-100°C, for 5 min, 30 min and 60 min.

GraphPad Prism software version 10.1.1 for macOS was used for plotting this graph and data analyses.

Based on the results presented, and the high L-theanine-to-caffeine ratio obtained in white tea, this tea was selected for subsequent experiments, and the emphasis was placed on L-theanine and caffeine levels and their ratios in the tea extracts.

4.2. Extraction of L-Theanine and Caffeine from Tea at Room Temperature

In this experiment, attempts were made to compare the extraction of L-theanine and caffeine at 10-11°C with their extraction at room temperature from white tea leaves.

The analysis of the HPLC data showed that at about 10-11°C, significantly (about 30%) more L-theanine was extracted compared to the room temperature extraction (~21°C). On the contrary, the caffeine level extracted at ~10-11°C was significantly (about 39%) lower than that extracted at room temperature (~21°C) ($P < 0.05$). These data are presented in Table 4 as means ± SD of 3 replicates.

In addition, at ~10-11°C significantly more L-theanine than caffeine was extracted from white tea leaves; whereas at room temperature (~21°C), significantly more caffeine than L-theanine was extracted ($P < 0.05$) (Figure 11).

Table 4- L-theanine and caffeine levels (mg/mL) and their ratios extracted from white tea at ~10-11°C and ~21°C after 5 min

Infusion temperature	L-theanine (mg/mL)	Caffeine (mg/mL)	L-theanine : caffeine
10-11°C	0.517 ± 0.037	0.344 ± 0.013	1.50 ± 0.13
21°C	0.399 ± 0.0005	0.562 ± 0.009	0.71 ± 0.01

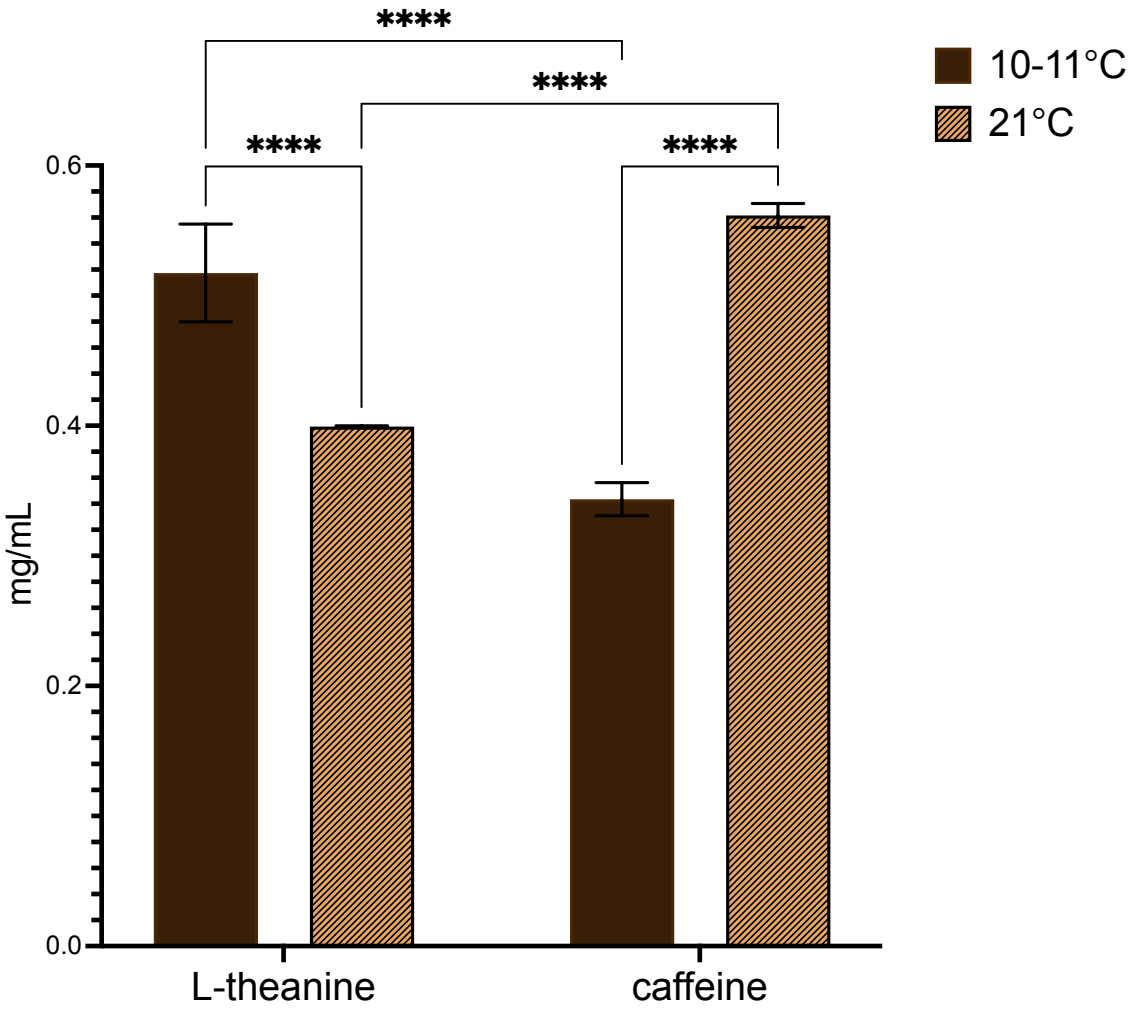


Figure 11- L-theanine and caffeine levels (mg/mL) extracted from white tea at cold temperature (~10-11°C) and room temperature (~21°C).

Two-way ANOVA. Data are means ± SD of samples analyzed in triplicate. Statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. **** indicates P < 0.0001.

The ratio of L-theanine concentration to caffeine concentration was determined for each sample. This ratio was significantly, and more than 2-fold, higher for the sample prepared at the colder temperature (~10-11°C) than that at the higher temperature (~21°C) (P-value < 0.05) (Figure 12 and Table 4).

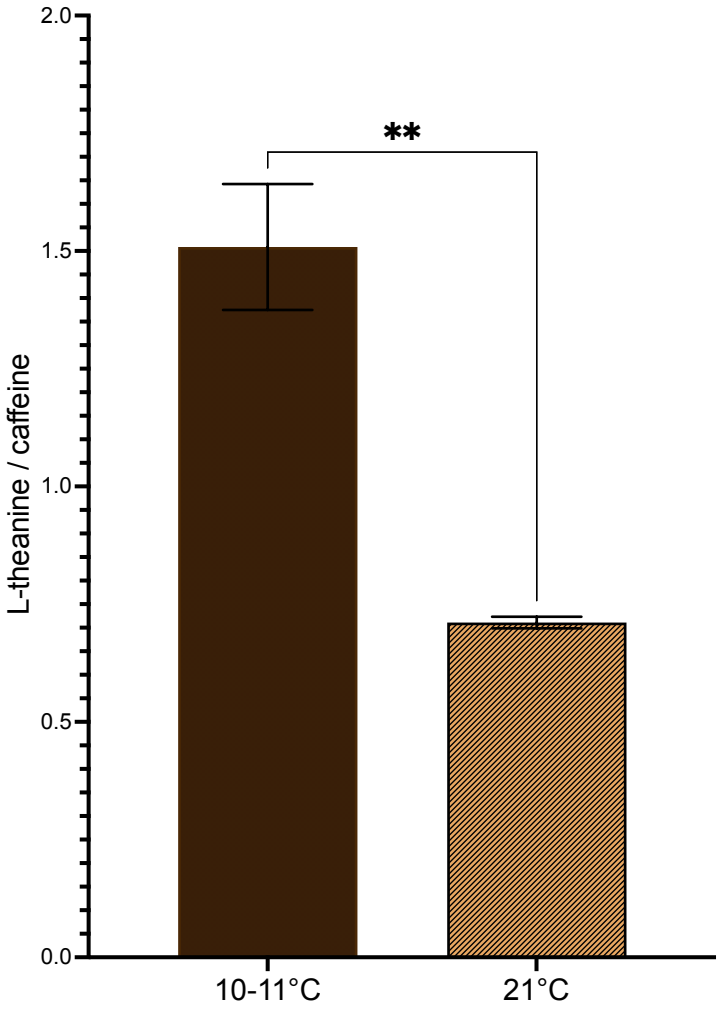


Figure 12- L-theanine-to-caffeine ratios for white tea extractions prepared at cold temperature (~10-11°C) and room temperature (~21°C).

Data are means ± SD of samples analyzed in triplicate. Paired t-test statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. ** indicates P-value 0.001 to 0.01.

4.3. Comparison of the DPPH Radical Scavenging Activity of White, Green and Black Tea Infusions

DPPH radical scavenging assay of white, green and black tea cold water extracts showed that black tea had significantly higher DPPH free radical scavenging activity (85 ± 0.23 %) followed by white tea (76 ± 0.13 %) and green tea (71 ± 0.35 %) ($P < 0.05$; means ± SD of 3 replicates) (Figure 13 and Table 5). DPPH radical scavenging activity of the positive control, ascorbic acid (0.37 mM to 3.78 mM), was between 98.09 ± 0.18 to 98.68 ± 0.17 % and about 15 to 16 % higher than for the black tea (data are means ± SD of 3 replicates).

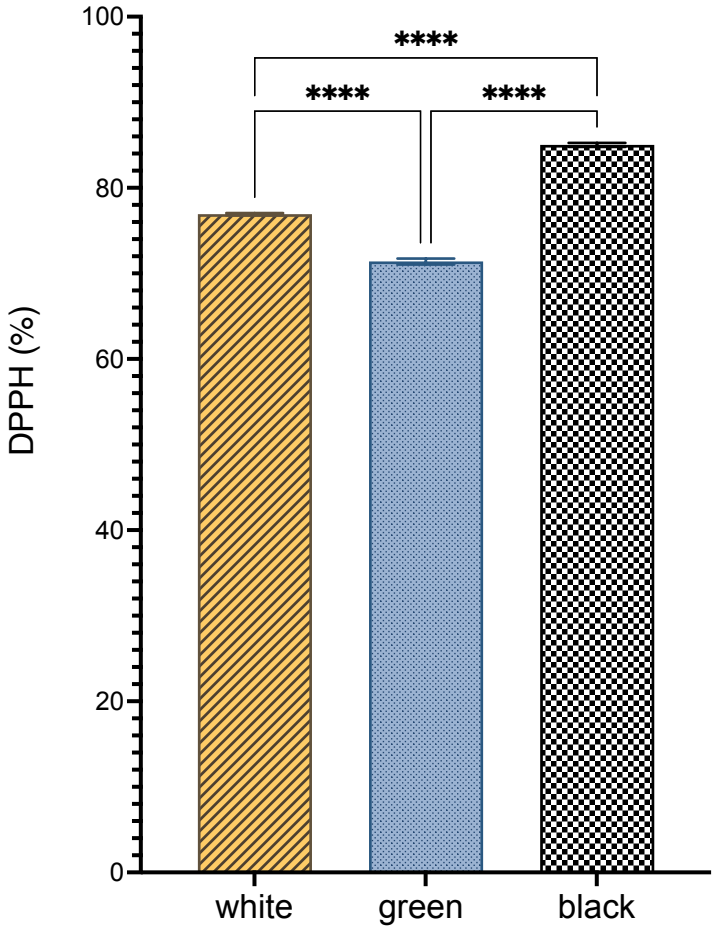


Figure 13- Comparison of the DPPH radical scavenging activity (%) of white, green and black tea infusions.

Data are means ± SD of samples analyzed in triplicate. Ordinary one-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. **** indicates $P < 0.0001$.

When comparing the L-theanine and caffeine levels infused in these tea samples, both L-theanine and caffeine levels were significantly lower in black tea than in green and white tea ($P < 0.05$). Additionally, as shown in Figure 14, green and white tea, had similar levels of L-theanine and caffeine.

L-theanine extracted from white and green tea was more than 40% higher than from black tea. Caffeine extracted from white and green tea was more than 4 folds higher than from black tea. Table 5 shows the mean ± SD of 3 replicates for L-theanine, caffeine and their ratios in the tea samples as well as the DPPH radical scavenging activity (%) of the tea samples.

Table 5- L-theanine (mg/mL), caffeine (mg/mL), L-theanine-to-caffeine ratio and DPPH (%) in the extracts of white, green and black teas

Tea sample	L-theanine (mg/mL)	Caffeine (mg/mL)	L-theanine:caffeine	DPPH (%)
White	0.448 ± 0.001	0.432 ± 0.016	1.039 ± 0.042	76.92 ± 0.13
Green	0.441 ± 0.034	0.448 ± 0.019	0.988 ± 0.106	71.39 ± 0.35
Black	0.310 ± 0.017	0.106 ± 0.004	2.94 ± 0.286	85.02 ± 0.23

Black tea infusions had significantly (about 3 folds) more L-theanine (0.31 ± 0.01 mg/mL) than caffeine (0.1 ± 0.004 mg/mL) ($P < 0.05$); whereas the L-theanine and caffeine levels were not significantly different from each other in both the green tea (0.44 ± 0.03 mg/mL and 0.44 ± 0.01 mg/mL respectively) and the white tea (0.44 ± 0.001 mg/mL and 0.43 ± 0.01 mg/mL respectively) infusions (data are means ± SD of 3 replicates) (Figure 14 and Table 5). Accordingly, the L-theanine-to-caffeine ratio was significantly higher for black tea ($P < 0.05$) compared to white and green teas. This ratio in black tea was about 3 folds higher than in white and green teas (Figure 15).

According to these results, there may be a correlation between DPPH radical scavenging activity and the L-theanine-to-caffeine ratios; since black tea, with the higher L-theanine-to-caffeine ratio, had a higher DPPH radical scavenging activity. The contribution of the polyphenols

of the different teas, which normally play a major role in the antioxidant property of the infusions, may have been less influential; because in this experiment a PVPP treatment was used which based on its saturation (Díaz et al., 2022), should have removed a major portion of the polyphenols from the tea infusions during preparation.

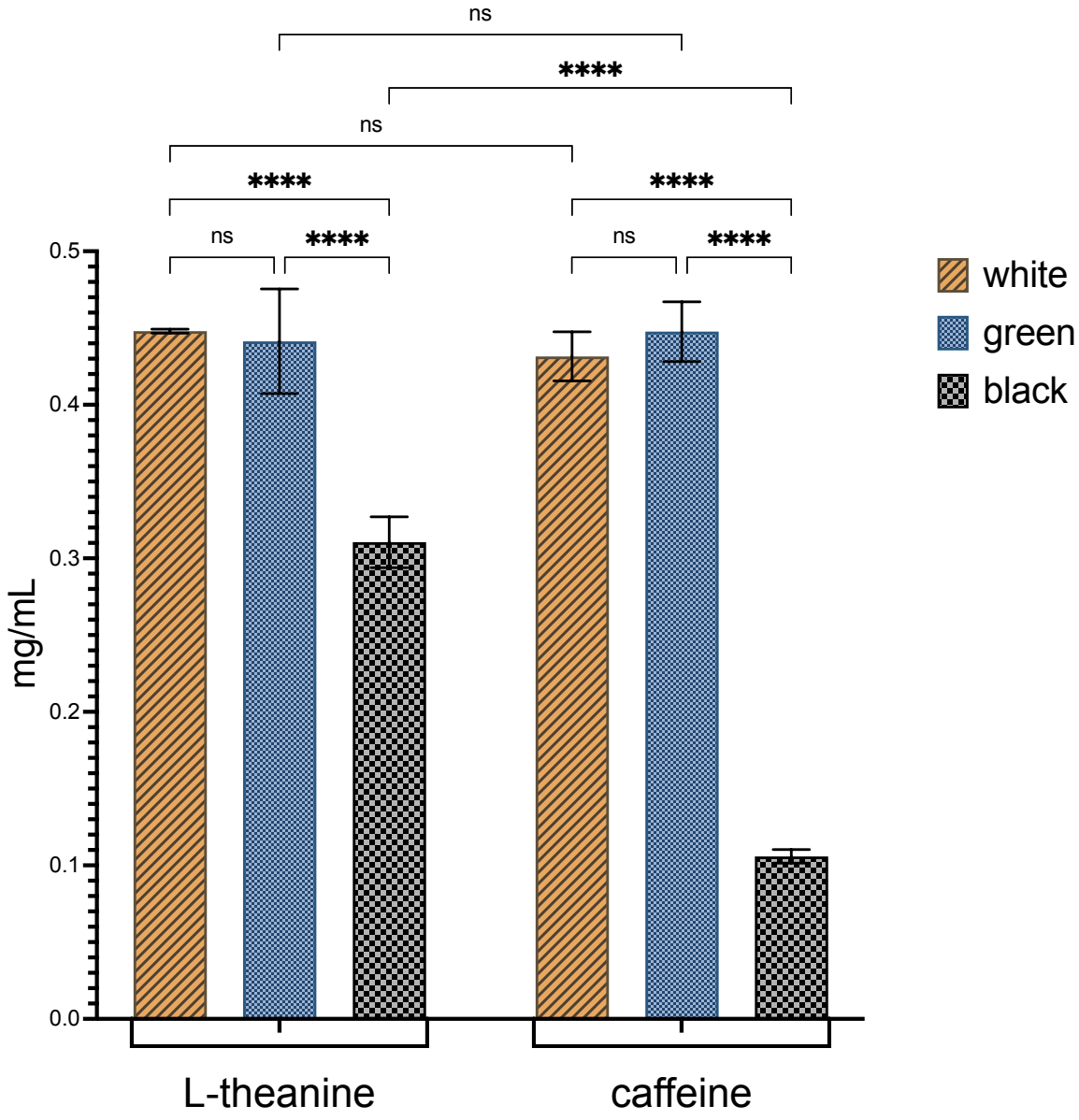


Figure 14- L-theanine and caffeine levels (mg/mL) in white, green and black tea infusions analyzed by HPLC.

Data are means ± SD of samples analyzed in triplicate. Two-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. **** indicates P < 0.0001; ns indicates P ≥ 0.05.

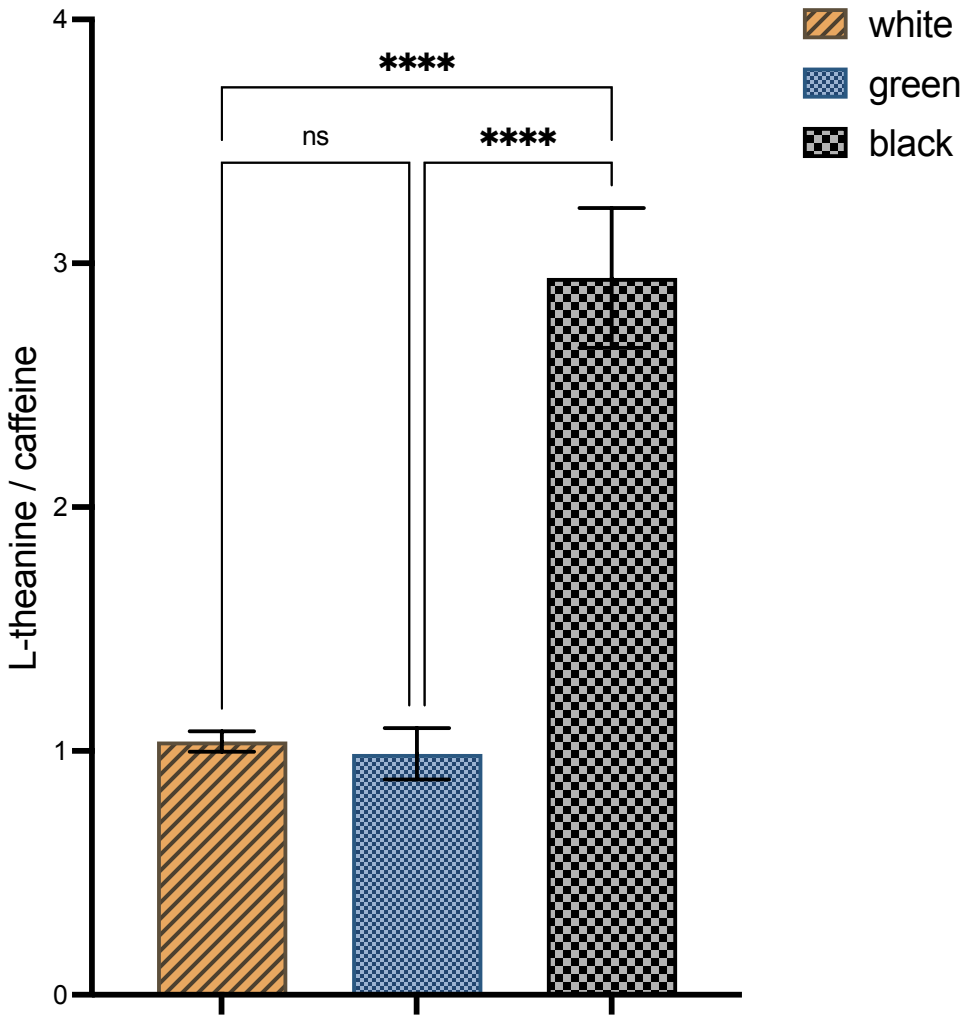


Figure 15- L-theanine-to-caffeine ratio for white, green and black tea infusions.

Data are means ± SD of samples analyzed in triplicate. Ordinary one-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. **** indicates P < 0.0001; ns indicates P ≥ 0.05.

4.4. Extraction of L-Theanine and Caffeine in Water, from Tea Leaves Pre-treated with Methanol

In this experiment, tea leaves were pre-treated with pure methanol. Aqueous methanol was not tested in this experiment, because according to the results of Jun (2009), the extraction of caffeine from tea leaves is not substantially different when either pure methanol or aqueous methanol is used (Jun, 2009). On the other hand, amino acids are more soluble in aqueous solvents than in pure solvents (Dey & Lahiri, 1986). Therefore, L-theanine would be more soluble in aqueous methanol than in pure methanol and be removed along with caffeine. This would reduce the selectivity of the methanol pre-treatment method for the preferential removal of caffeine.

HPLC analysis of tea samples prepared by extraction of methanol-treated white tea leaves in water, as described in the methodology section, showed that compared to the control, more L-theanine could be infused in water from tea leaves when they were first infused in methanol for 15 min. Significantly lower levels of L-theanine (about 32% less) were infused in methanol than in water (the control) ($P < 0.05$). When tea was infused in methanol, 0.42 ± 0.04 mg/mL L-theanine was extracted; whereas, when the same tea biomass was again infused in water (2-step extraction), 0.73 ± 0.13 mg/mL L-theanine was extracted. In the control sample, which involved a single infusion of tea leaves in water, 0.62 ± 0.05 mg/mL L-theanine was extracted indicating that L-theanine is more soluble in water than in methanol (data are means ± SD of 3 replicates). Overall, about 18% more L-theanine was extracted from the methanol-treated white tea leaves than from the control (Table 6 and Figure 16).

Table 6- L-theanine and caffeine levels (mg/mL) and L-theanine-to-caffeine ratios in the extracts of methanol-treated and methanol-untreated white tea leaves (means ± SD of 3 replicates)

Sample	L-theanine (mg/mL)	Caffeine (mg/mL)	L-theanine : caffeine
Methanol treated (2-step extraction)	0.736 ± 0.133	0.511 ± 0.049	1.446 ± 0.26
Methanol untreated (control)	0.624 ± 0.051	0.633 ± 0.100	0.995 ± 0.082
Methanolic fraction/eluate	0.424 ± 0.041	0.552 ± 0.014	0.77 ± 0.094

The HPLC analysis also showed that the extraction solvent did not significantly influence the level of caffeine infusion from white tea. However, about 19% less caffeine was infused in the water after being treated with methanol (0.51 ± 0.04 mg/mL) than in water alone (the control) (0.63 ± 0.1 mg/mL). In addition, the HPLC analysis of the separated methanolic fraction showed that about 8% more caffeine was extracted from tea in methanol alone (0.55 ± 0.01 mg/mL) compared to the 2-step extraction when the methanol-treated tea biomass was infused in water (0.51 ± 0.04 mg/mL) (data are means ± SD of 3 replicates) (Figure 16).

Figure 16 also shows that the tea sample obtained by water extraction of methanol-treated tea (2-step extraction) is the only sample with significantly higher L-theanine than caffeine levels

(about 44% more L-theanine than caffeine) ($P < 0.05$). Whereas, the control and the methanolic fraction had higher caffeine than L-theanine levels (Figure 16).

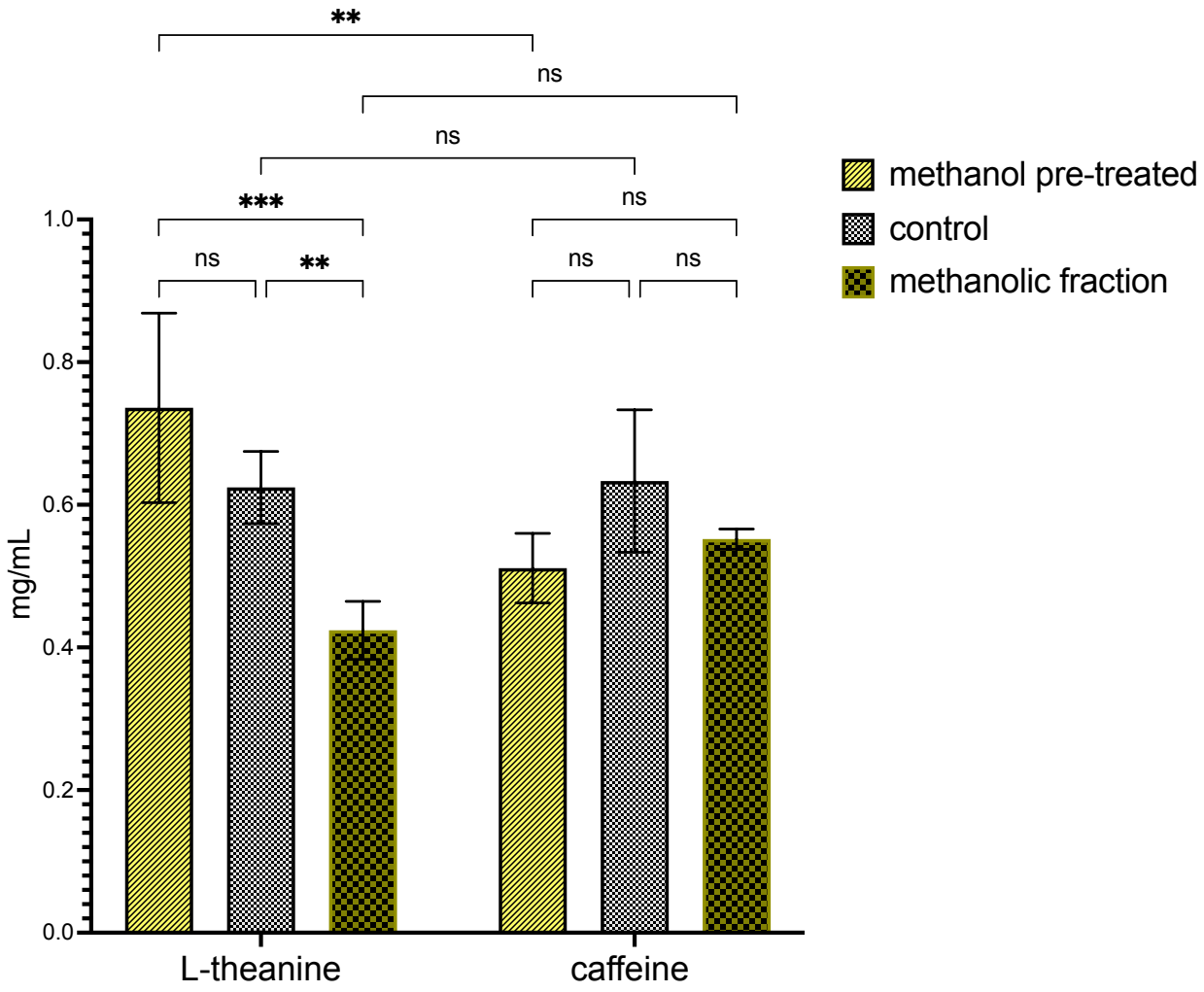


Figure 16- L-theanine and caffeine levels (mg/mL) extracted from white tea leaves with methanol pre-treatment and without methanol pre-treatment (control).

Data are means ± SD of samples analyzed in triplicate. Two-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. ** indicates P-value 0.001 to 0.01; *** indicates P-value 0.0001 to 0.001; ^{ns} indicates $P \geq 0.05$.

The L-theanine-to-caffeine ratios of the white tea samples indicate that this ratio was significantly (about 45%) higher when the methanol pre-treatment was conducted before extraction in water (1.44 ± 0.26), compared to the control (0.99 ± 0.08) ($P < 0.05$). This ratio was the lowest in the separated methanolic fraction (0.77 ± 0.09) (data are means ± SD of 3 replicates) (Figure 17).

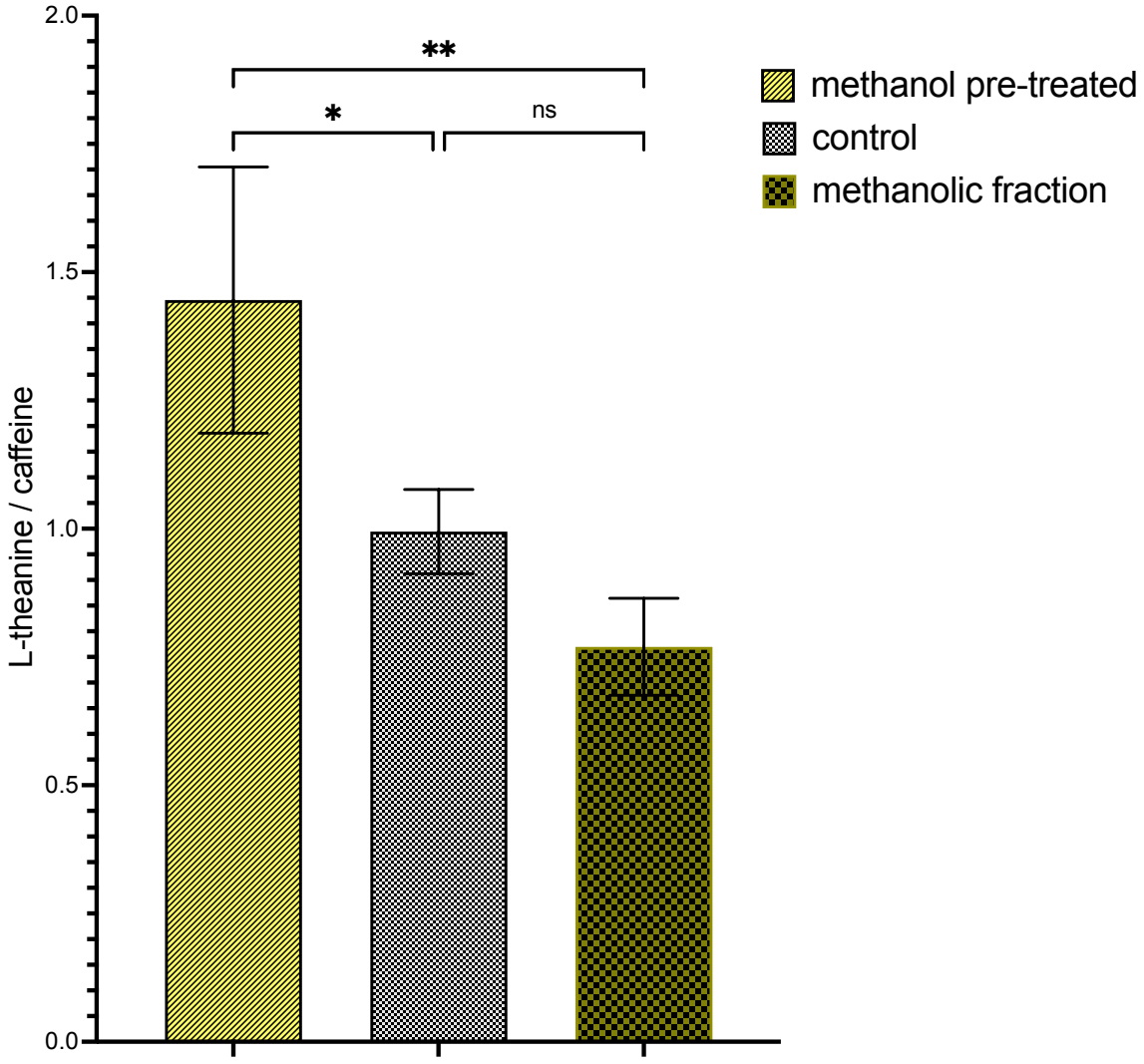
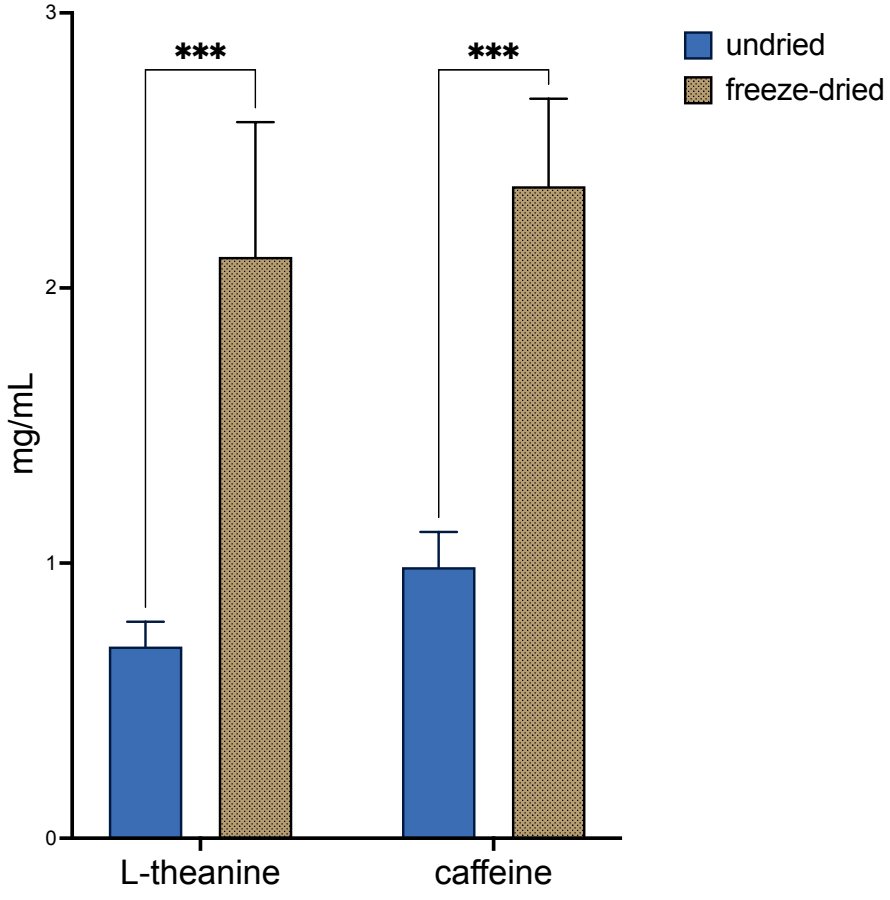


Figure 17- L-theanine-to-caffeine ratios for white tea extractions prepared after methanol pre-treatment of tea leaves compared to the control.

Data are means ± SD of samples analyzed in triplicate. Ordinary one-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. * indicates P-value 0.01 to 0.05; ** indicates P-value 0.001 to 0.01; ^{ns} indicates $P \geq 0.05$.

4.5. Effect of Drying on the Preservation of L-Theanine and Caffeine in Tea Infusions

HPLC analysis of the tea infusions before and after drying showed that after freeze-drying significantly more L-theanine and caffeine could be detected in the dried infusion (respectively about 200% and 140% more than before drying) (Figure 18a). Whereas, after spray-drying, significantly less L-theanine and caffeine (respectively about 30% and 45% less than before drying) could be detected in the dried infusion ($P < 0.05$) (Figure 18b).



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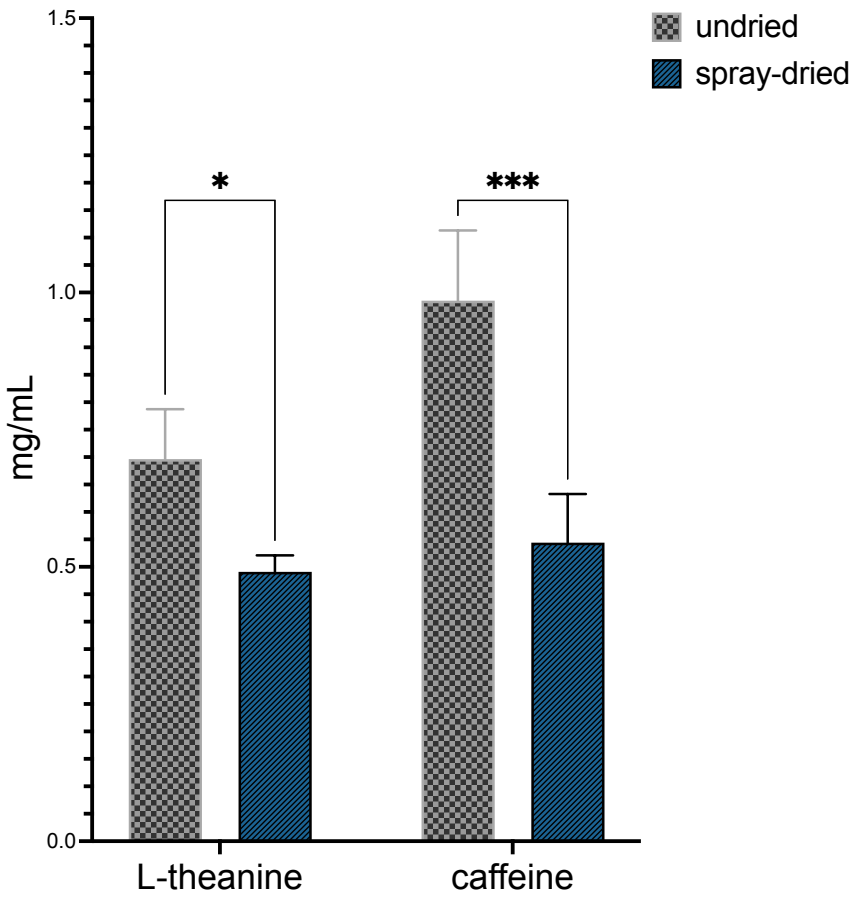
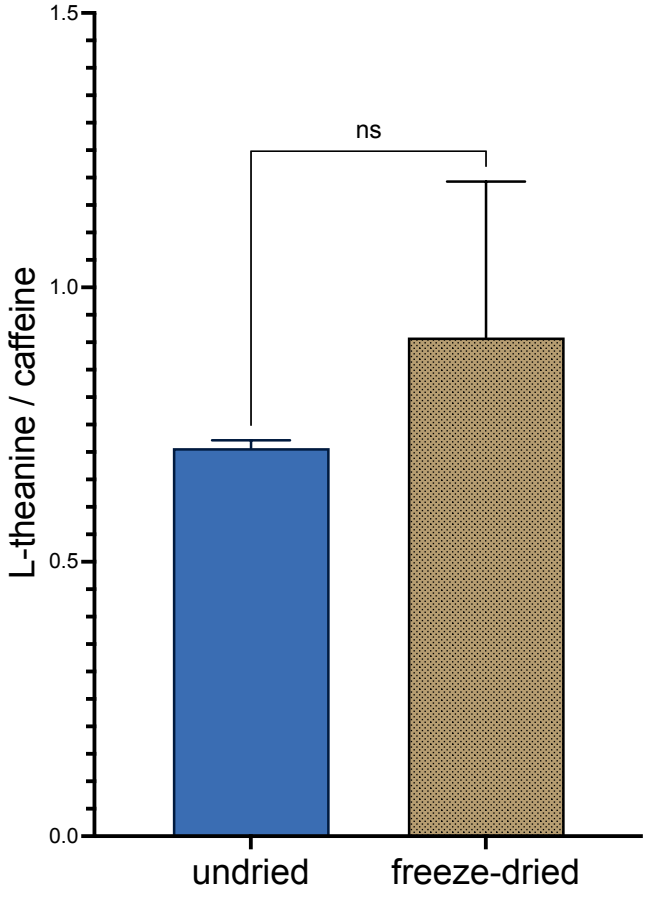


b

Figure 18- L-theanine and caffeine levels (mg/mL) in white tea infusions after being a) freeze-dried and b) spray-dried compared to the control (undried/liquid samples).

Data are means ± SD of samples analyzed in triplicate. Two-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. * indicates P-value 0.01 to 0.05; *** indicates P value 0.0001 to 0.001.

The L-theanine-to-caffeine ratios for freeze-dried and spray-dried tea samples were respectively about 29% and 31% more than that before drying although these differences were not statistically significant ($P \geq 0.05$). These data are shown in Figure 19a and b.



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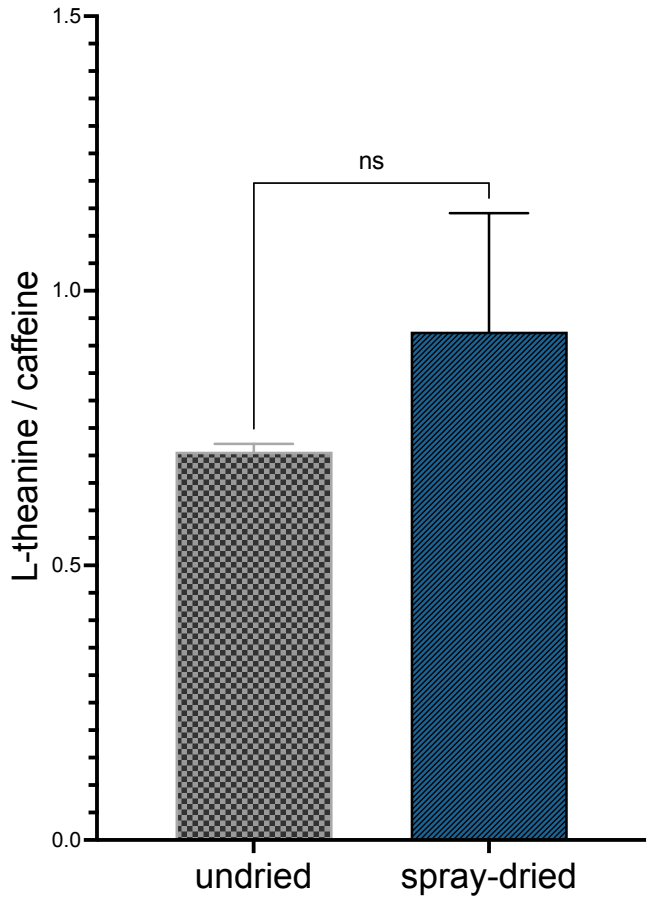


b

Figure 19- L-theanine-to-caffeine ratios in a) freeze-dried and b) spray-dried white tea infusions compared to the control (undried/liquid samples).

Data are means ± SD of samples analyzed in triplicate. Paired t-test statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. ^{ns} indicates $P \geq 0.05$.

4.6. Effect of US-Assisted Extraction on the Levels of L-Theanine and Caffeine in Tea Infusions

The HPLC analysis of US-treated and US-untreated white tea samples showed that the extraction of L-theanine and caffeine from tea increased with a time dependency in both treatments. The L-theanine level extracted from white tea without applying ultrasound (US) was 0.21 ± 0.03 mg/mL after 5 min extraction. After 20 min extraction, the L-theanine level in US-untreated samples increased by about 2 folds to 0.41 ± 0.002 mg/mL. In US-treated samples, the level of L-theanine was 0.51 ± 0.09 mg/mL after 5 min US treatment. After 20 min of treatment with US, L-theanine level increased by about 1.6 fold to 0.81 ± 0.06 mg/mL (data are means ± SD of 3 replicates) (Table 7 and Figure 20).

The caffeine level extracted from white tea without applying US was 0.16 ± 0.06 mg/mL after 5 min extraction. After 20 min extraction the caffeine level in the US-untreated samples increased by more than 3 folds to 0.59 ± 0.02 mg/mL. In US-treated samples, after 5 min US treatment 0.68 ± 0.16 mg/mL caffeine level was extracted. After 20 min US treatment caffeine level increased by about 2 folds to 1.34 ± 0.07 mg/mL (Table 7 and Figure 20) (data are means ± SD of 3 replicates).

Table 7- L-theanine and caffeine levels (mg/mL) and their ratios extracted from white tea with and without applying ultrasound (US)

Treatment	Time (min)	L-theanine (mg/mL)	Caffeine (mg/mL)	L-theanine : caffeine
US-treated	5	0.518 ± 0.095	0.687 ± 0.165	0.762 ± 0.069
	20	0.812 ± 0.06	1.349 ± 0.072	0.602 ± 0.015
US-untreated	5	0.216 ± 0.033	0.167 ± 0.062	1.358 ± 0.258
	20	0.41 ± 0.002	0.594 ± 0.024	0.691 ± 0.024

These data also clearly show that the conventional infusion of tea for 5 min (i.e. the 5 min US-untreated samples) is the only treatment that extracted more L-theanine than caffeine; whereas, all the other treatments resulted in the extraction of more caffeine than L-theanine (Figure 20).

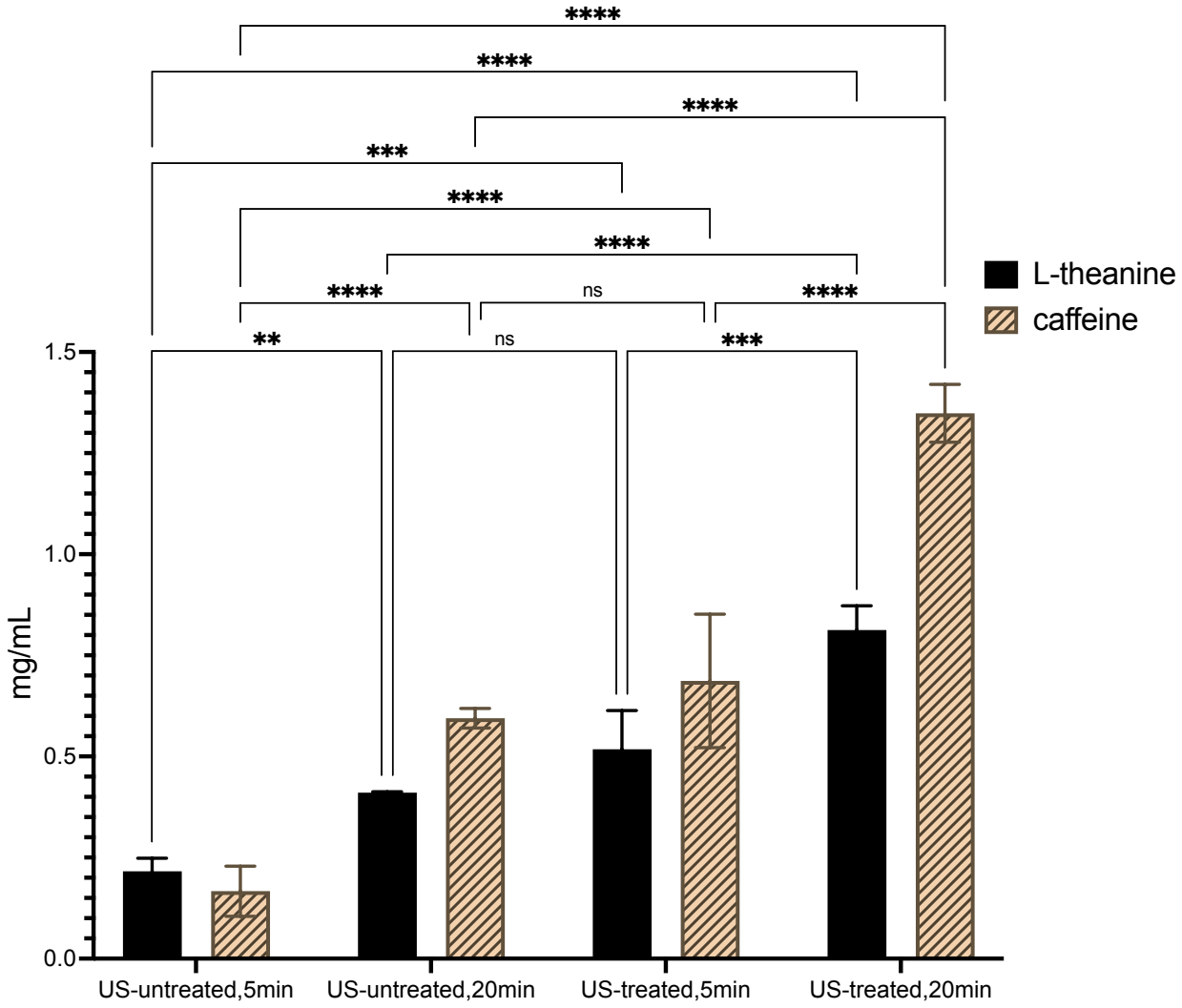


Figure 20- L-theanine and caffeine levels (mg/mL) extracted from white tea with and without US treatment for 5 and 20 min.

Data are means ± SD of 3 samples. Two-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. ** indicates P-values between 0.001 to 0.01; *** indicates P-values between 0.0001 to 0.001; **** indicates $P < 0.0001$; ^{ns} indicates $P \geq 0.05$.

The ratios between L-theanine concentrations and caffeine concentrations were determined and showed that the tea infusion prepared without US for 5 min had a significantly higher L-

theanine-to-caffeine ratio compared to other treatments ($P < 0.05$). The L-theanine-to-caffeine ratio for the 5 min US-untreated sample was about 2 folds higher than for other treatments. This ratio was not significantly different among other treatments (Figure 21 and Table 7).

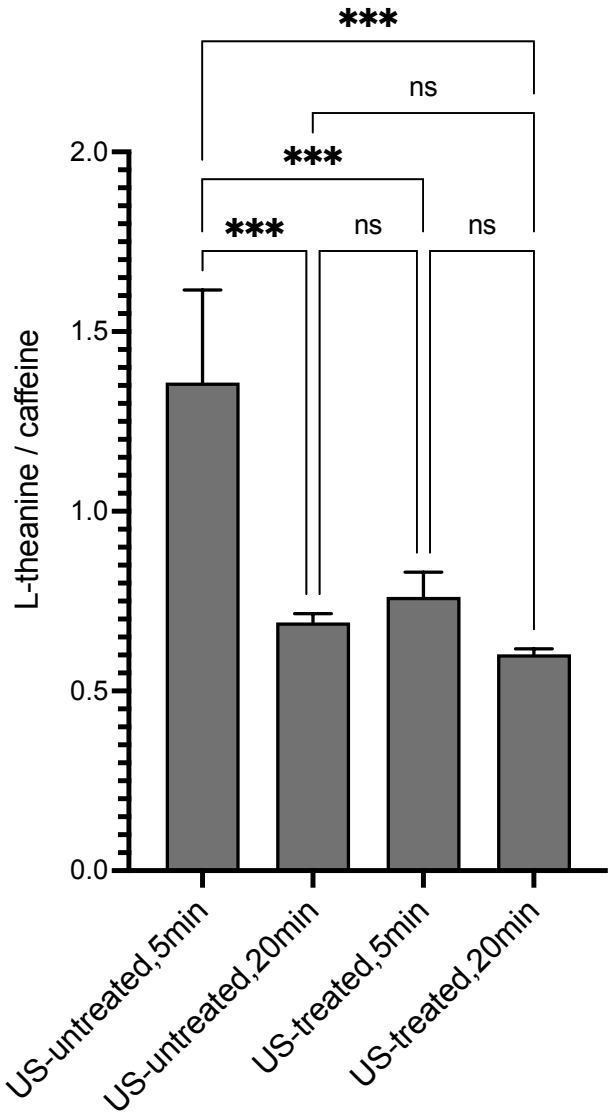


Figure 21- L-theanine-to-caffeine ratios for white tea samples with or without US treatment after 5 and 20 min.

Results are means ± SD of three replicates. Ordinary one-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was

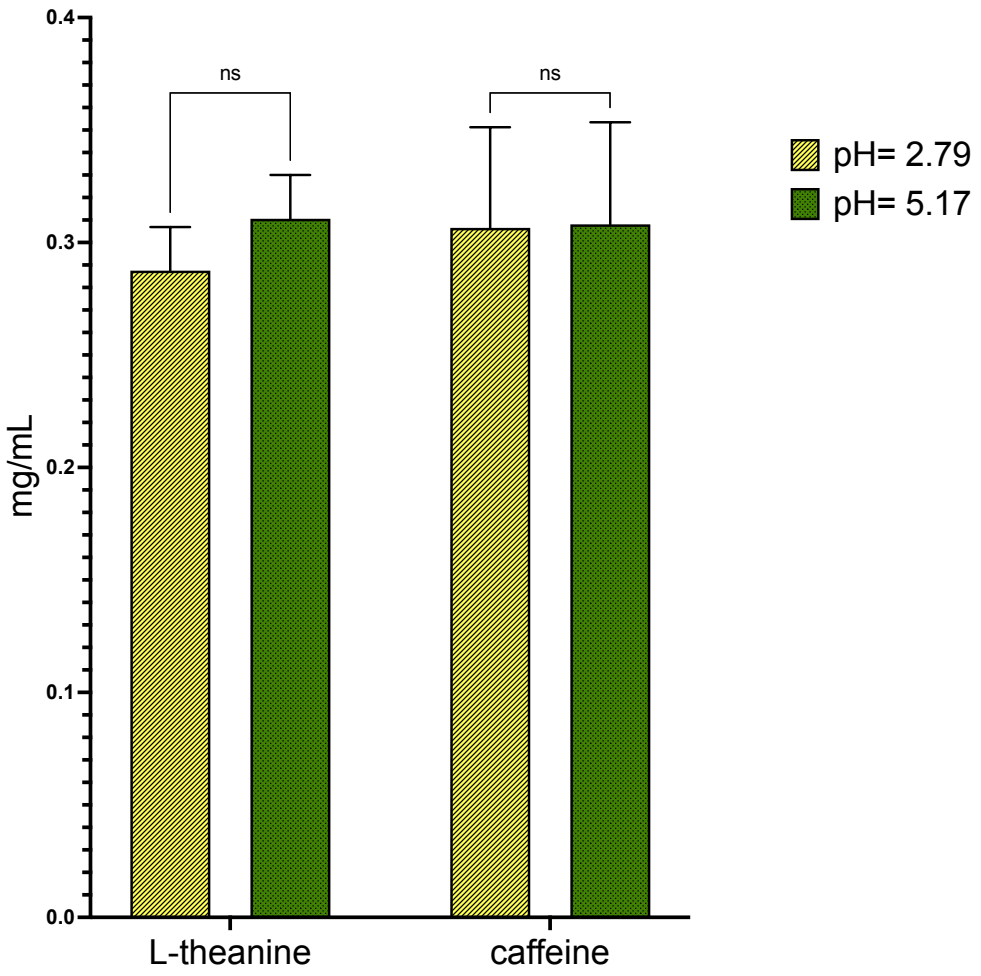
considered statistically significant. *** indicates P-values between 0.0001 to 0.001; ^{ns} indicates P $\geq$ 0.05.

4.7. Effects of Common Additives on the Levels of L-Theanine and Caffeine in Tea Infusions

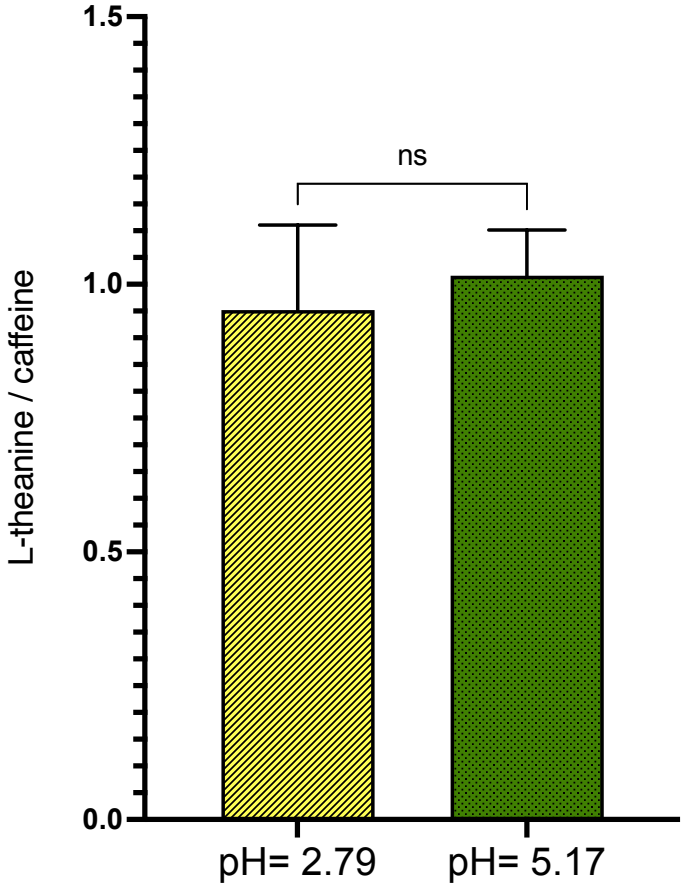
4.7.1. Effect of Adding Lemon Juice During Infusion on L-Theanine and Caffeine Levels of Tea Infusions

The HPLC analysis of the lemon-juice-treated and untreated samples revealed that lowering the pH did not significantly affect the levels of L-theanine and caffeine in tea infusions. When lemon juice was added to the tea sample, 0.288 ± 0.019 mg/mL L-theanine was extracted and when no lemon juice was added to the tea sample 0.311 ± 0.019 mg/mL L-theanine was extracted. The caffeine level in the lemon-juice-treated and untreated tea samples was 0.307 ± 0.045 mg/mL and 0.308 ± 0.045 mg/mL respectively (data are means ± SD of 3 replicates) (Figure 22a).

Consequently, the L-theanine-to-caffeine ratios in the lemon-juice-treated and lemon-juice-untreated samples were not significantly different; although, this ratio in the lemon-juice-untreated sample (control; pH = 5.17) was slightly higher (L-theanine : caffeine = 1.01 ± 0.08) than in the sample with lowered pH (lemon-juice-treated; pH = 2.79) (L-theanine : caffeine = 0.95 ± 0.15) (data are means ± SD of 3 replicates) (Figure 22b).



a



b

Figure 22- a) L-theanine and caffeine levels (mg/mL) extracted from white tea leaves in the presence (pH = 2.79) and absence (pH = 5.17) of lemon juice; b) L-theanine-to-caffeine ratios in white tea samples treated (pH = 2.79) and untreated (pH = 5.17) with lemon juice.

Two-way ANOVA statistical analysis was performed for data in Figure 22a and paired t-test statistical analysis was performed for data in Figure 22b using GraphPad Prism software version 10.1.1 for macOS. Data are means ± SD of 3 replicates. P-values < 0.05 were considered statistically significant. ^{ns} indicates $P \geq 0.05$.

4.7.2. Effect of Adding Milk to Tea Extracts/Infusions on Their L-Theanine and Caffeine Levels

The HPLC analysis of the tea infusion samples treated with different amounts of milk permitted to determine the levels of L-theanine and caffeine in each sample. It was observed that adding 10% and 20% (V/V) milk to tea did not have a significant effect on the level of L-theanine. L-theanine levels in 0%, 10% and 20% (V/V) milk-treated samples were respectively 0.648 ± 0.007 mg/mL, 0.628 ± 0.005 mg/mL and 0.647 ± 0.024 mg/mL (P-values ≥ 0.05). However, adding 50% (V/V) milk significantly reduced the level of L-theanine in tea samples by about 25% lower than the control (0% milk V/V) at 0.487 ± 0.03 mg/mL ($P < 0.05$) (data are means ± SD of 3 replicates) (Figure 23 and Table 8).

For the case of caffeine, adding milk to tea significantly decreased the level of caffeine in a concentration-dependent manner and the caffeine level in tea samples had a reverse relationship with the amount of added milk to the tea solution sample. Caffeine in tea added with 0%, 10%, 20% and 50% (V/V) milk was 0.478 ± 0.017 mg/mL, 0.396 ± 0.056 mg/mL, 0.279 ± 0.051 mg/mL and 0.24 ± 0.027 mg/mL respectively (data are means ± SD of 3 replicates) (Figure 23 and Table 8). Compared to the control (0% V/V milk), the caffeine level was about 17%, 42% and 50% lower in the 10%, 20% and 50% V/V milk-added samples, respectively (P-values < 0.05) (Figure 23).

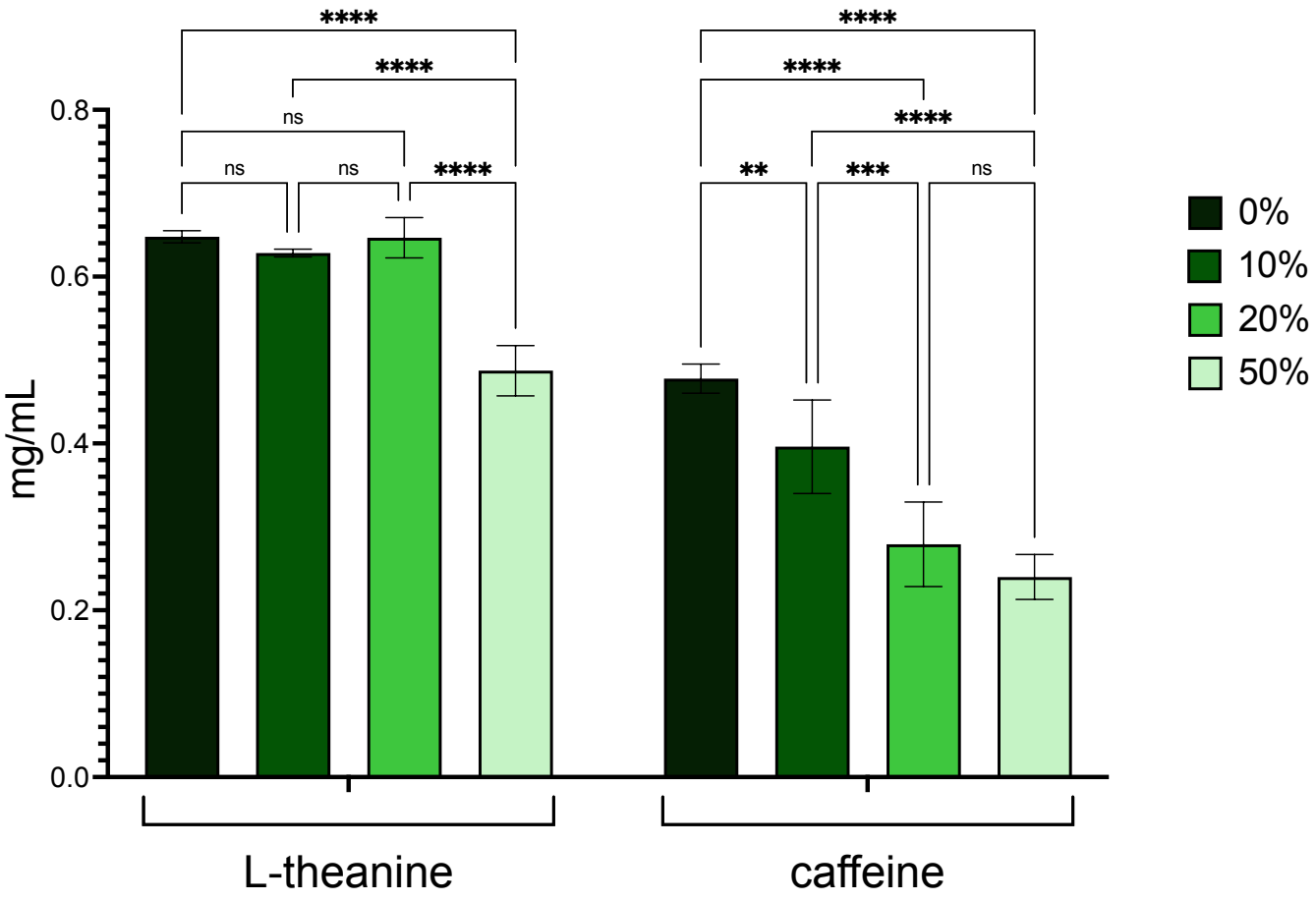


Figure 23- L-theanine and caffeine levels (mg/mL) in white tea samples added with 0%, 10%, 20% and 50% (V/V) milk.

Data are means ± SD of samples analyzed in triplicate. Two-way ANOVA statistical analyses were performed using GraphPad Prism Software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. ** indicates P-value 0.001 to 0.01; *** indicates P-value 0.0001 to 0.001; **** indicates $P < 0.0001$; ^{ns} indicates $P \geq 0.05$.

Table 8- L-theanine and caffeine levels (mg/mL) and their ratios in white tea infusions treated with or without milk

Treatment (milk V/V%)	L-theanine (mg/mL)	Caffeine (mg/mL)	L-theanine : caffeine
0	0.648 ± 0.007	0.478 ± 0.017	1.357 ± 0.047
10	0.628 ± 0.005	0.396 ± 0.056	1.61 ± 0.252
20	0.647 ± 0.024	0.279 ± 0.051	2.374 ± 0.468
50	0.487 ± 0.03	0.24 ± 0.027	2.045 ± 0.196

The L-theanine-to-caffeine ratios in the tea samples added with 0% to 50% V/V milk are shown in Figure 24 and indicate that this ratio was highest when 20% milk was added to the tea (2.37 ± 0.46), while the lowest ratio belonged to the tea sample without milk (1.35 ± 0.04) (data are means ± SD of 3 replicates) (Figure 24).

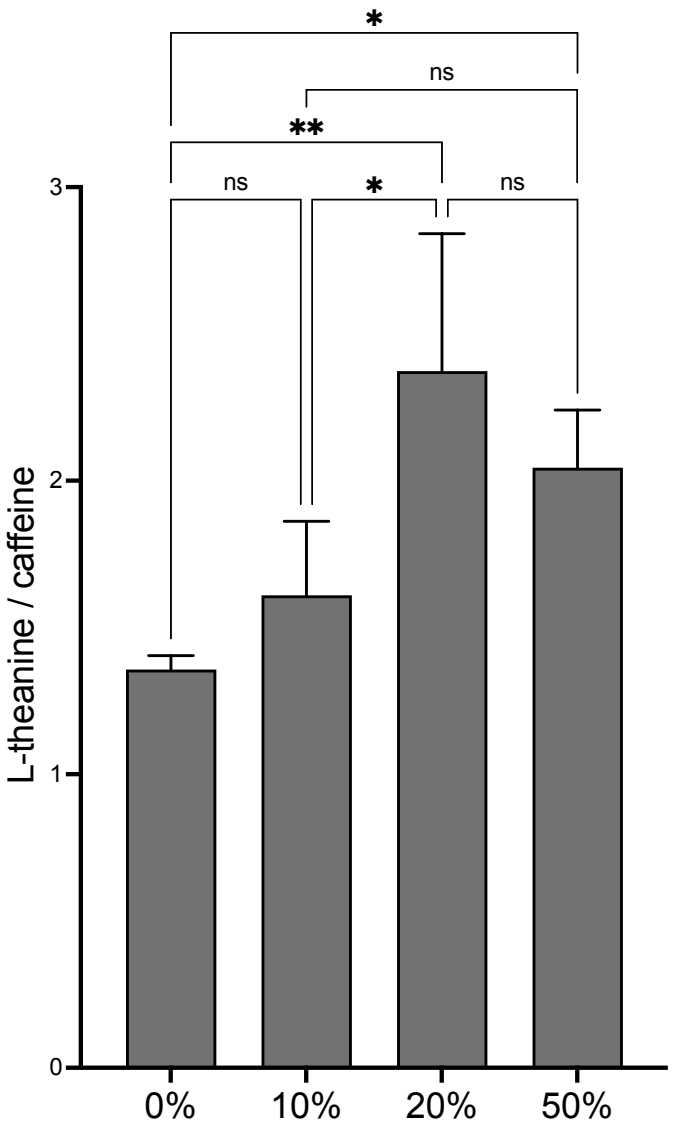


Figure 24- L-theanine-to-caffeine ratios in white tea samples added with 0%, 10%, 20% and 50% (V/V) milk.

Data are means ± SD of samples analyzed in triplicate. Ordinary one-way ANOVA statistical analyses were performed using GraphPad Prism software version 10.1.1 for macOS. P-value < 0.05 was considered statistically significant. * indicates P-value 0.01 to 0.05; ** indicates P-value 0.001 to 0.01; ^{ns} indicates $P \geq 0.05$.

4.8. Storage of Freeze-Dried, Spray-Dried and Liquid Tea Preparations

The spray-dried, freeze-dried and undried white tea samples were prepared and stored for 60 days at 39 ± 1°C. The samples were analyzed every 20 days (on days 0, 20, 40 and 60) as described in the methodology detailed in Chapter 3. DPPH radical scavenging activity and L-theanine and caffeine levels of all the samples were assessed on these days. The moisture contents of the dried samples were also assessed.

4.8.1. DPPH Radical Scavenging Activities of the Dried and Undried Tea Samples During the Storage Period

DPPH radical scavenging assay of the tea samples during 60 days of storage showed that both dried tea samples (freeze-dried and spray-dried) maintained a high antioxidant activity for at least 60 days. On the other hand, the undried tea samples lost most of their DPPH radical scavenging activity during this same storage period.

The DPPH radical scavenging activity of both dried tea samples followed the same trend during the 60 days of storage. In both dried samples the DPPH radical scavenging activity increased until it reached a peak on day 40 of storage and then it slightly decreased (Figure 25).

The DPPH radical scavenging activity of the freeze-dried tea increased by about 5% up to day 20 and then by about 2% from day 20 to day 40. This value then decreased by about 1.3% from day 40 to day 60 (P-values ≥ 0.05; two-way ANOVA) (Figure 25).

The DPPH radical scavenging activity of the spray-dried tea increased by about 2% up to day 20 and then by about 5% from day 20 to day 40. This value then decreased by about 2% from day 40 to day 60 (P-values ≥ 0.05; two-way ANOVA) (Figure 25).

The DPPH radical scavenging activity of the undried tea samples did not follow the same trend as the dried tea samples. The DPPH radical scavenging activity in the undried tea samples dropped by about 30% up to day 40 ($P \geq 0.05$) and then dropped significantly by more than 70% from day 40 to day 60 ($P < 0.05$) (two-way ANOVA). Overall, about 80% of the DPPH radical scavenging activity was lost in the undried tea samples from day 0 to day 60 of storage ($P < 0.05$; two-way ANOVA) (Figure 25).

DPPH radical scavenging activity of the positive control, ascorbic acid (1.5 mM), was 98.36 ± 0.1 % (mean ± SD of 3 replicates) and about 13%, 15% and 30% higher than the day 0 activity of the freeze-dried, spray-dried and undried tea samples respectively.

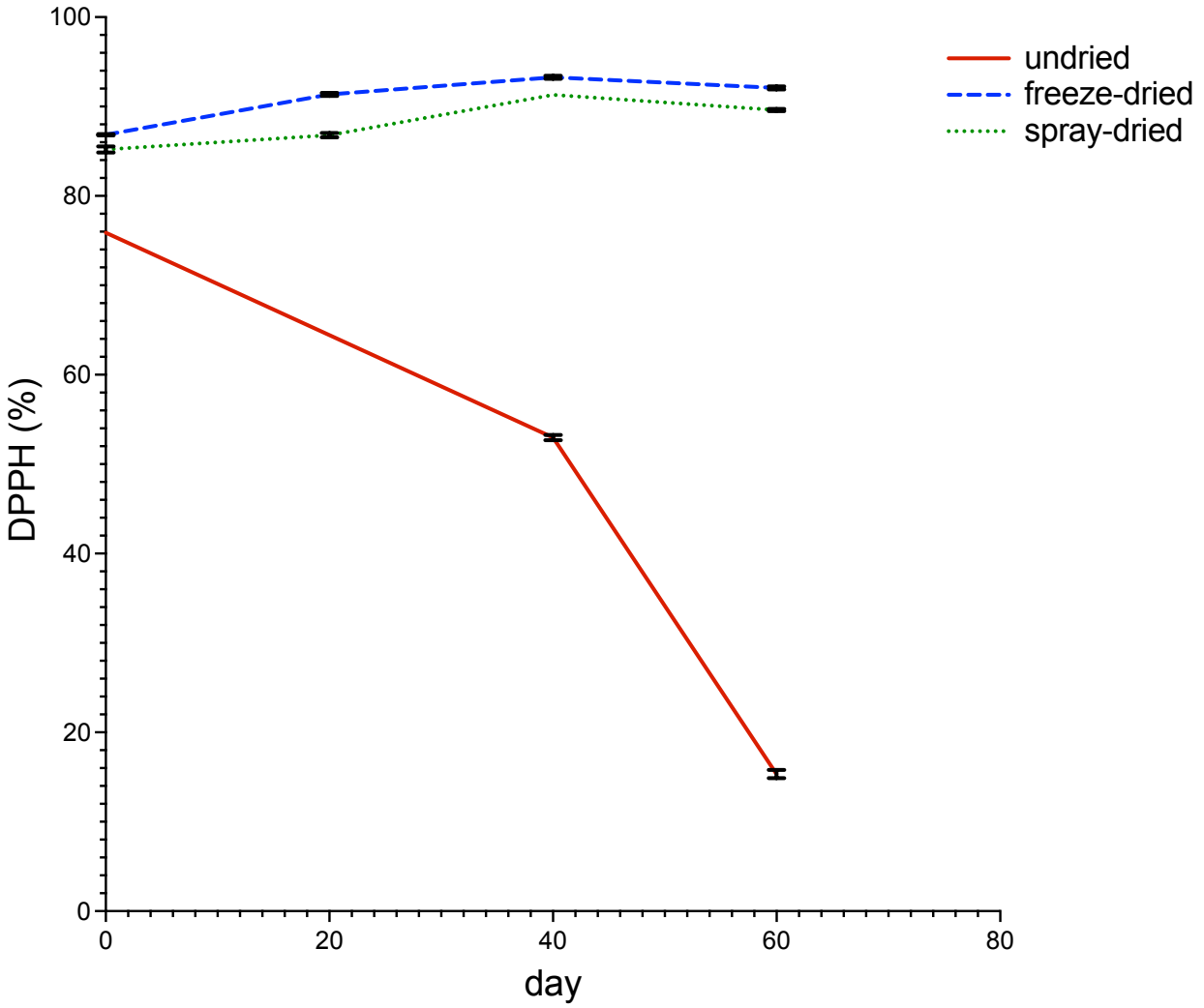


Figure 25- DPPH radical scavenging activity of freeze-dried, spray-dried and undried/liquid tea samples during 60 days of storage.

Data are means ± SD of samples analyzed in triplicate. Statistical analyses were performed and the graph was plotted using GraphPad Prism software version 10.1.1 for macOS.

4.8.2. L-Theanine and Caffeine Contents of the Dried and Undried Tea Samples During Storage, Determined by HPLC

HPLC analyses of each of the tea samples (freeze-dried, spray-dried and undried) on days 0, 20, 40 and 60 of storage showed a changing trend in their L-theanine and caffeine contents throughout the storage period.

L-theanine (mg/mL) in the freeze-dried tea, increased significantly by more than 40% up to day 20 ($P < 0.05$), decreased by more than 18% from day 20 to day 40 ($P < 0.05$) and increased again by about 8% from day 40 to day 60 ($P \geq 0.05$) (two-way ANOVA) (Table 9 and Figure 26).

In the spray-dried tea, L-theanine decreased by about 15% and then 10% until day 20 and then from day 20 to 40 respectively (P-values ≥ 0.05). L-theanine then increased by more than 17% from day 40 to 60 ($P \geq 0.05$) (two-way ANOVA) (Table 9 and Figure 26).

In the undried/liquid tea samples, L-theanine decreased by about 48% ($P < 0.05$) and then 1.3% ($P \geq 0.05$) from day 0 to day 20 and from day 20 to 40 respectively; it then increased by about 32% from day 40 to day 60 ($P \geq 0.05$) (two-way ANOVA) (Table 9 and Figure 26).

Table 9- L-theanine and caffeine levels (mg/mL) and their ratios in white tea preparations stored for 60 days (means ± SD of samples analyzed in triplicate)

Tea preparation	Time (day)	L-theanine (mg/mL)	Caffeine (mg/mL)	L-theanine : caffeine
Freeze-dried	0	1.169 ± 0.038	1.977 ± 0.017	0.59 ± 0.01
	20	1.651 ± 0.265	2.594 ± 0.059	0.63 ± 0.11
	40	1.347 ± 0.064	2.636 ± 0.031	0.51 ± 0.03
	60	1.455 ± 0.031	2.238 ± 0.047	0.65 ± 0.008
Spray-dried	0	0.366 ± 0.021	0.349 ± 0.038	1.05 ± 0.13
	20	0.311 ± 0.02	0.354 ± 0.004	0.87 ± 0.05
	40	0.279 ± 0.005	0.36 ± 0.051	0.78 ± 0.1
	60	0.328 ± 0.013	0.354 ± 0.085	0.9 ± 0.28
Liquid/undried	0	0.298 ± 0.0004	0.337 ± 0.011	0.88 ± 0.03
	20	0.155 ± 0.003	0.291 ± 0.014	0.53 ± 0.03
	40	0.153 ± 0.0008	0.284 ± 0.021	0.53 ± 0.03
	60	0.203 ± 0.001	0.263 ± 0.02	0.77 ± 0.06

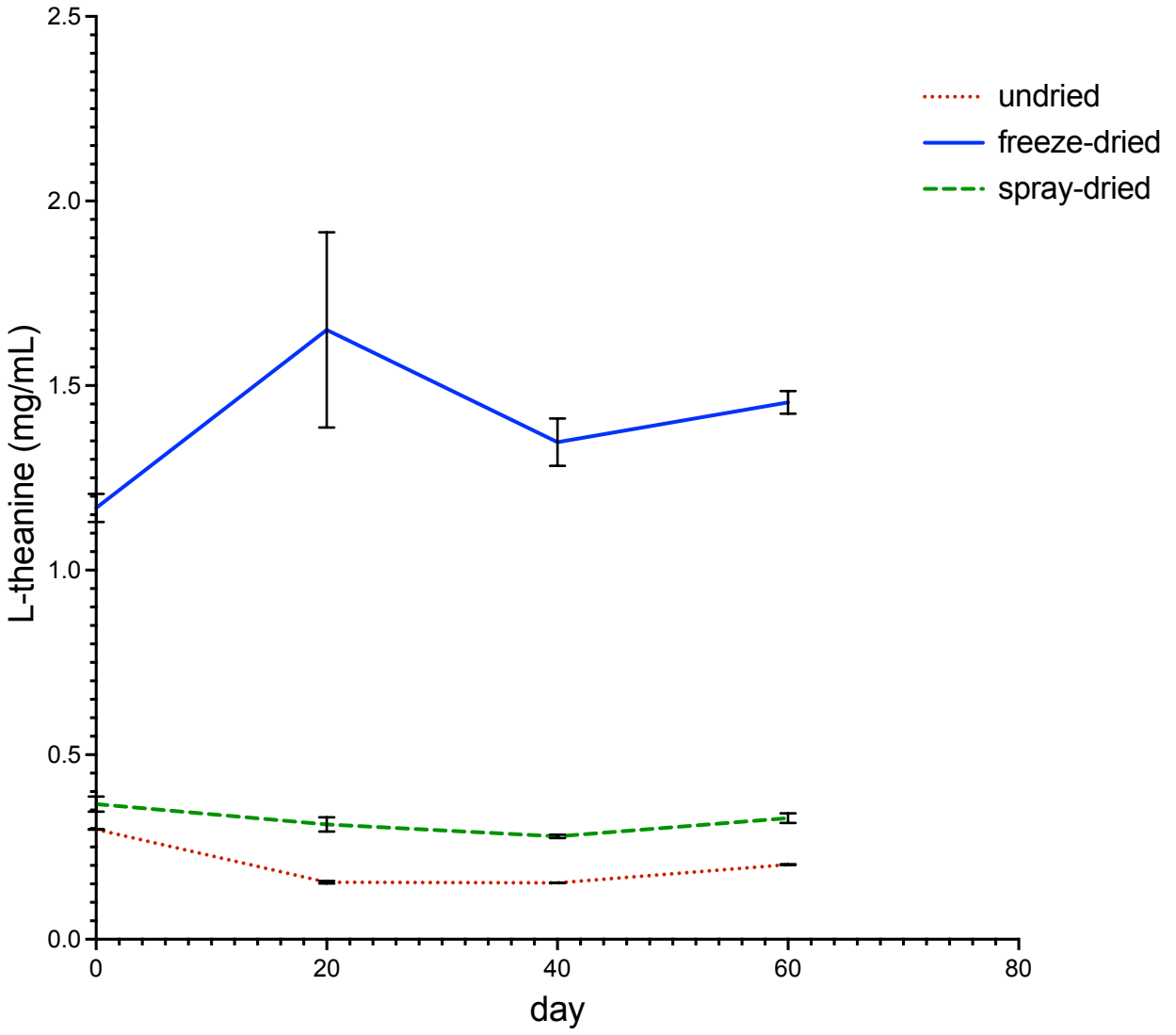


Figure 26- L-theanine levels (mg/mL) in freeze-dried, spray-dried and undried/liquid tea samples during 60 days of storage analyzed by HPLC.

Data are means ± SD of samples analyzed in triplicate. Statistical analyses were performed and the graph was plotted using GraphPad Prism software version 10.1.1 for macOS.

Similarly, caffeine (mg/mL) in the freeze-dried tea increased by about 31% ($P < 0.05$) and 1.6% ($P \geq 0.05$) from day 0 to day 20 and then from day 20 to day 40, respectively. Caffeine then

decreased by about 15% from day 40 to day 60 ($P < 0.05$) (two-way ANOVA) (Table 9 and Figure 27).

In the spray-dried tea, caffeine slightly increased by about 1.4% and then 1.7% from day 0 to 20 and then from day 20 to 40, respectively. It then decreased by about 1.6% from day 40 to 60 (P-values ≥ 0.05) (two-way ANOVA) (Table 9 and Figure 27).

In the undried tea, the caffeine level decreased from day 0 to 20, 20 to 40 and 40 to 60 by about 13%, 2% and 7% respectively (P-values ≥ 0.05) (two-way ANOVA) (Table 9 and Figure 27).

The ratios between L-theanine and caffeine concentrations in all the tea samples during the 60-day storage period are presented in Table 9 (means ± SD of samples analyzed in triplicate).

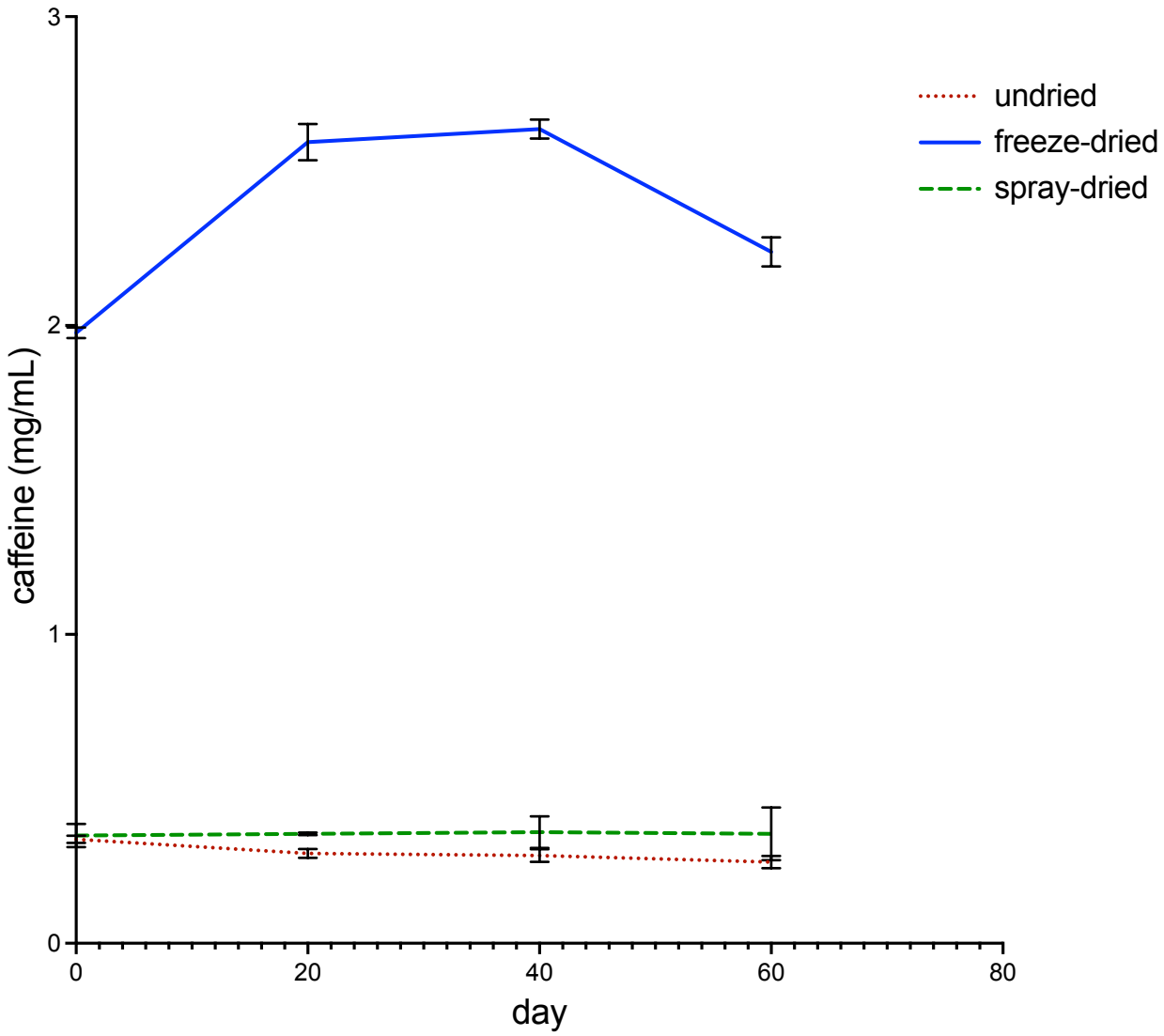


Figure 27- Caffeine levels (mg/mL) in freeze-dried, spray-dried and undried/liquid tea samples during 60 days of storage analyzed by HPLC.

Data are means ± SD of samples analyzed in triplicate. Statistical analyses were performed and the graph was plotted using GraphPad Prism software version 10.1.1 for macOS.

The data for both L-theanine and caffeine levels in the freeze-dried, spray-dried and undried/liquid tea samples during 60 days of storage are presented on a single graph as depicted in Figure 28 to illustrate their respective composition.

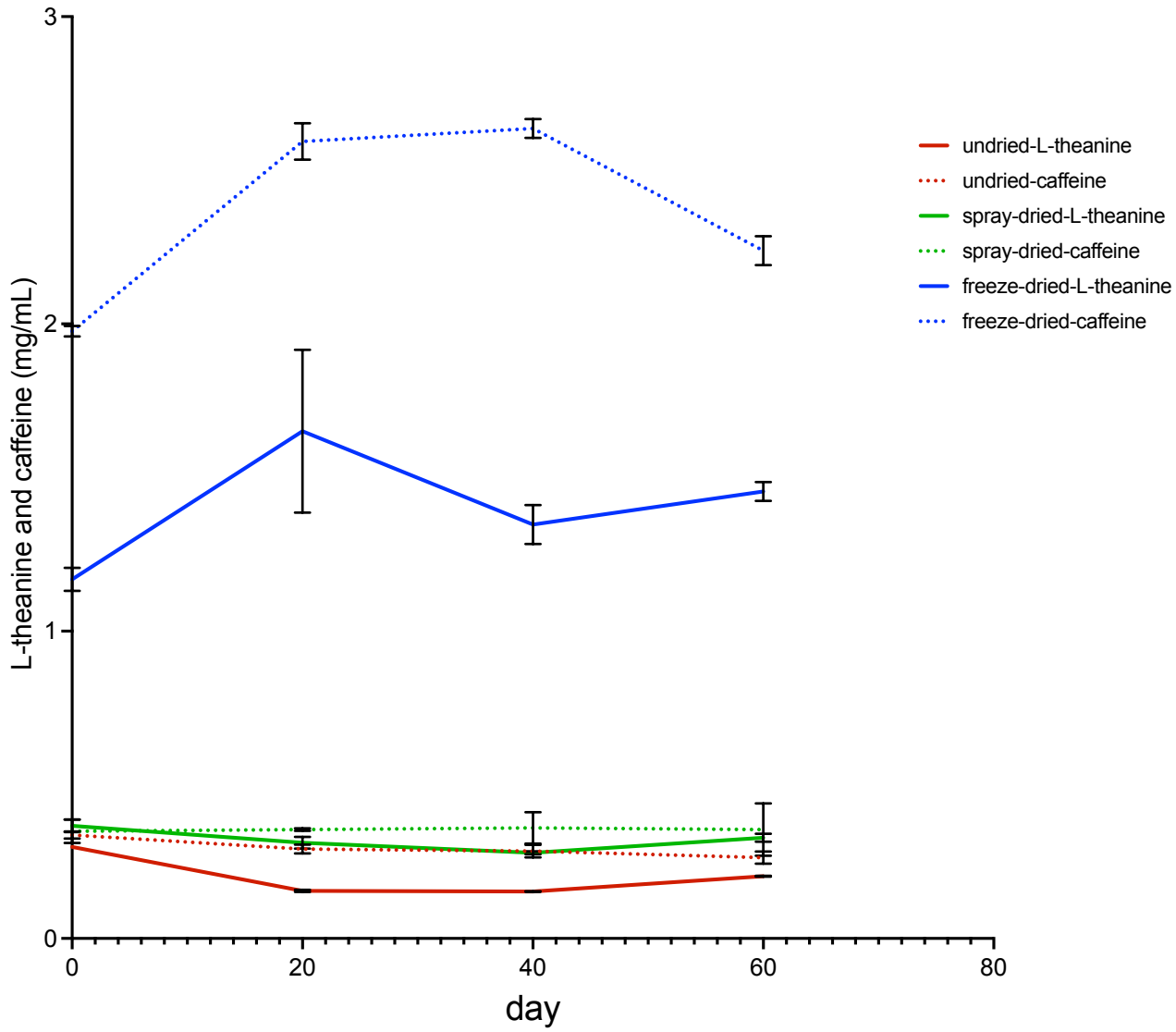


Figure 28- L-theanine and caffeine levels (mg/mL) in freeze-dried, spray-dried and undried/liquid tea samples during 60 days of storage analyzed by HPLC.

Data are means ± SD of samples analyzed in triplicate. Statistical analyses were performed and the graph was plotted using GraphPad Prism software version 10.1.1 for macOS.

4.8.3. Moisture Contents of the Dried Tea Infusion Samples During Storage

Moisture content analysis of the dried tea samples revealed that the change in moisture content (%) of both freeze-dried and spray-dried tea samples followed a similar trend during the

60 days of storage, being influenced by relative humidity with variations in their moisture content with time.

In freeze-dried tea, moisture content increased by about 13% from day 0 to 20; it then decreased by about 16% from day 20 to 40 and increased by about 16% from day 40 to 60 ($P \geq 0.05$; two-way ANOVA) (Figure 29). The average moisture content of the freeze-dried samples stayed within the range of ~ 6-7% throughout the storage period.

In spray-dried tea, moisture content increased by about 13% from day 0 to 20. It decreased by about 16% from day 20 to 40 and then increased by about 4% from day 40 to 60 ($P \geq 0.05$; two-way ANOVA) (Figure 29). The average moisture content of the spray-dried samples stayed within the range of ~ 3-4% throughout the storage period.

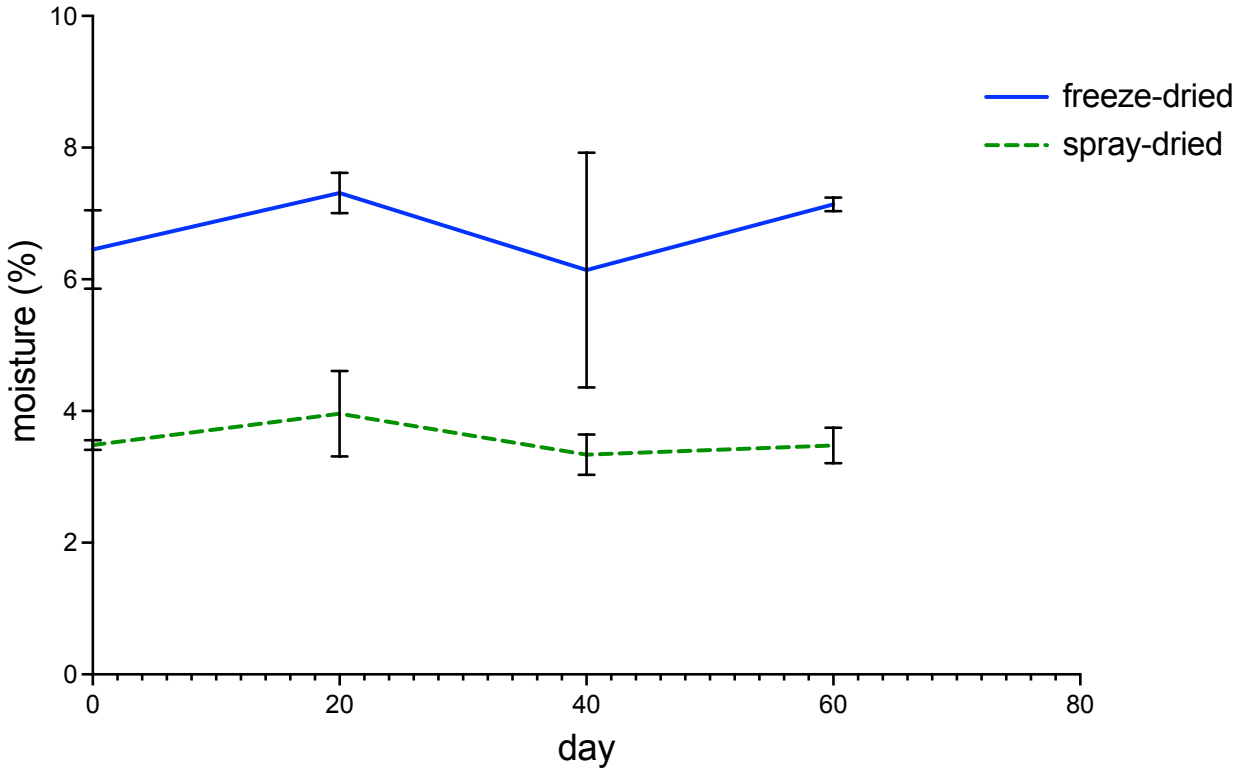


Figure 29- Moisture content (%) of freeze-dried and spray-dried tea infusions during 60 days of storage.

Data are means ± SD of samples analyzed in triplicate. Statistical analyses were performed and the graph was plotted using GraphPad Prism software version 10.1.1 for macOS.

Chapter 5: Scholarly Discussion of the Findings

Camellia sinensis is the main ingredient used in the preparation of the traditional tea beverage, one of the oldest and most popular caffeine-containing drinks. It has been used for centuries not only as a medicine but also as a recreational beverage. Fresh tea leaves are processed to produce a variety of commercially available teas providing different flavor profiles and potency. The different processing steps not only define the appearance and taste of the final product but also affect its chemical composition (Dairpoosh, 2023, Mondal, 2020; Snell, 2023).

L-theanine and caffeine are two important bioactive compounds of tea. The level of these compounds not only determines the taste and quality of tea but also its physiological and health-promoting effects. L-theanine is a potent relaxant which is always co-extracted with the stimulant caffeine. Caffeine has been shown to inhibit the relaxing effects of L-theanine. Using different approaches to decrease the extraction of caffeine relative to that of L-theanine would be a beneficial alternative to the decaffeination of tea. Of the many different factors that can affect the extractability of these two compounds, the influence of tea type, time and temperature of infusion, use of organic solvent, ultrasounds and commonly used additives (lemon juice and milk) was examined. The impacts of drying infused teas and storage of produced extracted tea powders and solutions on the levels of L-theanine and caffeine were analyzed and the stability of the tea preparations was determined based on their antioxidant activity and moisture content variations with storage time. The DPPH radical scavenging activity of different types of tea was determined and compared with their levels of L-theanine and caffeine. A rapid and efficient HPLC method was developed to simultaneously determine the exact amounts of L-theanine and caffeine in the extracts prepared in all the experiments. The ratio of L-theanine to caffeine was proposed as an

indicator of the potential beneficial effects of tea infusions and powdered tea extracts based on their high L-theanine content, not being suppressed by a high caffeine content.

In the first experiment, three different types of tea namely, white, green and black tea, were each infused for 5, 30 and 60 min in water at about 10-11°C, 50°C and 90-100°C. It was observed that when infused at ~50°C and ~90-100°C, the caffeine extraction from white tea was higher than from black and green tea, infused at the same temperature and for the same duration (Figure 9 b and c). Similarly, the L-theanine extraction at ~50°C and ~90-100°C was higher from white tea compared to black and green tea extracted at similar temperatures and durations (Figure 8 b and c). Cold tea infusions, however, showed a different pattern. At ~10-11°C, more L-theanine and caffeine were extracted from black and green tea than from white tea under similar temperature and infusion duration conditions (Figures 8a and 9a).

The observed lower extraction of L-theanine and caffeine from white tea at 10-11°C (Figures 8a and 9a) is justified by the fact that white tea is minimally processed and the structure of the leaf cells is mostly intact (Damiani et al., 2014); hence, at lower temperatures, lower levels of bioactive molecules can be extracted.

To manufacture different types of tea, fresh tea leaves undergo different levels of fermentation/oxidation. It has been reported that non-fermented teas contain more caffeine than fermented ones. Suteerapataranon et al. (2009) compared the caffeine levels of green tea and oolong tea and showed that non-fermented green tea contained more caffeine than semi-fermented oolong tea (Suteerapataranon et al., 2009). It has also been reported that non-fermented teas such as white and green teas contain more L-theanine than fermented teas such as black teas (Alcázar et al., 2007; Baptista et al., 2012). This is due to the degradation of L-theanine (Feldheim et al.,

1986) and caffeine (Lee, Lee, Chung, et al., 2011) during the fermentation/oxidation process of tea leaves.

The present study showed that on average the caffeine extraction was highest from white tea, followed by black tea; while on average the lowest caffeine content was extracted from green tea (the average caffeine levels for all the temperatures and durations tested) (Table 3). The same pattern was observed for L-theanine and the average highest L-theanine extraction was from white tea, followed by black and green tea respectively (the overall average of L-theanine levels for all the temperatures and durations tested) (Table 3). The average lower levels of these compounds infused from black tea compared to white tea can be explained by the fact that L-theanine and caffeine degrade during the fermentation/oxidation process of tea leaves.

Another important factor contributing to different levels of bioactive molecules in the tea plant's leaves is the age of the leaf, also known as the plucking position or leaf position. White tea consists of tea buds and exceptionally young leaves (Damiani et al., 2014; Song et al., 2012). Y.-S. Lin et al. (2003) showed that young tea leaves (bud and first to third leaves) contain more caffeine than older leaves (Y.-S. Lin et al., 2003). Similarly, it has been shown that younger tea leaves contain more L-theanine than older ones (Baptista et al., 2012). Figure 30 illustrates the plucking position (or leaf position) of the tea plant.

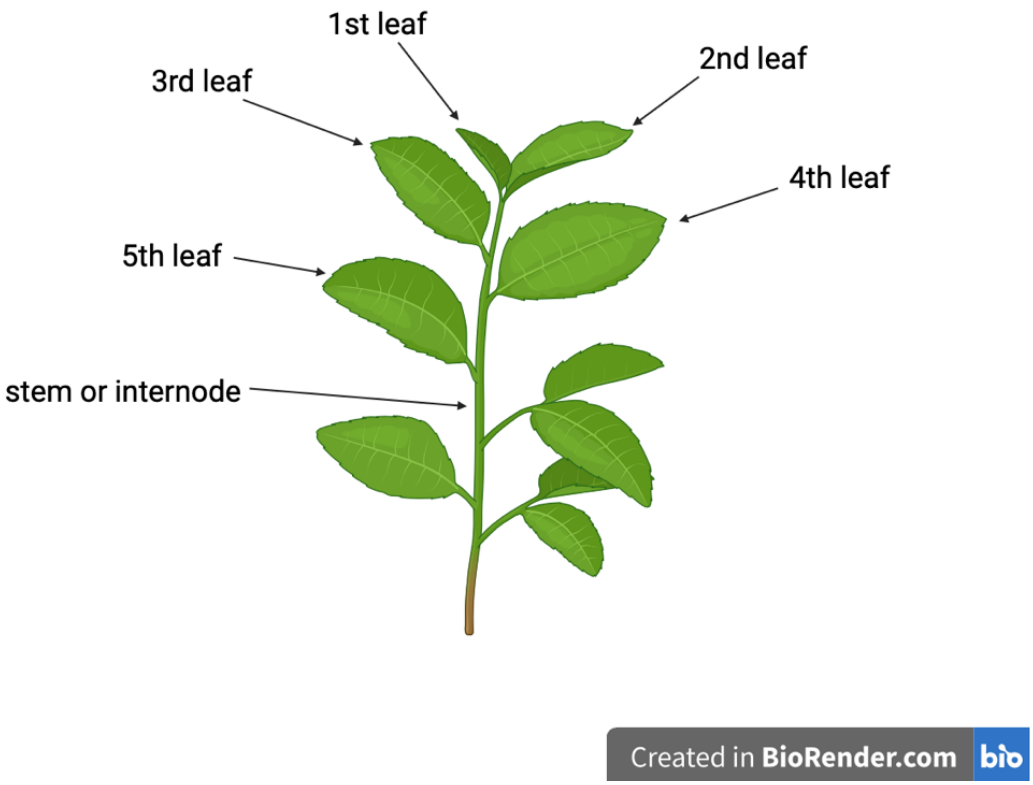


Figure 30- Plucking position or leaf position on a tea plant (*Camellia sinensis*).

It has been shown that naturally occurring methylxanthines, including caffeine, have insecticide activities (Nathanson, 1984) and the higher level of caffeine in the younger leaves has been justified as a means to protect themselves from insects (Baptista et al., 2012). Hence, caffeine is produced in the young leaves and accumulates as the leaves mature to a maximum and then the levels decrease (Ashihara et al., 2008).

The effect of leaf age on different levels of L-theanine and caffeine can be further explained by the fact that caffeine and L-theanine are major nitrogenous compounds in tea leaves (Z.-X. Li et al., 2016; Y. Zhang et al., 2020) and the total nitrogen concentration of tea leaves has been shown to decrease as the leaves mature (Z.-X. Li et al., 2016). Z.-X. Li et al. (2016) also observed a general declining pattern for the concentration of caffeine in tea leaves as they mature. In the

same report Z.-X. Li et al. (2016), showed that the concentration of total amino acids (including L-theanine, the most abundant amino acid found in tea) increased in the first three leaves and decreased in older leaves (Alcázar et al., 2007; Horanni & Engelhardt, 2013; Z.-X. Li et al., 2016). The reduction in L-theanine and caffeine levels by aging may thus be due to a reduction in the total nitrogen.

Another explanation for different L-theanine and caffeine levels in younger versus older tea leaves is that, as the tea leaves mature, their photosynthetic capacity increases (Z.-X. Li et al., 2016) and the lower L-theanine observed in older leaves may be due to the inhibition of L-theanine biosynthesis when the plant starts to photosynthesize more as it grows. On the other hand, the young leaves and buds which are not able to photosynthesize, use L-theanine as a carbon source to produce carbon-based polymeric compounds (Feldheim et al., 1986). In addition, the lower level of L-theanine in older leaves can be attributed to the contribution of L-theanine in the biosynthesis of tea polyphenols, as the *N*-ethyl group of L-theanine has been shown to contribute to the biosynthesis of tea polyphenols (Feldheim et al., 1986; Lee, Lee, Hwang, et al., 2011). Song et al. (2012) further confirmed this by showing that L-theanine and caffeine levels decreased as the age of the tea leaves increased whereas, the levels of tea catechins increased as the leaf age increased (Song et al., 2012). Therefore, the higher caffeine and L-theanine extracted from white tea in the present study is in part because white tea consists of tea buds and exceptionally young leaves which have been shown to contain more L-theanine and caffeine than older leaves. The white tea sample used in this study, bai hao yin zhen or silver needle, is exclusively made from unopened tea buds with no leaves (Damiani et al., 2014; Song et al., 2012).

The highest caffeine and L-theanine levels in this study were extracted from white tea (1.91 ± 0.18 mg/mL and 21.52 ± 2.64 mg/mL respectively, at ~90-100°C after 5 min) supporting that

white tea is naturally rich in these compounds. On the other hand, green tea was shown to contain the lowest levels of caffeine and L-theanine. The maximum level of L-theanine and caffeine extracted from green tea was only 2.84 ± 0.15 mg/mL and 0.83 ± 0.18 mg/mL respectively which were extracted at ~50ºC after 60 min and ~90-100°C after 5 min of extraction, respectively (Table 3). The low levels of L-theanine and caffeine in green tea can be explained by the fact that, unlike white and black tea, green tea leaves do not undergo withering during production. The withering process increases the amount of caffeine and amino acids in tea leaves (Baldi et al., 2020).

In the second experiment, the L-theanine and caffeine contents of white tea infusions prepared for 5 min at about 10-11°C and at room temperature (about 21°C) were compared. The results showed that at the lower temperature (~10-11°C), about 30% more L-theanine and about 39% less caffeine were extracted compared to the higher temperature (~21°C) (Figure 11 and Table 4).

The extraction results of both the first and second experiments can be partially explained by the molecular differences between L-theanine and caffeine. L-theanine ($C_7H_{14}N_2O_3$) with a molecular mass of 174.2 g mol^{-1} (Mu et al., 2015) is a smaller molecule than caffeine ($C_8H_{10}N_4O_2$; 194.19 g mol^{-1}) (Table 2) (Carvalho et al., 2012). The different molecular masses of these compounds affect their water solubility and this is a contributing reason for the rapid extraction of L-theanine at lower temperatures compared to caffeine and the higher L-theanine-to-caffeine ratio obtained at ~10-11ºC after 5 min (Figures 10 and 12).

Overall the differences observed in the levels of L-theanine and caffeine in the tea samples examined in the first and second experiments, are a cumulative result of several factors including

the leaf age, level of fermentation and withering and molecular differences of these two compounds.

However, the lower L-theanine extracted at room temperature (~21°C), observed in the second experiment, can also be explained by the results of a recent study conducted by Y. Xia et al. (2022). In their study, Y. Xia et al. (2022) showed that L-theanine and catechin bind spontaneously together by two hydrogen bonds in aqueous solutions and form molecular clusters which reduce the diffusion coefficient of these compounds in the solution. Y. Xia et al. (2022) demonstrated that the binding constant between catechin and L-theanine at 284 K (10.85°C) was 1.6 while at 294 K (20.85°C) this binding constant was 4.75 (Y. Xia et al., 2022), supporting the lower detection of L-theanine in the tea sample prepared at room temperature, observed in the present research (Figure 11).

The binding constant, also known as the association constant, indicates the strength and stability of the formed complexes relative to the stability of the free molecules in the solution. In simple terms, this constant can be defined as the ratio of the concentration of the complex formed by the two molecules, to the product of the concentrations of unbound free molecules: [catechin-L-theanine complex]/[catechin]×[L-theanine] (Wagner, 2020). Therefore, L-theanine and catechin at room temperature are more strongly bound which explains the lower level of L-theanine determined at this temperature by the HPLC analysis.

In the following experiment, the DPPH radical scavenging activities of white, green and black tea infusions in cold water were compared and infusions of black tea were shown to have about 10% and 20% higher radical scavenging activity than white tea and green tea respectively

(Figure 13). The DPPH radical scavenging activity of ascorbic acid was about 15 to 16 % higher than the DPPH radical scavenging activity of black tea.

The difference in the antioxidant activities of these teas, measured by DPPH radical scavenging assay, can be attributed to their different composition due to the different processing steps they underwent during production. The higher antioxidant activity of black tea compared to white and green tea may be due to compounds that are formed during fermentation (oxidation).

Some of the most important products of tea fermentation are theaflavins and thearubigins, which are both polyphenolic pigments (Koch, 2021). Even though, in this experiment, PVPP was used for the removal of polyphenols, the antioxidative effects observed may be explained partly by the possible incomplete removal of these potent compounds; since the adsorption rate of polyphenols onto PVPP depends on the particle surface of PVPP and the concentration of polyphenols in the solution (Dong et al., 2011). Theaflavins (TFs) are orange or orange-red compounds (Koch, 2021) with several health benefits including antimutagenic (Halder et al., 2005), total cholesterol-reducing (Maron et al., 2003), anti-obesity (Cai et al., 2021) and anticancer effects (Imran et al., 2019). Thearubigins (TRs) are red-brown or dark-brown compounds (Koch, 2021) with antimutagenic (Halder et al., 2005) and anticancer effects (Imran et al., 2019). Both of these compounds are proven to be potent antioxidants (Fatima & Rizvi, 2015; Imran et al., 2018; Leung et al., 2001; Sinha & Ghaskadbi, 2013; Z. Yang et al., 2008). Some studies reported that theaflavins have similar antioxidative activity to catechins (Leung et al., 2001) or higher antioxidative activity than catechins (Z. Yang et al., 2008).

Despite fermentation/oxidation, black tea still contains a significant amount of catechins, which are largely responsible for the known antioxidant activity of black tea (W. Wang et al.,

2023), in addition to the polyphenols formed during fermentation (the most important being theaflavins and thearubigins). The higher antioxidative activity observed in black tea may therefore be a cumulative result of theaflavins, thearubigins and catechins.

Withering is another important step in tea processing that significantly affects the chemical composition of the final product. Withering, which involves tea leaves losing some moisture under forced air circulation, catalyzes the oxidative reactions (Baldi et al., 2020). Dissimilar to black and white tea, green tea leaves are not withered during production. This means that white tea which is known as a non-fermented (unoxidized) tea, is in fact slightly oxidized due to withering. Therefore, white tea contains some levels of oxidation products including theaflavins and thearubigins (Damiani et al., 2014). This is a reason for the higher antioxidant activity of white tea compared to green tea, observed in this study.

The results of this experiment also show that there is an association between the antioxidant activity of tea extracts with the ratio of L-theanine to caffeine. It was shown that black tea samples with the highest DPPH (%) value contain significantly higher L-theanine than caffeine and about a 3-fold higher L-theanine-to-caffeine ratio than green and white teas. Accordingly, green and white tea samples with similar L-theanine and caffeine levels and lower L-theanine-to-caffeine ratios than black tea samples, possess lower DPPH radical scavenging activity (Figure 15).

Also, a reverse relationship between L-theanine level and the antioxidant activity of tea can be inferred. Black tea with lower L-theanine levels was shown to have the highest DPPH free radical scavenging activity (Figures 13 and 14). This result is in support of the results obtained by Y. Xia et al. (2022) who showed that L-theanine and catechin bind spontaneously by two hydrogen bonds and form clusters. This binding reduced the diffusion coefficient of the catechin in solution

and slowed the release of the antioxidant capacity of the catechin (Y. Xia et al., 2022). Therefore, since the black tea samples contain less L-theanine they could exhibit higher antioxidant activity.

In the fourth experiment, according to the solubility data of L-theanine and caffeine found in the literature as discussed in Chapter 1, it was hypothesized that soaking tea leaves in methanol would preferentially extract caffeine. Differences in the solubility of L-theanine and caffeine in methanol would be helpful in the removal of caffeine from tea leaves in a preliminary extraction, and after the complete separation of methanol, low-caffeine tea biomass with high L-theanine levels would be obtained to be further extracted in water.

This hypothesis was proven to be accurate since significantly less L-theanine was extracted in methanol (as the preliminary methanol extraction) when compared with the water extraction alone (the control). Therefore, pre-treatment of tea leaves with methanol successfully increased the subsequent water extraction of L-theanine by about 18% and decreased the water extraction of caffeine by about 19% compared to the control extraction process (Figure 16).

The higher solubility of L-theanine in water compared to methanol is justified by knowing that higher hydrogen bond formation occurs between the water and L-theanine molecules. L-theanine is very polar and since water is more polar than methanol, L-theanine is more soluble in water (Zhou et al., 2017).

L-theanine is an amino acid and, similar to other amino acids, L-theanine is very soluble in water with the water solubility decreasing when the hydrophobicity of the solvent increases (Dey & Lahiri, 1986). As an amino acid, L-theanine predominantly has a zwitterionic form (Dey & Lahiri, 1986; Kang et al., 2011), meaning that it has both positive and negative groups (i.e. both cation and anion) simultaneously in its chemical structure while maintaining a total neutral charge.

These compounds are, therefore, extremely polar and this chemical structure significantly affects their behaviour in the presence of different solvents (Jesus et al., 2021; Wu et al., 2019; Yoshizawa-Fujita et al., 2010) and due to the higher polarity of water, compared to methanol (Reichardt & Welton, 2011), the amino acid L-theanine is more soluble in water.

In the next experiment, the white tea infusions were spray-dried and freeze-dried and the results indicated that the freeze-dried tea contained about 200% more L-theanine and about 140% more caffeine than the undried tea (Figure 18a) with the concentration effect of the drying processes, making it an appealing option for delivering concentrated L-theanine. On the other hand, the spray-dried tea had about 30% less L-theanine and about 45% less caffeine than the undried tea infusion (Figure 18b).

The lower level of L-theanine and caffeine in the spray-dried tea infusion powder is likely due to the thermal degradation of these compounds. L-theanine has been shown to thermally degrade at 180°C to other compounds including *N*-ethyl-formamide, ethyl amine, propyl amine, 2-pyrrolidone, *N*-ethyl-succinimide and l-ethyl-3,4-dehydro-pyrrolidone (Yamanishi et al., 1989). Caffeine however is more thermally stable and has a melting range between 235°C and 237.5°C (Zubair et al., 1986), while another study showed that caffeine completely decomposed at about 285°C (R. Wang et al., 2019).

However, another reason for lower amounts of these compounds in the spray-dried tea extracts may be due to the degradation of these compounds through bioactive oxidation which is enhanced during the atomization process in the drying air (Jafari & Rashidinejad, 2021). Freeze-drying however operates under vacuum as well as low temperatures hence it is useful in protecting

the bioactive compounds from oxidation and thermal degradation (Ratti, 2024), supporting the current results.

The sixth experiment on the impact of ultrasounds (US) on L-theanine and caffeine extraction, showed that the application of US significantly increased the aqueous low-temperature extraction of both L-theanine and caffeine from tea leaves. After 5 min, this technique increased L-theanine and caffeine levels in the solution respectively by about 140% and 311% compared to the untreated samples. After 20 min, US application increased L-theanine and caffeine levels by about 98% and 127% respectively compared to the untreated samples (Figure 20).

This technique uses acoustic cavitation which improves cell disruption and consequently increases the penetration of the solvent into the cells and the mass transfer of bioactive compounds into the solvent (Tiwari, 2015). Several studies reported the efficiency of using an ultrasound technique in the extraction of bioactive materials. Recently this technique has been used for the extraction of antioxidant compounds (polyphenols and flavonoids) from apple pomace (Egüés et al., 2021), polysaccharides from *Ginkgo biloba* (J. Li et al., 2023), total phenolics content and total flavonoids content from tea (*Camellia sinensis*) (Afroz Bakht et al., 2019) and collagen from tuna tendons (Chanmangkang et al., 2024). However, this technique was not able to increase the L-theanine-to-caffeine ratio (Figure 21), because sonication enhances cell disruption and mass transfer by cavitation, which affected all the bioactive compounds in an equal manner. A higher L-theanine-to-caffeine ratio was only obtained without using UAE due to the extraction of more L-theanine than caffeine under this condition. The higher L-theanine-to-caffeine ratio obtained after 5 min extraction without applying US can be attributed to the molecular differences of L-theanine and caffeine leading to the preferential release of L-theanine into the solvent. This confirmed that a short conventional extraction (without US) combined with low temperatures can

yield a higher L-theanine-to-caffeine ratio. Under these conditions, about 30% more L-theanine than caffeine was extracted (Table 7).

In the next study, two separate experiments were conducted and the levels of L-theanine and caffeine were determined in tea preparations containing milk and lemon juice as popular tea additives.

Although adding lemon juice decreased the pH of the solution from 5.17 to 2.79, it did not have a significant effect on the extraction of L-theanine and caffeine (Figure 22a). Being an amino acid, L-theanine is an amphoteric compound; meaning that it exhibits both acidic and basic properties. Because amino acids including L-theanine, contain the carboxyl group (COOH) which exhibits acidic properties, and the amino group (NH_2) which exhibits basic properties, in their structure (Obodovskiy, 2019; Ye et al., 2011). The isoelectric point of L-theanine is at pH 5.6 (Ye et al., 2011). The isoelectric point (PI) is the pH point where the amino acid has an equal number of cations and anions, in other words, where the molecule has no electrical charge (Moldoveanu & David, 2017; Tseng et al., 2009). The isoelectric point of an amino acid is the average of pK_a for its amine group and its carboxyl group (Moldoveanu & David, 2017). The unchanged solubility of L-theanine in the presence or absence of lemon juice (pH = 2.79 and pH = 5.17 respectively) is because at a certain range of pH values before and after its isoelectric point, the solubility of an amino acid does not change. At this range of pH, the solubility of an amino acid is at a minimum, whereas at pH values lower or higher than this range (isoelectric band), the amino acid is charged and hence becomes more soluble. Needham Jr. et al. (1971) called this pH range “an isoelectric band of minimum solubility” and “an invariant band of similar solubility” (Needham Jr. et al., 1971; Pradhan & Vera, 1998).

Caffeine, on the other hand, displays various structures in the presence of acids and bases (Raczyńska et al., 2020). The unaffected solubility of caffeine in the presence of acidic conditions observed in this experiment can be explained by the pK_a of caffeine. The pK_a controls the state of ionization of a compound in a solution (Hale & Abbey, 2017). The pK_a of caffeine is 8.3 (Barbas et al., 2000; Couto Jr. et al., 2015). In the presence of an acid, caffeine becomes partially protonated and at pH values lower than pK_a, protonated caffeine is the predominant form (X. Wang et al., 2022). The initial and final pH values used in this study were respectively 5.17 and 2.79, which are both lower than the pK_a of caffeine (8.3). Thus, the predominant positively charged protonated (H^+) caffeine is repulsed by the acidic (also positively charged) media (Bachmann et al., 2021; Law & Rennie, 2020), explaining the unchanged solubility of caffeine observed.

When 10%, 20% or 50% (V/V) partly skimmed milk (2% milk fat) was added to the tea samples, up to 20% (V/V) milk did not significantly change the level of L-theanine. However, 50% (V/V) milk reduced the level of L-theanine in the tea solution by about 25% compared to when no milk was added (0% V/V). The effect of milk on caffeine, however, was more pronounced. Compared to the tea sample with no added milk (0% V/V), the caffeine levels in the 10%, 20% and 50% (V/V) milk-added samples decreased by about 17%, 42% and 50% respectively (Figure 23 and Table 8).

The reducing effect of adding milk on caffeine concentration can be explained by the formation of tea cream. Tea creaming is the formation of precipitates or suspensions in a tea infusion, through complexations of polyphenols with caffeine and proteins (Chao & Chiang, 1999). Tea creaming causes turbidity in the tea infusion which is very undesirable as it compromises the appearance of the beverage and causes the loss of active components upon removal of the cream (Y. Kim & Talcott, 2012). The addition of calcium increases tea creaming

by neutralizing the charges of colloidal particles and consequently stabilizing those particles (Jöbstl et al., 2005). Caffeine is one of the primary components that precipitate in tea cream along with catechins. This is because caffeine can form complexes with tea phenolics through hydrogen bonds (Chao & Chiang, 1999; Hayashi et al., 2004; Ishizu et al., 2016; Y. Liang et al., 2002). The bindings between caffeine and catechins result in the formation of insoluble particles (Y. Kim & Talcott, 2012). L-theanine also binds with catechin through hydrogen bonds (Y. Xia et al., 2022). Therefore, L-theanine also has been identified in tea cream, although in much lower amounts (Chao & Chiang, 1999; Vuong et al., 2013). Hence, in the present study, only a larger concentration of milk could reduce the level of L-theanine in tea. Overall, milk proteins and calcium may be responsible for increased tea creaming and consequently, the decreased caffeine and L-theanine observed in the solution.

In the last study, spray-dried, freeze-dried and liquid white tea samples were prepared and stored for 60 days at 39 ± 1°C. This higher temperature of storage was applied to accelerate the possible chemical changes that may happen during the storage-induced deterioration of the tea samples. Three experiments were conducted to establish the stability and functionality of these tea samples.

After storing the spray-dried, freeze-dried, and liquid white tea samples in airtight containers, at 39 ± 1°C for 60 days, both dried samples showed good storability in terms of DPPH free radical scavenging activity and moisture content, under these storage conditions.

Generally, the DPPH free radical scavenging activity of the freeze-dried tea samples increased from day 0 to day 60 by about 6%. The same increasing pattern was also observed for the DPPH free radical scavenging activity of the spray-dried samples with an overall increase of

about 5% from day 0 to day 60. The liquid tea samples, on the other hand, lost their DPPH radical scavenging activity by about 80% from day 0 to day 60 (Figure 25).

Similarly, the L-theanine and caffeine levels in the freeze-dried tea samples had an overall increasing trend. In the freeze-dried samples, the L-theanine increased by about 24% and caffeine increased by about 13% from day 0 to day 60. However, in the spray-dried tea samples, L-theanine decreased by about 10% from day 0 to day 60, while caffeine remained almost unchanged. L-theanine and caffeine in the liquid tea samples, both decreased from day 0 to 60, by about 32% and 22% respectively (Table 9 and Figures 26 to 28).

The moisture content of the freeze-dried tea samples was about 10% higher on day 60 compared to day 0. While the moisture contents of the spray-dried tea samples were similar on day 0 and day 60 (Figure 29). These results indicate that both produced tea infusion powders (spray-dried and freeze-dried) have good stability and will be easy to handle with less chance of clumping.

Both L-theanine and caffeine have been shown to possess antioxidative activities as discussed in Chapter 1 (Deng et al., 2016; Devasagayam et al., 1996; Jo et al., 2011; G. Li et al., 2012; Nagai et al., 2018; X. Shi et al., 1991; Zeng et al., 2020). On the other hand, the liquid (undried) tea samples lost about 22% caffeine and 32% L-theanine from day 0 to 60. Therefore, the significant loss of DPPH radical scavenging activity in the liquid (undried) tea samples may be due to the considerable loss of caffeine and L-theanine observed in this study. The loss of DPPH radical scavenging activity in the liquid tea samples may equally be due to the loss and degradation of other antioxidative compounds in tea.

As explained, both L-theanine and caffeine bind with polyphenols (Chao & Chiang, 1999; Hayashi et al., 2004; Ishizu et al., 2016; Y. Kim & Talcott, 2012; Y. Liang et al., 2002; Y. Xia et

al., 2022). Despite the removal of polyphenols by PVPP treatment in this experiment, a possible incomplete removal of tea polyphenols may have happened. The increase in the DPPH radical scavenging activity of freeze-dried and spray-dried tea samples observed in this experiment could be due to the gradual release of L-theanine, caffeine and polyphenols from various complexes formed between these compounds. Similarly, it has been shown in several studies that, processing practices and storage may lead to the formation of new antioxidant compounds. Jayabalan et al. (2008) observed that during storage of kombucha tea, EGCG, EGC and catechin initially decreased and subsequently increased (Jayabalan et al., 2008). Klimczak et al. (2007) reported that the DPPH radical scavenging activity of orange juice stored at 18°C and 28°C slightly increased in the first 2 months before subsequently decreasing. They attributed this increase to the products of the Maillard reaction (Klimczak et al., 2007). Silva et al. (2017) reported higher levels of total phenolics, total flavonoids and pectin content in freeze-dried yellow passion fruit residues than in the fresh residues (Silva et al., 2017). Similarly, Chang et al. (2006) showed that total phenolics, total flavonoids and DPPH radical scavenging activity increased in tomatoes after being freeze-dried or hot-air-dried compared to fresh tomatoes. Also, they reported an increase in the level of lycopene present in hot-air-dried tomatoes compared to fresh tomatoes (Chang et al., 2006). Pérez-Gregorio et al. (2011) observed an increase in flavonols and anthocyanins after freeze-drying onions compared to fresh onions (Pérez-Gregorio et al., 2011).

The most important limitation of this study is the numerous factors that affect the level of bioactive compounds in tea leaves and infusions. Some of these factors are difficult to control, such as those during the plant growth including the intensity of the sunlight and the natural shade level (Song et al., 2012; Too et al., 2015); while others can be controlled such as process parameters of fresh tea leaves during the manufacture including drying time and temperature

(Baptista et al., 2012) and withering time (Hung et al., 2010). Other factors such as growing altitude (Y. Chen et al., 2010), growing regions (W. Wang et al., 2023), brand and batch of tea and the season of production (W. Xu et al., 2012) also affect the levels of bioactive compounds in tea leaves and infusions.

Another challenge is the production of standardized and reproducible extracts which relies on controlling for every aspect of preparation, from plant cultivar, cultivation and processing to the type and condition of the water used for infusing. This level of control is necessary for obtaining reproducible results and minimal variations in the chemical compositions of the extracts.

The results of this study can help develop L-theanine-rich tea-based functional food ingredients with defined health benefits to be incorporated into a range of food matrices. This can be achieved by knowing the possible interaction of a tea extract and its bioactive molecules with other food ingredients. Another approach is to produce low-caffeine powdered instant tea or ready-to-drink (RTD) tea and tea-based products, targeted at people sensitive to caffeine, who want to benefit from tea's soothing and calming health effects and favourable flavour. Finally, a food-grade tea-based antioxidant additive with anxiolytic properties can be developed using the results presented here.

A few of the recent applications of tea and L-theanine in food processing are showing promise. In one study, up to 6% (W/W) green tea extract was successfully incorporated into butter without degrading the sensory properties of the butter, and significantly increased the butter’s antioxidant and antimicrobial properties (Thakaeng et al., 2020). In another study, cheese was supplemented with orthodox black tea and it was reported that adding 0.5 to 2 % W/W orthodox black tea to cheese increased the antioxidant activity but negatively affected some of the sensory

properties of the cheese (Fadhlurrohman et al., 2023). Another research team fed broiler chickens different amounts of L-theanine for 42 days and observed that L-theanine treatment improved meat quality by decreasing the oxidative stress level of the muscle and increasing the concentrations of essential, nonessential and flavour amino acids in the muscle (C. Zhang et al., 2020). In another study, Moawad et al., (2020) added green tea extract to raw chicken sausages which increased the storage life without negatively affecting the sensory properties of the sausages (Moawad et al., 2020).

Chapter 6: Summary and Conclusions

Tea (*Camellia sinensis*), is a popular drink that is a rich source of several bioactive compounds. L-theanine and caffeine, two of the most abundant components found in this plant are always extracted simultaneously in tea infusions. These compounds have opposite effects on the response of the central nervous system (CNS).

Under certain circumstances, some individuals are intolerant to caffeine intake and low-caffeine or decaffeinated drinks are recommended to them. There are different approaches to adjusting the caffeine and L-theanine proportion in tea-based products. To this end, knowing about the differences in the levels of L-theanine and caffeine in three types of tea (black, green and white tea) can provide useful information for the selection of an appropriate type of tea. Another approach is to optimize the infusion/extraction conditions to have products with higher L-theanine content versus lower caffeine content. Optimization of time and temperature of extraction, as well as factors such as extraction method, solvent and pH, amount of milk added, drying technique and storage conditions, are important parameters which can help increase overall extraction efficiency and quantity of active components in the final tea preparations or modify the proportion of these components. By adjusting the extraction parameters, different extractability of both L-theanine and caffeine can be better understood and the influential factors in their extraction and stability can be determined. To limit the negative effects of caffeine on the body, decaffeinated tea is widely produced. However, this research proposed a low-caffeine, high-L-theanine tea as a healthier and more environmentally friendly alternative to decaffeination.

First, three different types of tea with distinctly different production processes, namely black, green and white tea, were extracted in water at three different temperatures (~10-11°C, 50°C

and 90-100°C) and different time periods (5 min, 30 min and 60 min). It was observed that at higher temperatures (50°C and 90-100°C) more L-theanine and caffeine were extracted from white tea than from black and green tea. Whereas, at lower temperatures (10-11°C), more of these compounds were extracted from black and green tea than from white tea. It was shown that overall, white tea is a richer source of L-theanine and caffeine than black tea and green tea. Also, white tea infusion prepared at ~10-11°C for 5 min had a negligible amount of caffeine and therefore a substantially high L-theanine-to-caffeine ratio.

Interestingly, a change in extraction temperature could discriminate L-theanine and caffeine as it was shown that, unlike caffeine, L-theanine is better isolated under low temperatures compared to room temperature. The extraction of caffeine from white tea increased by increasing the temperature from about 10-11°C to about 21°C. Whereas, there was a decrease in the level of L-theanine when the temperature of the solution increased to room temperature. The white tea sample prepared at ~10-11°C contained significantly lower caffeine than L-theanine and therefore, a significantly higher L-theanine-to-caffeine ratio compared to the tea sample prepared at ~21°C. This confirmed the results of the first experiment and the advantage of cold brewing tea.

Cold brewing is a new and interesting idea that originated in Taiwan (Damiani et al., 2014) and tea steeped in cold water has been shown to possess several benefits. H. Ma et al. (2020) showed that green tea brewed in water at 30°C has anti-obesity effects in mice and can improve metabolic disorders (H. Ma et al., 2020). Also, cold water extracts of green tea have been shown to have some higher antioxidant activities than hot water extracts likely due to the different components effectively extracted into the infusion as a function of the infusing temperature (S.-D. Lin et al., 2008).

In this study, the relationship between L-theanine and caffeine in tea extracts with antioxidant status (the DPPH radical scavenging activity) was also elucidated. The white, green and black tea samples with different levels of L-theanine and caffeine possess different DPPH radical scavenging activities. Black tea was shown to have the highest DPPH radical scavenging activity followed by white tea and then green tea. Differences in DPPH radical scavenging activities are assigned to the compositions of active components. For example, black tea contains fermentation products, theaflavins and thearubigins, both of which are potent antioxidants. White tea, on the other hand, not only contains high L-theanine and caffeine but also some level of these fermentation products, due to undergoing withering during production.

This research confirmed that the temperature of the extraction solvent has a differentiating effect on the extraction of L-theanine and caffeine from tea, and attempted to increase this differentiating effect by manipulating the extraction conditions.

Using a solvent such as methanol for treating tea leaves was found to facilitate the somewhat preferential removal of caffeine from tea leaves. In this experiment, after methanol pre-treatment followed by a second infusion with water, more L-theanine and less caffeine were extracted from tea leaves compared to the control. This behaviour can be explained by the higher polarity of water than methanol which favours the solubility of the extremely polar L-theanine; also, the higher formation of hydrogen bonds between L-theanine molecules and water compared to methanol. These results are in agreement with the proposed hypothesis suggesting that pre-treatment of tea leaves with methanol can result in more L-theanine and less caffeine extraction in water and a significantly higher L-theanine-to-caffeine ratio compared to the control, offering an opportunity to produce an L-theanine-rich, low-caffeine extract.

Drying techniques are important factors in preserving the quality and active components of tea products and specialty extracts. The results of the drying experiment showed that freeze-drying concentrated and retained both L-theanine and caffeine very well.

Generally, it is believed that ultrasounds can increase the efficiency of the extraction of active components such as L-theanine and caffeine from plant biomass. This technique significantly increased the efficiency of cold-water extraction and simultaneously increased the extraction of both L-theanine and caffeine. Therefore, ultimately, it is only with conventional water extraction/infusion (without applying ultrasound) for 5 min, that a higher L-theanine than caffeine content and thus a high L-theanine-to-caffeine ratio was obtained.

The addition of lemon juice to the tea solution was less effective in enhancing the recovery of active components. Vuong et al. (2013) extracted green tea at 80°C for 30 min under a range of controlled pH values and similar to the results reported here, they observed no significant change in the levels of L-theanine and caffeine (Vuong et al., 2013).

The addition of 10% and 20% (V/V) milk to the tea infusion did not significantly change the level of L-theanine; however, by increasing the amount of milk to 50% V/V there was a significant reduction in the level of L-theanine. On the other hand, adding only a small concentration of milk (10% V/V) to the tea infusion significantly reduced the level of caffeine. Higher levels of milk in the tea infusion solution were effective in changing the proportion of active components as it was shown that, 50% V/V milk reduced the L-theanine level by about 25% while the caffeine level was reduced by about 50% compared to no milk (0% V/V), hence improving the L-theanine-to-caffeine ratio. Adding 20% and 50% (V/V) partly skimmed milk to the cold brew of white tea, could significantly increase the L-theanine-to-caffeine ratio compared

to the control (i.e. 0% V/V milk). The addition of milk to the tea infusion is a good approach to reducing caffeine without significantly/negatively affecting the L-theanine content.

Moreover, the accelerated storage stability of L-theanine and caffeine was examined in three different tea preparations, spray-dried, freeze-dried and liquid tea infusions, stored in airtight glass vials for 60 days and at exaggerated, higher than ambient temperatures (39 ± 1°C). The stability and functionality of each of these tea infusion preparations were also investigated in terms of their DPPH free radical scavenging activity and the moisture content of the dried samples under storage conditions. Throughout the 60-day storage period, both dried samples showed stable DPPH radical scavenging activity and moisture content (with the spray-dried samples having more stable moisture levels). However, the liquid tea samples quickly lost most of their DPPH radical scavenging activity. The L-theanine and caffeine were most stable in the freeze-dried samples, while the caffeine was also stable in the spray-dried samples. The L-theanine content, however, decreased slightly in the spray-dried samples. Both L-theanine and caffeine decreased in the liquid tea samples.

In conclusion, by adjusting the extraction procedure and choosing an appropriate drying method, the L-theanine-to-caffeine ratio can be increased which helps minimize the stimulating effects of caffeine while ensuring the anti-anxiety effects of L-theanine. The data presented in this dissertation can be helpful in better understanding the behaviour of L-theanine and caffeine, their differences and similarities during and after the tea infusion process. These data show that natural tea-based drinks (preparations) can be produced with higher levels of L-theanine and low levels of caffeine. Such preparations contain relatively high antioxidant activities which can be sustained for at least two months following drying and storage.

Suggestions for Further Study

Future studies can be conducted on freshly plucked unprocessed tea leaves, as a source of L-theanine and caffeine. Similarly, other parts of the tea plants such as stems and roots can be studied for their L-theanine and caffeine levels, and as potential sources of these bioactive compounds. The waste generated in tea factories consisting of discarded tea leaves, buds and stems may be a sustainable and rich source of bioactive compounds including L-theanine and caffeine. Studying this waste can help find a valuable, renewable, and cheap source of several bioactive compounds, especially L-theanine.

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