THEANINE AND CATECHIN EXTRACTION FROM GREEN TEA WASTE USING WATER AND SUBCRITICAL WATER

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THEANINE AND CATECHIN EXTRACTION FROM GREEN TEA WASTE USING WATER AND SUBCRITICAL WATER

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To my parents

with all my love and respect…

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ABSTRACT

THEANINE AND CATECHIN EXTRACTION FROM GREEN TEA WASTE USING WATER AND SUBCRITICAL WATER

Tea *(Camellia Sinensis),* a drink with a pleasant taste and aroma, is widely consumed in Turkey and in the World. Green tea contains bioactive compounds, such as theanine (0.1-2% dry wt), an amino acid that is responsible for the "umami taste" of tea, and catechins (30-42% dry wt), phenolic compounds with antioxidant activity. Therefore tea processing waste, which has no commercial value, can be utilized as a source of catechins and theanine with potential commercial applications in food, dieatary supplement, nutraceutical, and cosmetic industries. In this thesis, water extraction yields of theanine and catechins from green tea processing waste were determined considering the effects of extraction temperature (30-90°C) and time (5-30 min). The potential of using subcritical water (SCW) extraction system for the recovery of theanine and catechins from green tea waste was also evaluated. The effects of extraction temperature (50-110°C), time (5-60 min) and flow rate (2 ml/min and 4 ml/min) of SCW extraction on extration yields were investigated. Theanine and catechin content of the extracts were determined using HPLC-PDA. Maximum extraction yields of theanine (4.21±0.12 mg/g dry wt) and total catechins (74.82±0.48 mg/g dry wt, 76% EGCG) were obtained at 90°C in 30 min and at 80°C in 15 min, respectively, using conventional water extraction. Higher yields (increased by 4%, 3%, and 12%) could be achieved using SCW for theanine, total catechins and EGCG at 90°C in 60 min with 2 ml/min (for theanine), 110°C in 45 min with 4 ml/min (for catechins). SCW extraction curves were constructed for theanine and catechins to investigate the effect of temperature, time, flow rate and degradation/epimerization reactions on extraction yield, recovery and rate. Recovery of theanine and catechins from green tea waste using a sustainable technology offers product development opportunities for various applications in food industry.

ÖZET

YEŞİL ÇAY ATIKLARINDAN TEANİN VE KATEŞİNİN SU VE SUBKRİTİK SU İLE EKSTRAKSİYONU

Çay *(Camellia Sinensis)* hoş tat ve aroması ile Türkiye'de ve dünyada yaygın olarak tüketilen bir içecektir. Yeşil çay, teanin (%0.1-2 kuru ağırlık) gibi çayın umami tadından sorumlu olan bir aminoasit ve antioksidan aktiviteden sorumlu olan kateşinleri (%30-42 kuru ağırlık), fenolik maddeleri içerir. Bu nedenle hiçbir ticari değeri olmayan çay üretim atıkları kateşin ve teanin kaynağı olarak potansiyel ticari uygulamalarda gıda, kimya ve kozmetik endüstrisinde kullanılabilirler. Bu tezde, yeşil çay üretim atıklarından teanin ve kateşinlerin su ekstraksiyon verimleri ekstraksiyon sıcaklık (30-90°C) ve süresi (5-30 dk) dikkate alınarak belirlenmiştir. Yeşil çay atıklarından teanin ve kateşinlerin geri kazanımları için subkritik su ekstraksiyon sistemin kullanılma potansiyeli değerlendirilmiştir. Subkritik su ekstraksiyon verimleri ekstraksiyon sıcaklık (50-110°C), süre (5-60 dk) ve akış hızı etkileri (2 ml/dk ve 4 ml/dk) belirlenmiştir. Ekstraktların theanine ve kateşin miktarları HPLC-PDA ile belirlenmiştir. Teanin (4.21±0.12 mg/g kuru ağırlık) ve toplam kateşinin (74.82±0.48 mg/g kuru ağırlık, %76 EGCG) maksimum ekstraksiyon verimleri geleneksel su ekstraksiyon yöntemi ile sırasıyla 90°C, 30 dakika ve 80°C, 15 dakikada olarak belirlenmiştir. Subkritik su ekstraksiyonu kullanılarak teanin, toplam kateşin ve EGCG, 90°C de 60 dakikada 2 ml/dk akış hızı ile (teanin) ve 110°C de 45 dakikada 4 ml/dk akış hızı ile daha yüksek verimlerde elde edilebilir (%4, %3 ve %12). Teanin ve kateşinler için sıcaklık, süre, akış hızı ve bozunma/epimerizasyon reaksiyonlarının ekstraksiyon verimi, geri kazanımı ve oranı üzerindeki etkisini belirlemek için subkritik su ekstraksiyon eğrileri oluşturuldu. Sürdürülebilir bir teknoloji kullanılarak yeşil çay atığından teanin ve kateşinlerin geri kazanımı gıda endüstrisinde çeşitli uygulamar için ürün geliştirme fırsatları sunmaktadır.

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LIST OF SYMBOLS/ABBREVIATIONS

ASE	Accelerated Solvent Extractor
C	Catechin
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FAO	Food and Agriculture Organization
GA	Gallic acid
GC	Gallocatechin
GCG	Gallocatechin gallate
HPLC	High Pressure Liquid Chromatography
ISO	International Organization for Standardization
MAE	Microwave assisted extraction
SCW	Subcritical water
TÜİK	Turkish Statistical Institute

1. INTRODUCTION

Tea processing waste shows high potential as a source of bioactive compounds. Theanine and catechins have commercial potential as ingredients in food, nutraceutical, dietary supplement and cosmetic applications. Due to its high catechin content and well characterized phenolic profile, green tea processing waste offers a good model system for research on utilization of tea waste as a source of bioactive compounds. While there are many extraction studies of catechins from green tea, research on extraction of theanine from tea is limited. Simultaneous extraction of catechins and theanine from green tea waste has not been investigated. Characterization of black tea processing waste as a source of antioxidant and antimicrobial phenolic compounds, and recovery of phenolics, and caffeine from black tea waste were investigated [1,2], however to the best of my knowledge utilization as a source of bioactive compounds of green tea processing waste has not been studied until now. Considering their water solubility, water was used as a solvent in this thesis for the recovery of theanine and catechins as it is a non-toxic, inexpensive and easy-to-find "green" solvent. The optimum extraction conditions of theanine and catechins from green tea processing waste were investigated based on the effects of extraction temperature (30°C, 50°C, 70°C and 90°C) and time (5 min-15 min-30 min) using conventional water extraction in a shaking water bath. SCW extraction technique, an alternative to conventional extraction method, involves keeping water liquid at temperatures higher than its normal boiling point with pressure. The solvent power of water is thus modified, and the solubility and mass transfer properties of water are improved. In this thesis, the potential of using SCW extraction system for the extraction of theanine and catechins from green tea waste and the effects of extraction temperature (50-110°C), time (5-60 min) and flow rate (2 ml/min and 4 ml/min) were determined. HPLC methods were used for the analyses of L-theanine [3] and catechins [4]. Theanine (ISO/DIS 19563, 2016) [5] and catechin (ISO14502-2, 2005) [4] contents of green tea processing waste were determined by standard ISO methods and the efficiency of SCW extraction technique was compared with those of water extraction and ISO extraction methods. SCW extraction behaviors of theanine and catechins were studied by plotting extraction curves to determine the effects of time, temperature and flow rate on extraction yield, extraction rate and recovery, and to interpret the effects of solubility, diffusivity and degradation/epimerization reactions.

2. LITERATURE REVIEW

2.1. TEA

Tea *(Camellia Sinensis)*, which belongs to the family of Theaceae, is mainly produced in Southeast Asia (China, Vietnam, Indonesia), East of Africa (Kenya), South of Asia (Sri Lanka, India) and Turkey [6]. Tea production in Turkey was fifth in the world after China, India, Kenya and Sri Lanka while consumption was third after China and India in 2013 (FAO, 2013) [7].

There are two varieties of tea: *Camellia Sinensis var. Sinensis* and *Camellia Sinensis var Assamica*. *Camellia Sinensis var. Sinensis* is grown in Turkey. Tea is usually grown at temperatures of 13-32°C in areas with annual rainfall of at least 2000 mm and the annual moisture content of region should be more than 70% [8]. The most important factors affecting the growth of tea plants are climate, the type of soil and cultivation of tea plant. The most suitable temperature should be in the range of 18-30°C and soil should not contain more than 5% lime and the acidity of soil should in between pH 4.5-6. Tea plant shows the best growth in the soil with pH in between 5-5.6 [9]. Also to obtain high quality of tea, it is preferrable to collect two leaves and one bud due to higher quality of young leaves and buds (catechin and caffeine content) [10]. This also provides easy processing of tea. But, this is not implemented in Turkey. All these parameters determine the quality of tea.

There are three kinds of tea (black tea, oolong tea and green tea) in terms of production techniques. Consumption of different types of tea may vary from region to region. While consumption of black tea is common in parts of India and the West of India, consumption of green tea is common in the Far East [11]. Although consumption of black tea is prevalent in Turkish culture, green tea has been increasing its market share in Turkey in recent years due to increased coverage of its health effects.

2.1.1. Production and Processing of Tea

Different processing methods are used for the production of different types of tea. While black tea is obtained after withering, rolling, fermentation and drying stages, withering and fermentation procedures are not applied in green tea production (Figure 2.1). The most important step that distinguishes green tea processing from those of black tea and oolong tea is the application of heat to stop enzymatic activity during production of green tea. In this way, the polyphenol oxidase enzyme, which causes oxidation, become inactivated, so there are no losses of phenolic compounds [12]. Because in the oxidation (fermentation) step, flavanols (epicatechins and gallocatechins) transform to o-quinones with the activity of polyphenol oxidase (with H_2O_2), and this process cause the changing of catechins to theaflavins and thearubigins, which are responsible for the color and aroma of tea [13,14].

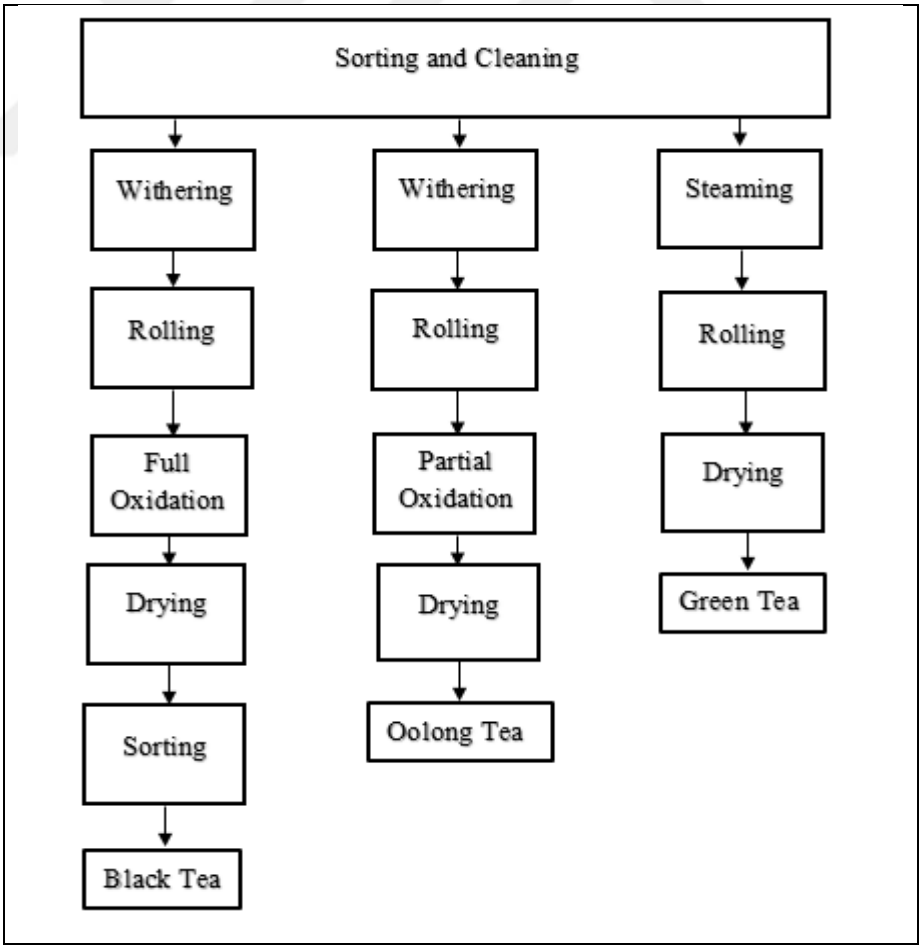

Figure 2.1. Production steps of different types of tea [15]

As seen in Figure 2.1, black tea and oolong tea are exposed to polyphenol oxidase enzyme due to fermentation during processing. For this reason, green tea contains higher amounts of catechins than black tea and oolong tea [12].

Turkey ranks 8th in terms of tea cultivation area, 6th in dry tea production (black, green and instant) and third in tea consumption with 228,000 tons after China and India (FAO, 2013) [7]. In Turkey, tea is mostly grown in the region of Black Sea mainly in Rize and Trabzon [8]. In some countries, germination of tea continues for 12 months. But, changing of climate does not allow growing of tea for 12 months in Turkey and harvesting term continues between May and October in three periods [16]. The first harvest season begins in the first weeks of May and it produces 40-45% of tea. The second harvest season begins in July and continue till the end of August and the third harvest season continues during September-October [17].

The amount of green tea leaves produced in Turkey between 2010 and 2016 based on data obtained from Turkish Statistical Institute (TÜİK) as 1,350,566 and 1,350,000 tonnes, respectively. In response to consumer demand annual production of green tea has increased in recent years in Turkey and worldwide which results in the increasing of production waste. The annual production rate of green tea increased by approximately 2.6% in the World in between 2000 and 2010 (FAO) [18]. For example, 681,000 tonnes was produced in 2000 as 900,000 tonnes in 2010 [18]. Production amount of green tea, which started as 57 tonnes in 2005, increased to 150 tonnes in 2014 and 2015 in Turkey (TÜİK). Also approximately 15% of the annual production was determined as production loss (TÜİK, 2016).

While green tea can be used as animal feed due to antioxidant activity and as a source of protein [19-21] no information was obtained about the content of waste which can be evaluated for commercial applications as a source of bioactive compounds.

2.1.2. Composition of Green Tea

Growing conditions of tea (climate, soil structure, arrival angle of sunlight), harvesting term and type of harvesting, production and processing conditions, play major roles on the amount and content of tea components [22-24]. The composition of green tea leaf is shown in Table 2.1 as % dry wt [25].

Table 2.1. Composition of fresh green tea leaves [25]

Components	**% dry weight**
Polyphenols	30
Polysaccharides	13
Cellulose	7
Lignin	6
Basic carbohydrates	4
Protein and amino acids	19
Lipids	3
Organic acids	0.5
Caffeine	3-4
Ash	5

Phenolic compounds found in green tea are aromatic compounds which has a benzene ring containing OH groups. Compounds that contain only one -OH group in their structure are called phenol, and that contain more than one OH group are called polyphenols [26]. Polyphenols are plant secondary metabolites, and for this reason plants are the most common sources for polyphenols [26,27].

The phenolic compounds found in plants can be separated into two groups as phenolic acids and flavonoids (flavanones, flavones, isoflavones, flavonols, flavanols and anthocyanidins). The most important phenolic acid found in green tea is gallic acid (GA) [22] (Figure 2.2). GA is a reducing compound, which shows fungicidal [28] and antioxidant activity [29].

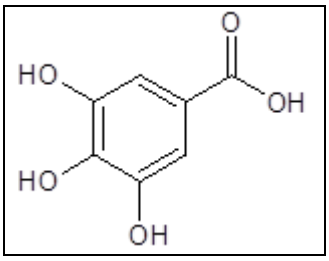

Figure 2.2. Structure of gallic acid

Different variety of green tea has different GA content [30]. For example, Meifoo green tea contains 0.74 mg/g GA, Shanghai green tea contains 0.37 mg/g and Jasmine green tea contains 1.13 mg/g [30]. Also different types of tea contain different amount of GA while 0.20±0.07 mg/100 mg dry tea leaves were found in green tea black tea contained 1.83±0.52 mg/100 mg dry tea leaves [31].

Flavonoids, the major group found in green tea, includes 2-phenylbenzofuran in the structure of $C_6C_3C_6$ (Figure 2.3) [21]. Flavanols (catechins, 17-30% dry wt) are major flavonoids and also flavonols (3-4% dry wt) are found in green tea [32].

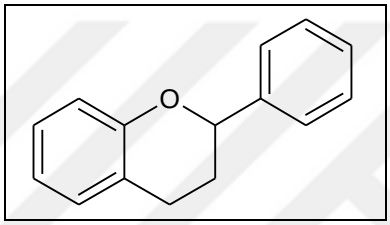

Figure 2.3. Basic structure of flavonoids

A large and significant portion of the phenolic compounds found in green tea are flavanols (catechins) which responsible for the antioxidant activity of green tea (EGCG) [20]. The flavanols (colorless) found in green tea are catechin (C), epicatechin (EC), epigallocatechin (EGC), gallocatechin (GC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epicatechin gallate (ECG) and catechin gallate (CG) which gives astringent taste in green tea.

Green tea catechins, their structure, amount, stability and antioxidant activity will be examined extensively in Section 2.2.2. Flavonols are quercetin, myricetin and kaempferol found in green tea [33], which conjugated with the ketone group present in the C ring (Figure 2.4), also exhibit antioxidant activity [33].

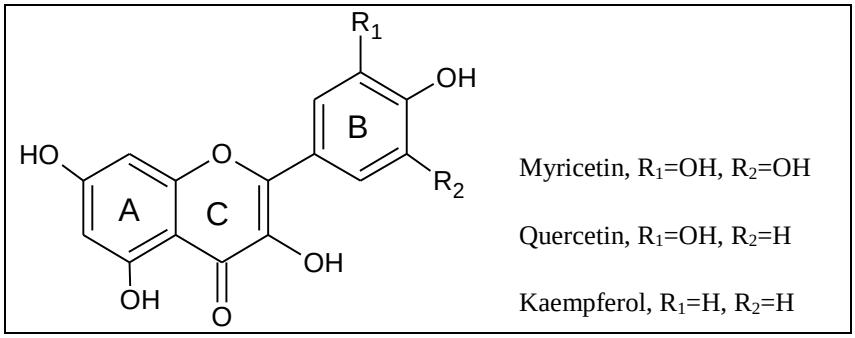

Figure 2.4. Structure of Flavonols

Tea includes higher than 20 amino acids (alanine, arginine, asparagine , histidine, isoleucine, leucine, lysine, proline, serine, threonine, theanine, valine, tyrosine,…etc.) [34]. Theanine is a water soluble non-protein amino acid (0.1-2% dry wt) found in green tea (50% of total free amino acid) that is responsible for the "umami" taste of green tea and shows bioactivity [5,35]. The structure, amount and stability of theanine will be examined in detailed in Section 2.2.1.

Caffeine is another compound as an alkaloid found in green tea (Figure 2.5) and green tea leaves contain 2-4% caffeine which responsible to the bitter taste of tea [36] and stimulates the central brain system.

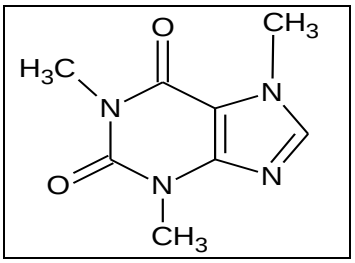

Figure 2.5. Structure of caffeine

Green tea cultivated in different regions contain different amounts of caffeine. While Indian green tea contains 19.5±0.3 mg/g dry matter, Chinese green tea contains 18.1±0.1 mg/g dry matter and Japanese green tea contains 14.7±0.2 mg/g dry matter [37]. While caffeine

content of green tea leaves was found as 24.23 mg/g [38], 1.29% caffeine content found in green tea waste which obtained from a beverage factory [19].

2.2. BIOACTIVE COMPOUNDS OF GREEN TEA

2.2.1. Theanine

Theanine (γ-glutamylethylamide, 5-N-ethylglutamine, $C_7H_{14}N_2O_3$, 174.2 g/mol, melting point: 217-218°C [35]) is a non protein aliphatic amino acid found in green tea (about 50% of the total amino acids), which includes nitrogen in its side chain (Figure 2.6) [39]. The buffering capacity of theanine amino group was determined as pK_a=8.9 and theanine does not react with other tea components during infusion. Theanine in tea can be analysed using HPLC, capillary electrophoresis and micellar electrokinetic chromatography [40-42].

Theanine was first isolated by Sakato in 1949 from tea leaf [43]. In addition to tea it is found in small amounts in Xerocomus badius mushroom in nature. Theanine gives the unique "umami" taste of tea [44], which is commonly associated with sodium glutamate [45] and elicits the umami taste receptors like T1R1 and T1R3 [46]. For this reason, theanine is directly related to the quality of green tea [47].

Theanine is synthesized from glutamic acid and ethylamine by theanine syntenase enzyme in the roots of tea plant [48,49]. It is further concentrated in the leaves and transformed into polyphenols with the help of sunlight [50]. Theanine is present mainly (98%) in the L form in nature and the D-isomer may also be present in small amounts [51].

L-theanine is a water soluble amino acid and it is insoluble in methanol, chloroform and ether [48,35]. While some studies reported higher theanine content for black tea [40,3], some studies showed that theanine content of green tea was higher due to the absence of withering and fermentation steps in green tea production [52,53].

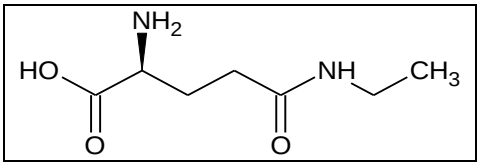

Figure 2.6. Structure of theanine

Theanine content of green and black tea can show a wide variation depending on variety of tea, climate, soil, angle of sunlight and harvesting conditions (Table 2.2). While total theanine content of Japanese fresh green tea leaves was 10.5 mg/g and Chinese green tea leaves was 17.8 mg/g [40], the total theanine content of dry green tea was in the range of 1.62 mg/g to 24 mg/g *(Pubilimba)* [54,3,55,53,56,52]. While the theanine content of Turkish green tea leaves was 18 mg/g dry wt [55], the theanine content of green tea was found as 3.1 mg/g [53], which is within the range of literature studies.

Theanine content of green tea leaves collected at the end of May (0.5%) was less than that collected by at the beginning of May (1.7%) [57]. The amount of theanine was determined in green tea (25 varieties) collected in three different harvesting periods. The highest amount of theanine was found as 0.54-1.50% dry wt in the first period (July-September), followed by 0.59-1.34% dry wt in the second period (October-December) and 0.14-1.09% dry wt in the third period (January-March) [58].

In another study, the amounts of theanine in different parts (cotyledon, leaves, root and stems) of the tea plant (six week old seedling) were examined [59]. The highest amount of theanine was found as 6.79 mg/g at the root of the shoot, followed by stems 5.89 mg/g, leaves 2.55 mg/g and cotyledons 1.98 mg/g. The position of the leaves might also affect theanine content. For example, theanine is found mostly in the bud and less in the other leaves such as second, fourth and over-wintered leaves [57]. Also, arrival angle of sunlight is another important parameter to obtain high amount of theanine. For example, Japanese Matcha tea (powdered green tea) contains low content of phenolic compounds although the amino acid content is high. The reason for this is the prevention of the photosynthesis of the Matcha tea leaves by the shadowing method. In this way, plant growth is slowed down and amino acid production is triggered. The amount of phenolic compounds in tea produced by the method of shadowing is thus less due to the inhibition of photosynthesis [60].

Theanine is stable in acidic but unstable in alkaline conditions [35]. Theanine was stable in neutral (pH=7) and acidic pH (pH=3) conditions for 12 months at 25°C in a green tea beverage [45]. But, while stable in acidic conditions, theanine can transform to glutamic acid and ethylamine by base hydrolysis [35].

Theanine is a thermally stable compound [61,48] which decomposes at temperatures higher than its melting point (217-218°C). But, there is no experimental study on the effect of temperature on decomposition of theanine. Theanine was stable in green tea beverages processed at 121°C for 5 min [45]. While an extraction study showed a decrease in yield at temperatures higher than 90°C (70-96°C) [55], another study showed that theanine was stable between 80°C and 90°C (5-90°C) [56].

After being orally ingested, theanine is absorbed into the bloodstream through intestinal absorbtion and easily transported to the brain [44]. Theanine (in tea infusion) affected the mental state by increasing the brain activity and the power of alpha band, which measured by electroencephalogram [45], resulting in a relaxation effect [38]. In addition to these direct administration of theanine into the brain by microinjection caused the increasing of dopamine in rats [62]. Also, theanine caused the release of inhibitory neurotransmitter such as γ-aminobutyric acid (GABA) which provides the regulation of dopamine and seratonin levels in the brain [40]. Thus theanine consumption provides relaxation and increase the learning ability.

Theanine has a number of potential applications in food, dietary supplement and nutraceutical industries due to its umami taste, flavor and health effects [48]. Theanine can be used in juices, beverages, mints, chewing gum, chocolate bars and specialty bottled waters as a food ingredient which determined as generally recognized as safety (GRAS) [63]. But, low theanine content of green tea results in an expensive product, making industrial scale production infeasible. For example, 60 capsules, 200 mg L-Theanine are sold at about $27 (GNC). Because of these factors, chemical or enzymatic synthesis of theanine were studied in order to determine its feasibility on industrial scale.

Table 2.2. Theanine contents of tea samples obtained using water extraction

Type of Tea	Theanine content (mg/ g)	Theanine content (mg/g dry wt)	Tea:Solvent	Temp (°C)	Time (min)	Note	Ref.
Green tea (Sencha)	10.5[a]		Pretreatment was performed	45	120		[40]
Green tea (Gunpowder)	17.8[a]						
Black tea (Lapsang Souchoung)- Black tea (Yunnan)	8.2-23.8[a]					13 dif. types of black tea	
Green tea	6.9[b]		0.1g:100 ml	90	30		[54]
Green tea	3.6		2g:200 ml	80	2	3dif. brand	[3]
Black tea	9.1					17dif. brand	
Green tea	2.2[c](25°C, 2 min)- 3.1[c] (85°C, 15 min)		3g:100 ml	25, 85	2, 5, 10, 15	From local market Infusion	[53]
Black tea	1.5[c] (25°C, 2 min)- 2.1[c] (85°C, 15 min)						
Green tea		1.62-3.37	0.25 g:10 ml	80	25	21 sample	[52]
Black tea		0.5-4.12				28 sample	
Black tea	4.0-4.8[d]		1g:10 ml	80	10	7 dif. grade of black tea	[64]
Green tea		8 (96°C, 25 min)- 18 (80°C, 25 min)	0.25g:50 ml	70, 80, 90, 96	5, 10, 15, 20, 25, 30		[55]
Green tea		13 (5°C, 30 min)- 24 (80°C, 30 min)	1g:100 ml	5-90	5-120		[56]

Converted from [a]g/100 g, [b]% w/w, [c]mg/l, [d]mg/100g for comparison purposes

2.2.2. Catechins

Catechins are compounds that give astringency and bitterness to tea. They also have antioxidant, antibacterial, antifungal activities and reduce blood pressure [14,65,66]. The catechins found in green tea are (-) epigallocatechin gallate (EGCG), (-) epigallocatechin (EGC), (-) epicatechin gallate (ECG), (-) epicatechin (EC). But, epimerization reactions cause the formation of epimers of epicatechins such as (±) catechin (C), (-) gallocatechin (GC) and (-) gallocatechin gallate (GCG), which are not found in green tea leaves as naturally [67,68]. The amount of individual catechins in green tea vary according to the type of tea, climate and the growing conditions [23,24].

Green tea grown in different countries or of different varieties had different total catechin content [37]. The yield of total catechin of Indian green tea was 53.5±1.5 mg/g dry wt, Japanese green tea was 69.5±1.8 mg/g dry wt and Chinese green tea was 51.5±1.4 mg/g dry wt [37]. Also different type of green tea grown in the same country showed different total catechin content. For example, total catechin content of as Chun mee green tea (72.9±1.7 mg/g dry wt) was 41% higher than that of other Chinese green tea (51.5±1.4 mg/g dry wt) [37].

Structures of catechins are shown in Figure 2.7 and if these catechins include three-OH groups by binding to B ring then gallocatechins occur (Figure 2.8) [22]. Catechin gallates are formed as a result of esterification of the OH group on the pyran ring with gallic acid (Figure 2.9).

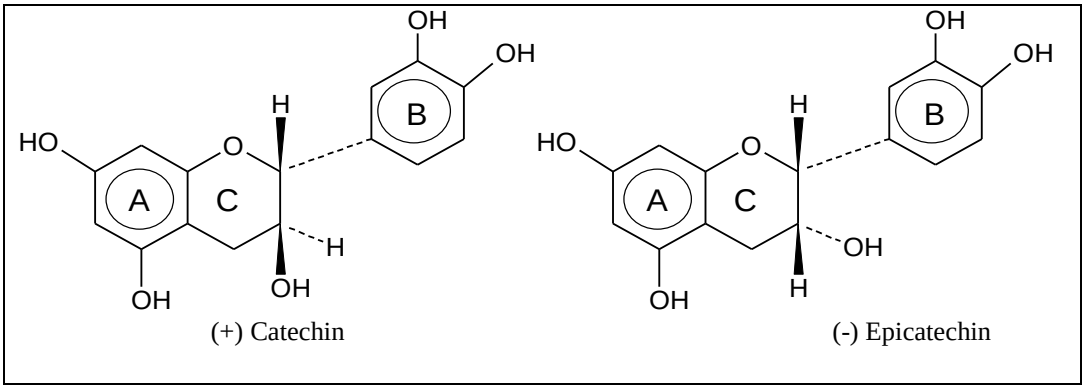

Figure 2.7. Basic catechin structures

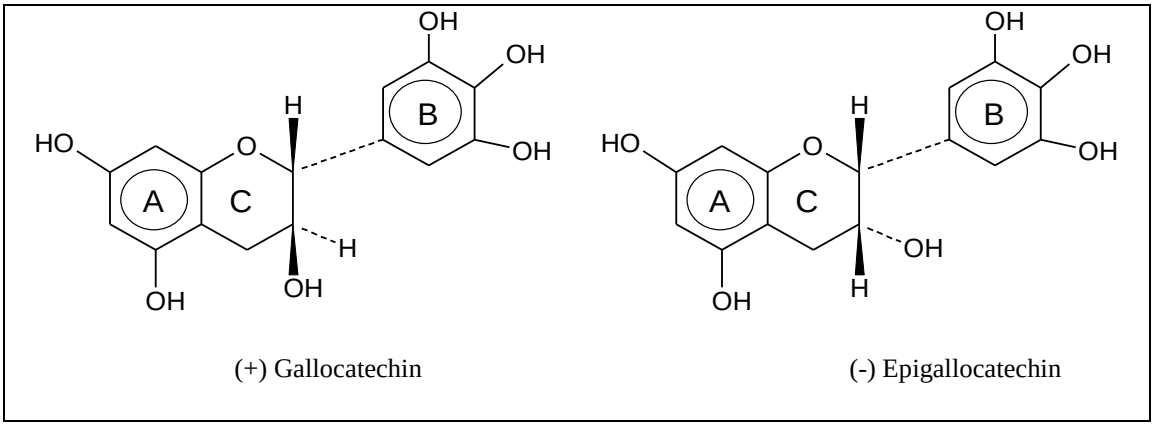

Figure 2.8. Structure of gallocatechins [22]

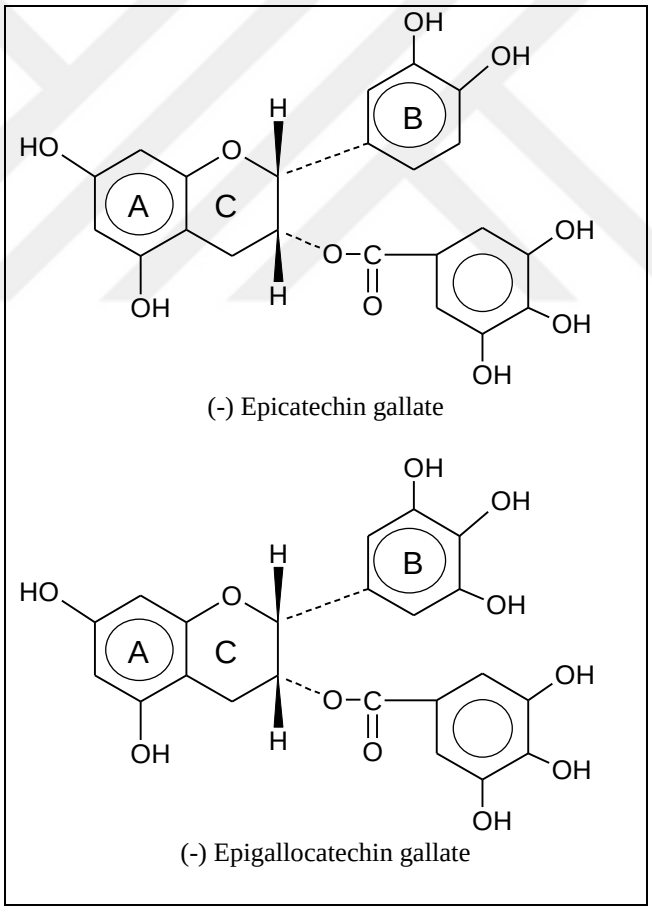

Figure 2.9. Structure of catechin gallates [22]

The free radical capture capacities of galloylated catechins were higher than those of non-galloylated catechins [69]. Tea catechins show high antioxidant activity due to reducing free

radicals, inhibiting oxidation reactions and chelating metal ions [68]. Free radical capture capacity is related to electron reduction potential. Compounds with low reduction potential require low energy to capture hydrogen and electron. EGCG has low reduction potential and this is an important factor to determine the antioxidant activity. Catechins effectively capture the singlet oxygen, O_2, -OH, peroxy radicals (-OOH), and the capture capacity of EGCG was determined to be higher than ECG, EGC or EC by electron spin resonance spectroscopy [70].

Turkish Food Codex specified the amount of total catechin in green tea as at least 7% on dry wt [71]. This shows that there is a correlation between the amount of catechin and quality of green tea. EGCG is found mostly in buds and first leaves of tea [72]. For this reason, it is known that the tea that is produced with young tea leaves contains more EGCG yielding higher quality tea.

Catechins can transform to their epimers (EGCG to GCG, EGC to GC, ECG to CG, EC to C) or can be degraded during production, processing or storage when exposed to oxygen, high temperatures, and pH changes [73,67]. Catechin has two asymmetric C atoms (C-2 and C-3) in a C ring and for this reason epimerization products can be form under the high temperature and time conditions [74].

Stability of catechins depends on temperature, pH, the presence of metal ions, oxygen and storage conditions of tea [22]. These differences can cause the transformation of cis-structured catechins (EGCG, EGC, ECG and EC) to non-epi catechins in the trans configuration (GCG, GC, CG and C) reversibly, respectively and there are differences in the bioactivities of these compounds [73].

The effect of temperature on the stability of catechins was investigated in water, and while epimerization reactions were not observed at temperatures below 40°C, it has been found that epimerization reactions occur at temperatures of 80°C and above [67,75-78].

Extractions were carried out at temperatures in between 5°C and 90°C for 30 min with water and the amounts of catechins increased up to 80°C (maximum EGCG content) and decreased at 90°C which can be related with the stability of catechins under high temperature conditions [78]. For example, the amount of EGCG was 5.1±0.7 mg/g at 5°C, 55.7±1.1 mg/g at 80°C and 52.0±2.9 mg/g at 90°C corresponding to a 6.6% decrease. Due to the decrease

in EGCG content, the amounts of non-epi catechins increased by 10% in between 80°C and 90°C [78]. In another study extractions were carried out with water for 45 min at 20°C, 40°C, 60°C, 80°C, 100°C using dry green tea [77]. Similar to the previous study, the highest amount of EGCG, ECG and EGC were reached at 80°C and the amount of GCG, GC and CG increased while the amounts of epicatechins decreased as temperature increased to 100°C. For example, the amount of GCG (epimer of EGCG) was 5.7 mg/g dry wt at 80ºC and 25.0 mg dry w at 100ºC [77]. The amount of epi catechins decreased at high temperatures (>80°C) due to epimerization and this causes the increasing of the amount of non-epi catechins [78,75,67,77].

Also, while epimerization was observed at 80°C and 100°C for 20 min for the catechin standards in water, no effect of epimerization was observed at 40°C and at room temperature for 20 min [67]. Another study showed that the thermal stability of catechins at 37°C and 98°C for 7 hours and 20% of the green tea catechins were reduced after 7 hours at 98°C and this reduction could be explained by epimerization and degradation reactions [76].

In another study catechins were extracted with pure water at 80°C for 5-120 min to determine the effect of extraction time on the stability of catechins [78]. The highest yield of epicatechins was reached in 30 min extraction time and extraction continued at about the same efficiency after 30 min. The yield of non-epi catechins continued to increase during 120 min. For example, the amount of GCG was 3.0±0.8 mg/g in 30 min and was 6.4±0.3 mg/g in 120 min with an increase of 113% [78]. This demonstrated that at 80°C extraction times higher than 30 minutes caused a negative effect on the stability of catechins [78]. In another study, which showed the negative effect of long times on the stability of catechins, extractions were carried out at 100°C with pure water and the stability of catechins were investigated in 20 min and 1, 3 and 5 hours. The amount of EGCG was 24. 2 mg/100 ml initially, 17.4 mg/100 ml at the end of the first hour and 12.1 mg/100 ml at the end of the third hour. The amount of GCG increased until the end of the 3rd hour due to epimerization and then decreased by 5.1% due to degradation [67]. Green tea catechins were degraded by 5% at 100°C in 30 min and the degradation rate was 29% at the end of the 3rd hour while the degradation rate was 25% at 70°C [79]. All these studies showed that the increasing of extraction temperature and time accelerate the epimerization reactions and also may cause degradation.

Tea includes Cu^{+2}, Fe^{+2}, Fe^{+3}, Na^{+}, K^{+} ions and these ions may bind with catechins to form complexes affecting their antioxidant activity. Extraction of tea leaves carried out with 50% acetonitrile at room temperature in a tea solution and the effects of antioxidant properties of metal ions were examined for 13 different metal ions [80]. Fe^{+2} ion increased the oxidation rate of catechin and decreased the antioxidative effect of catechin while the Cu^{+2} and Mn^{+2} ions increase the antioxidative effect of EGCG.

The effect of metal ions on the stability of catechins was investigated by extraction with tap water and distilled water. Tap water shows differences from pure water because of the pH= 7.1-7.9 and ions. The amount of EGCG was reduced in tap water infusions without epimerization within a few hours due to the ions contained [67]. Also this study showed that boiling tap water caused the epimerization reactions more readily when compared with purified water.

Another important factor affecting the stability of catechins is the pH of the solvent. Studies showed that catechins are less stable where solution pH is basic [76,75,81,82]. According to the studies performed in solutions with pH 3, 4, 5 and 6 it was observed that increasing of pH caused the degradation of catechins (80%) [76]. Another study showed that the amounts of epicatechins decreased when autoclaved at 120°C for 30 min and the amount of non-epicatechins increased which was related with the epimerization [75]. Similarly, the total amount of catechin decreased by 75% at the end of 3 hours in a solution with pH 7.4, while no degradation was observed in the range of pH from 1 to 5 solution for 3 hours [82]. As a result, studies showed that catechins are more stable in acidic conditions.

2.3. EXTRACTION OF THEANINE AND CATECHIN FROM GREEN TEA

Extraction methods of theanine and catechins from green tea can be divided into traditional extraction techniques and modern extraction techniques. Traditional extraction techniques are water extraction, maceration, organic solvent extraction and mechanical extraction (pressing). The need for a long time to prepare the sample, excessive consumption of organic solvents, environmental pollution associated with their disposal and their high price consequence can be given as some disadvantages of traditional extraction methods [83]. As a result of these disadvantages, researchers have looked for modern extraction techniques in

recent years. Development of modern extraction techniques have provided some advantages such as shorter extraction times, working at higher temperatures, less solvent consumption and higher extraction efficiency [84]. Modern extraction techniques include subcritical water extraction, supercritical fluid extraction (SCF), ultrasonic extraction and microwave assisted extraction (MAE). While only MAE was used for the recovery of theanine, effects of all other new extraction techniques were determined for green tea catechins.

2.3.1. Extraction of Theanine

Generally traditional extraction techniques were used to obtain theanine mostly using water from green tea while the use of modern extraction techniques are limited. Theanine shows high solubility in water and insolubility in ethanol, methanol, chloroform and ether [35]. While extraction of theanine from green tea was carried out with traditional extraction techniques mostly using water, solubility of standard L-theanine increased when water content of aqueous organic solvents (methanol, ethanol and acetone) increased and the solubility in water was much higher than in other solvents [85] whereas theanine yield of column chromatography with cation exchange resin from green tea was similar with 60% ethanol and water [86]. The optimum extraction conditions to obtain theanine from green tea mainly depends on the extraction temperature and time.

Theanine extraction from green tea leaves was carried out at 70°C, 80°C, 90°C and 96°C for 25 min and 80°C was found as optimum extraction temperature (18 mg/g dry wt) [55]. Also the same study determined the effect of extraction time (5-30 min) at 80°C and 25 min was found as optimum.

In another study on the effect of extraction temperature (5-90°C) for 30 min and time (5-120 min) at 80°C on theanine recovery from green tea, theanine yield increased significantly from 13 mg/g at 5°C to 24 mg/g at 80°C (30 min), a further increase in temperature to 90 °C did not affect theanine yield [56]. While 80°C was the optimum extraction temperature, theanine yield decreased from 90°C to 96°C in 25 min [55].

While there are only two studies on the effects of extraction temperature on theanin yield, determinations of theanine content were carried out at 85°C for 15 min for Turkish green tea [53], at 80°C in 10 min for Turkish black tea [64], at 80°C in 2 min [3], 80°C in 25 min [52],

90°C in 30 min [54], 45-55°C in 1.5 h [87], 90°C in 20 min [88] and 80°C in 3 min[89] for green tea.

In another study green tea infusions prepared by adding hot water (at 85°C) to green tea were kept either at 85°C or at room temperature for 2 min, 5 min, 10 min and 15 min [53]. While theanine content of green tea infusion at room temperature was 2.88±0.14 mg/g after 15 min, it was 3.14±0.08 mg/g at 85°C.

The effect of extraction time on theanine yield was investigated at 80°C for 5-120 min [56]. A significant increase in yield was observed with extraction time up to 30th min and similar yield (not significant) was obtained up to 120 min [56]. In another study at 80°C, theanine yield increased with extraction time from 5th to 25th min followed by a decrease at the 30th min [55]. Theanine yield from green tea increased by 10% when extraction time increased from 5 min to 15 min at 85°C [53].

Microwave oven extraction was the only new extraction technique used for the extraction of theanine from green tea [90]. But, extraction with microwave oven (500 W, 30-45-60 s) not affect theanine yield (10 mg/g) when compared with the efficiency of water extraction at 80°C for 30 min [90].

2.3.2. Extraction of Catechins

2.3.2.1. The Effect of Solvent

Green tea catechins are generally obtained by solvent extraction. There are many solvents that have been used in the literature in order to obtain green tea catechins such as hot water (mainly), acetone, ethanol, acetonitrile, ethyl acetate and methanol [37,91-93]. Some studies showed that water extraction was more effective in order to obtain green tea catechins [37,92,93] but on the other hand some studies suggested that extraction with organic solvents were more effective. For example, acetonitrile and ethanol are good solvents to obtain green tea catechins [91,94]. But, the use of these solvents is restricted due to the harmful effects except food grade ethanol.

In a study conducted in 2006 higher catechin yield was obtained with water extraction compared with 100% ethanol extraction [92]. Extractions were carried out for 5 min with 80% methanol, 70% ethanol and water at 60°C, 80°C and 100°C, respectively and results showed that maximum catechin yield was obtained with water at 100°C [34]. Similarly, water extraction of catechins provided higher efficiency than ethyl acetate [93], 80% methanol and 70% ethanol [37] extraction. In an extraction study which used different solvents (acetone, methanol, ethanol, acetonitrile, water) highest catechin yield (99.8%) was obtained using 50% acetonitrile and the lowest yield was obtained using pure acetonitrile [91]. However, in the same study, catechin yield was 534 mg/g, 565 mg/g, 569 mg/g dry extract when different concentrations of aqueous acetone (25%, 50%, 80%, respectively) were used, whereas it was found as 492 mg/g, 476 mg/g, 491 mg/g and 500 mg/g for methanol and 394 mg/g, 445 mg/g, 493 mg/g and 522 mg/g dry extract for ethanol extractions (25%, 50%, 80%, 100%, respectively). The catechin content was obtained as 448 mg/g at 80°C in 20 min and 258 mg/g dry extract at 95°C in 90 min [91].

One of the most studied solvents after water is ethanol. Water (boiling point, 5 min) and ethanol extraction (60°C, 15 min) of green tea catechins (24 different tea bags) was investigated and it was found that total catechin yields obtained with 80% ethanol extraction was higher (12.3 mg/g and 136.3 mg/g) than those of water extractions (4.4 mg/g and 100 mg/g) [94]. In a study which supported higher solvent power of aqueous ethanol, extractions were carried out using water and aqueous ethanol (10%, 40% and 70%) and the highest catechin yield was obtained with 40% ethanol at 80°C for 30 min [95]. The highest catechin yield was obtained with 40% ethanol in another extraction study which used water and aqueous ethanol (10%, 20%, 30%, 40%, 50%, 80%) at 25°C for 12 h [96]. Another extraction study using water and aqueous ethanol (25%, 50%, 75%, 100%) showed that 50% ethanol extraction provided 91% higher total catechin content (124.44±4.17 mg/g) compared with water extraction (65.21±1.97 mg/g) at room temperature for 2.5 h [97].

In the context of traditional extraction methods, solvents such as water, ethanol, methanol, ethyl acetate are used as solvents for catechins. But, using ethanol, methanol and ethyl acetate causes increasing cost to removing of these organic solvents, the harm effect to the environment, and high price.

2.3.2.2. The Effects of Extraction Temperature and Time

Type of solvent plays an important role in efficient extraction of catechins from green tea. But, also the effects of extraction temperature and extraction time are very important parameters to obtain catechins effectively.

In a study, catechins were extracted with water at 50°C for 4 h, at 80°C for 40 min and at 100°C for 15 min. Maximum catechin content was reached with water extraction at 80°C for 40 min [92].The effect of extraction temperature was determined from green tea at 5-90°C for 30 min and 80°C was found as optimum to obtain optimum EGCG content (55.7±1.1 mg/g) and total catechin yield [78]. Also same study showed that the effect of extraction time (5-120 min) at 80°C and 30 min was optimum and higher than 30 min the difference in yield was not significant.

Green tea catechins were extracted at different temperatures (20ºC, 40ºC, 60ºC, 80ºC, 100ºC) for 45 min using water and it was observed that the yields of GCG, GC and CG increased due to epimerization between 80°C-100°C [77]. Total catechin yield was 104.6 mg/g dry wt at the optimum temperature (80˚C) [77]. The same study, showed the effect of ethanol concentration (10-90%) for 45 min and maximum total catechin yield was found as 118 mg/g dry tea with 50% ethanol extraction which was 13% higher than that of water extraction at 80°C. In another study, extraction was carried out with 10%, 40% and 70% ethanolic water for 5, 15 and 30 min at 80°C and the highest catechin content was obtained as 40% ethanol extraction in 30 min [95].

Besides these traditional extractions, modern extraction techniques such as ultrasonic extraction, microwave extraction, supercritical carbon dioxide extraction and subcritical water extraction methods were examined for green tea catechins.

Ultrasonic extraction reduces the particle size while helping the components inside the cell to get out of the cell more quickly [98] and considering the epimerization of catechins can enable extraction at lower temperatures. Higher extraction yields of catechins were obtained using ultrasonic extraction of green tea due to an increase in surface area when compared with conventional extraction methods (aqueous ethanol) [99]. However, in another study catechin yield of extraction at room temperature (131.27 mg/g) was higher than that of ultrasonic extraction (125.21 mg/g) in 2% phosphoric acid-40% ethanol solution [96].

Another extraction technique that can be used for the extraction of catechins from green tea is MAE, which uses microwave energy for heating the sample matrix and the solvent. The choice of solvent is important to achieve successful extraction because of the absorption of the microwave by the solvent and the interaction of solvent with the sample. The solvent that has a larger dipole moment will heat up faster under microwave radiation and if the selected solvent causes very strong heating, sample degradation can not be avoided [100]. Extraction is usually carried out in a closed container. In this case pressure increases and higher temperatures can be used. The advantage of this method is the reduction in solvent and energy consumption. MAE was used to obtain polyphenols and caffeine from green tea leaves (0.5-8 min) with pre-leaching time (0-90 min) at room temperature [101]. Polyphenol and caffeine extraction yield increased with extraction time up to 4 min and after 4 min caffeine yield decreased while the yield of polyphenol was stable. The ethanol ratio in the solvent was another important parameter and increasing of the ethanol ratio to 50% increased polyphenol and caffeine yield. Compared with ultrasonic extraction (90 min) this method provided higher extraction yield of polyphenols and caffeine (30%, 4%) in a shorter time (4 min). Also microwave oven was used to obtain polyphenols from green tea, differently from the other study yields of all individual catechins (mg/g tea extract) were determined and the effects of microwave assisted water extraction were compared with conventional extraction methods [102]. The temperatures of 80°C and 100°C were used for 60 min for MAE and traditional extraction. EGCG was the predominant catechin and maximum yield was obtained at 80°C in 30 min with MAE, which was 17% less than that obtained by traditional water extraction at 80°C for 45 min [102]. Also 21% less catechin content was obtained by MAE (500W, 1 min) when compared with the catechin content obtained by water at 80°C for 30 min in green tea bags [90].

Another new extraction method is the supercritical fluid extraction. Every fluid has a critical temperature and pressure and at values higher than these it is called a supercritical fluids [103]. The diffusivity and viscosity of a supercritical fluid are in between gas and liquid like values, which provides faster mass transfer. Solvent power of a supercritical fluid can be modified by changing temperature and pressure due to the large compressibility near the critical temperature and pressure [103].

The high diffusion rate and low viscosity provide easy penetration of solvent into the sample when compared with other solvents [100]. The density of supercritical fluids can be changed

by changing temperature and pressure and supercritical fluids show better dissolution properties at higher density. Generally CO_2, which has a low critical temperature and pressure (31.1°C and 1100 psi) (Figure 2.10), is used in food applications due to its low cost and non-toxic properties [104]. Also, food materials are not degraded due to extraction at low temperature. This process reduces solvent consumption and provides shorter extraction time. Also, CO_2 can be used repeatedly. But, since it is nonpolar, it may be necessary to add modifiers in order to increase the extraction of polar analytes [105].

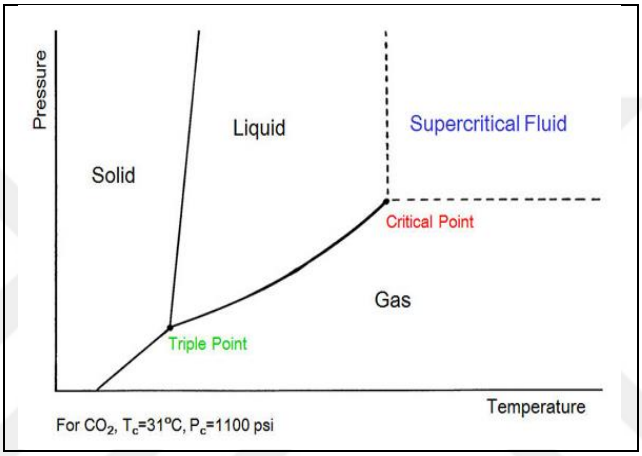

Figure 2.10. Phase diagram of supercritical carbon dioxide

Supercritical carbon dioxide extraction of tea waste was carried out using water and 10% ethanol as modifiers to obtain caffeine [106,107] and green tea catechins [108]. An extraction study which carried with water as modifier showed that the maximum yields of caffeine from stalk and fiber were found as 14.9 mg/g and 19.2 mg/g, respectively and the increase in yield was 61.9% and 65.5%, respectively when compared with solvent extraction (chloroform extraction) [106]. The yields of EGCG and caffeine were determined by supercritical carbon dioxide extraction at 40-80°C under the 200-400 bar pressure conditions with water (4-7%). This study showed that high selectivity (caffeine yield/EGCG yield, w/w%) as 2.57 can be obtained at 40ºC, 400 bar with 7% water while selectivity of traditional extraction was very low (by water 0.88, by ethanol 0.24) [107]. But, differently water or aqueous ethanol (18%, 70%, 95%, 99.8%) was used as modifiers with different ratios (at 4496 psi, 60°C) in supercritical carbon dioxide extraction and this method was ineffective for the extraction of green tea catechins and caffeine compared to solvent extraction [108].

Subcritical water extraction is an alternative new extraction method to conventional extraction techniques. Subcritical phase of water (Figure 2.11) includes temperatures from 100°C to 374°C and up to 218 atm [109].

Subcritical water is kept in liquid phase at temperatures higher than its boiling point using pressure and the dielectric constant of water, which is a measure of its polarity decrease with temperature and this increases diffusion and solvent properties of water. Solvent power of water can be improved by changing temperature of the system and this can provide the dissolution of non-polar components besides polar components [110].

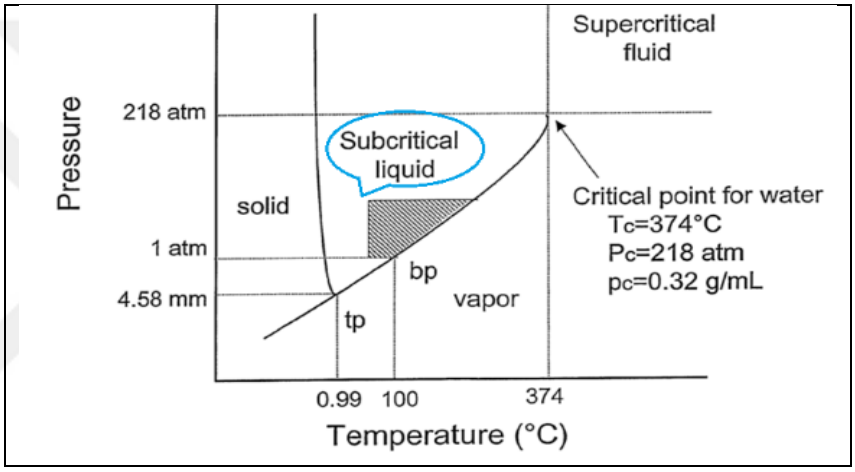

Figure 2.11. Phase diagram of subcritical water extraction [111]

While this method allows utilization of higher extarction temperatures, application of high temperature disrupt the Van Der Waals bonds, hydrogen bonds and dipole pulling forces disrupting the interaction between the analyte and the sample and this increases the diffusion rate and extraction efficiency [112]. However the application of high temperatures may not always result in improved efficiency due to sample degradation.

Water is an economical, easy to find, environmentally friendly and non toxic solvent. All these properties indicate the importance of the subcritical water extraction method. Despite the fact that research on the subcritical water extraction of catechins from tea and tea wastes have begun in recent years, literature studies in this area are still rather limited.

Subcritical water extraction of green tea leaves [38], black tea leaves [113] black tea waste [2] and black tea processing waste [114] were studied, and the effects of extraction parameters such as temperature, time, flow rate, particle size of tea on extraction efficiency efficiency were investigated.

The temperature effect was investigated using 2 g of black tea processing waste at 90°C, 120°C, 150°C and 180°C for 30 min at a flow rate of 2 ml/min using a laboratory scale extraction system. Maximum yield of EGCG was found as 4.92±0.54 mg/g dry extract at 120°C, which was higher (10%) than that obtained with standard ISO extraction method [114]. The total catechin content was maximum at 90˚C, which was lower (31%) than that obtained with ISO standard extraction method [114]. Also, while GCG, the epimer of EGCG, was not observed at 90°C, GCG yield increased with temperature up to 150°C, which was attributed to epimerization reactions of EGCG. A dramatic decrease was then observed at 180°C, which is indicative of degradation reactions [114]. Gallic acid yield increased with temperature and the maximum caffeine content (68.87±0.98 mg/g dry extract) was obtained at 90˚C.

In another study, subcritical water extraction was used to to obtain caffeine from black tea leaves and to determine the effects of temperature (100˚C, 125˚C, 150˚C, 175˚C), flow rate (1, 2, 4 g/min) and particle size (0.5, 1, 2 mm) [113]. The optimum yield of caffeine was obtained at 175˚C, at a flow rate of 4 g/min with 0.5 mm particle size in 120 min. So, the the yield of caffeine increased with temperature and flow rate [113]. In addition, the effect of extraction temperature (100˚C, 125˚C, 150˚C, 175˚C, 200˚C), flow rate (1 g/min, 2 g/min, 4 g/min), particle size (0.25 mm, 0.5 mm, 1 mm, 2 mm) and pressure of the system (20 bar, 30 bar, 40 bar) were studied from tea waste with subcritical water extraction to obtain caffeine [2]. The optimum extraction conditions were found at 175˚C at a flow rate of 4 g/min with a particle size of 0.5 mm and pressure did not have a significant effect on the efficiency of caffeine extraction [2]. According to this study, the yield of caffeine increased until 175˚C and decreased dramatically at 200˚C. This decrease was explained by the effect of degradation on caffeine yield.

In another study, subcritical water extraction of catechins were studied using an Accelated Solvent Extraction system (ASE) and the effect of extraction temperature (110˚C, 130˚C, 150˚C, 170˚C, 190˚C) and time (5 min, 10 min, 15 min) were investigated [38]. The

maximum yield of total flavanols and EGCG were obtained at 150˚C in 5 min and it was observed that epimerization reactions began at higher extraction times. For example, the EGCG yield (% flavanol) was 94% at 110°C in 5 min and 75.97% in 15 min extraction. Also, GCG yield increased with temperature and time. For example, at 110°C and 5.70% 24.03% GCG was obtained in 5 min and 15 min, respectively.

In addition to catechins and caffeine, flavonols were extracted with subcritical water from black tea at 110-200°C for 5-15 min at 10MPa and the maximum yield of myricetin and quercetin was obtained at 170°C for 5 min (0.57 mg/g dry wt and 1.18 mg/g dry wt, respectively) while the yield of kaempferol was obtained at 200°C for 15 min (2.77 mg/g dry wt) [110].

2.4. AIM OF THE STUDY

The aim of this study was to investigate value added utilization of green tea processing waste for the recovery of theanine and catechins using conventional water and subcritical water extraction. The effects of extraction parameters (temperature (30-90°C) and time (5-30 min)) on efficiency of conventional water extraction were determined. The effects of extraction temperature (50-110°C), time (5-60 min) and flow rate (2 ml/min and 4 ml/min) on subcritical water extraction behavior of theanine and catechins (yield, extraction rate, and recovery) were also investigated.

3. MATERIAL AND METHODS

3.1. Samples

Green tea and green tea processing waste were obtained from a black tea processing factory at the Black Sea Region of Turkey during the third harvest season in September 2013 and stored at -20°C until further analysis.

3.2. Chemicals

Phosphoric acid (HPLC grade) and sodium dodecyl sulphate were purchased from Fluka (USA). L-Theanine standard was acquired from Santa Cruz (USA). HPLC grade catechins ((+) catechin (C), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechin (GC), epicatechin (EC) and organic solvents (methanol, ethanol and acetonitrile) were obtained from Sigma Aldrich (USA). All standards was >95% purity. Milli-Q grade water was used for all of the analyses.

3.3. Extraction of Green Tea Waste

3.3.1. Water Extraction of Theanine and Catechins

Green tea processing waste (400 mg), ground using IKA Analytical Mill (Staufen, Germany) for 10 s was used for conventional water extractions. Green tea waste (400 mg) was extracted with water (40 mL) at 30°C, 50°C, 70°C, 90°C in a shaking water bath at 150 rpm for 5 min, 15 min and 30 min in triplicate to obtain theanine and catechins. Additional to these extraction conditions the extraction was carried at 80°C to determine the effect of epimerization reactions on catechins. Before place the glass bottle which including the sample into the water bath, water was heated to the desired temperature in another water bath. Extracts were centrifuged at 3500 rpm for 10 min and supernatants were separated.

3.3.2. Theanine and Catechin Extraction by Standard ISO Extraction Methods

Green tea and green tea waste were extracted according to ISO/DIS 19563 “Determination of theanine in tea and instant tea in solid form using high-performance liquid chromatography” with water [5] and ISO 14502-2:2005 “Determination of substances characteristic of green and black tea” with 70% methanol [4] to determine the theanine and phenolic contents of samples and to compare the results of analyses.

3.3.3. Subcritical Water Extraction of Theanine and Catechins

A subcritical water extraction system (Figure 3.1) which was built in-house, was used for the extraction of theanine and catechins from green tea waste. Green tea waste sample (2 g) was placed into the extraction column (1/2” o.d x 20 cm) equipped with 2 μm frits. After the column was connected to the system in an oven (Venticell, LSIS-B2V/ VC 111), the system was pressurized by pumping water using an HPLC pump (Dionex, Ultimate 3000). After system was filled flow was then stopped and the system was heated. After temperature of the water, which was controlled by two thermocouples inserted at the inlet and outlet of the column, reached the desired temperature extraction was started by pumping water and fractional extracts were collected as a function of time. The pressure of the system was kept at 1000 psi using a back pressure regulator. All extractions were carried out in duplicate. After each extraction, extraction system was cleaned with 70% methanol.

Fractional extraction of green tea processing waste was carried out at 50°C, 70°C, 90°C and 110°C (0-5 min, 5-15 min, 15-30 min), and at 90°C and 110°C (60 min, 5 fractions (0-5 min, 5-15 min, 15-30 min, 30-45 min and 45-60 min) using a flow rate of 2 ml/min to investigate the effect of extraction temperature and time on the extraction yield/recovery of theanine and catechins. Fractional extraction (0-5 min, 5-10 min, 10-15 min, 15-20 min, 20-30 min and 30-45 min) was also carried out at 4 ml/min to determine the effect of flow rate on extraction behaviour. Theanine and catechin yield of the extractions were determined using HPLC analysis as described in the next section. Recovery of theanine was calculated based on the maximum yield of conventional water extraction and catechins were calculated based

on the catechin contents of green tea processing waste determined by ISO standard method for green tea (ISO14502-2, 2005)[4].

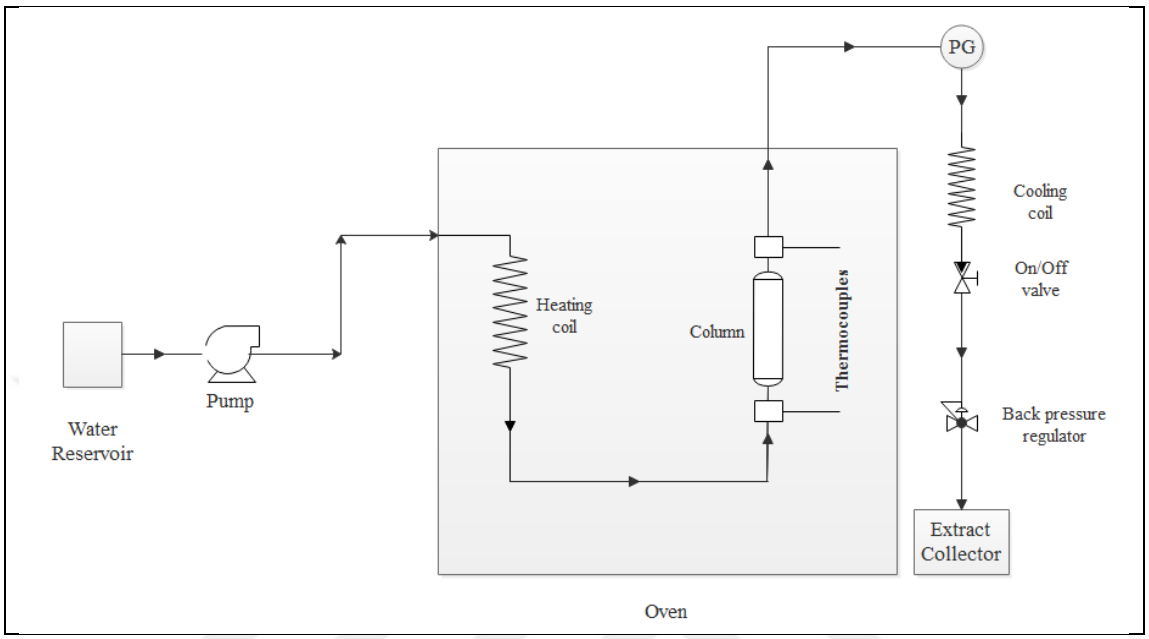

Figure 3.1. Schematic diagram of subcritical water extractor

3.4. Analytical Methods

3.4.1. HPLC Analysis of Theanine

The theanine content of the extracts were determined from green tea waste according to the method which was developed by Keenan et al. (2011) [3] using HPLC system (Thermo Scientific, USA), which includes a HPLC pump (Accela 600), a Diode Array Detector (DAD) and 5µ Hypersil ODS column (250 mm x 4.6 mm).

Mobile phase A included 0.01M SDS dissolved in 65% deionised water, 35% acetonitrile and 0.1% phosphoric acid and mobile phase B included 5% acetonitrile. The change of mobile phase versus time shown in Table 3.1.

Table 3.1.Mobile phase conditions

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow
0-1.5	100	0	isocratic
1.5-2	95	5	isocratic
2-17	95⟶ 5	5⟶ 95	linear gradient
17-20	5 ⟶ 95	95 ⟶ 5	linear gradient
20-20.5	100	0	isocratic
20.5-30.5	100	0	isocratic

205 nm wavelength (DAD) was used for detection of theanine peaks and the volume of injection was 10 µL. In addition, flow rate of mobile phase was 1 ml/min. Extracts were filtered using a 0.45 µm PTFE filter prior to HPLC analysis and 2 ml of extracts were put into amber HPLC vial. Identification of theanine peak was done using retention time of the standard compound. Theanine content was quantified based on the theanine peak area by using calibration curve and results were expressed as mg/g dry wt.

3.4.2. HPLC Analysis of Catechins

Catechin content of green tea waste was determined using standard ISO method by HPLC [4]. HPLC system (Thermo Scientific, USA) consisted of a HPLC pump (Accela 600), a Diode Array Detector (DAD). Luna 5µ Phenyl-Hexyl column (250 mm x 4.6 mm) was used at 35 °C. 278 nm wavelength was used to determine the phenolic profile. Mobile phase A was 9% acetonitrile, 2% acetic acid with 20 µg/ml EDTA and mobile phase B was 80% acetonitrile, 2% acetic acid with 20 µg/ml EDTA. 100% mobile phase A was used for 10 min followed by a linear gradient to 68% A in 15 minutes, and an isocratic flow with 68% A for 10 min. Afterwards 100% mobile phase A was used for 10 min before another injection to reequilibrate. Flow rate of mobile phase was 1 ml during analysis and all of the extracts were passed through a 0.45 µm RC filter before HPLC analysis and 1 ml of extracts were put into the amber HPLC vial. Identification of phenolics (gallic acid, C, EC, ECG, EGC, GC, EGCG and GCG) was done using to the retention times of individual standards.

Quantification of phenolics were done by calibration curves and results were expressed as mg/g dry wt.

3.5. Statistical Analysis

In this study, General Linear Model (GLM) and interaction with two factors (temperature x time) and Tukey test were used to compare the results of conventional water extraction at a significance level of $p<0.05$. Simple effect test was carried out when interaction was significant. All statistical analyses were done using SPSS. Results were expressed as mean±standard deviation.

4. RESULTS AND DISCUSSION

4.1. Theanine and Catechin Content of Green Tea Waste

Theanine and catechin (EGCG, EGC, EC, ECG, GCG, GC, C) contents of green tea waste were determined by standard ISO methods for green tea using water and 70% methanol extraction (ISO/DIS 19563, 2016 [5] and ISO14502-2, 2005 [4]), respectively and results were expressed as mg/g dry green tea waste (Tables 4.1 and 4.2).

Theanine content of green tea waste, which includes stem, stalk, leaves, fiber and dust, was determined as 3.45±0.45 mg/g dry wt, which was 25% lower than that of green tea (Table 4.1). Although green tea leaves and cotyledons contain less theanine than the root of shoots and stems [59], the presence of lower quality fiberous parts and dusty particles might decrease the theanine content of green tea waste.

While theanine content of green tea waste has not been reported in literature, theanine contents of different types of tea (black tea and green tea) from various countries (Japan, China, Vietnam and Turkey) were reported using different units, which were converted to mg/g and for comparison purposes (Table 2.4).

Theanine content of green tea waste (3.45±0.45 mg/g dry wt) was in the literature range with green tea (Table 2.4). Theanine content of green tea waste was similar to that of Turkish green tea [53].

Total catechin content of green tea waste was found as 87.63±3.62 mg/g dry wt (Table 4.2). EGCG was the predominant catechin (52%) followed by EGC (25%), EC (12%), ECG (7%), GCG (2%), GC (2%), C (1%). Similar to green tea waste, EGCG was the most dominant catechin (58%) in green tea followed by EGC (22%), EC (8%), ECG (8%), GCG (2%), GC (1%), C (1%) (Table 4.2). The total catechin content of green tea waste was 38% less than that of green tea, the yield of EGCG was 45% less, the yield of caffeine was 22% less and the yield of GA was only 8% less in green tea waste. It was expected that the amount of catechins found in green tea waste would be lower than the green tea. Because green tea waste includes stem, stalk, fiber and dust, which can have lower catechin content than green

tea leaves. For example, the total catechin content of black tea processing waste, which contained fibrous materials, was 27% less than that of black tea [1].

While there are no studies on the extraction or content of catechins from green tea processing waste, green tea was widely studied. Total catechin content of green tea was between 21.38±0.21 mg/g dry wt and 228.20±3.32 mg/g dry wt and EGCG was dominant catechin [37,115,77,78]. Similar to theanine content, the differences between catechin contents of green tea may come from the variety of green tea, growing conditions (variety of soil, sun's angle of incidence), region (climate) and harvesting period [37,116-118].

4.2. Water Extraction of Green Tea Waste

Water extraction of green tea waste was carried out at 30˚C, 50°C, 70°C and 90°C for 5 min, 15 min and 30 min for the recovery of theanine and catechins. 80°C was also tested for the recovery of catechins as it was. The optimum temperature to obtain catechins from green tea due to the epimerization reactions [78,95]. Results were expressed as mg/g green tea waste (Table 4.1 and 4.2).

4.2.1. Theanine Yield of Green Tea Waste

Theanine yield was found in the range of 3.38±0.43 mg/g dry wt (30°C, 15 min) and 4.21±0.12 mg/g dry wt (90 °C, 30 min) (Table 4.1).

The extraction yield of theanine was significantly less only at 30°C and no significant effect was observed with increasing of temperature up to 90°C. The effect of extraction time was not significant ($p>0.05$) for the extraction of theanine.

Table 4.1. Water extraction yields of theanine from green tea waste by water extraction (mg/g dry wt)

Temperature (°C)	**Extraction Time (min)**								
	5			**15**			**30**		
30	3.40	±	0.09	3.38	±	0.43	3.72	±	0.42
50	4.00	±	0.36	3.63	±	0.38	3.98	±	0.16
70	3.95	±	0.14	4.05	±	0.33	4.05	±	0.40
90	4.11	±	0.18	4.01	±	0.33	4.21	±	0.12
ISO-GTW[a]	3.45±0.45								
ISO-GT[b]	4.58±0.25								

[a]ISO-GTW: Theanine content of green tea waste, [b]:ISO-GT: Theanine content of green tea determined by standard ISO method

Standard ISO method was found inadequate for quantitative recovery of theanine from green tea waste such that water extraction of green tea waste (90°C, 30 min) resulted in 22% higher theanine yield than ISO extraction (3.45±0.45 mg/g dry wt). This may be due to the fact that the ISO method is optimized for green tea, which has a different composition than green tea waste, which contains stalk, stem, fiber, dust in addition to green tea leaves. As there is no study on the extraction of green tea waste, results were compared with the green tea extraction studies.

There are only two studies in the literature that investigated the effect of extraction temperature on theanine yield [55,56]. Extraction of theanine from dried green tea leaves was carried out at 70°C, 80°C, 90°C and 96°C for 25 min [55]. Although, the highest yield (18 mg/g dry wt) was obtained at 80°C, similar to our results there was no significant difference between 70-80-90°C for 25 min [55]. Also the effect of extraction temperature was extensively determined (5-90°C) for 30 min from Shan variety green tea (Vietnam) [56]. While theanine content of 12 mg/g dried green tea (50%) was obtained at 5°C, 24 mg/g dried green tea was obtained at 80°C. While a significant temperature effect was observed at temperatures lower than 80°C, the yield of theanine obtained at 90°C was not significantly different that that of 80°C. Also theanine yield of green tea was found as 18 mg/g dried green tea at 50°C in 30 min, the yield increased by 15% at 70°C and constant at 90°C, this was interpreted as stability of theanine [56].

While in this study theanine yield did not increase significantly with time (5-30 min), increase in theanine yield from green tea leaves and green tea with extraction time was reported in literature [56,55]. The effect of extraction time on theanine content of green tea leaves was reported for 5-30 min at 80˚C and yield increased with extraction time up to the 25th min [55]. Theanine yield of green tea increased by 10% with extraction time from 5 min to 15 min at 85˚C (infusion) [53]. An increase (4 fold) in the yield was observed at 80°C when extraction time increased from 5 min to 30 min and no further change was observed up to 120th min [56]. But, similar to the findings of this study which obtained 4.11±0.18 mg/g dried wt (3.99±0.17 mg/g wet basis) theanine from green tea waste at 90°C for 5 min, a study showed that it was possible to obtain 3.6 mg/g theanine yield from green tea using shorter time (in 2 min) at 80°C [3].

4.2.2. Catechin Yield of Green Tea Waste

Catechin (EGCG, EGC, EC, ECG, GCG, GC, C, total catechin) yields of green tea waste extracts were determined as mg/g dry wt green tea waste (Table 4.2). While the effect of extraction temperature was significant for all compounds ($p<0.05$), the effect of extraction time was significant for only EC ($p<0.05$). The interaction of extraction temperature and time were significant for GCG, GC and C ($p<0.05$).

The yields of total catechin, EGCG and EC were in between 42.25±4.02 mg/g dry wt (30˚C, 5 min)-74.82±0.48 mg/g dry wt (80˚C, 15 min), 14.69±0.70 mg/g dry wt (30°C, 5 min)-34.42±1.08 mg/g dry wt (80°C, 15 min) and 5.65±0.95 mg/g (30°C, 15 min)-8.05±0.67 mg/g dry wt (80°C, 5 min), respectively. The yields of total catechin, EGCG and EC were significantly higher at 80°C ($p<0.05$) while no differences was observed at 50°C, 70°C and 90°C ($p>0.05$). Minimum extraction yields were obtained at 30°C ($p<0.05$) (Figure 4.1-4.3). The yields of EGC and ECG were in the range of 16.55±2.60 mg/g dry wt (30°C, 15 min)-22.67±1.22 mg/g dry wt (80°C, 15 min) and 1.99±0.14 mg/g dry wt (30˚C, 5 min)-4.29±0.18 mg/g dry wt (90˚C, 30 min).

A significant decrease was observed in extraction yield at 90°C for total catechin, EGCG and EC which can be related to the epimerization of catechins. While EGC yield similarly decreased at 90°C (Figure 4.4) which can be related with the epimerization reactions, a

decrease was not observed in ECG yield. While all (105%) EGC content was recovered at 80°C for 15 min, 71% of ECG was recovered at 90°C for 30 min based on the ISO extraction method (70% methanol).

Table 4.2. Water extraction yield of catechins (EGCG, EGC, ECG, EC, GCG, GC, C and Total C) from green tea waste (mg/g dry wt)

Temperature (°C)	Time (min)	EGCG			EGC			ECG			EC			GCG			GC		
30	5	14.69	±	0.7	17.04	±	2.12	1.99	±	0.14	5.67	±	0.77	0.20	±	0.05	1.85	±	0.27
	15	15.49	±	2.71	16.55	±	2.60	2.08	±	0.34	5.65	±	0.95	0.14	±	0.04	1.77	±	0.27
	30	17.89	±	0.64	18.23	±	0.21	2.37	±	0.09	6.30	±	0.49	0.18	±	0.02	1.89	±	0.27
50	5	25.41	±	1.61	21.64	±	1.23	3.33	±	0.22	6.58	±	0.20	0.28	±	0.02	1.97	±	0.36
	15	24.7	±	2.41	20.23	±	2.10	3.24	±	0.35	6.57	±	0.49	0.26	±	0.03	2.43	±	0.58
	30	27.62	±	2.11	21.32	±	1.60	3.64	±	0.29	7.29	±	0.73	0.30	±	0.03	2.07	±	0.31
70	5	28.77	±	3.28	21.15	±	2.36	3.81	±	0.42	6.86	±	0.37	0.34	±	0.04	2.36	±	0.49
	15	29.6	±	2.72	20.3	±	1.70	4.05	±	0.27	6.81	±	0.20	0.40	±	0.04	1.94	±	0.21
	30	25.79	±	2.53	18.15	±	1.44	3.55	±	0.18	7.07	±	0.58	0.40	±	0.03	1.96	±	0.12
80	5	33.28	±	3.41	22.00	±	2.20	3.90	±	0.40	8.05	±	0.67	0.66	±	0.04	2.84	±	0.23
	15	34.42	±	1.08	22.67	±	1.22	4.22	±	0.10	7.91	±	0.40	0.89	±	0.03	3.29	±	0.27
	30	31.86	±	0.85	20.74	±	0.35	4.02	±	0.13	8.03	±	0.40	1.26	±	0.09	3.54	±	0.05
90	5	27.02	±	4.68	19.25	±	2.70	3.80	±	0.69	6.94	±	0.43	0.52	±	0.11	2.11	±	0.30
	15	27.24	±	3.31	18.38	±	1.80	4.00	±	0.36	6.77	±	0.15	1.08	±	0.14	2.67	±	0.32
	30	28.25	±	1.6	18.76	±	0.88	4.29	±	0.18	7.49	±	0.29	1.88	±	0.14	4.03	±	0.22
ISO-GTW		45.58	±	10.14	21.66	±	3.70	6.06	±	1.39	10.56	±	1.04	1.34	±	0.64	1.54	±	0.10
ISO-GT		82.51	±	0.29	31.26	±	0.39	12.02	±	0.46	11.57	±	0.69	2.38	±	0.09	1.57	±	1.04

EGCG: Epigallocatechin gallate, EGC: Epigallocatechin, ECG: Epicatechin gallate, EC: Epicatechin, GCG: Gallocatechin gallate, GC: Gallocatechin, C: Catechin, Total C: Total catechin, GA: Gallic acid, CAF: Caffeine

Table 4.2 continued

Table 4.3. Water extraction yield of catechins (EGCG, EGC, ECG, EC, GCG, GC, C and Total C) from green tea waste (mg/g dry wt)

Temperature (°C)	Time (min)	C			Total C			GA			CAF		
30	5	0.81	±	0.08	42.25	±	4.02	0.26	±	0.15	9.34	±	0.74
	15	0.85	±	0.12	42.55	±	6.99	0.16	±	0.02	9.41	±	1.30
	30	0.92	±	0.04	47.76	±	1.04	0.18	±	0.01	10.23	±	0.16
50	5	1.03	±	0.05	60.24	±	0.63	0.20	±	0.02	11.67	±	0.70
	15	1.16	±	0.13	58.59	±	0.97	0.18	±	0.06	11.12	±	0.99
	30	1.13	±	0.07	63.36	±	0.81	0.19	±	0.01	12.20	±	1.01
70	5	1.18	±	0.09	64.47	±	1.28	0.15	±	0.04	11.81	±	1.03
	15	1.14	±	0.05	64.25	±	1.05	0.12	±	0.01	11.65	±	0.82
	30	1.14	±	0.07	58.07	±	0.94	0.11	±	0.01	10.73	±	0.96
80	5	1.22	±	0.08	71.95	±	1.30	0.23	±	0.01	12.53	±	1.11
	15	1.42	±	0.13	74.82	±	0.48	0.28	±	0.02	12.72	±	0.35
	30	1.52	±	0.12	70.96	±	0.28	0.28	±	0.01	12.16	±	0.18
90	5	1.22	±	0.11	60.85	±	1.74	0.12	±	0.01	11.31	±	1.45
	15	1.40	±	0.09	61.54	±	1.23	0.15	±	0.01	11.37	±	1.07
	30	1.77	±	0.13	66.47	±	0.56	0.20	±	0.02	12.22	±	0.54
ISO-GTW		0.90	±	0.06	87.63	±	3.62	0.12	±	0.02	12.73	±	2.02
ISO-GT		0.83	±	0.05	142.13	±	0.34	0.13	±	0.02	16.26	±	12.08

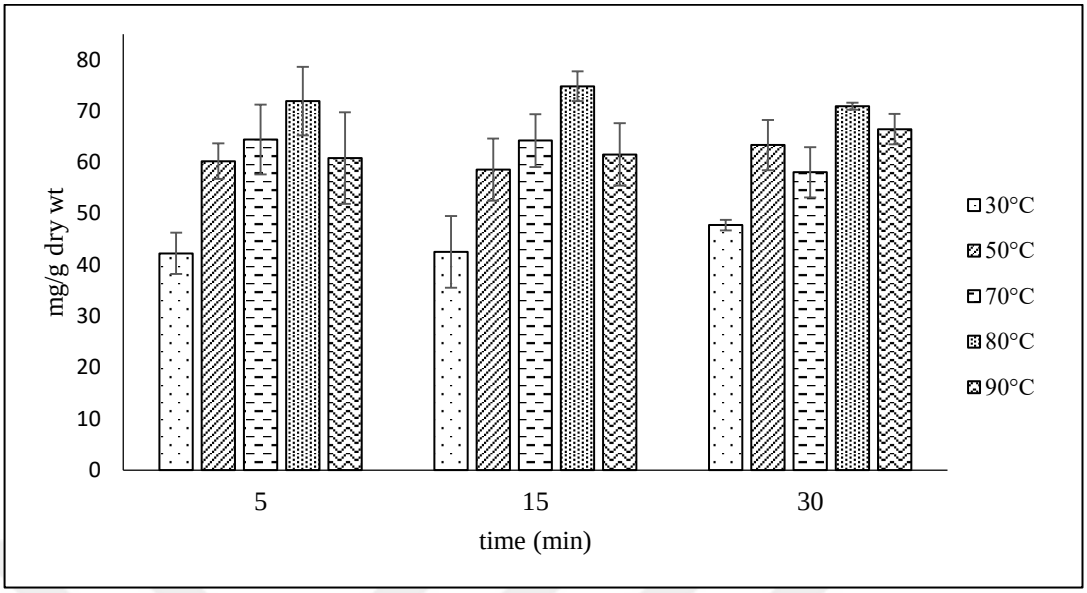

Figure 4.1. Water extraction yield of total catechins obtained at 30-90°C in 5-30 min

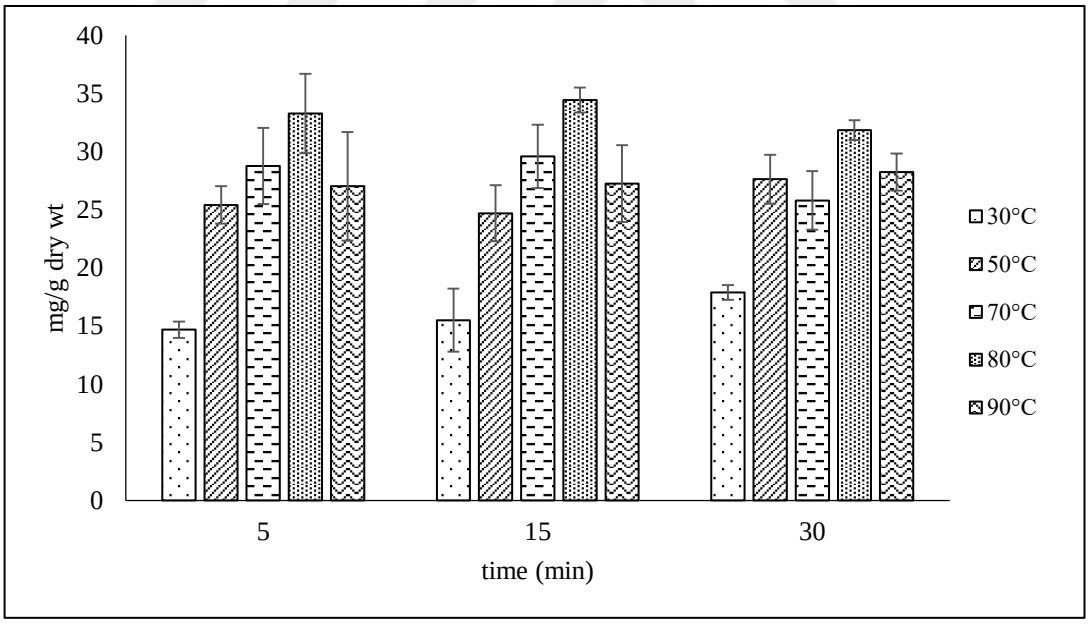

Figure 4.2. Water extraction yield of EGCG obtained at 30-90°C in 5-30 min

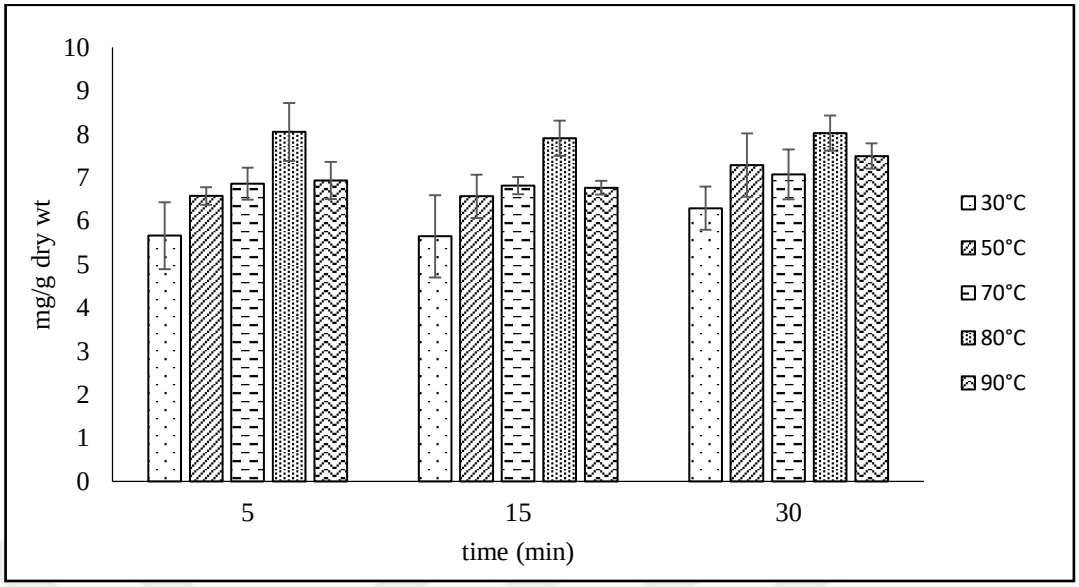

Figure 4.3. Water extraction yield of EC obtained at 30-90°C in 5-30 min

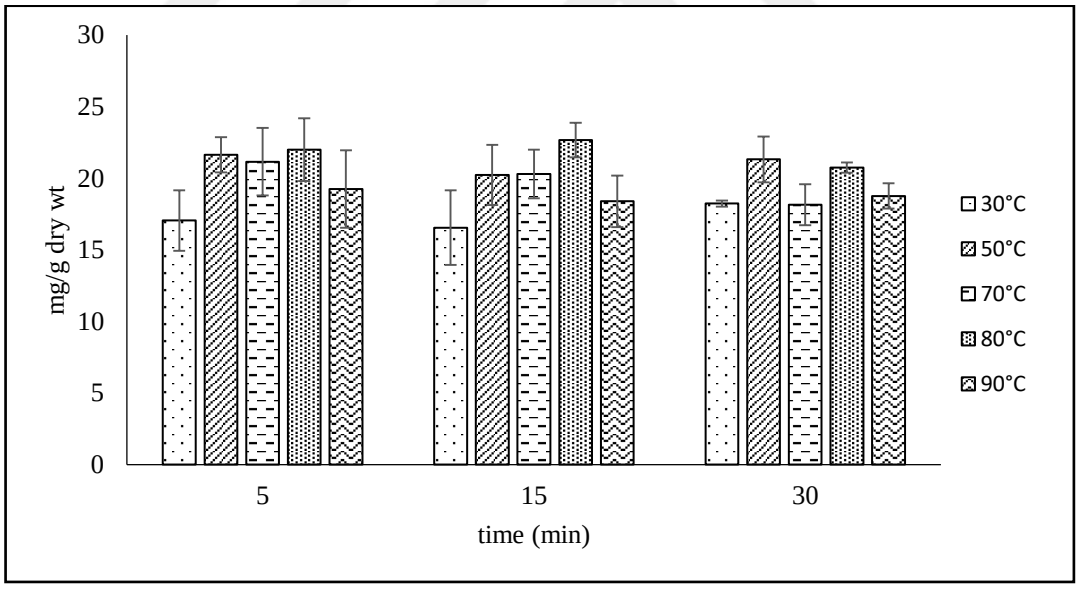

Figure 4.4. Water extraction yield of EGC obtained at 30-90°C in 5-30 min

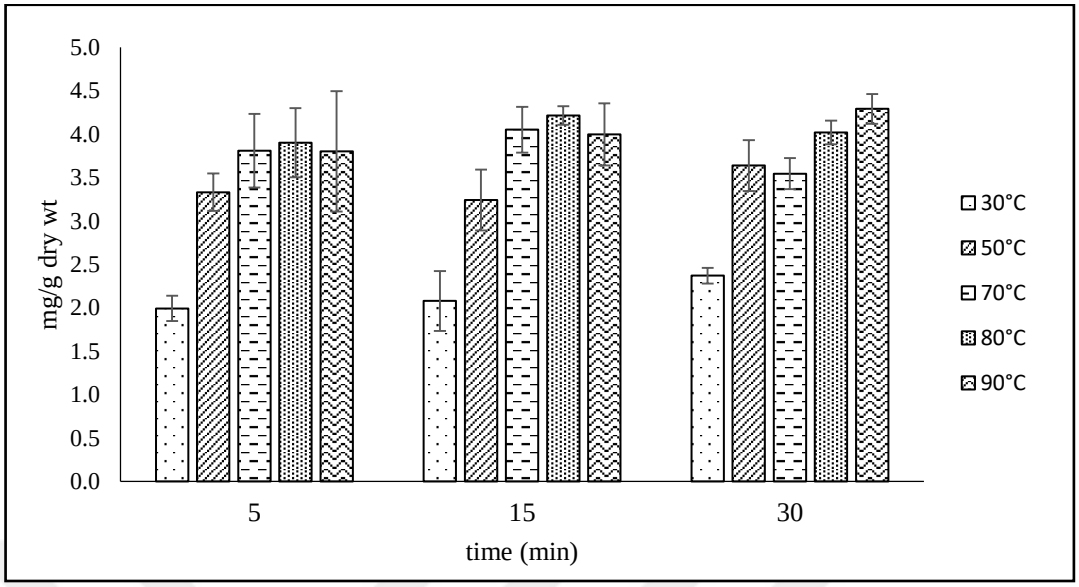

Figure 4.5. Water extraction yield of ECG obtained at 30-90°C in 5-30 min

A similar temperature effect was observed by Vuong et al. [78], while extraction yield of all catechin (EGCG, EGC, ECG, EC, GCG, GC, CG and C) from green tea increased with extraction temperature (5-80°C) in 30 min, a decrease was observed for EGCG (7%) when temperature was increased from 80°C to 90°C while no differences were observed for EGC, ECG and EC [78]. Similar to the findings of this study (i.e. no differences in the yields of total catechin, EGCG and EC between 50°C-70°C), there were no differences in total catechin yield between 40°C-60°C in 45 min for Longjing tea [77]. The maximum yields of EGCG, EGC and ECG were obtained at 80°C while the maximum EC content was reported at 60°C in 45 min [77]. While in this study there was no effect of a temperature increase in between 50°C and 70°C for total catechin, EGCG and EC, an increase in extraction temperature from 50°C to 70°C (30 min) increased EGCG, EGC, ECG and EC yields by 87%, 27%, 115%, 24%, respectively [78].

While this study showed that 5 min was sufficient to obtain catechins except EC, extraction time (5-30 min) was also not significant for the extraction of EGCG at 80°C using water from green tea bags and found significant for water extraction of loose green tea leaves, for which a 37% increase was observed when extraction time increased from 5 min to 15 min [95].

Interaction between extraction temperature and time was investigated for the yields of GCG, GC and C using interaction plots (extraction yield versus temperature and extraction yield versus time-Figures 4.6-4.8). The yield of GCG was in between 0.14±0.04 mg/g dry wt (30°C, 15 min)-1.88±0.14 mg/g dry wt (90°C, 30 min). Based on the yield versus temperature plot (Figure 4.6a), while extraction time did not have an effect on yield up to 70°C, at higher temperatures yield increased with extraction time, and this effect was more pronounced at 90 °C. Also while no time effect was observed at 30°C-70°C, at 80°C and 90°C GCG yield increased with time (Figure 4.6b).

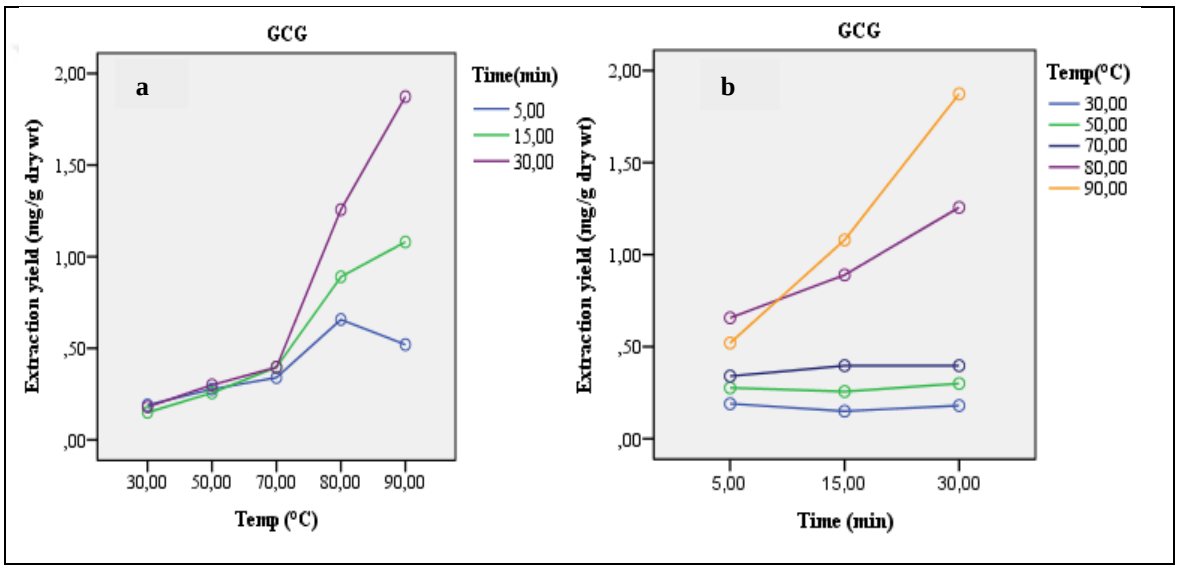

Figure 4.6. Interaction plots for GCG a) extraction yield versus temperature and b) extraction yield versus time

The extraction yield versus temperature and the extraction yield versus time plots of GC showed that temperature effect was dependent on time and time effect was dependent on extraction temperature (Figure 4.7). At temperatures higher than 70°C, the extraction yield increased with time from 5 min to 30 min (Figure 4.7b).

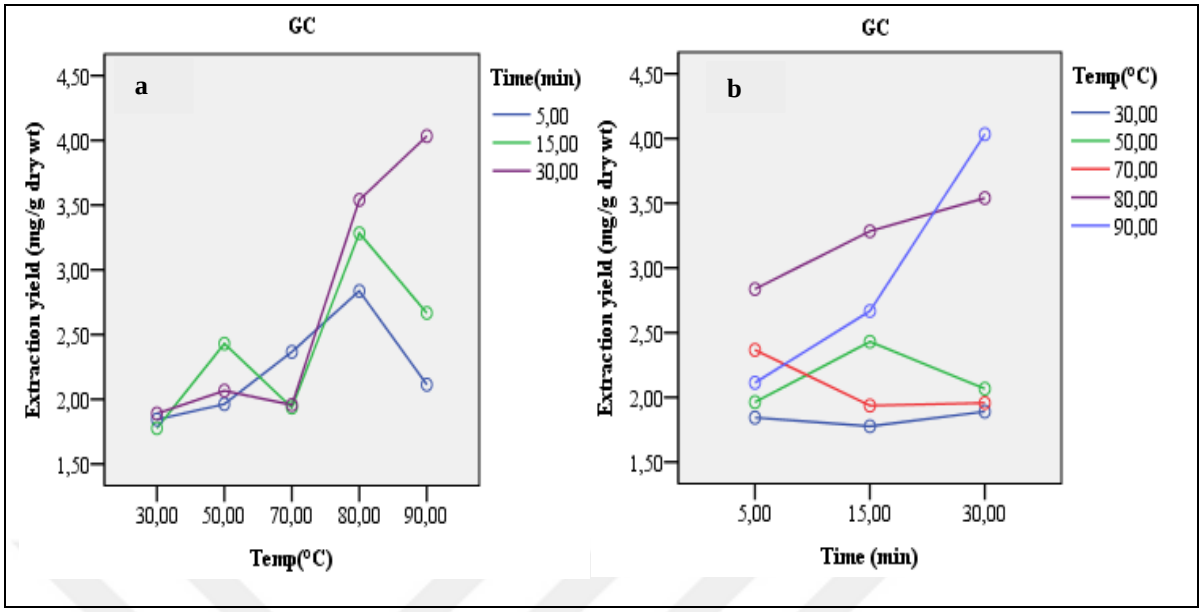

Figure 4.7. Interaction plots for GC a) extraction yield versus temperature and b) extraction yield versus time

Similarly the interaction plots of C (Figure 4.8) showed that at temperatures higher than 70°C extraction yield increased with time and reached maximum at 90°C for 30 min.

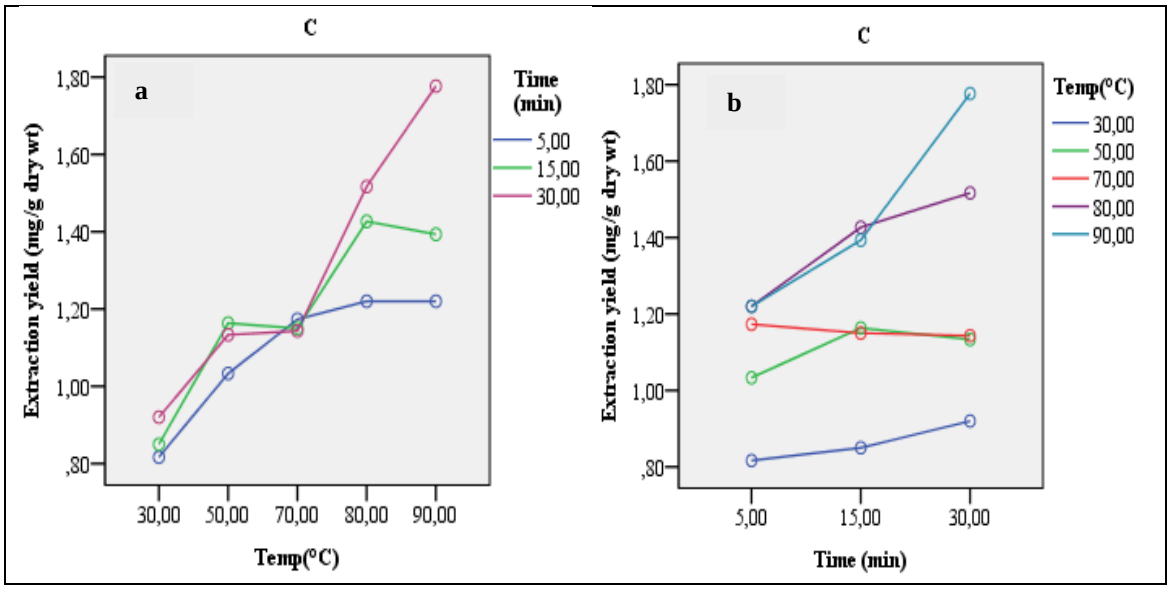

Figure 4.8. Interaction plots for C a) extraction yield versus temperature and b) extraction yield versus time

In general, extraction yield increases with extraction temperature and time due to increases in solubility and diffusivity of compounds [119]. But, an increase in extraction temperature and time may cause undesirable epimerization reactions which was related to the transformation of epicatechins (EGCG, EGC and EC) to their epimers (GCG, GC and C), respectively and degradation [120,77,78,73].

This study showed that the yields of epicatechins (EGCG, EGC and EC) decreased with temperature at temperature higher than 80°C and besides, increasing trend was observed in the yields of non-epicatechins (GCG, GC and C) with temperature and time particularly at higher temperatures. For example, while the extraction yield of EGCG decreased by 11% as the extraction temperature increased from 80°C to 90°C in 30 min, a 49% increase was observed in the yield of GCG, the epimer of EGCG, under the same conditions (Figure 4.9). This can be attributed to the presence of epimerization reactions at 90°C. Similar to the findings of this study, the EGCG yield decreased by 7% and GCG yield increased by 40% when temperature increased from 80°C to 90°C for 30 min [78]. Also in this study the yield of EGC decreased by 10% while temperature increased from 80°C to 90°C for 30 min and GC increased by 14% at the same extraction conditions. These results show the effect of epimerization reactions on the yield of EGC at temperatures higher than 80°C. Finally, the yield of EC decreased by 7% and the epimer of EC, which is C, increased by 16% when temperature increased from 80°C to 90°C for 30 min. Similar to GCG and GC, this shows the effect of epimerization reactions on the yield of EC at temperatures higher than 80°C. Extraction yields of GCG, GC and C increased with temperature between 80°C-90°C for 30 min [78]. Also GCG and CG content of green tea extracts increased with temperature in between 80˚C-100˚C for 45 min ($p<0.05$) [77].

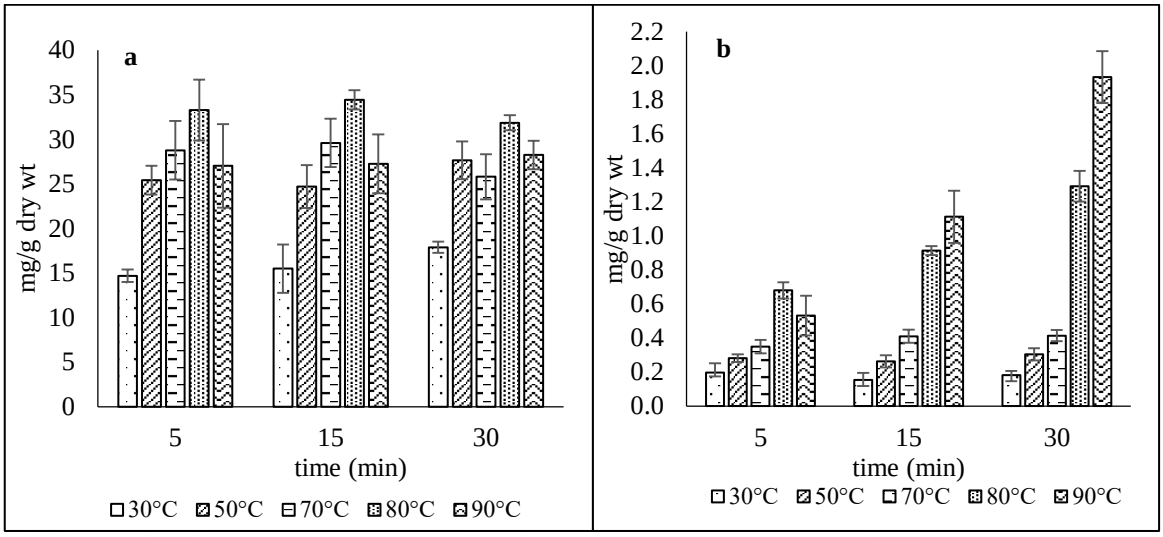

Figure 4.9. Water extraction yields of a) EGCG and b) GCG at 30-90°C for 5-30 min

4.2.3. Subcritical Water Extraction of Theanine and Catechins from Green Tea Waste

4.2.3.1. Subcritical Water Extraction of Green Tea Waste Theanine

The effects of extraction temperature, time and flow rate on the extraction behavior (yield, extraction rate, and recovery) of theanine were investigated by plotting extraction curves (yield versus time) (Figures 4.10 and 4.11). Recovery values were calculated based on theanine content of green tea waste as determined by water extraction at 90°C for 30 min (4.21±0.12 mg/g dry wt) as the ISO standard method for green tea was not adequate for quantitative recovery from green tea waste. Maximum extraction yield of theanine was obtained at 90°C in 60 min with 2 ml/min flow rate as 4.38±0.48 mg/g dry wt (%104 recovery) (Table 4.3).

Fractional extraction of theanine at 50-110°C (30 min, 2 ml/min, Figure 4.10a) showed that the extraction curves obtained at 90°C and 110°C were linear up to around 10 min (corresponding to a constant extraction rate) followed by a decreasing rate period. However, at 50°C and 70°C a clear linear phase could not be observed with extraction rate decreasing thoughout the extraction. Temperature effect on extraction yield was observed at extraction times higher than 10 min such that extraction yield increased with temperature particularly in the range 50-90°C. Recovery values of 67%, 74%, 86%, 89% were obtained at 50°C,

70°C, 90°C and 110°C, respectively, after 30 min of extraction. The shape of the extraction curves indicated that extraction could not be completed in 30 min, therefore fractional extractions up to 60 min were carried out at 90°C and 110°C to determine the maximum recovery of subcritical water extraction of theanine (Figure 4.10b). Theanine recovery increased to 101% as the extraction time was extended to 45 min at 90°C, however no further increase was observed at the 60th min. Theanine yield decreased with temperature after 30 min of extraction such that 9% lower yield was observed at 110°C (3.89±0.01 mg/g dry wt) compared to 90°C (4.27±0.03 mg/g dry wt) at the 45th min. A decrease was observed in theanine yield of water extraction of green tea when temperature increased from 80°C to 96°C in 25 min [52]. While L-theanine was stable at 121°C for 5 min in tea drinks [41] and in between 80°C and 90°C for 30 min [53], degradation might occur as a function of extraction time.

Table 4.4. Extraction yields of theanine from green tea waste by subcritical water extraction

Temp. (°C)	Flow Rate	Time (min)	Theanine		
50	2 ml/min	5	1.08	±	0.02
		15	2.24	±	0.18
		30	2.84	±	0.01
70		5	1.11	±	0.10
		15	2.47	±	0.09
		30	3.13	±	0.03
90		5	0.99	±	0.26
		15	2.58	±	0.19
		30	3.62	±	0.05
110		5	0.89	±	0.06
		15	2.58	±	0.02
		30	3.74	±	0.12
90	2 ml/min	5	1.06	±	0.19
		15	2.68	±	0.20
		30	3.81	±	0.11
		45	4.27	±	0.03
		60	4.38	±	0.48
110		5	1.06	±	0.19
		15	2.67	±	0.16
		30	3.74	±	0.10
		45	3.89	±	0.01
		60	3.89	±	0.01
90	4 ml/min	5	1.65	±	0.33
		10	2.51	±	0.05
		15	2.98	±	0.07
		20	3.28	±	0.04
		30	3.64	±	0.04
		45	3.86	±	0.09
110		5	1.73	±	0.02
		10	2.81	±	0.02
		15	3.35	±	0.01
		20	3.65	±	0.06
		30	3.98	±	0.06
		45	3.98	±	0.06
ISO-GTW	3.45±0.45				

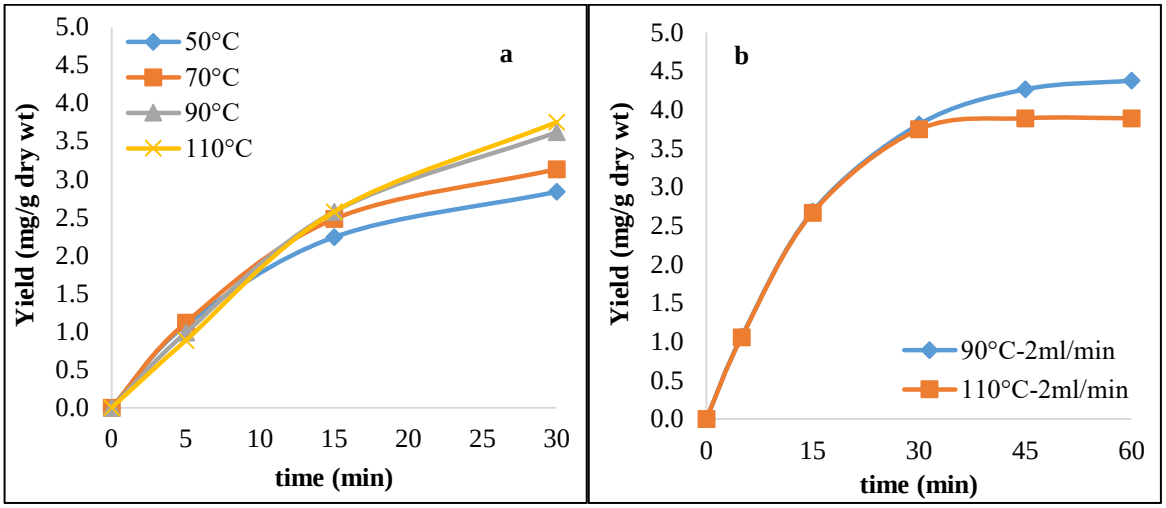

Figure 4.10. Subcritical water extraction curves of theanine from green tea processing waste a) at 50-110°C in 30 min (at 2 ml/min) and b) at 90-110°C in 60 min (at 2 ml/min)

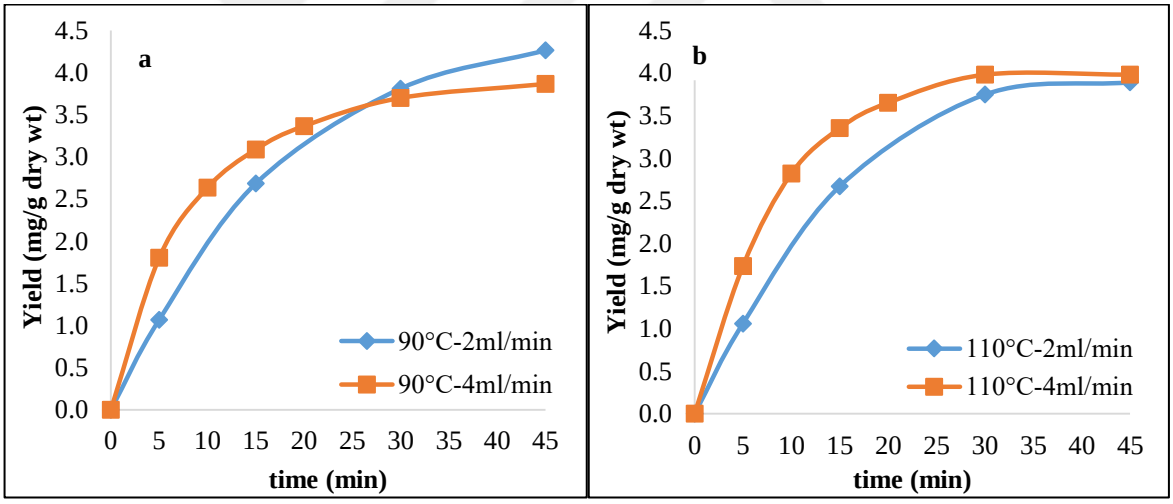

Figure 4.11. Effect of flow rate (2 ml/min and 4 ml/min) on the subcritical water extraction yield of theanine from green tea waste at (a) 90°C and (b)110°C

Increasing the flow rate to 4 ml/min resulted in an approximately proportional increase in initial extraction rate at 90°C and 110°C (Figure 4.11a and b). Extraction rates decreased with flow rate as the extraction proceeded at both temperatures. At 90°C, a crossover was observed at the 30th min such that a 10% higher yield was obtained using 2 ml/min at the 45th min. At 110°C the flow rate effect decreased with time resulting in similar yield values

at the end of 45 min, which is prevalent in these type of extractions [121]. A proportional increase with flow rate is indicative of solubility as the dominant extraction mechanism and has been commonly observed for subcritical water extractions [121]. The different extraction behavior observed at 90 °C can be attributed to a change in the dominant extraction mechanism with extraction time.

Similar theanine recovery was reached by water extraction at 90°C for 30 min and by subcritical water extraction at 90°C for 45 min at a flow rate of 2 ml/min. The differences in particle size of the samples should be considered while interpreting these results. While green tea waste was milled before water extraction, milling was not applied before subcritical water extraction as it resulted in blockage of the SCW extraction system.

4.2.3.2.Subcritical Water Extraction of Green Tea Waste Catechins

Subcritical water extraction yields of green tea waste catechins (EGCG, EGC, EC, GCG, GC, C) as a function of extraction temperature, time and flow rate are presented in Table 4.4 and extraction recoveries were evaluated using catechin contents determined by standard ISO method for green tea. Total extraction yield of catechins increased with temperature, time and flow rate such that maximum yields were obtained at 90°C and 60 min at 2 ml/min and 110°C and 45 min at 4 ml/min (Table 4.4).

Table 4.5. Subcritical water extraction yields of catechins (mg/g dry wt)

Temp. (°C)	Flow Rate	Time (min)	EGCG			EGC			EC			ECG			GCG			GC		
50	**2 ml/min**	5	0.57	±	0.13	0.80	±	0.38	0.26	±	0.10	0.05	±	0.00	0.00	±	0.00	0.05	±	0.02
		15	6.75	±	0.41	6.65	±	0.76	2.28	±	0.20	0.62	±	0.03	0.05	±	0.00	0.47	±	0.09
		30	12.41	±	0.21	10.57	±	0.37	3.70	±	0.09	1.23	±	0.01	0.09	±	0.00	0.75	±	0.00
70		5	2.59	±	0.82	4.89	±	0.63	1.36	±	0.24	0.22	±	0.06	0.02	±	0.00	0.29	±	0.01
		15	11.33	±	0.15	13.26	±	0.29	3.94	±	0.07	1.29	±	0.02	0.21	±	0.00	0.91	±	0.03
		30	20.33	±	5.49	17.89	±	3.37	5.81	±	1.14	2.41	±	0.65	0.35	±	0.03	1.33	±	0.25
90		5	3.12	±	0.13	3.32	±	0.08	1.08	±	0.04	0.32	±	0.01	0.03	±	0.00	0.24	±	0.01
		15	15.24	±	1.37	10.41	±	0.47	3.67	±	0.15	1.74	±	0.19	0.26	±	0.05	0.86	±	0.11
		30	26.81	±	1.49	15.78	±	0.43	5.66	±	0.00	3.19	±	0.16	0.65	±	0.08	1.49	±	0.09
110		5	2.91	±	0.07	3.52	±	0.04	1.04	±	0.00	0.32	±	0.01	0.12	±	0.00	0.40	±	0.00
		15	17.80	±	0.80	11.56	±	0.14	3.85	±	0.06	2.27	±	0.13	1.07	±	0.06	1.62	±	0.12
		30	28.25	±	0.40	14.94	±	0.21	5.51	±	0.02	3.98	±	0.06	2.96	±	0.10	3.10	±	0.15
90	**2 ml/min**	5	4.00	±	0.73	4.20	±	0.00	1.27	±	0.13	0.43	±	0.09	0.07	±	0.01	0.31	±	0.03
		15	16.57	±	0.61	10.85	±	0.39	3.77	±	0.07	1.99	±	0.07	0.39	±	0.01	0.93	±	0.00
		30	27.91	±	1.25	15.44	±	0.54	5.79	±	0.22	3.42	±	0.20	0.86	±	0.01	1.51	±	0.02
		45	33.80	±	1.09	17.38	±	0.53	6.73	±	0.16	4.29	±	0.13	1.12	±	0.03	1.85	±	0.04
		60	36.51	±	0.06	17.92	±	0.03	7.09	±	0.03	4.72	±	0.01	1.32	±	0.01	1.96	±	0.03
110		5	4.47	±	0.43	4.08	±	0.10	1.39	±	0.11	0.55	±	0.06	0.21	±	0.03	0.51	±	0.06
		15	18.12	±	2.31	11.26	±	1.41	4.28	±	0.34	2.43	±	0.34	1.10	±	0.14	1.66	±	0.19
		30	28.73	±	1.94	14.66	±	0.30	6.08	±	0.04	4.22	±	0.34	2.90	±	0.34	3.02	±	0.11
		45	31.42	±	0.42	15.18	±	0.00	6.54	±	0.07	4.79	±	0.11	3.64	±	0.20	3.38	±	0.02
		60	32.47	±	0.12	15.32	±	0.03	6.70	±	0.05	5.05	±	0.04	3.87	±	0.26	3.48	±	0.00

EGCG: Epigallocatechin gallate, EGC: Epigallocatechin, EC: Epicatechin, ECG: Epicatechin gallate, GCG: Gallocatechin gallate, GC: Gallocatechin, C: Catechin, Total C: Total catechin, GA: Gallic acid, CAF: Caffeine

Table 4.4. continued

Table 4.6. Subcritical water extraction yields of catechins (mg/g dry wt)

90	**4 ml/min**	5	8.05	±	1.38	6.15	±	1.20	2.20	±	0.37	0.89	±	0.19	0.12	±	0.03	0.52	±	0.09
		10	17.22	±	0.65	10.61	±	0.21	3.98	±	0.08	1.97	±	0.10	0.30	±	0.02	0.92	±	0.03
		15	23.37	±	0.32	13.12	±	0.14	5.09	±	0.09	2.74	±	0.03	0.46	±	0.03	1.18	±	0.04
		20	27.55	±	0.22	14.67	±	0.06	5.79	±	0.06	3.28	±	0.03	0.59	±	0.04	1.34	±	0.04
		30	32.68	±	0.16	16.35	±	0.11	6.63	±	0.04	4.00	±	0.02	0.81	±	0.05	1.54	±	0.05
		45	36.48	±	0.20	17.45	±	0.16	7.25	±	0.06	4.56	±	0.01	0.99	±	0.04	1.72	±	0.09
110		5	9.78	±	1.48	6.92	±	0.66	2.42	±	0.20	1.16	±	0.20	0.23	±	0.04	0.71	±	0.07
		10	21.52	±	1.77	12.39	±	0.31	4.54	±	0.13	2.72	±	0.33	0.77	±	0.15	1.47	±	0.09
		15	28.39	±	0.43	15.06	±	0.03	5.69	±	0.05	3.70	±	0.13	1.38	±	0.21	2.08	±	0.12
		20	32.34	±	0.28	16.30	±	0.24	6.32	±	0.13	4.34	±	0.04	1.92	±	0.18	2.53	±	0.05
		30	36.32	±	0.46	17.28	±	0.23	6.96	±	0.14	5.04	±	0.01	2.72	±	0.21	3.05	±	0.05
		45	38.68	±	0.57	17.75	±	0.18	7.35	±	0.15	5.49	±	0.05	3.28	±	0.03	3.38	±	0.01
ISO-GTW			45.58	±	10.14	21.66	±	3.70	10.56	±	1.04	6.06	±	1.39	1.34	±	0.64	1.54	±	0.10

Table 4.4 continued

Table 4.7. Subcritical water extraction yields of catechins (mg/g dry wt)

Temp. (°C)	Flow Rate	Time (min)	C	Total C	CAF	GA
50	**2 ml/min**	5	n.d	1.80 ± 0.54	0.34 ± 0.05	n.d
		15	n.d	17.48 ± 1.34	3.61 ± 0.18	0.06 ± 0.02
		30	n.d	29.85 ± 0.55	6.43 ± 0.03	0.08 ± 0.01
70		5	n.d	9.92 ± 1.76	1.71 ± 0.31	0.07 ± 0.00
		15	0.56 ± 0.04	32.45 ± 0.81	5.47 ± 0.19	0.12 ± 0.00
		30	0.77 ± 0.13	50.41 ± 11.07	8.93 ± 0.21	0.15 ± 0.00
90		5	0.11 ± 0.00	8.49 ± 0.28	1.98 ± 0.33	0.03 ± 0.00
		15	0.41 ± 0.00	33.60 ± 2.35	7.24 ± 0.27	0.08 ± 0.01
		30	0.67 ± 0.03	55.95 ± 2.28	11.67 ± 0.31	0.14 ± 0.00
110		5	0.16 ± 0.00	8.73 ± 0.04	2.00 ± 0.08	0.03 ± 0.00
		15	0.67 ± 0.05	40.04 ± 0.79	8.19 ± 0.29	0.10 ± 0.00
		30	1.20 ± 0.06	61.77 ± 0.37	12.14 ± 0.01	0.18 ± 0.00
90	**2 ml/min**	5	0.14 ± 0.02	10.43 ± 1.02	2.30 ± 0.34	0.03 ± 0.00
		15	0.42 ± 0.00	34.93 ± 1.15	7.46 ± 0.09	0.07 ± 0.00
		30	0.68 ± 0.02	55.59 ± 2.23	11.19 ± 0.29	0.11 ± 0.00
		45	0.82 ± 0.03	66.01 ± 2.02	12.83 ± 0.28	0.14 ± 0.00
		60	0.89 ± 0.01	70.39 ± 0.03	13.32 ± 0.07	0.15 ± 0.00
110		5	0.24 ± 0.02	11.44 ± 0.80	2.56 ± 0.23	0.04 ± 0.01
		15	0.78 ± 0.09	39.65 ± 4.84	8.02 ± 0.73	0.10 ± 0.02
		30	1.34 ± 0.02	60.94 ± 2.99	11.73 ± 0.52	0.18 ± 0.02
		45	1.54 ± 0.02	66.48 ± 0.65	12.33 ± 0.02	0.23 ± 0.01
		60	1.62 ± 0.02	68.51 ± 0.32	12.46 ± 0.01	0.26 ± 0.01

Table 4.4 continued

Table 4.8. Subcritical water extraction yields of catechins (mg/g dry wt)

90	**4 ml/min**	5	0.23	±	0.03	18.15	±	3.29	4.17	±	0.65	0.05	±	0.01
		10	0.42	±	0.02	35.41	±	0.96	7.78	±	0.14	0.08	±	0.00
		15	0.54	±	0.02	46.51	±	0.68	9.88	±	0.14	0.10	±	0.00
		20	0.62	±	0.01	53.85	±	0.45	11.16	±	0.07	0.12	±	0.00
		30	0.71	±	0.02	62.71	±	0.28	12.54	±	0.11	0.14	±	0.00
		45	0.79	±	0.02	69.25	±	0.54	13.46	±	0.15	0.16	±	0.01
110		5	0.37	±	0.16	21.61	±	2.82	4.73	±	0.45	0.05	±	0.00
		10	0.76	±	0.19	44.16	±	2.97	8.99	±	0.35	0.09	±	0.00
		15	1.02	±	0.09	57.32	±	0.91	11.31	±	0.04	0.13	±	0.00
		20	1.19	±	0.02	64.93	±	0.37	12.57	±	0.14	0.16	±	0.00
		30	1.39	±	0.04	72.74	±	0.54	13.69	±	0.12	0.20	±	0.01
		45	1.54	±	0.00	77.48	±	0.91	14.27	±	0.13	0.25	±	0.00
ISO-GTW			0.90	±	0.06	87.63	±	3.62	12.73	±	2.02	0.13	±	0.02

nd : not determined

Extraction curves of the predominant catechins in green tea waste, EGCG (Figures 4.12-4.14) and EGC (Figure 4.15-4.17) were further investigated to determine the effect of temperature, time and flow rate on extraction behavior. EGCG recovery increased with temperature during 30 min of extraction using a flow rate of 2 ml/min (27% at 50°C-62% at 110°C, Figure 4.12a).

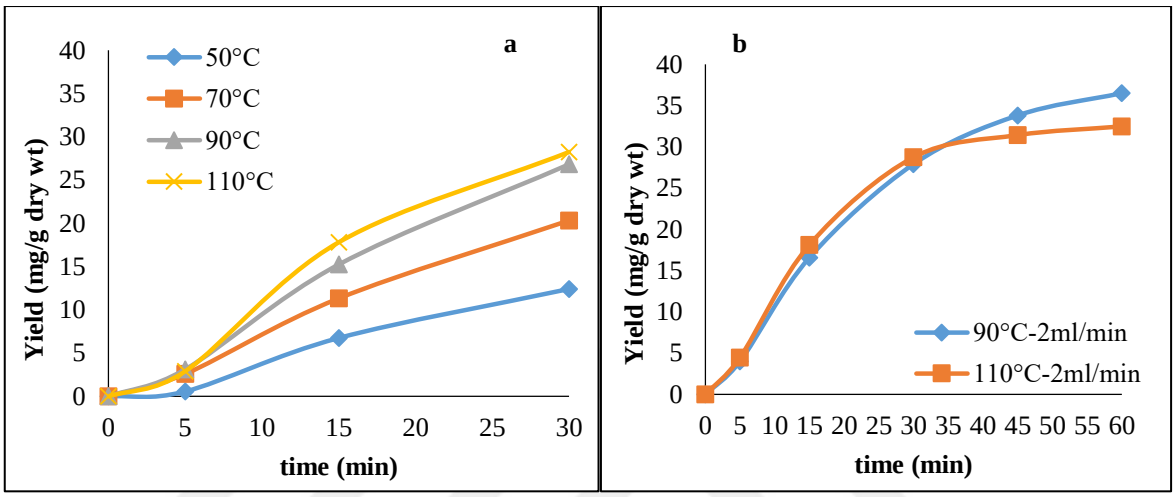

Figure 4.12. Extraction curves of EGCG from green tea processing waste a) at 50-110°C in 30 min (at 2 ml/min) and b) at 90-110 °C in 60 min (at 2 ml/min)

Similar to theanine extraction, increasing the extraction time to 60 min at 90°C and 110°C increased EGCG recovery (to 80% and 71% at 90°C and 110°C, respectively) (Figure 4.12b). Similar to the our findings 81% of EGCG was recovered from green tea leaves by subcritical water extraction (150°C for 5 min) [38].

A lower yield was observed at 110°C (11%) compared to 90°C for 60 min of extraction. The decrease in EGCG yield with temperature was accompanied by an increase in GCG yield (193%) (Figure 4.13) indicating epimerization reactions at 110°C.

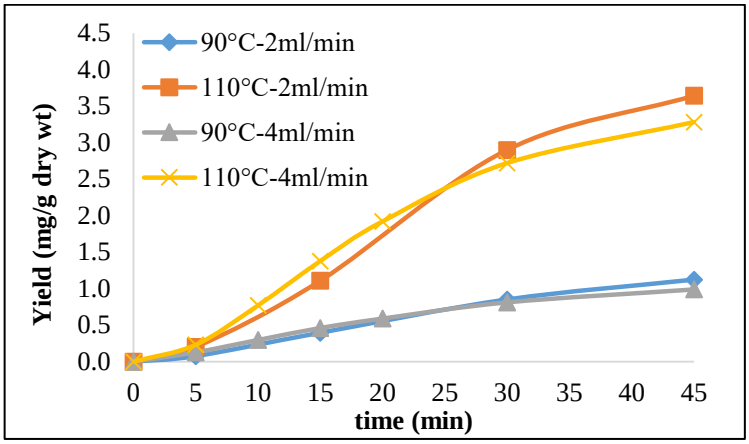

Figure 4.13. Effect of extraction temperature (90-110°C) and flow rate (2 ml/min-4 ml/min) on the subcritical water extraction yield of GCG

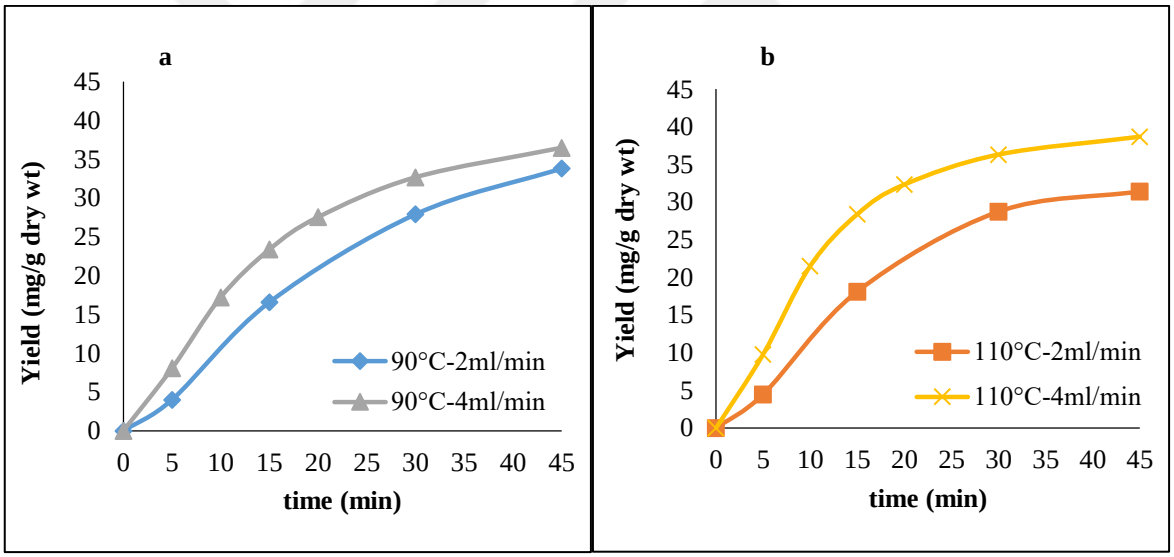

Figure 4.14. Effect of extraction flow rate on the yield of EGCG at 90°C (a) and 110°C (b)

Flow rate increased EGCG yield at both temperatures, however the effect was more pronounced at 110°C (Figure 4.14), which can be related to the limiting effect of the higher flow rate on epimerization reactions. Flow rate had no effect (at 90°C) or a small effect (at 110°C) on GCG yield (Figure 4.13).

The recovery of EGC increased up to 70°C for 30 min followed by a decrease with temperature and the recovery values were 49%, 83%, 73%, 69% at 50°C, 70°C, 90°C and

110°C, respectively for 30 min (Figure 4.15a). While extraction curve showed that extraction was not completed in 30 min and higher yield obtained at 70°C, extraction time extended up to 60 min at 90°C and 110°C due to the high EGCG (predominant catechin) content at these temperatures with 2ml/min (Figure 4.15b).

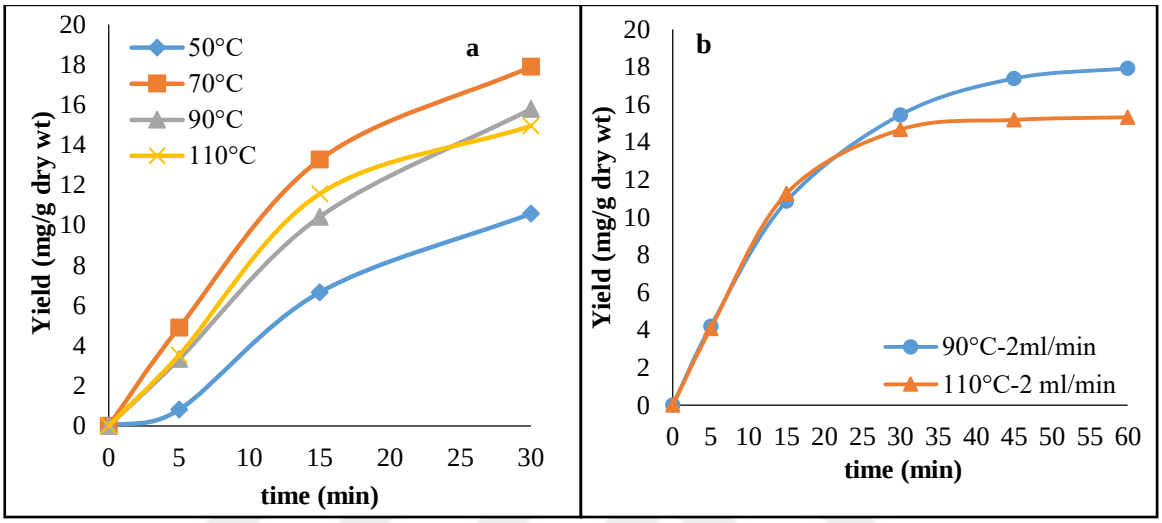

Figure 4.15. Extraction curves of EGC from green tea processing waste a) at 50-110°C in 30 min (at 2 ml/min) and b) at 90-110°C in 60 min (at 2 ml/min)

Similar to EGCG yield, a decrease in EGC yield with temperature (at 110°C) was observed after 20 min, which can be related with an increase in GC yield due to the epimerization reactions at 110°C (Figure 4.15b and 4.17). The decrease was 15% in EGC yield and increase was 78% in GC yield at 110°C for 60 min when compared with 90°C at a flow rate of 2 ml/min which can be related with the effect of epimerization.

An increase in yield was observed at 90°C and 110°C with flow rate while the effect of flow rate was marked at 110°C (Figure 4.16), similar to EGCG, which can be related with epimerization of EGC.

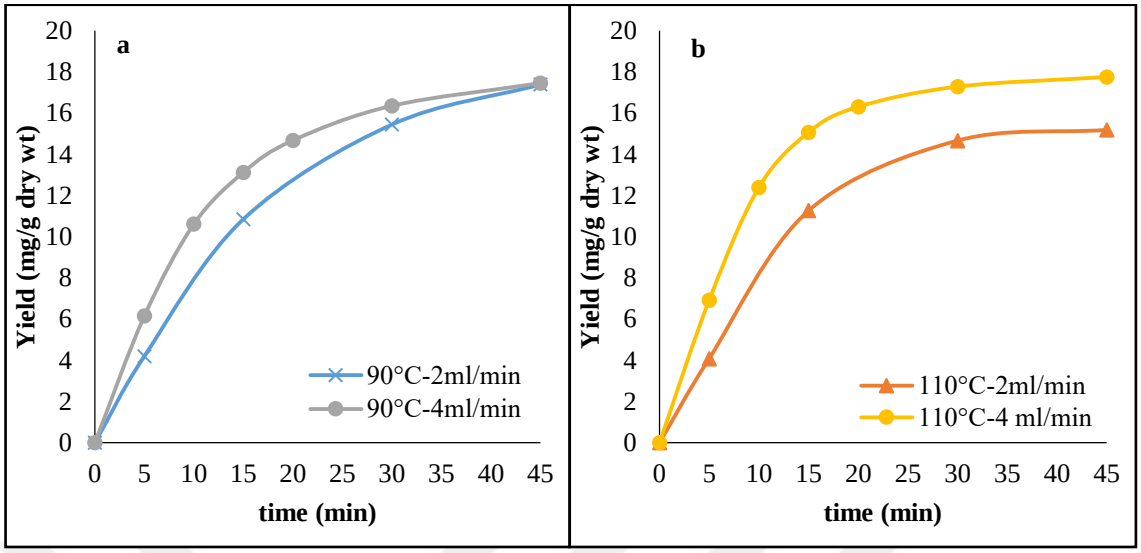

Figure 4.16. Effect of extraction flow rate on the yield of EGC at 90°C (a) and 110°C (b)

While the yield of GC increased with temperature and time, no effect of flow rate was observed after 30 min for the yield of GC at both temperatures (Figure 4.17). Catechin epimerization was also observed in a study on subcritical water extraction of black tea processing waste, which determined the effect of extraction temperature (90-180°C) and at temperatures higher than 90°C epimerization of EGCG was observed [114]. The effect of extraction temperature (110-190°C) and time (5-15 min) were determined for the extraction of catechins from green tea leaves [38] and epicatechins (EGCG and ECG) decreased higher than 150°C in 5 min due to the epimerization reactions [38].

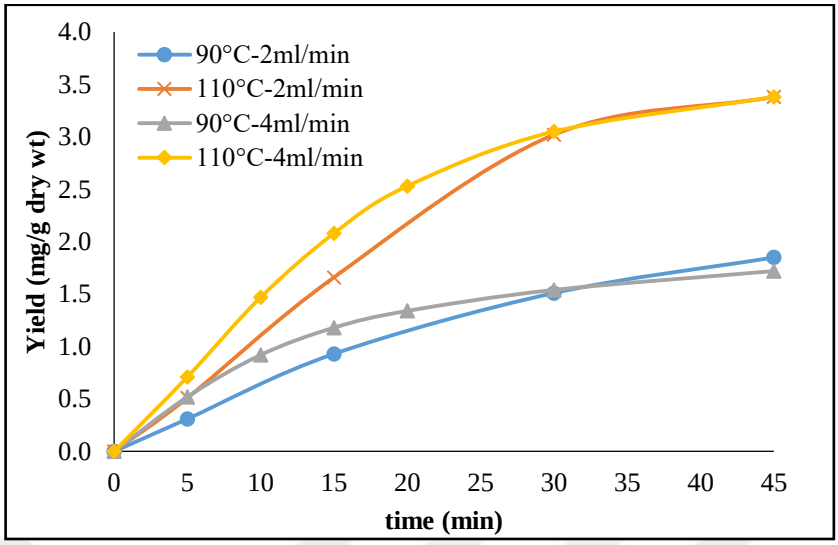

Figure 4.17. Effect of extraction temperature (90-110°C) and flow rate (2 ml/min-4 ml/min) on the subcritical water extraction yield of GC

Similar to catechins, subcritical water extraction of caffeine was not completed in 30 min (Figure 4.18a). Maximum recovery (112%) of caffeine was obtained at 110°C, in 45 min at a flow rate of 4 ml/min (Figure 4.18b) while recoveries of caffeine were similar at 90°C (105%) and 110°C (98%) for 60 min at 2 ml/min (Table 4.5).

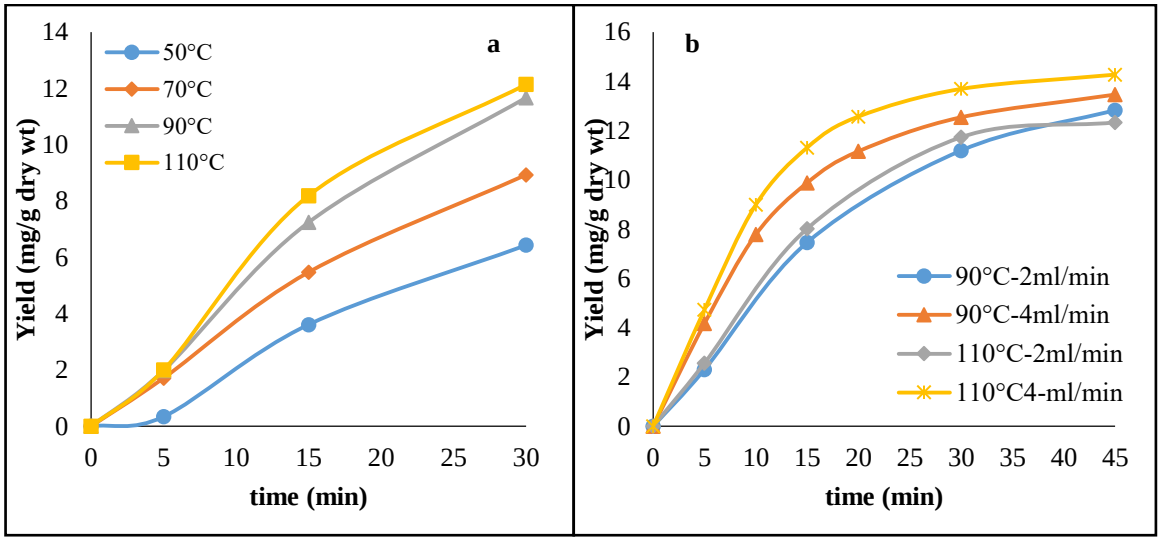

Figure 4.18. Extraction curves of caffeine from green tea processing waste a) at 50-110°C in 30 min (at 2 ml/min) and b) at 90-110°C in 45 min (at 2 ml/min, 4 ml/min)

The extraction behaviour of gallic acid was determined based on the effect of extraction temperature, time and flow rate (Figure 4.19). Two times higher GA yield was obtained at 110°C compared to 90°C for 45 min at a flow rate of 4 ml/min while extraction was not completed even after 60 min.

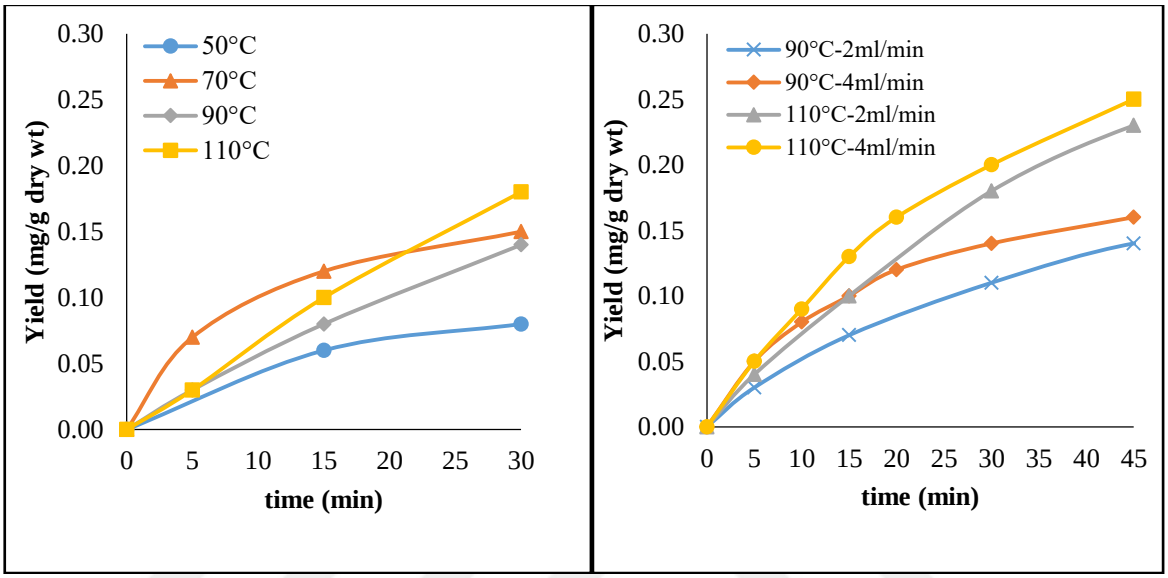

Figure 4.19. Extraction curves of gallic acid from green tea processing waste a) at 50-110°C in 30 min (at 2 ml/min) and b) at 90-110°C in 45 min (at 2 ml/min, 4 ml/min)

While the maximum recovery of theanine (104%) was obtained at 90°C for 60 min at a flow rate of 2 ml/min, extraction was completed in 45 min, which provides 101% recovery of theanine. Maximum recoveries of total catechin (88%), EGCG (85%), EC (70%), ECG (91%), caffeine (112%) and GA (200%) from green tea waste by subcritical water extraction were obtained at 110°C at a flow rate of 4 ml/min for 45 min.

While maximum recovery of total catechin (85%) and EGCG (76%) were obtained at 80°C for 15 min by water extraction, a slight increase was obtained in the recovery of total catechin (88%) at 110°C for 45 min with 4 ml/min by SCW extraction. Besides these, 12% higher extraction yield of EGCG, which is the predominant catechin in green tea waste was obtained by SCW extraction (110°C, 45 min, 4 ml/min,) when compared with water extraction (80°C, 15 min). Therefore, quantitative recovery of catechins could not be achieved using water or subcritical water extraction. Similar to our findings, the maximum extraction yield of

subcritical water extraction of total catechins (150°C, 5 min) from green tea leaves was slightly higher than traditional extraction techniques using ethanol and methanol (60°C, 2 h) [38].

5. CONCLUSION

Water, a green, non-toxic, cheap and easy to find solvent, can be used for value added utilization of green tea waste as a source of catechins and theanine with applications in food, nutraceutical, dietary supplement and cosmetic industry. In order to fully utilize the potential of subcritical water as an extraction solvent for the recovery of theanine and catechins from green tea waste, system modifications, such as changing the heating mode, can be investigated to limit degradation/epimerization reactions. Oven heating used in this study exposes the sample to high temperatures while water is being heated. Higher flow rates can also have a limitng effect on degradation/epimerization reactions. Additionally, flow direction (which was opposite to gravity in this study) can be changed [123] or length of column can be extended [121] to improve extraction efficiency. Sample pretreatment can be used to increase surface area to obtain higher extraction yield in shorter time as particle size/shape is an important parameter to obtain high extraction efficiency [2]. However, the pretreatment step should be optimized considering the blockage of the system that occurred at small particle size. Smaller frit sizes can also be considered to avoid blockage.

In addition to theanine and catechins green tea waste includes other components such as carbohydrates (sugars, cellulose and lignin). Reovery of these compounds with subcritical water can also be investigated. Subcritical water extraction can be used for for the recovery of theanine in addition to catechins black tea processing waste, which is not utilized commercially, due to the high amount of black tea production in Turkey which has no commercial value.

The ISO extraction method used for the determination of theanine in green tea needs to be modified to improve mass transfer of theanine to achieve quantitative recovery of theanine from green tea processing waste. Research on thermal stability of theanine as affected by temperature and time is needed to fully understand the subcritical water extraction behavior of theanine.

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