Optimization of ultrasound assisted extraction of cold brewed black tea

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Dedication

To my parents Raghunath Santhanam and Sindhuja Raghunath, for believing in me even when I doubted myself and instilling a belief that I am capable of accomplishing anything which I dreamt off.

"*And we know that all things work together for good for those who love God, to those who are called according to his purpose"*.

-Romans 8:28

Abstract

The tea is a general term used for a beverage that originate from the tea plant, *Camellia sinensis*. Among different types of teas, black tea is always considered as a rich source of antioxidants, phenolic compounds, essential oils, dietary fiber and other natural bio-actives, which have been shown to exhibit health-promoting effects. Generally, a hot brew is the mechanism of using hot water for brewing the tea leaves. However, application of this technique leads to degradation of flavor and heat sensitive bioactive compounds after being released from the cell membranes.

Cold brewing is an alternative method, which can be used to preserve the flavors and other components in the tea. However, application of this method is limited due to the low extraction efficiency and long extraction time. The aim of this project is to evaluate the feasibility of using emerging processing technologies like ultrasonication for improving the extraction of bio-active compounds from black tea and to optimize the processing conditions. This work provides insight on understanding the comparative analysis of OVAT (One variable at a time) vs RSM (Response surface methodology) modeling for cold brewed black tea. This research would make efforts of utilizing alternative process technologies like ultrasound to improve the extraction yield of cold brewed black tea.

Keywords: black tea, cold brew, ultrasound, OVAT, RSM, Optimization

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Introduction, Justification and Objectives

1.1. Introduction

In recent years, the consumption of teas has increased globally. Between 2003 and 2013, the consumption of teas in the United States has increased by more than 10% (Tea Association of USA, 2013). Teas can be prepared from different parts of the plants such as roots, flowers, seeds, berries or bark, depending on the solubility of the active compounds (Apak, Güçlü, Özyürek, Esin Karademir, & Erçağ, 2006). A tea beverage is usually prepared by steeping the particular plant material in water for a few minutes. Leafy teas are widely known to comprise of polyphenols that help to reduce the risks involved in insomnia, intestinal disorders, high blood pressure, and various other chronic diseases (Craig, 1999). Various parts of the world have popularized teas as a beverage that is believed to exert known beneficial effects.

Brewing is a crucial step carried out to extract the bio-active compounds from the tea leaves. Teas are generally brewed either in hot or cold water. Hot brewing of tea is one of the most straightforward processes. The leaves are compressed and subjected to hot water or steam, to extract essences and produce a beverage. However, application of this technique may cause degradation of naturally occurring flavors and heat-sensitive bio-active compounds. For instance, (Campanella, Bonanni, & Tomassetti, 2003) reported that the infusion time of 5 min with hot water resulted in excellent antioxidants extraction from tea while steeping more than this time causes the antioxidants either precipitate or form micelles reducing both the antioxidant capacity and polyphenol content of the infusion.

Moreover, although consumers assume that hot water brewing could reduce the microbial contamination, brewing herbal teas in hot water may present a false sense of safety when one relies on the potentially high temperature (90°C) of the brewing water as

the tea bags were found to be highly contaminated of *Bacillus* with a maximum of 3.9 x 10(5) CFU/100 ml and possible risk of nosocomial infection. In practice, the tea is prepared with heated water instead of boiling water, which favors microbial contamination (Wilson, Dettenkofer, Jonas, & Daschner, 2004).On the other hand, the extraction and processing of herbal teas at low temperatures have been reported as a useful method to maintain the flavor, aroma and the nutritive value of tea beverages (Wang, Sun, Cao, Tian, & Li, 2008). Generally, samples prepared by cold brewing have a strong flavor, smooth texture, lesser caffeine, and reduced bitterness. However, it is time-consuming and costly to extract tea compounds using cold brewing, which limits the application of this technique in the food industry. Certain emerging processing technologies such as ultra-sonication as a method of extraction can be used to overcome these limitations in cold brewing.

Ultrasound is used in various sectors of the food industry to extract lipids, phenolic compounds, carotenoids from micro-organisms, oil and antioxidants from seeds and various antioxidants, natural color and carotenoids from fruit and vegetables. The use of ultrasound is mainly due to the significant effect that could be implemented by this technology when compared to conventional processes. The efficiency of extraction mainly relies on the effect of cavitation, which is generated by the power ultrasound (Wang et al., 2008). According to Zhu et al., 2017, "Cavitation is the result of creation, growth, and implosion of gas bubbles generated during ultrasonic treatment. These bubbles collapse on the surface of plant material and release high pressure and generate heat and shock waves, which lead to the micro-fractures formation". It, therefore, allows better penetration of the solvent into the sample of interest and increase the area of contact between the surfaces (Koubaa et al., 2016). Thus, this leads to an increase of mass transfer from the cells to the

solvent. It was previously reported that the use of ultrasound had improved the extraction of tea solids at 60°C by nearly 20% (Mason & Zhao, 1994a). After extraction, different technologies such as membrane filtration or reversed osmosis are used to concentrate the extracts before drying. According to the literature review (Raghunath et al., 2019), there are only fewer researches being published on the application of ultrasound technology for improving the cold extraction of bio-active compounds from the black teas.

The tea is one of the best sources of polyphenols, which consists of significant compounds like flavonoids, phenolic acids, and flavan-3-ols. The Green and oolong teas are mostly consumed in Japan, and Black tea is usually preferred more in the United States and certain parts of Europe (Fujihara, Nakagawa-Izumi, Ozawa, & Numata, 2007). Both green and black tea is rich in antioxidant activities due to the polyphenols present in the leaves. These polyphenols are generally associated with lowering heart diseases and oxidative stress(Cheng, Sheen, Hu, & Hung, 2017; Mao, Gu, Chen, Yu, & He, 2017). However, this concentration of polyphenols is entirely dependent on the type of plant and other environmental conditions (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Usually, water is used at higher temperatures for brewing tea; however, to extract valuable bio-active compounds; the process needs to be performed at lower temperatures (Banerjee & Chatterjee, 2015).

In order to increase the extractability of bio-active compounds from teas different emerging processing technologies like supercritical fluid extraction, pulsed electric field, pressurized liquid extraction, microwave-assisted extraction, and ultrasound-assisted extraction (Raghunath et al., 2019) have been used. However, most of the research work

has not concentrated on improving cold brewing. This study mainly focuses on using ultrasound to improve cold brewing of black tea.

The cost of setting-up an ultra-sonication machine in the industry would be very high; however, the amount of energy spent will be relatively low (Chemat et al., 2017). This consequently will have tremendous benefits in the food industry, such as reducing processing costs, generating highly pure product, and eliminating some of the downstream purification steps.

1.2. Justification

The scope of the research was to evaluate the application of ultrasound for improving the cold extraction of bio-active compounds from black tea. Black tea is generally completed fermented tea leaves. Previous studies suggested that the effect of the ultra-sonication process affects the amounts of bio-active compounds extracted from black tea. However, it would be useful to test these effects of ultra-sonication on cold brewing of black tea. The total phenolic content, antioxidant activities, and total tannin content were studied in detail during the cold brewing of black tea with water as a solvent. Tannins are one of the water-soluble polyphenols present in the tea, (Khasnabis, Rai, & Roy, 2015) and we want to limit the tannin content due to its inhibitory effect on iron absorption (Delimont, Haub, & Lindshield, 2017) although tannins have a beneficiary effect of antioxidant properties (Bizuayehu, Atlabachew, & Ali, 2016). However, the absorption of iron and inhibition of absorption is more dependent on an individual's response to the food product and the type of meal they consume. Thus, this study will also focus on limiting the extraction of tannins from the system and also increasing the extractability of other secondary metabolites (polyphenols) with antioxidant effects.

This present research looked at the influence of various processing parameters of ultrasound on total phenolic content and antioxidant activities of cold brewed black tea. The optimization of the processing parameters is aimed at maximizing phenolic content, maximizing antioxidant activity while minimizing tannin content. One of the limitations of this optimized process is that the minimum extraction of tannins may lead to a reduction in antioxidant activity. From the results of this study, it is anticipated that the beverage manufactures, mainly the tea manufacturers will have better process methods for cold brewing of black tea.

1.3. Objectives

The main objectives of this research study are to

1. Investigate the effect of process parameters of ultrasound-assisted extraction for cold brewing of black tea.
2. Optimization of the process conditions for maximum total phenols and antioxidant capacity in the cold brewed black tea while minimizing the tannin content, using response surface methodology.

Chapter 2
Literature Review

2.1. Tea: An Introduction

"Tea is one of the most consumed beverages" (Goldbohm, Hertog, Brants, van Poppel, & van den Brandt, 1996, p.93) with caffeine worldwide, which contains a high polyphenol and antioxidant activity. There is a histrionic predicted growth rate to 15% in the area of nutrition and food businesses of plant products. Tea (*Camellia sinensis)* is widely cultivated and has influenced history in various parts of the world and most importantly China and India. The origin of tea traces back to the southern part of China, the so-called Yunan Province in the southwest. *Camellia sinensis* var. *sinensis* and *Camellia assamica* var. *assamica* are two different types of tea, which are differentiated by the size of leaves, and the type of plant (bush or tree). The *Camellia sinensis* var. *sinensis* is mostly preferred for its flavor, however, *Camellia assamica* var. *assamica* is commonly used in the production of black tea due to higher tannin and catechin content (Li, Lo, Pan, Lai, & Ho, 2013). The per capita consumption was higher in the India and United States during the 19^{th} century. It is also considered as a commercial beverage throughout the world (Harbowy, Balentine, Davies, & Cai, 1997).

2.2. History

2.2.1. History of Tea trade

According to Harbowy et al., 1997, The history of tea trade traces back to 2700 B.C. Emperor Shen Nung, was considered to be the first person to have discovered tea. The tea in China was not prevalent until 780 A.D as the tea was considered as a medicine rather than a commercialized product. The tea was then imported to various parts of the world, including Britain during the 16th century, and the demand for tea started to increase as many people started getting addicted to the taste of tea. From the 16th century to this very day, tea was influential and has grown as a commercial beverage throughout the world (Fullick, 1999).

2.2.2. History of brewing techniques

Harbowy, Balentine, Davies, & Cai, 1997 briefly talks about the history of brewing techniques. "The tea was initially consumed as a soup with vegetables. One of the most notable technique brewing tea was the production of brick tea which involved the steaming of tea and was compressed into bricks. Slowly the idea of brick tea started to fade off and was being replaced by powdered tea" (Harbowy, Balentine, Davies, & Cai, 1997, p.94). From the 13th century to the modern-day, the technique of hot brewing tea is being used. "There have been many differences in the brewing techniques to improve flavor and taste"(Harbowy, Balentine, Davies, & Cai, 1997, p.95), but the oldest known method for brewing is hot brewing. Hot brewing refers to steeping of leaves in hot water for a few

minutes. This hot brewing was commercialized throughout the world and still being used today (Harbowy, Balentine, Davies, & Cai, 1997)

2.3. Types of tea and Manufacturing

2.3.1. Types of Tea

Tea, which is known to be a popular beverage worldwide is from a plant called *Camellia sinensis,* which belongs to the Theaceae family. These plants are typically shrubs, which can grow only a few meters (10-15) tall (Ross, 2005; Walker & Sutherby, 2003). It grows in the tropics where there is rainfall annually and with slightly acidic soil (Chan, Lim, & Chew, 2007). This plant has been cultivated for more than 2000 years (Graham, 1992). The newest and the youngest leaves of the plants are used in the manufacturing of tea. The most excellent quality of tea is obtained from the youngest leaves and the buds of the tea plant (Chan et al., 2007). There are three types of tea based on the manufacturing process as green tea, black tea, and oolong tea, respectively (Chen, Qu, Fu, Dong, & Zhang, 2009).

Green tea has recently attracted attention due to its purported health benefits like anticancer and antioxidant activities (Cooper, Morré, & Morré, 2005; Dube, Nicolazzo, & Larson, 2010). About 22%, 78%, and 2% account for the amount of green tea, black tea and oolong tea that is being manufactured annually throughout the world (Cabrera, Giménez, & López, 2003).

Figure 1: Types of tea (Jackson, 2015)

2.3.2. Manufacturing of tea- A detailed overview

The type of manufacturing process defines the type of tea, which is being produced namely unfermented (green tea), fermented (black tea) and partially fermented (oolong tea) which in turn depends on the degree of the oxidation process. Pou, 2016 explains that oxidation process of the leaves generally refers to the natural browning reaction which takes place in the tea leaves. These reactions are further catalyzed by natural enzymes. (Sarkar, Chowdhury, Mandal, & Chowdhury, (2016) explained that in order to produce oolong and black tea, tea leaves undergo a process called withering to reduce the moisture content. This step is very critical for aroma development (Sarkar, Chowdhury, Mandal, & Chowdhury, (2016) and the next step involves rolling and crushing of the leaves to begin the oxidation of tea polyphenols. This fully fermented or oxidized tea from rolling and crushing is called black tea (Pou, 2016). The final quality and grade of tea depends on the type of maceration process used, namely orthodox rolling (Large leaf teas) and crush, tear, curl (CTC) (small leaf teas) (Sarkar, Chowdhury, Mandal, & Chowdhury, (2016). Black tea usually fully fermented the tea, and the fermentation is separately done with the circulation of cold air through the crushed and rolled leaves to moderate the oxidation/fermentation reaction (Pou, 2016).

2.4. Black tea

2.4.1. An Introduction

Black tea is one of the major varieties of tea wherein the oxidation or the fermentation process is responsible for alteration of the flavor profile of the tea leaves (Ruan, Berichterstatter, & Berichterstatter, 2005). Witono, Kang, & Mananda, (2016) explains that the fermentation by with the cold air through the crushed and rolled leaves to moderate the reaction. Witono, Kang, & Mananda, (2016) further adds that this process results in the oxidation his process results in the oxidation of the simple polyphenols into more complex polyphenol compounds (Li, Lo, Pan, Lai, & Ho, 2013). The oxidized leaves are then subjected to fire to stop the process and inactivate the enzymes (Witono, Kang, & Mananda, 2016). The leaves are dried and bio-active compounds undergo final chemical reactions and transformations (Witono, Kang, & Mananda, 2016). Li, Lo, Pan, Lai, & Ho, (2013) reports that the major polyphenols in black tea includes theaflavins, thearubigins and other catechin polymers. Catechins are always present in minor amounts in black tea as monomeric compounds since the oxidation process doesn't convert the either catechin compounds into complex polymers (Li, Lo, Pan, Lai, & Ho, 2013).

2.4.2. Origin and Chemistry

The formation of black tea comprises of two main steps, namely (Li et al., 2013) "oxidation and polymerization" (Matsuo, Tanaka, & Kouno, 2009). Li et al., 2013 further explains that the catechins present in the tea are converted into quinones in the presence of polyphenoloxidase, which is a natural enzyme present in the plants. The second reaction is a nucleophilic addition reaction or polymerization reaction, where the gallocatechins are

converted to catechin polymers (Li et al., 2013). This polymerization reaction takes place in the presence of oxygen or hydrogen with the elimination of carbon dioxide. This polymerization reaction simply involves the rearrangement of molecules for the synthesis of "benzotropolone" (Matsuo, Tanaka, & Kouno, 2009). Li et al., 2013 states that the benzotropolone is the main core molecule in the black tea.

2.5. Components of Black Tea

The composition of black tea depends on the origin and type of manufacturing applied (Ruan et al., 2005). The chemical composition of black tea includes compounds like polyphenols, flavonoids, pro-anthocyanidins, theaflavins, thearubins, and tannins (Li, Lo, Pan, Lai, & Ho, 2013).

The polyphenols are the leading group of chemical compounds in black tea, followed by carbohydrates and proteins. Caffeine belongs to a group called Xanthenes and one of the stable molecules which remains unchanged during the fermentation process (Li, Lo, Pan, Lai, & Ho, 2013). Polyphenols have been regarded as a pool of bio-active compounds with potential health benefits and therefore, the most interesting part of the black tea compounds(Khan & Mukhtar, 2011).

2.5.1. Polyphenols

The polyphenols are organic acids and molecules which comprise a 2-phenulbenzopyran skeleton. They are considered as molecules with aromatic rings with multiple hydroxy groups (Harbowy, Balentine, Davies, & Cai, 1997; Mary, Bradford, & Mrpharms, 1999). The resonance due to the electrons from the lone pair of oxygen in the aromatic ring the phenols are acidic (Mary et al., 1999). Mary et al., (1999) also explain

that it helps in the proton loss or help the polyphenols to function as a hydrogen donor as it reduces the density of electron around the oxygen molecule and decreases the strength of the OH bond. This donor behavior "induces resonance, which in turn leads to negative charges in ortho and para positions" of the molecule (Mary et al., 1999, p.29). Thus, these molecules are more prone to electrophilic attack and the polyphenolics change when the tea is being processed (Mary et al., 1999).

More than 30% of the leaf is composed of catechins or favan-3-ols, galloyl esters and other glycosides and phenolics are a part of secondary defense mechanisms (Harbowy et al., 1997). One of the major source of dietary phenols are represented by tea (Mojzer, Hrncic, Škerget, Knez, & Bren, 2016). Generally, an estimated amount of 250 to 350 mg of phenolics are consumed per tea cup (Khan & Mukhtar, 2011). The tea has various constituents of phenolics (Harbowy et al., 1997). Plants generally synthesize primary and secondary metabolites are typically called organic compounds. The primary metabolites like nucleotides, phytosterols, acyl lipids, amino acids and organic acids are compounds that are responsible for preforming photosynthesis, respiration, growth and development (Pagare, Bhatia, Tripathi, Pagare, & Bansal, 2015). Secondary metabolites are generally accumulated in higher concentration in the plant cells and these compounds are structurally diverse and play a major role in defense mechanisms (Pandey & Rizvi, 2009) to protect the plant from infections, herbivores, signal molecules, seed dispersion, pollination and UV protection (Crozier, Jaganath, & Clifford, 2007).

Based on their origin, the secondary metabolites are divided into three main groups namely 1. Phenolic and polyphenolic compounds 2. Terpenoids 3. Nitrogen containing alkaloids and Sulphur containing groups (Anulika, Ignatius, Raymond, Osasere, & Abiola,

2016). Phenolic compounds are divided into: flavonoids and non-flavonoids based on the number and the arrangement of carbon atom (Libro, Giacoppo, Rajan, Bramanti, & Mazzon, 2016). The polyphenols are the largest group with many other different compounds which are more commonly found in tea. These polyphenolics are found in varies other plants and vegetables. One of the major group of polyphenolics present in tea are flavanols which are commonly called as catechins. The phenolics are derived from a combination of shikimate and acetate-malonate bio-synthetic pathway. Catechins are water soluble compounds that are perceived for the bitter taste and astringency of tea leaves (Tronnes, 2012).

The major tea catechins are "epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), gallocatechin (GC), epicatechin (EC)" (Reygaert, 2018).

The structures of the following compounds are summarized below:

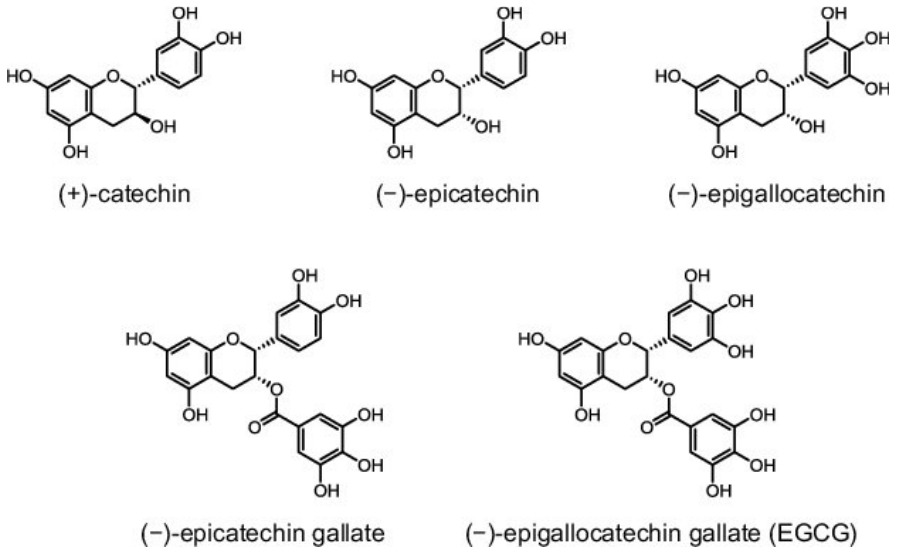

Figure 2 : Structure of the major catechins in green tea (Retrived from Isemura et al., 2015)

The green tea is rich in epigallocatechin-3-gallate and few of them are stereoisomers and epimers like catechin and epicatechin (Johnson, 1999). The polyphenolic compounds are commonly known for its antioxidant propertied and are used for therapeutics for various diseases (Dube et al., 2010).

2.5.1.2. Antioxidants

Any atom or a molecule, which consists of an unpaired electron, is called a free Radical. The free radicals are highly reactive radicals (Tronnes, 2012). The human body produces these free radicals because of response to some stress or condition in the environment. The body uses these free radicals when are present in moderate concentrations as mediators for cell signals (Phaniendra, Jestadi, & Periyasamy, 2015). Excessive concentration of these free radicals are considered to be a hazard and can lead to cell damage (Dröge, 2002). There are certain conditions in which the body produces excessive radicals like the oxidative stress conditions and the antioxidants that are already present in the cells are not able to moderate these free radicals (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011) This stress conditions have led to many inflammatory diseases (Cabrera et al., 2003; Higdon & Frei, 2003). These radicals can be scavenged by the polyphenols present in tea and can help in the treatment as well as prevention of diseases (Cory, Passarelli, Szeto, Tamez, & Mattei, 2018) due to their antioxidant properties (Higdon, Frei, & Blumberg, 2003).

The radical scavenging activity of polyphenolic antioxidants are categorized as hydrogen- atom transfer and electron transfer mechanisms (Leopoldini, Marino, Russo, & Toscano, 2004). The hydrogen transfer is based on the principle that the free radical is scavenged by the hydrogen atom. The phenolic group acts as a hydrogen donor in the case

of polyphenols (Quideau et al., 2011; Tronnes, 2012) .The effectiveness of this reaction depends on two important reactions namely :the speed at which the H-atom is getting transferred and the stability of the product formed after the transfer (Prior, Xianli, & Schaich§, 2005; Quideau et al., 2011; Tronnes, 2012)

According to Tronnes, (2012) the electron transfer mechanism works on the principle of single – electron transfer from polyphenols to the free radicals which results in the formation of a stable radical cation. The polyphenol compounds in the tea is known to exhibit the antioxidant activity in these two mechanisms mentioned able. The polyphenols can also act as a chelating agent (Bhullar & Rupasinghe, 2013). It also act as an enzyme inhibitor of pro-oxidants like xanthine oxidase and protein kinase which are responsible for catalyzing the reaction to produce free radicals (Quideau et al., 2011; Tronnes, 2012).

2.5.1.3. Oxidative stress and antioxidants

Oxidative stress is generally brought by both internal and external factors and this can lead to the development of non-communicable diseases which are most probably controlled by following healthy lifestyle and supplying the body with dietary antioxidants. Antioxidants are predominantly found in plants and particularly in tea (Dragland, Senoo, Wake, Holte, & Blomhoff, 2003). Numerous studies indicate that the tea, in general, is a healthy beverage to consume due to the antioxidants present (Higdon et al., 2003; Kris-Etherton & Keen, 2002; Siddiqui, Afaq, Adhami, Ahmad, & Mukhtar, 2004). This is one of the major reasons why tea is a popular beverage world-wide (Yang, Lu, Wu, Wu, & Chang, 2004). Other studies have indicated that if the tea was consumed in required amounts it can help reduce cancer and CVD (Dufresne & Farnworth, 2001). Black and

green teas have been studied extensively and these teas are known to contain high amounts of antioxidants (Peterson et al., 2005).

There are several factors which play a significant role in influencing the polyphenolic content and the antioxidant capacity which includes the type of tea, processing method, and the method of preparation (brewing) (Turkmen, Sarı, & Sedat Velioglu, 2009).

2.5.1.4. Theaflavins and thearubigins

Black tea is a rich source of theaflavins and thearubigins (Menet, Sang, Yang, Ho, & Rosen, 2004). The enzymatic oxidation followed by the condensation of di or tri hydroxylated flavan-3-ols forms theaflavins (Mary et al., 1999). These theaflavins contains a benzotropolone ring (Collier et al., 1973) and have an absorption band at three different wavelengths 280nm, 365 and 450 nm depending the type of molecule and constitute only about 2% by the weight of black tea (Mary et al., 1999) . In the year 1958, Roberts termed a group of heterogeneous substance as thearubigins as it was hypnotized that these are breakdown products of theaflavins. The thearubigins account for about 20% of the weight of black and is mostly responsible for the formation of the color in black tea (Mary et al., 1999; Roberts, 1958).

2.5.1.5. Phenolics and beverage quality

Polyphenols help in determining the quality of black tea. But there are other factors such as the size, shape, particle density and volatiles which play a very critical role in quality (Mary et al., 1999). Interactions between the tea and its compounds determines their flavor and with varying amount of theaflavins and caffeine the astringency and bitterness can be altered (Mary et al., 1999). The color of tea can be influenced by other factors such

as the addition of milk, protein, water, minerals, temperature and the mode of addition of the milk (Smith & White, 1965). The changes are caused by various interactions like chelation, hydrogen bonding and hydrophobic bonding. Astringency is more attributed to the presence of galloyl group (Luck et al., 1994). The molecular size influences the solubility and ability to interact with the protein and the hence acts as a factor that contributes to the astringency (Mary et al., 1999). Most of the astringent molecules lie in the range of "500 to 3000 daltons"(Clifford & Ohiokpehai, 1983). Polyphenols precipitate with the proteins as they enter the uncoiled sections of a protein and thus reducing the hydrophilic nature of the protein (Haslam, 1979). The oligomers with the conformational mobility, rather than polymers are always responsible for astringency (Hemingway, 1998).

2.5.2. Flavonoids

Flavonoids are polyphenolics which consists of 15 carbon atoms with 2 aromatic rings connected by a three-carbon bridge (Kumar, Pandey, Lu, & Sastre, 2013). The major classifications of flavonoids include flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins are found throughout the plant kingdom and The flavoon-3-ol is the most important subclass of flavonoids in tea (Panche, Diwan, & Chandra, 2016). It ranges from the simplest monomer called catechin to the most complex polymer called proanthocyanidins (Monagas et al., 2010). The flavon-3-ols are non-planar molecules (Monagas et al., 2010) with a saturated C3 element in the heterocyclic carbon ring. The compounds are hydroxylated to form gallocatechins and are esterified with gallic acid (Crozier et al., 2007).

2.5.3. Catechins

Catechins are flavonoids, which are the major phytochemical present in tea (Dwyer & Peterson, 2013). There are present as monomers in green tea (Leung et al., 2001) and to a considerable amount in black tea and oolong tea. However, the major part of it is being converted to polymeric and oligomeric forms in black tea and oolong tea due to fermentation process (Ponmurugan, Kavitha, Suganya, & Mythili Gnanamangai, 2019). The structure of catechins which are important to exhibit antioxidant properties include the presence of OH group in the carbon ring in the 3rd position (Tronnes, 2012), a ring for epigallocatechin and epigallo catechingallate (Higdon & Frei, 2003) and 2 Hydroxyl or OH groups which plays a major role in the stabilization and the delocalization of the radical form on the B ring (orthodiphenolic arrangement) (Tronnes, 2012)

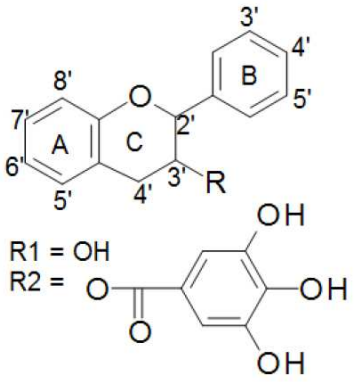

Figure 3 : General structure of catechins

Catechins also help in cell signaling (Mandel et al., 2008) like transduction pathways which are very helpful in anti-inflammatory properties (Fan, Sang, Jiang, & McPhee, 2017). According to Higdon & Frei, (2003) the role of catechins in the human system were as follows, inhibition of the activation of the transcription factors like Nuclear factor – kappa B. This transcription factor helps in the regulation products of pro-

inflammatory gene, inhibition of enzyme lipoxygenase activity, which are responsible for elevating the oxidative stress in the cells and elevating the activity of enzymes like peroxidase, superoxide and catalase, which acts as antioxidants in the body (Higdon & Frei, 2003).

The catechins from tea leaves have been shown in various studies as a treatment and as a preventive measure for a variety of diseases, which are linked to oxidative damage and stress in the cellular level of the human body (Bernatoniene & Kopustinskiene, 2018; Fan et al., 2017). They are also reported to have antibacterial and antiviral properties (Cabrera, Artacho, & Giménez, 2006; Tronnes, 2012)

2.5.4. Proanthocyanidins

The proanthocyanidins are further classified into two types names type A and type B and are polymeric units can extend up to 50 units (Spencer & Crozier, 2012). The proanthocyanidins are formed by the reaction between monomers of epicatechin and catechin by oxidative coupling occurring between C4 of the heterocycle to C6 or C8 positions of the adjacent monomeric unit to create polymers or oligomers of the compound (Barreca, Smeriglio, Bellocco, & Trombetta, 2017). The type A differs from type B with an additional ether bond formed between C2 and C7 (Spencer & Crozier, 2012). The majority of monomeric units consists of epicatechin units which are called procyanidins (He, Pan, Shi, & Duan, 2008) which are abundant type of proanthocyanidins. The tea contains high levels of these flavan-3-ols epigallocatechin, epigallocatechin gallate and epicatechin gallate. During the fermentation process of the levels of catechins decreases and the major compound of black tea are high molecular weight compounds called thearubigins and theaflavins (Rio et al., 2004). Theaflavins are derived from flavan-3-ols

units and thearubigins are derived from flavonoid units. These compounds are commonly referred to as “Tannins”. (Mary et al., 1999)

2.5.5. Theaflavins

Theaflavins are important compounds in black tea and are responsible for imparting the reddish orange pigments. The absorption of theaflavins in digestive tract are low when compared to catechins (Pereira-Caro et al., 2017). It is reported to have rate inhibition of α- amylase the digestive system against amylase (Hara & Honda, 1990) and sucrase (Honda & Hara, 1993) which tends to reduce the blood glucose after meals.

2.5.6. Tannins

Seguin in 1796 used the term “tannins” to classify the bitter capacity of oak to tan leather (Foo, Lu, McNabb, Waghorn, & Ulyatt, 1997) . In food, generally the presence of tannins in not considered to be a healthy thing though it is rich in antioxidants. It also has adverse health effects. The tannins are a group of polyphenols which belong to the class of high molecular weight compounds. They generally have a molecular mass of 500 - 3000 Da and are more widely distributed in the food system (Naczk, Amarowicz, Pink, & Shahidi, 2000). Salivary amylase is rich in the amino acid proline and interacts with the tannins to make them inactive (Savolainen, 1992).

The chemistry of tannins is very complex, and the tannins are classified into two different types’ namely hydrolysable tannins and condensed tannins (Khanbabaee & Van Ree, 2001). The condensed tannins are mostly considered as derivatives of flavanols and the hydrolysable tannins are generally esters of carbohydrates. These compounds are responsible for the astringent taste of foods (Ashok & Upadhyaya, 2012). Tea contains a mixture of both hydrolysable tannins and condensed tannins (Lau, Luk, & Huang, 1989). Tannins are considered to be the most important secondary metabolite (Hung, Chen, Chen,

& Cheng, 2010) and catechins are the monomeric flavan-3-ols found in the green tea leaves and the oxidized form of catechins are more abundant in oolong tea and black tea (Leung et al., 2001). Tannins in foods are considered to be undesirable because they lead to off colors (Maxon & Roowney, 1972), but not in the case of tea as it defines the color of the product.

Tannins can form soluble complexes with proteins, and they are present in various plants used for food. The interaction between the proteins and the tannins play a major role in the anti-nutritional effect of tannin- containing foods (Arts et al., 2002). Tannins are generally high molecular weight polymers which have the ability to bind with the protein molecule (Adamczyk, Simon, Kitunen, Adamczyk, & Smolander, 2017). However, the term tannin is more commonly used to refer to the polyphenolic compounds (Maxon & Roowney, 1972).

Tannins widely exists in the plants are considered to have antioxidant properties (Gong, Li, & Qu, 2014). In recent years, the production of tannins has become an alarming issue in the field of pharmaceutical, food and nutraceutical industries due to effect of inhibition in iron absorption (Delimont et al., 2017). The increasing interest in tannins is due to beneficial effect as they can act as antioxidants. Tannins in green tea are known to be a very strong natural antioxidants in our diet. A study by El-Din et al., (2015) suggests that the catechins in tea can reduce the risk of diseases.

2.6. Experimental designs

2.6.1. Design of experiments

One of the common methods of experimental designs is one-variable-at-a-time or OVAT analysis, where we vary one of the variables at a time and keep the other variables constant. This is mostly based on guesswork, experience and luck for the process. However, often the results concluded from the OVAT are not reliable, time consuming, and may lead to false interpretation of results from the study (Antony, 2007).

Ronald Fisher in London, England developed the design of experiments in the year 1920 (Durakovic, 2017; Lye, 2002). He used DOE to determine effects of different fertilizers on different acres of land but his final results was mostly dependent on various other factors like moisture, soil condition and not only the fertilizers. DOE has been widely accepted and used worldwide for various purposes and they have been successful in the implementation of these experimental designs and results. The experiments involve the sequence of activities: hypothesis, experiments, analysis, interpretation and conclusion.

2.6.2. Experimental setup

The experimental design always plays a critical role in both academia as well as industry. An experimentation of a process involves the application of various treatments to the experimental units and is believed to be a part of specific methodology based on the measurement of the responses. It is more important to observe the all the process operations and the system as well. In order to be consistent and obtain a final result, an experimenter must plan and design experiments for the analysis of the results. One of the most commonly used methods for experimental designs is Response Surface Methodology. The RSM is

most use as it allows the use of evaluating multiple factors and interaction of these factors on the response variables (Aydar, 2018).

2.6.3. Response surface design

The experimental designs are always explained generally in three most important steps. The initial step is detecting the parameters that affect the responses; the next step of the experimental design to make sure that design in such a way to minimize the effects of other factors which cannot be controlled. The thirds step is the usage of statistical analysis to separate the effects of other parameters (Shahavi, Hosseini, Jahanshahi, & Najafpour, 2015). RSM was first introduced by Box and Wilson in the year 1951 as a technique that was established as a mean to figure out the optimal settings that will maximize or minimize the target of the measured responses (Box & Wilson, 1951). The RSM models can be described as a mathematical representation as first order model without interactions or a first order model with interactions and second order which is a quadratic model (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008).

In the year 1990, Cornell specified about experimental models can be fitted with RSM in-order to a) Monitor and screen out the most important parameters that are influencing the responses into consideration. b) Figure the region accompanied by the parameter or the factor space which can fairly approximated by RSM modelling and is most commonly referred to as empirical modeling. c) Minimizing the cost of experimentation and the time taken an initial approximation of the surface in a simple model can be obtained by RSM. d) Acquire a sequential process or a procedure to pinpoint more desirable values of the response under consideration (Preece & Cornell, 1982)(Peterson, Cahya, & Castillo, 2002).

Response surface design is an experimental design for the optimization of various parameters and for obtaining the best set of factor levels to achieve the target goals (Nwabueze, 2010). RSM involves a sequential nature for the optimization process to achieve a maximum or minimum response (Peterson, Cahya, & Castillo, 2002). In the present study, the experimental designs and statistical analysis were performed using Design-Expert software (Design of experiments, Stat-ease Inc, Minneapolis, MN, USA). Central composite designs and Box-Behnken designs are the two major designs for Response surface methodology. Central composite designs are one of the favorite designs considered for the second order model. RSM is more a sequential form of experimentation which is used to help predict or optimize the response which can be dependent, or outcome variables made up of a mathematical statistical model of several input which can be independent or predictor factors.

2.6.4. Central composite design

The central composite design consists of center points that are amplified with a group of star point. The star point is efficient way to determine the coefficients of the 2^{nd} degree polynomial equation.

In the face-centered design, the points are located at the center of each face (in factorial space), so that alpha = ± 1. This variety requires three levels of each factor to be central composite face centered design (Leiviskä, n.d.). It is said to be an ideal method and solution for fitting the second-order response surface model ("Central Composite Designs (CCD)," n.d.; Leiviskä, n.d.). The CCD involves many of variables, and hence, this method is suitable for application in the later stage of RSM application where the total number of process variables is lowered to an acceptable figure. A CCD design generally requires more

runs at the center point, so the design usually exceeds the total number of runs (Wu, 2013). The central composite face-centered design has been a more useful methodology for modeling various process experiments on a small scale when compared to the one variable at a time model (Savic, Gajic, Stojiljkovic, Savic, & Gennaro, 2014). These are a particular type of response surface designs that can fit into a quadratic model (Breyfogle, 1992). A typical CCD design can be represented as a cube with corners which represent product levels at -1 and 1 respectively, the star or the axial points along the axes or outside the cube and the center point is considered as the point of origin.

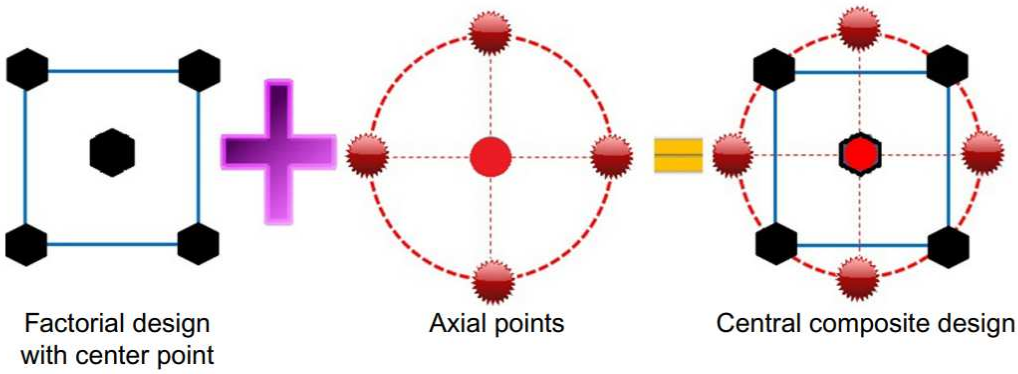

Figure 4 : A graphical 2D design of a central composite design for response surface modelling

(Retrived from Das & Saikat Dewanjee, 2018)

2.6.5. Ultrasound cell disruptor®

Ultrasound refers to the high frequency sound waves that are inaudible to the human ear (Zhang, Wang, Zeng, Han, & Brennan, 2019). It works on the basic principle of cavitation effect and aids in the penetration of the solvent into the system for higher extraction of the bio-active compounds (Raghunath et al., 2019). The instrumentation used for the ultrasonication process is called the sonifier cell disrupter® (Model: SLPe EDP 100-214-254, Branson Ultrasonics Corporation, Danbury, Connecticut). It works on the

simple principle of converting the electrical energy into high frequency (40Hz) mechanical vibrations. These vibrations are then transmitted into the horn or the probe. This leads to the creation of bubbles in the system. Then leads to the development of the bubble (Majid, Nayik, & Nanda, 2015a) and finally causes an implosion due to pressure and temperature. This causes an intense agitation of the solution leading to the extraction of the bioactive compound.

The ultrasound assisted extraction system has various operating parameters which are important like amplitude, sonication time and solvent volume. Amplitude refers to the height of the wave (Medina-Torres, Ayora-Talavera, Espinosa-Andrews, Sánchez-Contreras, & Pacheco, 2017) and frequency refers to number of repetitions of the wave (compression and rarefaction)(Medina-Torres et al., 2017a). Sonication time refers to the time period to which the sonication is being applied to the system.

The ultrasonic system used in the study was Digital Sonifier model Slpe 120 volt, Model 4C15, EDP: 101-135-126r and s/n: WCN09193361, tool type: EDP 100-214-254 Rev A and tip used 1/8" microtip P/N 109-122-1065 – 0.125" diameter tapered tip. The maximum power reading was 150 Watts with 68 micron meters. The amplitude settings used was 10% (12 microns), 50% (34 microns) and 70% (68 microns).

2.6.5.1 Ultra-sound assisted extraction

Ultrasound is one of the alternative technologies for the extraction of compounds (Chemat & Khan, 2011). Ultrasound-assisted extraction can be used to overcome many limitations of the conventional methods of extraction. The application of the ultrasonication generates cavitation, which leads to the creation of bubbles in the system. This cavitation phenomenon helps in increasing the rate of mass transfer between the plant

material and solvent which acts as the medium by the generation of currents inside the liquid system (Da Porto & Decorti, 2009; Stadnik, Dolatowski, & Baranowska, 2008). Cavitation on the surface results in the disruption of cell and breakdown the particle (Chemat et al., 2017a; Paniwnyk, Cai, Albu, Mason, & Cole, 2009) which also increases mass transfer from the samples to the solvent by increasing the surface area. A detailed study of the ultra-sonication and other novel technologies used for extraction of bio-active compounds from tea has been discussed in Chapter 3 (Raghunath et al., 2019)**.**

Figure 5 : Branson ultrasonic sonifier cell disrupter

2.7. Methodology and principle of assays

2.7.1. Total phenolic content

In order to estimate the total polyphenols Folin Ciolcateau assay was used for the estimation of total polyphenols, in the black tea sample. This assay is based mainly on the movement of the electrons from the phenolic compounds to the molybdenum, which in turn results in the formation of blue-colored complexes. A spectrophotometer monitors this reaction at 760 to 765 nanometers (nm) (Magalhães, Segundo, Reis, & Lima, 2008).This assay is specific to the reaction between the Folin reagent and the aromatic group of the phenolic compounds, which are polyphenolics (Singleton. & Rossi, 1965). The inference

of compounds in the sample is minimized due to the absorption of at a longer wavelength. Magalhães et al., (2008) and Piek, (2016) explains that the continuous analysis is difficult as is that it involves a long incubation time of 60 minutes. Another limitation is the use of water as the solvent, and therefore, it is not suitable for lipophilic compounds (Magalhães et al., 2008; Piek, 2016). The assay represents the total phenolic content of the sample in milligrams of gallic acid equivalence (GAE).

2.7.2. Total antioxidant activity

There are various steps involved in the determination of the total antioxidant capacity of cold brewed black tea. Pérez-Jiménez et al., (2008) explains the steps involved in the total antioxidant activity as "preparation," "extraction" and "measuring the antioxidant capacity of the sample" (p.274) and the most importantly used procedures for total antioxidant capacity for 2,2'-azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and DPPH. Both the ABTS and DPPH measure the sample's free radical scavenging activity.

2.7.2.1. ABTS OR TEAC Assay for antioxidant capacity

The ABTS assay is a decolorization assay which is suitable for determining the antioxidant capacity for any type of the sample (Floegel, Kim, Chung, Koo, & Chun, 2011; Re et al., 1999) including all the flavonoids. The ABTS reaction is created by the oxidation of the ABTS with potassium persulfate to create ABTS radicals. These radicals are in turn react with the hydrogen donating antioxidants from the sample. It has been reported by

Piek, (2016) that the ABTS method gives a better understanding of the antioxidant capacity of the compound in comparison with the DPPH assay.

Zulueta, Esteve, & Frígola, (2009) explains that ABTS is "the most economical" and easy to use methods for the determination of antioxidant capacity. It allows studying at a wide range of pH conditions i.e., the method remains reliable when subject to pH changes (Karadag, Ozcelik, & Saner, 2009). However, there are few limitations in the assay, such as preparation of the free radicals before the assay ($ABTS^{+}$). The assay is not a standardized procedure; hence, the values cannot be compared to a large extent across laboratories.

2.7.2.2. DPPH Radical scavenging activity

"The DPPH is a free radical scavenging assay" (Kedare & Singh, 2011, p.412) where the DPPH has an organic nitrogen radical which has a rich purple color. This purple complex formed is transformed into lighter yellow hydrazine by the antioxidant compounds available in the sample (Kedare & Singh, 2011). This ability of the antioxidant to reduce the DPPH radical can be estimated by monitoring the decrease in the absorbance at 515 nanometers (nm) in the spectrophotometer until it is stable (Karadag et al., 2009; Kedare & Singh, 2011; Piek, 2016). It is generally a stable free radical which accepts hydrogen from the donor and loses the characteristic purple color complex color. The DPPH is "a stable free radical and the molecules do not dimerize like others" (Kedare & Singh, 2011, p.412). The delocalization is also responsible for the purple color complex with absorbance at 515 nm. On mixing the DPPH with the solution with antioxidants, which acts as a hydrogen donor, it gives rise to reduced form with the loss of purple color. The reaction between DPPH and antioxidant can be represented as follows:

DPPH*(radical) + AH (Antioxidant) = DPPH-H + A*

The color vanishes as the electron pairs with DPPH. Commonly used as a method to quantify in food, biological systems for both liquids as well as solid samples (Blois, 1958; Kedare & Singh, 2011; Parry et al., 2005; Sendra, Sentandreu, & Navarro, 2006; Yu, 2001). The method is unique and selective to determine the antioxidant capacity of a sample or an extract. DPPH method can be utilized to examine even weak antioxidants and both hydrophilic and lipophilic antioxidants (Prior, Wu, & Schaich, 2005). The results of the test are highly reproducible to other antioxidant scavenging methods (Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000). One of the major limitations of using this assay is that interference of other molecules in the same absorption range. Arnao, 2000 states the limitations with the DPPH method could be the sensitivity of the DPPH radical to the surrounding environmental conditions. Even a minor alteration in the pH can modify the values of the antioxidant activity to a huge extent. It is also only soluble in organic solvents, and interference can be an issue in the case of quantitative analysis (Arnao, 2000).

2.7.3. Total tannin content with protein precipitation

The tannins, which are phenolic compounds, have a unique ability to precipitate caseins as well as whey proteins (Hagerman & Butler, 1978; El-Din et al., 2015). Yuksel, Avci, & Erdem, (2010) explained the hydrophobic interaction between milk proteins and green tea flavonoids. El-Din et al., (2015) also described that this interaction, in general, is influenced by the structure of the phenolic compound. “The peptide bonds are stabilized by electrostatic interaction and the pi – OH bonds are observed in stabilization” (Madhan et al., 2001, p.334). One of the milk proteins, caseins has a significant “hydrophobic reaction between the amino acid proline and phenolic group” (El-Din et al., 2015, p.19).

The interaction is stabilized by hydrogen bonding. In a more specific manner, “the phenol ring groups interact with the bis-alkyl substituted amide nitrogen present in the proline imine group” (El-Din et al., 2015, p.19). The interaction with casein results in an insoluble complex, which can be filtered out from the solution (Luck et al., 1994; Mohamed et al., 2015; Yuksel et al., 2010). The precipitation of tannins would depend on various factors such as pH, ionic strength and concentration of other compounds (Adamczyk, Salminen, Smolander, & Kitunen, 2012).

Chapter 3: Application of innovative processing technologies for the extraction of value-added compounds from tea: A review

Application of innovative processing technologies for the extraction of value-added compounds from tea: A review

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Abstract

Tea is the most widely consumed beverage in the world with an excellent source of bio-active compounds such as catechins, caffeine, and epigallocatechins. There is an increasing trend to extract these bioactive compounds for the purpose of delivering them as value added products. Generally, the extraction of polyphenols and other functional compounds from different parts of tea is carried out using different solvents (e.g., water, water-ethanol, ethanol, methanol, acetone, ethyl acetate, and acetonitrile). The extraction efficiency of functional compounds from tea depends on the type and polarity of solvent as well as the applied process. Several conventional techniques such as boiling, heating, Soxhlet®, and cold extraction methods are used for the extraction of bio-active ingredients. However, these procedures are not suitable for achieving high yields and biological activities due to various reasons like long extraction times for cold brewing and the high temperatures in boiling. Many efforts have been carried out in food and pharmaceutical industries to replace conventional extraction techniques by innovative ones (e.g. microwave, ultrasonic, ultra-high pressure, pressurized liquid, pulsed electric field, and supercritical fluid), which are fast, safe, energy-saving and present eco-friendly characteristics. In this study, the application of novel processing technologies for the extraction of value-added compounds from tea leaves and by-products are reviewed. The advantages and drawbacks of using these technologies will be also highlighted.

Keywords: tea, tea waste, bio-active compounds, conventional extraction methods, innovative extraction method

3.1. Introduction

Tea (*Camellia sinensis* L.) is an ancient tree crop belonging to the Theaceae family. Although this evergreen plant originates from southeastern China, it has been widely distributed in over 52 countries with tropical and subtropical climate changes throughout the world. Tea from China spread to India and Japan, and then to Russia and other European countries (Sharangi, 2009; FAOSTAT, 2015). In 2015, China and India were the major producers of tea with about 36.3% and 22.6% of the total global production, respectively (FAOSTAT, 2015). Even though there are various kinds of tea (e.g., green, white, black, oolong, Pu'er or Pu-erh, and Roobios or red bush) depending on the processing method of fresh leaves, two tea types of green and black are extensively consumed in all around the world (Sharangi, 2009; Khan & Mukhtar, 2013). There are thousands of chemical constituents in tea, where amounts of them can be substantially affected by the different heredity (e.g., genetic strain), environmental factors (e.g., weather, soil, irrigation method, growth altitude, and harvest season), horticultural practices, as well as processing technologies and conditions (Sultana et al., 2008).

Recently, there is an increasing trend towards extraction of bio active compounds from tea with the purpose to produce value added products such as health supplements. In addition there is also a demand for cold brewed tea products due to the consumer's perception of a healthy beverage. In general, the extraction of polyphenols and other functional compounds from the different parts of tea is carried out using a variety of solvents. Although water as a traditional solvent has been applied in most of the studies to extract polyphenols from green and black teas (Larger, Jones, & Dacombe, 1998; Obanda, Okinda Owuor, & Mang'oka, 2001; Khokhar & Magnusdottir, 2002; Liang, Lu, Zhang,

Wu, & Wu, 2003; Perva-Uzunalić et al., 2006), the use of other solvents such as water-ethanol (Sökmen, Demir, & Alomar, 2018), ethanol (Opie, Robertson, & Clifford, 1990; Asadi et al., 2013), methanol (Yao et al., 2004; Kerio, Wachira, Wanyoko, & Rotich, 2013), acetone (Wang & Helliwell, 2001; Perva-Uzunalić et al., 2006), ethyl acetate (Farhoosh, Golmovahhed, & Khodaparast, 2007), and acetonitrile (Perva-Uzunalić et al., 2006) has been also reported.

The extraction efficiency of bio-actives without any chemical modification is not only a function of type and polarity of the solvent used but also influenced by the applied processing techniques (Zuo, Chen, & Deng, 2002). Boiling, heating, and reflux distillation are conventional techniques that are used for the extraction of bio-active ingredients from tea and tea by-products (Jun, Deji, Ye, & Rui, 2011). Processing under an optimal combination of tea/water ratio, particle size, agitation rate, and time/temperature can significantly lead to improved extraction of bio-active compounds from tea (Rostami & Gharibzahedi, 2017). Generally, these conventional procedures are not suitable to achieve high yields and biological activities due to the long extraction times and the high temperatures used (Spigno & De Faveri, 2009).

Many efforts have been carried out to promote the use of innovative technologies (e.g., microwave, ultrasonic, ultra-high pressure, pressurized liquid, pulsed electric field, and supercritical fluid) with fast, safe, energy-saving and eco-friendly characteristics instead of the conventional solvent extraction methods (e.g., heat reflux) in food and pharmaceutical industries (Nkhili et al., 2009; Spigno & De Faveri, 2009; Zhao, Yang, Wang, & Lu, 2009; Jun et al., 2011; Villanueva Bermejo et al., 2015; Xi, He, & Yan, 2015; Zderic & Zondervan, 2016). The application of these novel technologies under gentle processing

conditions can not only decrease the impurity and structural changes of polyphenols sensitive to epimerization and oxidative oligomerization reactions, but can also significantly enhance the extraction yield with an increase in the solvent permeability rate into plant cells and the mass transfer coefficient of the target secondary metabolites (Nkhili et al., 2009). In addition, nowadays there is a serious concern about tea by-products getting accumulated in the environment. Therefore, the reuse of such agricultural waste using economic and environmental approaches seems to be necessary for the food industry.

The application of innovative extraction and separation systems such as nano-filtration membranes (Nwuha, 2000), supercritical carbon dioxide (Chang, Chiu, Chen, & Yang, 2001), microwaves (Pan, Niu, & Liu, 2003b), ultrasounds (Xia, Shi, & Wan, 2006), and pressurized liquids (Piñeiro, Palma, & Barroso, 2004) has been reported to significantly enhance the extraction of bio-active compounds from tea waste for the fortification of different foods such as bakery products (Culetu, Héritier, & Andlauer, 2015). Furthermore, some researchers explored that the tea waste can be a suitable substrate to produce activated carbon using combinations of chemical activation and microwave energy (Yagmur, Ozmak, & Aktas, 2008), as well as microwave and infrared energies (Leonelli & Mason, 2010). Moreover, the discoloration process of dye wastewater by pulsed discharge plasma combined with charcoal derived from tea waste has been previously reported (Wang, Qu, Pei, Liang, & Hu, 2016). This paper provides a comprehensive summary of the literature published on the application of innovative processing technologies for the extraction and recovery of bio-active compounds from tea and tea waste.

3.2. Tea and tea waste: Nutrition and health properties

Tea as the most popular and oldest non-alcoholic beverage has a unique flavor with some health benefit effects (Xu et al., 2017). The global average consumption of this healthy functional drink is about 120 mL per day per person, while this value for Great Britain's inhabitants is 4.5 times higher (≈540 mL/day) (Gardner, Ruxton, & Leeds, 2007). Polyphenols are the most important nutritional compounds present in the chemical structure of tea. Catechins and theaflavins are primary and secondary polyphenols present in tea, respectively. Catechins that are present in green tea leaves are oxidized during the fermentation process to theaflavins through the enzymatic browning by polyphenol oxidase (Astill, Birch, Dacombe, Humphrey, & Martin, 2001; Tanaka, Inoue, Betsumiya, Mine, & Kouno, 2001). There are two optical isomers for each geometrical isomer of catechin (*trans*-catechins and *cis*-epicatechins) including (+,–)-catechin and (+,–)-epicatechin. Esterification of (–)-catechin with gallic acid (GA) can lead to the synthesis of (–)-gallocatechin-3-gallate (GCG), (–)-catechin-3-gallate (CG), epicatechin-3-gallate (ECG), and (–)-epigallocatechin-3-gallate (EGCG). Moreover, four different kinds of theaflavin namely theaflavin (TF), theaflavin-3-gallate (TF3G), theaflavin-3'-gallate (TF3'G), and theaflavin-3,3'-digallate (TF33'G) can be formed with the polymerization through the oxidative coupling (Friedman et al., 2005; Zhang, Suen, Yang, & Quek, 2018). The presence of other flavonoids (e.g., quercetin), alkaloids (theophylline, theobromine and caffeine), long-chain aliphatic alcohols (e.g., policosanols), amino acids (e.g., glutamic acid, aspartic acid, and theanine), and minerals (e.g., fluorine, chlorine, calcium, and manganese, etc.) in various tea products has been demonstrated (Sharangi, 2009; Choi, Park, Park, Park, & Jung, 2016).

The daily drinking of tea can significantly reduce the incidence rate of cancer types such as skin (Saha & Das, 2002; Mantena, Meeran, Elmets, & Katiyar, 2005; Rees et al., 2007; Katiyar, 2011), breast (Kavanagh et al., 2001; Sun, Yuan, Koh, & Yu, 2006; Thangapazham et al., 2006; Deb, Thakur, Limaye, & Gupta, 2015), ovarian (Gosvig et al., 2015; Gao, Rankin, Tu, & Chen, 2016), prostate (Wang, Henning, Heber, & Vadgama, 2015; Lee et al., 2017), lung (Fu et al., 2009; Hudlikar et al., 2017), oral (Wang, Yang, Zhang, & Wu, 2014; Chen et al., 2017), colon (Su & Arab, 2002; Henning et al., 2013), stomach (Yang, Du, & Yang, 2016; Chen et al., 2017), and pancreatic (Bimonte et al., 2017; Lai, Bautista, Rodriguez, & Bolivar, 2017) cancers induced by the consumption of alcohol and tobacco. The presence of polyphenols such as EGCG can notably inhibit the activation of carcinogens and consequently cancer initiation due to its antiradical and antioxidant activities, as well as its implication in the activation of detoxification system. This strong mechanism associated with the modulation in membrane organization, the formation of intercellular interactions with some functional macromolecules (e.g., proteins and nucleic acids), the epigenetic alteration and the regulation of cellular replicative potential can highly limit the progress of carcinogenesis by preventing the self-renewal, the proliferation and the viability of the predominant tumor-initiating clones, and thus the consequent growth (Sur & Panda, 2017). Earlier, the effects of anti-mutagenic, anti-diabetic, anti-inflammation, anti-bacterial, anti-viral, anti-arthritis, anti-obesity and neuro-protective of tea polyphenols have been comprehensively reported by other researchers (Xiao, Yang, Shi, Liu, & Chen, 2008; Moon, Akbar, Yun, & Cho, 2009; Cheng et al., 2009; Osterburg, Gardner, Hyon, Neely, & Babcock, 2009; Danesi, Philpott,

Huebner, Bordoni, & Ferguson, 2010; Singh, Akhtar, & Haqqi, 2010; Smith et al., 2010; Zhang, Li, Liang, Dai, Ding, Wang, & Li, 2010).

Regarding tea waste, a high number of bio-active compounds such as polyphenols and caffeine can be extracted using conventional and novel extraction systems (Senol & Aydin, 2006; Farhoosh et al., 2007). The tea dust generally contains 2.5% of decaffeinated tea, which leads to the production of 80 tons of tea dust from 3300 tons/year (corresponding to the annual average production) that serve as a value-added source to extract bio-active compounds such as theanine (Culetu et al., 2015). This amino acid has many health benefits such as relaxing and anti-tumor effects, enhancement of learning capability, decrease of weight and nervousness, reduction of blood pressure, triglyceride and cholesterol levels, promotion of immune system, and inhibition of tobacco and nicotine addiction (Owen, Parnell, De Bruin, & Rycroft, 2008; Wang et al., 2010; Yan et al., 2010; Higashiyama, Htay, Ozeki, Juneja, & Kapoor, 2011; Vuong, Bowyer, & Roach, 2011; Culetu et al., 2015).

3.3. Conventional extraction of bio-active compounds from tea

Selecting a proper extraction technique is an essential step to recover the maximum amount of bio-actives from tea and tea waste. The conventional solid-liquid extraction (CSLE) methods are commonly used due to their ease and broad applications (Li, Smith, & Hossain, 2006) and liability (Bonoli, Marconi, & Caboni, 2004; Li et al., 2006; Teixeira, Patão, Coelho, & da Costa, 2006; Vatai, Škerget, & Knez, 2009; Guo & Beta, 2013). Soxhlet extraction is a standard technique for the extraction of phenolic compounds from tea and tea waste using organic solvents such as methanol, ethanol, acetone, diethyl ether, and ethyl acetate. Flavonoids can be extracted with polar solvents such as ethanol,

methanol, water and/or combinations of these solvents (Stalikas, 2007; Kalia, Sharma, Singh, & Singh, 2008; Kumar, Kumar, Sivakumar, & Kaushik, 2009). The choice of solvent depends on the number of factors including the solvent's ability to solubilize the solute, the extraction temperature, and the particle size of the solute.

Although water is the most commonly used solvent for the extraction of phenolics from tea, the application of other non-polar green solvents has been reported for the extraction of other bio-actives such as catechins, as well as for decaffeinating. For instance, the potential of using various co-solvents combined with carbon dioxide-assisted Soxhlet extraction for the extraction of phenolics from green tea was reported by Chiehming, Chang, Chiu, Chen, and Chang (2000). In another study, the application of a novel packed-column extractor with a strong absorption system was reported to improve the quality of oils extracted from green tea (Chiehming et al., 2002). Moreover, the results have shown that the level of phenolic compounds extracted using 95% ethanol was 4.4-fold higher than that extracted by water.

Ethyl acetate compared to *n*-butanol and *n*-hexane was found to be a better solvent to isolate catechins from green tea (Dong, Ye, Lu, Zheng, & Liang, 2011). The optimum extraction conditions with water were reported to be at the solid to solvent ratio of 1:30, temperature of 80°C, and extraction time of 40 min for the extraction of catechins which were then isolated using ethyl acetate and decaffeinated using citric acid. The authors reported that this treatment could lead to a substantial reduction in the caffeine content up to 78.8%. The application of liquefied dimethyl ether resulted in removing the total caffeine from dried green tea leaves before extraction, whereas catechins were retained up to 56% (Kanda, Li, & Makino, 2013). Goksu and Poyrazoglu (2013) investigated the effect of

using 80% methyl alcohol on the extraction of total phenolic content (TPC) from caffeinated- and non-caffeinated green and black teas. There was a significant difference in TPC contents between caffeinated (159.4 mg/kg) and non-caffeinated (32.81 mg/kg) black teas. Similar results were observed for caffeinated (128.22 mg/kg) and non-caffeinated (43.16 mg/kg) green teas.

In another study, the green deep eutectic solvent (DES) was used to extract catechins from Chinese green tea (Heng & Kyungho, 2014). The results showed that the efficiency values of catechins, (+) epicatechin gallate, and (-) epigallocatechin gallate were 82.7%, 92.3%, and 97.0%, respectively. Nadiah, Nadiah, and Uthumporn (2015) characterized catechins, caffeine, and GA present in the leaves of tea and spent tea. They evaluated the effect of various extraction conditions such as the use of boiling water, 50% ethanol concentration, and different extraction times. Compared to water, the use of ethanol resulted in higher extraction efficiency of phenolic compounds from tea extracts, probably due to the higher polarity of ethanol that influenced the extractability rate.

The effect of particle size and solvent type on the TPC, total flavonoid content (TFC), tannin content, and antioxidant activities of leaves of yellow, green and black tea was studied by Kopjar, Tadić, and Piližota (2015). Pulverized tea leaves treated with acidified methanol exhibited the highest values of functionalities among the different tea leaves. The yellow tea leaves had a higher bio-activity than the leaves of green and black teas. The antioxidant activities of extracts obtained from the yellow and green tea leaves by the quencher method were higher than those obtained from black tea leaves. Nibir, Sumit, Akhand, Ahsan, and Hossain (2017) have recently studied the antioxidant and antimicrobial properties of aqueous extracts of flowery broken orange pekoe, broken

orange pekoe, red dust and green tea prepared with a solid to water ratio of 1:6. The aqueous extract of green tea exhibited promising antibacterial properties with a maximum level of phenolic content corresponding to 26.33 mg GA equivalent (GAE)/g extract.

The optimization of the operating parameters involved in the extraction process is important to obtain the maximum efficiency and functionality of bio-active compounds from plant-based food matrices (Rostami & Gharibzahedi, 2016). The optimization of phenolic compounds extracted from tea fruit peel biomass (TFPB) was carried out by Xu et al. (2012). The highest TPC (47.5 mg GAE/g) was obtained at the optimum conditions of 43% ethanol, 60°C extraction temperature and 33 min extraction time. Gallocatechin and epigallocatechin were the major phenolic compounds of TFPB. In another study, Kim et al. (2016) evaluated the optimization of the TPC, antioxidant activity, and epigallocatechin gallate (EGCG) of green tea leaves at different ethanol concentrations (0-100%), extraction times (3-15 min), and extraction temperatures (10-70°C). The maximum antioxidant activity (88.4%) was obtained using 57.7% ethanol at 70°C for 15 min. Zielinski, Haminiuk, & Beta (2016) optimized the extraction process of phenolic compounds from white tea. The optimum conditions for the extraction of phenolic compounds were reported to be the extraction time of 10 min, a temperature of 66°C, and a concentration of 30% ethanol solution.

Although the conventional extraction of bio-active compounds from tea and tea waste materials is easy and convenient, the application of these methods implies negative thermal effects on the extraction yield and quality with a large expenditure of solvents and energy. Thus, the potential of using innovative extraction methods such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pulsed electric field (PEF),

supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), and solid-phase microextraction (SPME) has been reported to be a good alternative to produce tea extracts at industrial scale with an optimal expenditure of energy and chemicals. The application of some of these techniques for the extraction of bio-active compounds from tea and tea waste are discussed in the subsequent sections.

3.4. Innovative methods of extraction

3.4.1. Ultrasound-assisted extraction (UAE)

UAE is one the most emerging, efficient and eco-friendly method used for the disruption of cells to extract the intracellular compounds from the cell matrix. The main principle of UAE is the cavitation phenomenon in which bubbles or micro-channels are formed in the sample by increasing the penetration rate of the solvent into the matrix (Ghasemzadeh-Mohammadi, Zamani, Afsharpour, & Mohammadi, 2017; Dolatowski, Stadnik, & Stasiak, 2007; Dimaki, Iatrou, & Lamari, 2017; Gharibzahedi & Jafari, 2018). It is also referred to as the mechanical waves that can accelerate the medium pressure leading to the formation of cavities (Horžić, Jambrak, Belščak-Cvitanović, Komes, & Lelas, 2012). Due to the increase in pressure, the cavities cannot absorb more energy beyond a certain limit and leads to the implosion of the bubbles when they attain maximum volume thus, aiding in the disruption of the cells (Horžić et al., 2012).

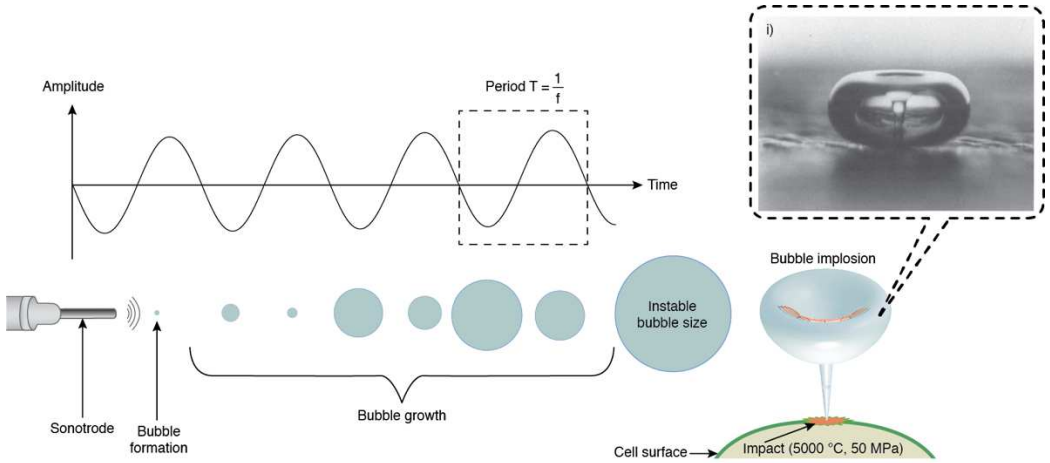

Figure 6: Graphical representation of the cavitation formation and bubbles collapse accelerating the release of bioactive compounds from the plant cells (Retrived from Roohinejad, Koubaa, Sant'Ana, & Greiner, 2018)

UAE is capable of enhancing the mass transfer rate of bio-active compounds and macromolecules during the extraction process from the plant tissues. The application of this method reduces the extraction time and energy consumption and provides higher extraction yield with higher antioxidant activity (Chemat, Zill-E-Huma, & Khan, 2011; Aybastier, Işik, Şahin, & Demir, 2013; Upadhyay, Nachiappan, & Mishra, 2015; Hossain et al., 2012; Pan, Yu, Zhu, & Qiao, 2012). Agitating the solvent using UAE leads to an increase in the surface area under contact between the solvent and the cell matrix (Shalmashi, 2009; Dimaki et al., 2017). Due to reduced extraction time and temperature, UAE can remarkably decrease the thermal degradation of heat sensitive bio-active compounds such as polyphenols (Saini, Panesar, & Bera, 2019).

UAE involves faster energy transfer, effective mixing, faster response to process control systems and lesser energy consumption with moderate extraction time and reduction in thermal degradation are the major advantages of using UAE compared to the conventional extraction methods (Das, Adsare, Das, Kulthe, & P., 2017).

The application of UAE results in the extraction of the targeted bio-active compounds present in tea at a lower cost, higher recovery, and better efficiency. Moreover, it is also easier to scale-up the process, which requires very little capital investment (Pasrija & Anandharamakrishnan 2015).

In 1994, Mason & Zhao extracted the tea solids using UAE with water as a solvent and studied the effect of temperature, sonication time, and power on the extraction efficiency. The optimum temperature conditions were found to be 60°C since it had an improved extraction efficiency of 20% for 10 min. Additionally, it was also reported that the extraction yield was improved up to 40% after the sonication was stopped and the entire mixture was heated up to 100°C. In general, increasing the sonication time was found to significantly impact the extraction yield.

Ultrasound can be also used as a method to improve the extraction efficiency at lower temperatures. The effect of UAE on the sensory and the chemical quality of the tea infusions was reported by Xia, Shi, & Wan (2006). UAE was found to have a better extraction yield of the chemical compounds present in tea at a lower temperature. Tea polyphenols, amino acid content and the caffeine amount in the tea infusions were higher in the samples treated by UAE compared to the conventional methods. The results showed that the application of UAE increased the extraction yields of aroma compounds and other glycosidic precursors. The sensorial properties of the UAE extracts were found to be better than that of the conventionally extracted ones. Based on an orthogonal design, the optimized conditions were reported to be 40 kHz frequency, 250 W power, 60°C temperature, and 40 min extraction time. However, Jacques et al. (2006) evaluated the chemical composition of the matte tea leaves extracted by different techniques (pressurized

liquid extraction (PLE), maceration, and UAE at 40 kHz, 90 W, 75°C, 180 min) using hexane and methanol as solvent and did not observe any significant difference in the quality of the extracts. However, the application of PLE was more effective than UAE and maceration in terms of extraction of caffeine, phytol, palmitic acid, and stearic acid.

Koiwai & Masuzawa (2007) have used ultrasonic irradiation as a method to extract catechins from green tea leaves. The ultrasonic pressure was identified as a critical parameter for the extraction of catechins. The results of the study showed that the ultrasonic irradiation was effective for increasing the amount of catechin extraction from green teas at low temperatures. The application of ultrasonication at 25.1 kHz, 28°C, and 30 min proved that the amount of the catechins extracted was proportional to the ultrasound applied. This might be due to the increased permeability of the tea cells, which was induced by ultrasonic irradiation. In another study, the extraction of catechins and caffeine from green and black teas was carried out by ultrasound at 40°C, 10 min, and using methanol: water (1:3 v/v) as solvent (Gu, Cai, & Zhang, 2007) using dynamic (constantly changing) UAE. This resulted in improving the extraction efficiency as well as in decreasing the extraction time and the solvent consumption. The application of dynamic UAE reduced the oxidation and the hydrolysis of the analysts due to the reason that the system was airtight. Following this research Saito et al., 2007 developed a method to compare the chemical content in the tea using 3 extraction systems namely UAE, hot water and different solvents. The UAE treatment was performed in 100 mL solvent (50% acetone, and 25% ethanol) for 30 min at 30°C. The 50% acetone with sonication provided the highest extraction yield in comparison to the other methods employed in the study.

Sonawane & Patil (2008) evaluated the effect of ultrasound to investigate the leaching of tannic acid from tea using different solvents (water and methanol) and compared the results with stirring at a constant speed and natural leaching process. The model of the leaching process was based on the thin film concept, which provided the resistance to transfer. The ultrasonication was performed at 50°C and 20 kHz. The sonication was conducted at intervals of 10-20 s and each time the tannic acid content of the solutions was measured. The results helped to prove that the methanol served as the best extraction solvent and had the highest extraction yield of tannic acid.

Shalmashi (2009) evaluated the effect of ultrasound conditions (e.g. power, time and temperature of the extraction, and the solvent to solid ratio) on the extraction of oil from the tea seeds. Compared to the conventional methods of extraction, UAE was performed at a shorter extraction time with a minimal usage of the solvent. Increasing the ultrasonic power from 10 to 50W resulted in increasing the extraction yield from 46.23 to 85.21%, respectively. The yield decreased by increasing the temperature. The optimum conditions were found to be the ultrasonic power of 50 W, at the temperature of 30°C with an extraction time of 30 min, and a solvent to solid ratio of 6:1.

Naşcu-Briciu, Cobzac, & Baciu, (2011) optimized the ultrasound-assisted extraction of flavonoids from green tea leaves. The optimization of the process was performed based on the composition of the extraction solvent, time, temperature, and type of organic modifier of the extraction mixture. The maximum extraction yield was obtained at 45°C for 50-60 min with 80% ethanol using UAE. The repeatability of the process was very high, and the relative standard deviation (RSD) was less than 5.5%. The results showed that the green teas had the highest amount of polyphenols. Horžić, Jambrak, Belščak-

Cvitanović, Komes, & Lelas (2012) compared the effect of conventional and UAE (bath and probe) techniques for the extraction of bio-actives (e.g. total flavonoids, non-flavonoids, polyphenolics, and methylxanthines) and antioxidant activity of the yellow tea samples using ethanol and water. The application of the ultrasonic probe (power of 20 kHz, 50% amplitude, and during 30 min) resulted in the highest extraction of total flavonoids using ethanol as solvent. Sereshti, Samadi, & Jalali-Heravi (2013) evaluated the feasibility of using UAE along with dispersive liquid-liquid microextraction for the extraction of 42 volatile compounds and caffeine from black tea, green tea, oolong tea, and white tea. The optimization of UAE was performed using central composite design (CCD). The optimized parameters were as follows: sonication time of 21 min, temperature at 32°C, volume of the solvent (methanol) of 27 μL, and salt concentration of 7.4%. The application of ultrasound resulted in releasing the volatiles from the matrix at a lower temperature.

Lante & Friso (2013) investigated the potential of ultrasound (60°C for 15 min) for the extraction of catechins from green tea leaves with higher EGCG contents. Subsequently, water-in-oil green tea nanoemulsions were prepared using different types of oil such as soy, peanut, sunflower and corn oils. The application of UAE enhanced the extraction yield of EGCG to 15% and the highest oxidative stability was observed in the nanoemulsion sample prepared with green tea/peanut oil. The efficiency of the process was influenced by several factors such as acoustic intensity, type of solvent, time and temperature of extraction.

A graphene oxide based ultrasonic-assisted dispersive micro solid phase extraction (SPE) method was developed by Sereshti, Khosraviani, Samadi, & Amini-Fazl (2014) to extract theophylline, theobromine, and caffeine from different infusion tea samples (black,

white, and oolong, green). The synthesized graphene oxide was dispersed ultrasonically in the sample solution (5 mL) and used as a sorbent (15 mg) in a batch SPE coupled with HPLC-UV technique. The desorption of the analysts from the graphene oxide was performed by the low volume of the organic solvent (100 μL). The authors claimed that they developed a simple procedure with low cost and eco-friendliness, which can be used for the isolation of polar and hydrophilic molecular compounds from the aqueous solutions.

A comparative study based on the isolation and the extraction efficiency of caffeine and catechins from green tea leaves was carried out using different extraction methods (UAE, room temperature or reflux extractions), solvent systems (ethanol, distilled water) and extraction times (0.5-24 h) (Choung et al., 2014). UAE was found to be more efficient than the other extraction methods with respect to time and productivity. The optimized conditions for UAE method were found to be as follows: ethanol concentration of 40%, extraction time of 2 h, and extraction temperature of 40°C. Compared to other isolation approaches, the recovery of catechins was higher when the combination of ethyl acetate and dichloromethane was used. UAE helped in decreasing the process time and process temperature and prevented the epimerization of catechin molecules caused by the extraction at high temperature.

Kotovicz, Wypych, & Zanoelo (2014) reported that compared to atmospheric pressure, the application of UAE (47 kHz and 16°C) at high pressure (91.4 to 338.2 kPa) enhanced the extraction yield of *Ilex paraguariensis* (matte leaves) to 200%, respectively. There was a reduction in the extraction time and the efficiency of the extraction was close to 74%. Wei & Yang (2015), evaluated the effect of ultrasound-assisted supercritical CO_2 (USC-CO_2) method on the extraction of triterpenic acids from *Hedyotis diffusa* and *Hedyotis*

corymbose, the major ingredients of healthy tea. Compared to the conventional SC-CO_2 and solvent extraction techniques, the application of USC-CO_2 extraction method resulted in increasing the extraction yields (15-16%), decreasing the extraction time to 95 min in comparison to 180 min of heat reflux extraction and 135 min of UAE, and minimizing the amount of solvent used (43 ml extraction vessel with a continuous flow).

See et al. (2016) investigated the effect of various methods such as UAE (Q500 sonicator, 20 kHz, 70% ethanol, 300 W, and 30 min), MAE, mechanical grinding, and sample pre-treatment with acids and alkali on the extraction of bio-active compounds from java tea. Both UAE and MAE induced the disruption of the cell walls and consequently improved the diffusion process of the bio-active compounds, which resulted in obtaining 86-95% of the extraction yield.

Zhang, Xie, Tian, Pu, & Qin (2016) optimized the extraction conditions of total flavonoids, dihydromyriceitin, myricitrin, and myricetin from *Ampelopsis grossedentata* (vine tea) using response surface methodology. The optimum UAE conditions were reported as follows: a concentration of the solvent of 80-87% methanol, an extraction time of 32 min, and a liquid to solid ratio of 41.64:1 mL/g. The application of UAE resulted in the isolation of the bio-actives on adequately large scale with high purities in a single operation.

Pavlić et al. (2017) found that the highest recovery of polyphenols and flavonoids from sage tea dust (*Salvia officinalis)* using UAE compared to MAE and maceration. Some biochemical properties such as the antimicrobial effect, as well as the polyphenol and flavonoid contents were higher in the UAE (40 kHz, 75°C, 80 min, sonication intensity of 43 W/L, 5 g of sample, 100 mL of 60% ethanol). UAE was hence one of the suitable

methods for the production of sage extracts from tea dusts in comparison with MAE, maceration, subcritical water extraction, and hydro-distillation.

The UAE was optimized to investigate the effects of enzymatic pre-treatment on mountain tea (aromatic plant). The usage of UAE as a pre-treatment method (40 kHz, 3 g of sample, 80 mL of petroleum ether, 30 min) increased the extraction yield of bio-active compounds. From the study, UAE has been regarded as the optimal method for the analysis of volatiles from *Sideritis* spp. (mountain tea) and concluded that UAE can be useful for the analysis of other aromatic plants (Dimaki, Iatrou, & Lamari, 2017).

Similarly, a comparative experimental study was performed by Ghasemzadeh-mohammadi, Zamani, Afsharpour, & Mohammadi (2017) who optimized two extraction methods: MAE and UAE of green tea leaves. The extraction efficiency (extraction yield) of UAE was found to be 85% when compared to the MAE, which was 95%. The optimum conditions for UAE were estimated based on the following parameters: extraction time of 57 min, 3 extraction cycles, extraction temperature of 65°C, and water as solvent. The Total phenolic content (TPC) was found to be 96±6 and 125±5 mg of Gallic acid equivalent per g of DW, for UAE and MAE, respectively. The green tea leaves were decaffeinated before the application of novel techniques to extract the bio-active compounds. The 50% inhibition of DPPH was obtained using 66 mg/g of phenol when UAE was applied, compared to 56 mg/g of phenol when using MAE. The caffeine extraction yield depended on the temperature. The study concluded that MAE was more efficient for the extraction of TPC and catechins from green tea. Jeong et al. (2017) optimized the conditions for UAE (extraction time of 6.4 min, and 80.7% for the solvent (BGG-4) content and solvent volume of 1.8 mL/100 g), was used for the extraction of catechins from green tea leaves and the

results concluded that the solvent with BGG-4 (betaine, glycerol and D(+)glucose) improved the extraction efficiency of the catechins from green tea. The catechins were found to be more stable in deep eutectic solvent (DES) extracts than in any other solvents (e.g. water, methanol, ethanol) used for the extraction process.

A comparative study on the usage of sage (type of tea made from *Salvia officinalis*) herbal by-product of filter tea factory to extract antioxidants was done using MAE and UAE (Zeković et al., 2017). The optimization of the process was done using a Box-Behnken experimental design and response surface methodology for a higher efficiency of total phenolic and flavonoid contents. The optimized parameters for the extraction process using UAE were as follows: temperature of 75.4°C, extraction time of 18.7 min, and an intensity of 42.5 W/L, whereas for MAE, the concentration of ethanol solution used was 46.2%, an extraction time of 18.7 min and a solvent to solid ratio of 40 mL/g. The temperature and the ethanol concentration were the critical factors for UAE and MAE. The authors concluded that the sage herbal by-product is a rich source of antioxidant molecules and could be efficiently extracted using MAE and UAE, compared to the conventional extraction methods. Luo, Yao, Liu, Zhang, & Ying (2018) performed an optimization using an ultrasound-assisted aqueous two-phase system for the extraction of saponins from *Coreopsis tinctoria*; an herbal tea. The optimization was done by response surface methodology (37.76% for $(NH_4)_2SO4$ and 35.62% for ethanol). The extraction yield was found to be 33.4 g/kg of raw material. The authors concluded that UAE under the conditions of 30 min extraction time, 250 W power, 40 kHz frequency, and a solid to liquid ratio of 21:1 was the one of the best method for the extraction of saponins.

The efficiency of the extraction usually differs from one tea species to another due to the differences in the structure and the composition of the leaf matrices. Some other factors such as the turbidity of the plant tissue (tea leaves) and the starch granules within the cytoplasm of the cell can be influenced by the ultrasound energy applied and thus the effectiveness of the extraction (Talmaciu, Volf, & Popa, 2015). The solvent used for the extraction process should be also properly selected based on its selectivity for the target bio-active compound. A summary of some experiments carried out by the UAE to obtain bio-active compounds from tea and tea waste is listed in Table 1.

3.4.2. Microwave-assisted extraction (MAE)

Microwave-assisted extraction has received an extensive attention as an alternative method for the extraction of bio-active compounds from plant-based food matrices (Spigno & De Faveri, 2009; Rahim, Nofrizal, & Saad, 2014; Talmaciu, Volf, & Popa, 2015; Li, Huang, Tang, & Deng, 2010). The application of MAE involves the propagation of non-ionizing electromagnetic waves (300 MHz to 300 GHz) situated between X-rays and infrared rays in the electromagnetic spectrum. These waves have the ability to penetrate into the sample, interact with the polar compounds, and generate heat, which subsequently leads to substantial changes in the structure of the cells (Chan, Yusoff, Ngoh, & Kung, 2011; Talmaciu et al., 2015). The synergistic combination of heat and mass transfer, as two transport phenomena working in a uniform direction, is the major cause for the process acceleration and the increased extraction yield (Dhobi et al., 2009; Talmaciu et al., 2015).

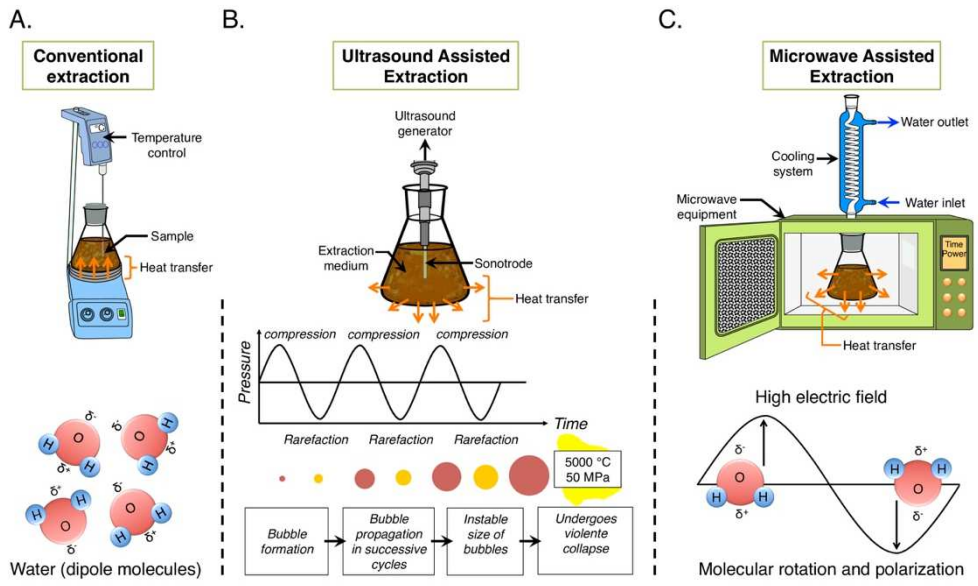

Figure 7: Comparative illustration of conventional, ultrasound and Multi-mode microwave applicator used to extract bioactive compounds from tea tissues (Retrived from Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016)

M AE is a sequential process wherein the solvent initially penetrates into the solid matrix (e.g., tea plant tissues), followed by the structural breakdown, which leads to transport the solutes rich in bio-active compounds out of the matrix. The solute migrates from the external solid surface to the bulk solution leading to the separation and the discharge of the extract containing bio-active compounds (Azmir et al., 2013; Talmaciu et al., 2015). Then, the solvent interacts with the free water molecules present in the plant cells resulting in the rupture of the cell wall and aids in the release of bio-active compounds from the cells to the solvent (Chen, Zhao, Liu, & Zuo, 2012; Xia et al., 2012; He et al., 2014). The solvent composition, solid to solvent ratio, extraction temperature and time, microwave power, stirring speed, and surface area of contact are the critical parameters affecting the extraction of bio-active compounds in a MAE process (Talmaciu et al., 2015).

The solvent used for the extraction depends on the targeted bio-active compound as the penetration and the interaction of the solvent with the targeted compound is the

deciding factor by excluding the other unfavorable matrix compounds (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017; Y. Li et al., 2017; Talmaciu et al., 2015). The solvent also should present a very good capacity to absorb the microwave energy and get heated up depending on the boiling points, dissipation, and the dielectric properties. The volume of the solvent used must be sufficient to ensure that the sample is completely immersed. This parameter is important to consider, as an excessive solvent used requires more energy to concentrate during the purification process. Since the exposure to the microwaves is non-uniformly distributed, the degree of recovery will be lower. However, the excessive exposure to the microwave radiation at a reduced temperature can also lead to a decrease in the extraction yield due to the decomposition of the bio-active compounds. The yield and efficiency of the extraction increase proportionally to the time and the microwave power applied, however, they start to decrease beyond a certain limit mainly due to the increase of temperature. Microwave power is a crucial parameter to set-up in order to minimize the time needed to reach the set temperature and also to ensure that the time of exposure doesn't affect the available bio-active compounds. The stirring also plays an important role in the optimization process since it is directly related to the mass transfer in the solvent phase, which in turn induces convection in the headspace available so that equilibrium is achieved quicker between the aqueous and the vapor phases. The surface area under contact also enhances the efficiency of the extraction process. The finely powered samples usually have a large surface area offering a better contact surface between the plant matrix and the solvent, thereby deepening the penetration of the microwave (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017; Y. Li et al., 2017; Talmaciu et al., 2015).

Many other researchers have reported that the MAE is more effective than the conventional extraction methods (solid liquid extraction, extraction at room temperature, maceration, reflux extraction) in extracting bio-active compounds from green teas at ambient temperature (Pan et al., 2003a,b; Liu, Ding, Zhang, Hu, & Bu, 2006; Sultana et al., 2008). The results of the bio-active extraction from tea leaves using different operating conditions of MAE are summarized in Table 2.

In a study conducted by Pan, Niu, & Liu (2003), the MAE extraction of polyphenols and caffeine from green tea leaves was optimized using different independent variables such as ethanol concentration (0-100%), extraction time (0.5-8 min), solvent/solid ratio (10:1-25:1 mL/g), pre leaching time (0-90 min), and different types of solvents (e.g. acetone, methanol, ethanol, water). The optimal conditions were 50% ethanol concentration, 1:1 v/v of ethanol/water solution, 5 g of solid, and 20:1 mL/g of liquid/solid ratio for 4 min with a pre-leaching time for 90 min. MAE was considered as the fastest method of extraction (polyphenols and caffeine) in 4 min, with a higher yield and less labor-intensive process compared to that observed for the other extraction methods (extraction at room temperature at 20°C, ultrasonic extraction at 20-40°C, and heat reflux extraction at 85°C). Sultana et al. (2008) conducted a qualitative and quantitative analysis of tea flavonoids using 1) MAE and ASE (accelerated solvent extraction), and 2) HPLC, respectively. The optimization process clearly proved that the MAE delivered the highest yield in terms of extraction in a short period of time. MAE was carried out at 150 W for 200 s with a ventilation period of 5 min and also at 700 W for 45 s, 0 W for 10 s, 250 W for 3 s, 0 W for 10 s. The comparison was made with respect to the total tea polyphenols showing that MAE was the best method with the highest extraction efficiency for the

recovery of catechins and derivatives within a short period of time. A year later, Nkhili et al. (2009) performed a newer version of microwave-assisted water extraction (MWE) to extract polyphenols from green tea with 6 g of sample, in 120 mL of water, at 80/100°C, during 60 min, and with a power of 600 W. The experimental conditions were optimized based on the temperature and the extraction time, whereas the efficiency of the process was determined based on the duration of the extraction, the total phenolic content, the chemical composition, and the antioxidant activity of the extracts obtained. The flavanol content, EGCG (epigallocatechin gallate) was found to be higher in the case of MWE with 97.6 (mg catechin/g) of flavanols and 77.14 (mg catechins/g) of EGCG in comparison with conventional heating and water extraction (CWE) with 83.06 (mg of catechins/g) of flavanols and 64.18 (mg of catechins/g) of EGCG. Moreover, MWE was comparatively more efficient at higher temperatures (80 or 100°C) than CWE. MWE was suitable for producing green tea extracts that are rich in polyphenols. The optimum extraction temperature was 80°C since the thermally sensitive compounds could be extracted along with some specific flavanols presenting high concentration of ECGC and high antioxidant activity. The use of MAE allowed reducing the extraction time, the energy consumption, and the environmental burden. Similar study was performed by Spigno & De Faveri (2009) for total phenols recovery from black tea powder, by studying the effect of microwave power (450, 600, and 900 W) and the duration of irradiation (30-210 s). The experiments were performed using an ordinary household microwave oven. The study concluded that MAE led to higher recovery of total phenolic compounds, in comparison with the normal brewing techniques, without affecting the antioxidant potential of the tea. The total phenol diffusion rate was studied at different water to tea ratios and the experimental data was

successfully predicted as a mass transfer model. MAE can be used for industrial applications with minor modifications with respect to the sample size, the solvent to solid ratio, and various other parameters. The enhancement of product recovery was due to the heating effect of the microwave. Higher solvent to solid ratios along with constant solvent volume led to higher extraction yield and recovery. Depending on the volume of the solvent used, the heating time was altered. Subsequently, higher the solid to solvent ratio, lower the recoveries were observed.

Similarly, Spingo, Tsubaki, Sakamoto, & Azuma (2010) performed an experimental study in order to extract the phenolic compounds from oolong, green, and black tea residues under auto hydrolytic conditions coupled with MAE. The conditions were maintained without using catalyst or an organic solvent. The results showed an increase in the extraction of phenols when the residues were heated at 230°C in the microwave. The extract's composition was different depending on the tea residue treated. For the green tea, the main constituents were pyrogallol (24.6%) and catechol (6.7%) (derived from the degradation of the catechins), for the oolong tea, the mains identified compounds were dihydroconiferyl alcohol (10.3%) and vanillin (8.1%) (both are derived from guaiacyl units of lignin), whereas for the black tea, the residue was rich in derivatives of both catechins and lignin. MAE was the fastest extraction method, involving a rapid extraction of phenols (within 2 min). The study concluded that these phenols could be chemical bio-based feedstocks, with high antioxidant activity. Nshimiyimana (2010) used MAE (450 W, 70°C and 120 s) for tea polyphenols' recovery from black and green tea, and studied their radical scavenging capacities. Green tea extracts were more concentrated in total polyphenols (26%) compared to black tea (16%). The higher concentration of polyphenols influenced

higher free radical scavenging activity. The result suggested that MAE (26%, 16%, respectively for green and black teas) was much more effective than the conventional decoction (21%, 14%, respectively for green and black teas) in terms of total phenolics recovery, scavenging activity, energy consumption, and extraction time.

MAE and CE (capillary electrophoresis) were combined to design a fast analysis method of catechin and epicatechin in green tea (Li, Huang, Tang, & Deng, 2010). The optimized MAE conditions were 1 min of extraction time and 400 W of microwave irradiation, providing the maximum extraction yields of catechin and epicatechin. The study concluded that the proposed method had a good recovery of catechins (118%) and (120%) epicatechin. MAE coupled with CE was proven to be easy to apply, convenient, fast, and reliable method for the determination of catechins and epicatechins in the green tea, along with reduced time, sample, and solvent consumption. In another study, **Li & Jiang (2010)** optimized the MAE conditions using an orthogonal array design to extract tea polyphenols from decaffeinated green tea. Four parameters were optimized; microwave intensity, irradiation time, irradiation number of times, and tea to water ratio. The optimal extraction conditions were recorded as 600 W power, irradiation time for 3 min, and with a ratio of 1:20. The order of influence of these parameters was deduced to be irradiation time > intensity > tea to water ratio > number of irradiation times. The authors concluded that MAE is an appropriate method for the extraction of polyphenols from tea matrices. In order to enhance the extraction efficiency, **Wang, Qin, & Hu (2010)** used an orthogonal design for the recovery of polyphenols from tea using MAE. The influential parameters affecting the extraction included the temperature (80°C), the microwave power (600 W), the concentration of solvent (60% ethanol), the extraction time (10 min), and the solid to

liquid ratio (1:12 g/mL), which provided a yield of 96.5% of the total polyphenols. The extraction time was reduced to more than 8 times when compared with HRE (hot reflux extraction), 2 times when compared to UAE (ultrasound-assisted extraction), and 5 times when compared to SFE (supercritical fluid extraction). The extraction yield was increased by 17.5% compared to HRE. The extraction time was reduced 5 times when compared to SFE, and the energy consumption was ¼ when compared to UAE with 40% increase in the total phenolics. The study concluded that MAE only required shorter time and lesser energy consumption and provides higher extraction selectivity and extraction yield.

In 2011, Wang et al. investigated a rapid method based on dynamic microwave assisted extraction for the extraction of caffeine from tea, and compared it with the SAME (static MAE) results. The parameters (microwave power, extraction solvent volume, flow rate) were optimized using a Box-Behnken design and the maximum extraction efficiency was obtained using 70 W microwave power, 3.5 mL of extraction solvent (ethanol 50:50), and 1 mL/min of extraction solvent flow rate with a limit of detection of 0.01 mg of caffeine/g. The caffeine recovery in the tea samples was found to be in the range of 88.2% to 99.3%. The Dynamic MAE (DMAE) was considered more selective and sensitive and could be achieved in considerably reduced time and labor for the extraction of caffeine compared to other conventional methods (e.g. Soxhlet and liquid-liquid extraction). The caffeine yield using DMAE (47 mg/g) was higher than SAME (37 mg/g) with reduced sample amount, volume of the organic solvent required, and time required for preparation. It showed the highest efficiency.

Rahim, Nofrizal, & Saad (2014) performed a reverse phase HPLC method to identify 8 catechin monomers and caffeine extracted from tea with the help of a monolithic column.

Water: acetonitrile:methanol (83:6:11, v/v) was used as the solvent or the mobile phase at a flow rate of 1.4 mL/min. The MAE was used in combination with HPLC in this research with 11 tea samples including 6 green, 3 black, and 2 oolong teas. The results showed higher level of caffeine in black tea whereas higher amount of catechins, mainly EGCG, was observed in green teas. The MAE was optimized to pressure: 350 psi, irradiation time: 6 min, and irradiation power: 600 W. The MAE played a major role in improving the extraction efficiency of catechins and caffeine. In this work, the extraction efficiency of the catechins and caffeine increased with the irradiation time up to 6 min and remained stable beyond 8-10 min. MAE was also used to extract the tea saponins from the oil - tea camellia seed cake. The effect of microwave power, irradiation time, temperature, ratio of solvent to the solid, and ethanol concentration were optimized using systematic orthogonal experiments. In comparison to the extraction methods (ultrasonic treatment, liquid-liquid extraction and reflux treatment), the MAE proved to be the best, as it reduced the extraction time from 6 h to 4 min, and the amount of organic solvent used down to 50%, along with the enhanced extraction yield by 14% (He et al., 2014). Following this, Liu et al. (2014) used MAE to improve the extraction of 1-deoxynojitimycin (DNJ) from mulberry tea. Response surface methodology was used to optimize the conditions, which were 602.28 W of microwave power and 11.41 min of extraction time. The extraction yield found under these conditions was 0.19%. According to the authors, the conditions used in MAE were found to be more convenient than that applied for the conventional extraction using hot water immersion.

In the same year, Bekdeşer, Durusoy, Özyürek, Güçlü, & Apak (2014) applied MAE for tea samples and investigated the effect of hypochlorous acid on the recovery of

polyphenols. The conditions of MAE were optimized based on the extraction time (0-10 min), the temperature (50-100°C), the solvent composition (ethanol:water at 20, 40, 60, 80 and 100%), the type of the solvent used (ethanol, methanol, water), and the solvent to solid ratio (10-40), with respect to the inhibition percentage (HOCl hypocholorous scavenging activity) of tea extracts using resorcinol method. The optimal extraction conditions found for MAE were: a temperature of 80°C, an extraction time of 3 min, a concentration of ethanol of 80% (using methanol as solvent), and a solvent to liquid ratio of 20:1. The authors concluded that MAE was the best method for the extraction of polyphenols from tea.

In a research article published in 2016 by Lam et al., the chemical characteristics of different parts of tea flower (*C. morifolium*) (flowers, bud, seeds, stems, and leaves) growing in China showed excellent antioxidant and antidiabetic properties, when applying MAE followed by HPLC for the detection of 13 major compounds. MAE was used as a method of sample preparation, wherein 0.1 g of sample was mixed with 2 mL of water and transferred into 5 mL extraction vessels, and then extracted at 400 W and 80°C for 5 min. The results obtained helped in improving the quality and helped in pharmaceutical application of different parts of *C. tinctoria*. In another study based on the use of wild apple fruit extract, which is a by-product from filter tea factory, MAE was used for the production of extracts rich in polyphenols (Pavlić et al., 2017). Box-Behnken experimental design was used at three different levels and for the three parameters: extraction time (15-35 min), ethanol concentration (40-80%), and irradiation power (400-800 W). The optimal conditions were 15.2 min extraction time, 40% ethanol concentration, and 400 W power. MAE was considered for the extraction due to its effectiveness in increasing the extraction

yield of some targeted compounds and enhancing the quality of the extracts in terms of antioxidant activity. The ethanol concentration was found to be the most influential parameter for the extraction of polyphenols. The extraction time and the irradiation power where found to be the parameters that must be reduced as much as possible to decrease the degradation of polyphenols. The study concluded that the by-product used has the potential to be used to produce value-added compounds.

In 2017, Ghasemzadeh-mohammadi et al. optimized the extraction of caffeine and catechins (EGCG and EGC) from Iranian green tea leaves (decaffeinated), using MAE and UAE techniques, and water as solvent. The MAE was carried out with a domestic microwave at 190 W. The efficiency of the extraction was found to be 95% for MAE (compared to 85% using UAE). The optimum extraction conditions for MAE were found to be 7.8 min and 3 extraction cycles. The total phenolic content was 125±5 g of GA equivalent/g DW, whereas the 50% inhibition of DPPH was found using 56 mg of GA equivalent/g DW. The MAE was comparatively good in extraction as well as the efficiency levels were higher when compared with UAE. The temperature was considered as the major variable for caffeine and catechin extraction using MAE. The extraction efficiency of caffeine was 90%. The study concluded that decaffeination of tea with water and MAE on the sample as a quicker method of preparation. MAE and UAE were used by Zeković et al. (2017) to extract phenolic antioxidants from sage herbal dust obtained from filter tea factory and the method was optimized based on the maximization of total phenols and total flavonoids. The optimization was done with both Box-Behnken and response surface methodology for the following parameters temperature (40, 60, and 80°C), extraction time (40, 60, and 80 min), and ultrasonic intensity (24, 42, and 60 W/L) in the case of UAE and

ethanol concentration (40, 60, and 80%), extraction time (10, 20, and 30 min), and liquid to solid ratio (20, 30 and 40 mL/g). The study concluded that UAE and MAE provided some advantages in the recovery process of sage polyphenols in comparison to the conventional methods and can be used as a raw material for polyphenolic extraction from tea factory by-product. The temperature for UAE and the ethanol concentration for MAE were identified as the most important parameters to be taken into account. The optimized parameters for MAE (46.2%, 18.7 min, 40 mL/g), and UAE (75.4 °C, 80 min, 42.54 W/L) were based on the influence of these factors on phenolic extraction.

3.4.3. Pulsed electric field (PEF)

Pulsed electric field (PEF) is among the non-thermal and non-invasive technologies used for the extraction of targeted bio-active compounds from tea. This method has high potential for the disruption of cell wall by increasing the electrical conductivity and thus enhancing the permeability of the cell structure for a better extraction of the intercellular bio-actives (Chen, Peng, Zhao, Liu, & Wang, 2016). PEF application don't induce severe damage to the aromatic compounds, polyphenols (Zhu, Zhang, Tsang, Huang, & Chen, 1997; Zhao, Yang, & Wang, 2009), color, taste and aroma of tea (Zhu et al., 1997; Xia et al., 2006; Zhao, Yang, Wang, et al., 2009; Ye, Zhang, Sun, Chen, & Fang, 2014; Zhu, Zhang, Tsang, Huang, & Chen, 1997; Zderic & Zondervan, 2017). It involves minimal energy consumption, uniform transmission, and quicker processing (Chen et al., 2016; Ting et al., 2016). It is also normally used as a preservation method for the inactivation of microorganisms due to the breakage of cell membranes (Castro, Barbosa-Cánovas, & Swanson, 1993; Wang et al., 2008; Zhao, Yang, & Wang, 2009; Gabrić et al.,

2018), which increases the shelf life of the food (Zhao, Yang, Wang, et al., 2009; Knorr et al., 2011; Zderic & Zondervan, 2017).

PEF is one of the emerging technologies in the process industries and is considered as competitive compared to the other processes when it comes to cost effectiveness. The extraction process in PEF is simple wherein the sample is placed between two electrodes followed by the application of high electric field pulses for a very short duration (ns to μs) (Zhao, Yang, Wang, et al., 2009; Zderic, Zondervan, & Meuldijk, 2013; Esser, Smith, Gowrishankar, Vasilkoskl, & Weaver, 2010; Zderic & Zondervan, 2017; Asavasanti, Ristenpart, Stroeve, & Barrett, 2011; Zderic & Zondervan, 2017). The size and formation of pores, which can be reversible or irreversible, depend on the pulse intensity, the electric field strength, the number of pulses, and the time period of treatment (Zderic & Zondervan, 2016).

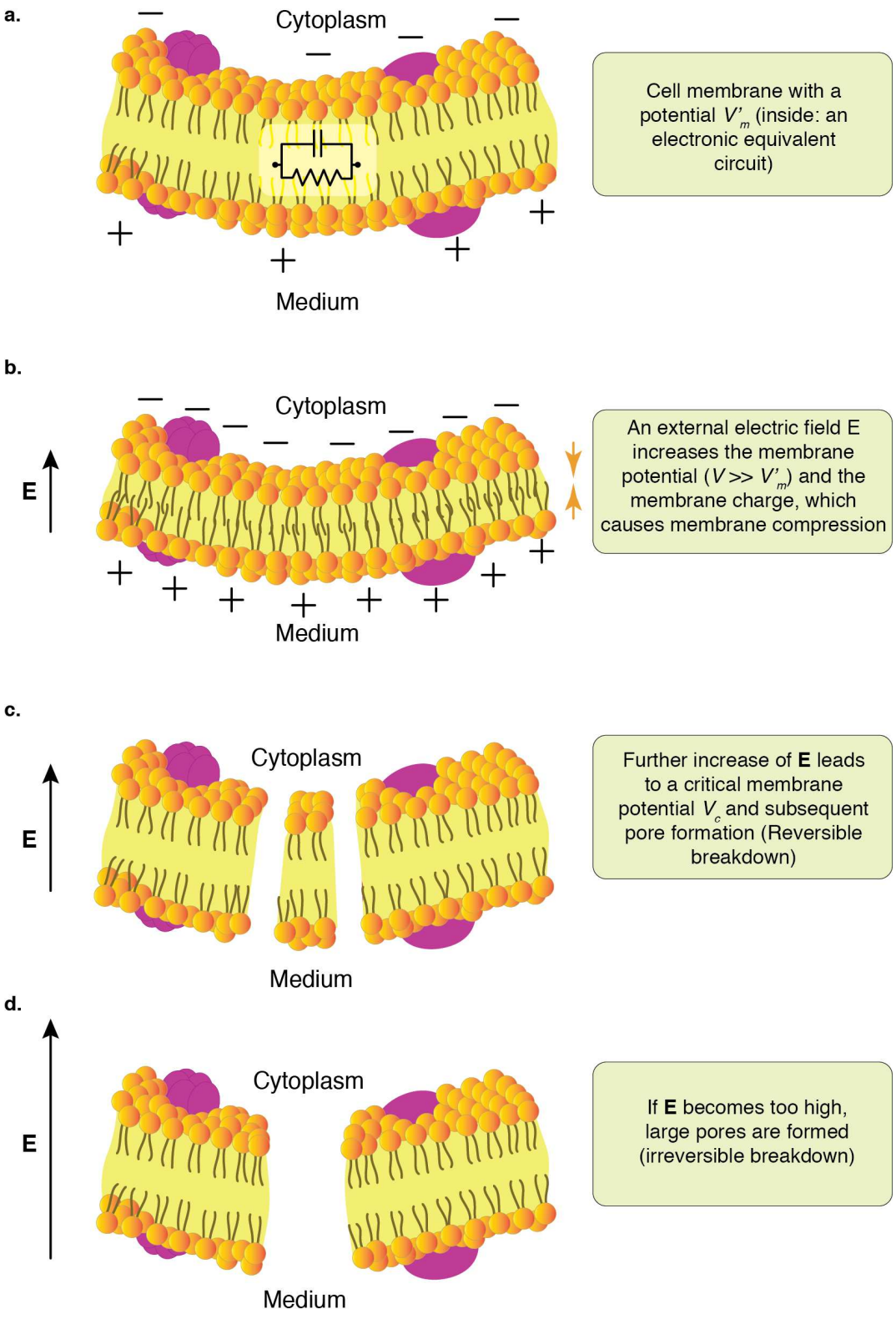

Figure 8: Schematic illustration of the electroporation mechanism in the cell membrane exposed to an electric filed (Retrived from Roohinejad, Koubaa, Sant'Ana, & Greiner, 2018)

The intensity of the electric field is one of the important factors involved in cell inactivation, which increases proportionally to the electric field intensity, beyond a certain value termed as critical field intensity. The critical field intensity is higher for the cells having larger size, and it is dependent on the pulse width. The extraction yield increases proportionally to the intensity of the electric field. The conductivity of the solvent and the solubility of the bio-active compounds in the solvent are the two main important factors that should be taken into consideration for the selection of the solvent. Increasing the conductivity of the solvent enhances the electroporation on the cell membrane. Thus, selecting a suitable solvent with higher conductivity enhances the extraction efficiency. The solid to solvent ratio is also crucial in ensuring an efficient solvent extraction. It has also been proven that if the solvent to solid ratio is increased, the extraction yield started to decrease, and higher energy will be required to remove the solvent from the solution after the treatment. In addition, He et al., (2014) studies report that increasing the solvent concentration can lead to reducing the concentration of the bio-active compounds bound to the sample and hence make it easier for the dissolution process. The pulse duration increases the permeation of the cells but leads to the decomposition of the extracts. The resistance of the chamber in the treatment is also an important factor in the process. The effect of the pulse width varies depending on the electric field strength, type, quality and contact parameters such as the geometry and the size of the samples (Zderic, Zondervan, & Meuldijk, 2013b; Zderic et al., 2013b).

The efficiency of electroporation is controlled by the electric field intensity. Lower intensities of electric fields involve longer time taken for the electroporation of the cellular membranes. Other parameters that need to be considered include the pulse duration, the

number of pulses, and the pause between the pulses. The disintegration degree depends on the time of the treatment and the strength of the electric field strength. Longer pulses were found to be more effective and pronounced effect at a moderate level. The extraction kinetics strongly depends on the interval between the pulses and higher the field strengths lead to better damage efficiency of plant tissues (Zderic et al., 2013b).

Polyphenols are the major extracted compound with respect to tea from PEF. In 2009, Zhao et al., studied the influence of PEF on the inactivation of microorganisms such as *Escherichia coli and Staphylococcus aureus*, as well as its effect on phenols, free amino acids and some properties such as the color. The tea samples were inoculated with *E. coli* and *S. aureus* before PEF treatment and were subjected to various field strengths (18.1, 27.4, 38.4 kV/cm) and treatment time (40, 80, 12, 160, 200 μs). The inactivation was found to be effective at 38.4 kV/cm, for 16 to 200 μs, and led to 5.6 to 4.9 log reductions of *E. coli and S. aureus*. The study also confirmed that the temperature of storage and the antimicrobial activity have a synergistic effect on the reduction of microorganisms. This led to increase the shelf life and to maintain the quality of the bio-active compounds as well as the color of the green tea. A comparative study of PEF was performed at 40 kV/cm electric field strength, 200 μs treatment time, 667 pps (pulse per second), and 2 μs of pulse duration, and was compared to a conventional heat treatment at 121°C for 3 min (Wang, Yang, & Zhao, 2008). With respect to the effect on bio-active compounds (polyphenols, catechins, and free amino acids) and color of green teas, PEF treatment was found to retain more bio-active compounds compared to that obtained by the heat treatment.

In another study of green tea infusions, the recovery of sub lethally injured microorganisms was determined after a PEF treatment of the leaves (38.4 kV/cm electric

field strength, 200 μs treatment time, pulse repetition rate of 667 Hz, flow rate of 29 mL/min, pulse width of 2 μs), and the use of different temperatures of storage (4, 25, and 37°C) (Zhao et al., 2009). The usage of a combined storage temperature at 4°C for few hours and then the storage at 37°C helped in delaying the repair mechanisms occurring after PEF treatment, and hence the infusions had a longer shelf life of about 90 days. Similarly, the influence of PEF (20 to 40 kV/cm electric field strength, 5-15°C temperature, 200 μs treatment time, 667 pps (pulse per second), 2 μs pulse width) on bio-active compounds recovery (e.g., catechins, polyphenols, free amino acids), and some properties (e.g., color and flavor) of the green tea infusions were studied by Zhao, Yang, Wang, & Lu (2009). The results obtained showed an increase in the amino acids content by 7.5% and a loss of the volatiles by 10% depending upon the treatment of PEF beyond the critical level. The PEF treatments were found to efficiently retain the bio-actives and the color with increased amino acids content.

In a research conducted by Zderic et al (2013), the breakage of fresh tissues was studied using PEF in order to evaluate the amounts of polyphenols obtained. The breakage of the tissue increased the amount of polyphenols extracted with a maximum extraction yield of 27% at optimum parameters (number of pulses (N) =30, width of the pulse (PD) = 0.05, pause between the pulses (PBP) =0.5 s, electric field=0.9 kV/cm / N=30, PD=0.05, interval between pulses = 3 s, electric field=1.1 kV/cm, PBP=3 s). The extraction yield was found to be dependent on the pause duration between the pulses, the number of pulses, and the electric field strength. Ye, Zhang, Sun, Chen, & Fang, (2014) investigated various novel non thermal processing technologies including PEF, freeze concentration, and vacuum freeze drying for the production of instant high aroma black tea powder with different

extraction techniques (PEF, cold water, and hot water). The extraction efficiency of PEF was higher under the optimal conditions with orthogonal experimental design (tea/water ratio of 1:16, electric field strength of 20 kV/cm, pulse frequency of 125 Hz), with an extraction yield of 22.7%. The freeze concentration and vacuum drying helped to retain the flavor and aroma compounds (61 aromatic compounds identified), thereby increasing the solubility of tea in cold water and reducing the tea cream. The combination of freeze concentration and PEF helped in maintaining the nutrients and color. It also helped in the reduction of loss of aromatic compounds due to volatilization of pure floral notes of the instant tea.

Chen et al., (2016) performed an experiment to study the effect of aging in unfermented Pu'er tea using high voltage PEF (electric field strength of 18 kV/cm, treatment time of 60 min, 200 pulses per second (pps), frequency of 120 Hz, and pulse width of 0.3 μs). PEF improvised the taste and the aroma of the tea samples, and the sensory parameters were similar to that of the natural aging effect of teas. Various parameters (voltage, frequency, and time) were employed to study the effect of PEF and led to a steady decrease in the polyphenols and the theanine content, thus changing the taste with a significant increase in the salutary effect. The high voltage of PEF helped in artificial aging thus improving the taste with an accelerated process of aging. It can also be used as a rapid method of aging of unfermented Puer tea providing a new method of enhancement of tea quality and safety.

Zderic & Zondervan (2016) extracted polyphenols from tea leaves using PEF and obtained a maximum extraction yield of polyphenols of 27%, which was obtained based on the strength of the electric field, the duration of the pulses, and number of pulses applied.

The energy input per unit of mass of the amount of the tea sample subjected to electric field strength of 0.9 kV/cm and an interval between the pulses ≥ 3 s was found to be 29.7 kJ/kg. The temperature was also recorded to be not more than 10°C for the process providing a proof that PEF is a non-thermal method of cell dispersion technique. On the whole, the strength of the electric field and the treatment time were identified as the key operational parameters for polyphenols' extraction from tea leaves.

More recently, Zderic & Zondervan (2017) performed another molecular level approach to optimize the parameters (electric field strength, pulse duration, and number of pulses) for the extraction and isolation of polyphenols from fresh tea using PEF. The optimized conditions found using a Box-Behnken design were 1.1 kV/cm of field strength, $0.1x10^{-3}$ s of pulse duration, and 50 pulses, resulting in the outcome of 32% extraction yield. The PEF technology provided the maximum extraction yield of polyphenols without destroying their activities, compared to the conventional hot brewing method. The effect of various operating parameters involved in PEF technology on the extraction efficiency and functionality of bio-active compounds of tea are summarized in Table 3.

3.4.4 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is one of the rising alternative technologies used for extraction of bio-active compounds from tea leaves (Raventós, Duarte, & Alarcón, 2002; Chen et al., 2014). This method doesn't involve heat and organic solvents, thus protecting the food from thermal degradation as well as from residues of an organic solvent (Saldaña, Mohamed, Baer, & Mazzafera, 1999). The supercritical fluid commonly used in SFE is CO_2 (with high purity) as it is non-toxic, non-flammable under low critical

pressure. Also, CO_2 is cost effective and allows easy removal of the supercritical fluid from the extracts (Saldaña et al., 1999; Park et al., 2007).

SFE works on the principle of the supercritical properties of fluids (e.g. CO_2). The extraction and separation are the two major steps in the SFE process. The SFE system consists of an extraction chamber, wherein the tea sample is placed inside along with a supercritical fluid. The tea samples are subjected to a specific temperature and pressure for the extraction of the bio-active compounds. After the extraction process, the fluid-bio-actives mixture passes through the separator wherein the separation takes place. The temperature and pressure are adjusted based on the dissolving power of the bio-active compounds (Raventós et al., 2002). Generally, the co-solvent used in the extraction process has an intermediate volatility between both the supercritical fluid as well as the bio-active compounds to be extracted. This helps in enhancing the solubility of the bio-active compound into the supercritical fluid. SFE has been employed for the removal of caffeine in green tea by avoiding the extraction of antioxidants from the tea matrix (Herrero, Mendiola, Cifuentes, & Ibáñez, 2010).

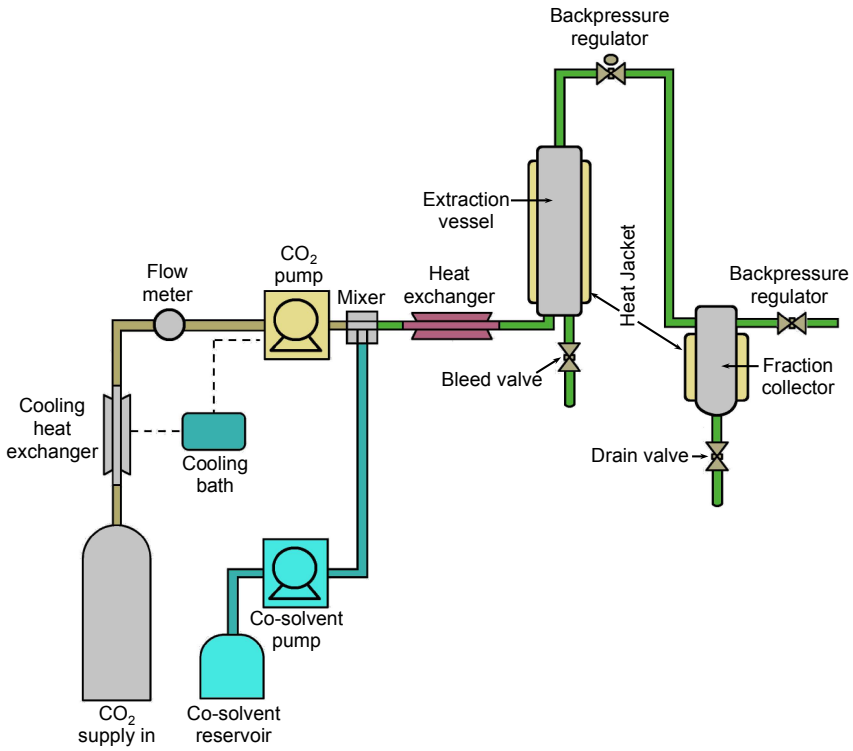

Figure 9 : Graphical illustration of SFE method used to extract bioactive compounds from tea (Retrived from Koubaa et al., 2015)

Hills, Hill, & Maeda (1991) experimented SFE of derivatized and underivatized samples of roasted Japanese tea at 80°C temperature, 405 bar pressure, methanol as solvent, and a flow rate of 450-500 mL/min during 10 min. Silylating agents (e.g. trimethylsilyl) were added after the extraction process to form a complex with the sample. The silylating served as both polar modifier and rivatizing reagent. The Simultaneous supercritical fluid derivatization (SFDE) had a higher extraction yield and efficiency when compared to the SFE. The optimized extraction conditions of safrole (a mutagenic agent) and other allylbenzenes from Sassafras teas (unbrewed) were at 690 bar pressure and 80°C temperature with methanol as co-solvent. The extraction left for 15 min and led to the recovery of 96% to 101% of safrole and other allylbenzenes, respectively (Heikes, 1994).

The SFE was compared to that of steam distillation and was proven to be more accurate and yielded better results within a short period of time. Saldaña, Mohamed, Baer, & Mazzafera (1999) extracted purine alkaloids (e.g. caffeine, theobromine, theophylline) from Mate (*IIex paraguariensis*) leaves using SFE. Caffeine showed a higher selectivity towards CO_2 and also had a retrograde behavior with the temperature when compared to theobromine and theophylline, which demonstrated a normal behavior. The extraction was done for 7 h at 70°C and 255 bars pressure with a flow rate of 0.9 to 1.2 g/min. The extractability was found to be 57%, 68% and 94% for theophylline, theobromine and caffeine from tea, respectively. Following this, in 2001, Wong, Wyllie, Cornwell, & Tronson used SFE to remove the 8 major monoterpenes from *Malaleuca alternifolia* Cheel leaves and the maximum removal was obtained at the optimum conditions of 0.25 g/mL of CO_2, 74 bar pressure, hexane as rinse solvent, for 10 min duration, and at 100°C. The sample matrix of the leaves played a fundamental role in the process and the study concluded that the extraction process would depend on the sample matrix. In 2002, Saldaña, Zetzl, Mohamed, & Brunner continued the study and tried to extracted caffeine from mate tea leaves using SFE and obtained a 98% extraction rate at 70°C, 400 bar pressure, ethanol as co solvent, 400 min extraction time, and at a flow rate of 5.7 g/min of carbon-dioxide. Ethanol, the co-solvent in the process was found to be effective for the extraction of methylxanthines. The application of SFE lowered the amount of solvent required and also enhanced the extraction efficiency of caffeine and methylxanthines. The effects of temperature and pressure were found to be critical for the extraction process, and the time period for the caffeine extraction was found to be shorter.

Kim, Kim, & Oh (2007) and Huang, Wu, Chiu, Lai, & Chang (2007) used SFE to extract caffeine from Korean tea and green tea with 66% extraction rate for Korean tea when the conditions were maintained at 50°C temperature, 400 bar pressure with 20.8% water and a flow rate of 28.08 kg CO_2/kg for 60 min and all these four parameters were considered as the critical part of the extraction process. Huang et al., (2007) studied the extraction of caffeine from green tea at 60°C, 300 bar pressure, using ethanol or water as solvent, for 10 min and at a flow rate of 12 mL/min. The results showed a maximum removal of caffeine (91.5%) and a retention of 80.8% of catechins. Park et al., (2007) applied the SFE at 70°C temperature, 300 bar pressure, 120 min extraction time, and using 95% ethanol for the decaffeination of tea. The type and the concentration of the co-solvent used were the critical parameters for the extraction process and the caffeine content was minimized to 2.6% of the initial concentration of the caffeine that was present in the green teas and 37.8% of ECGC (Epigallocatechingallate) was lost during the process. Similarly, Lee, Park, Kim, & Kim (2007) studied the effect of the SFE at 70°C, 300 bar pressure, ethanol as solvent, during 51 min, and at a flow rate of 1.25 kg of CO_2/min on the extraction of volatile compounds from green tea. The study concluded that the increased amount of caffeine extracted using SFE was accompanied by a decreased amount of volatiles present in the tea.

A year later, Kim, Kim, Kim, Oh, & Lee (2008) performed a selective extraction of caffeine and EGCG with SFE using water as co-solvent, at 40°C, 400 bar pressure, during 300 min, and using a flow rate of 28.08 kg of CO_2/h. The optimization of the parameters was based on the maximum extraction of caffeine (54%) and EGCG (21%) from the tea. Water was experimentally proved to be the best solvent for the selective extraction of

caffeine from the green teas. In addition, the selectivity was equal to 0.88, which was higher than that obtained with ethanol (0.24).

Cassel et al., (2010) used SFE to extract alkaloids from *Ilex paraguariensis* St. Hil leaves at different temperatures and pressures. The optimum conditions for maximum extraction of caffeine using SFE were as follows: 50°C temperature, 150 bar pressure, and using methanol as solvent. The study concluded that the SFE was efficient for the extraction of caffeine and theobromine and not a better method for the other polyphenolics from tea. SFE was used as a decaffeination method of green teas by Park, Im, & Kim (2012) and the process parameters (temperature, pressure, concentration of the solvent (ethanol)) were optimized using response surface methodology (RSM) for the maximum efficiency of the extraction process with a constant flow rate of CO_2. The optimized conditions were a temperature of 63°C, a pressure of 23 MPa, a 95% ethanol concentration/100 g of CO_2, and a duration of 120 min/10 g of green tea. The extraction rate was 36.1% for caffeine and 40.6% for catechins. Some amount of chlorophyll was also co-extracted along with caffeine, but the research concluded that further processing needs to be done in order to recover the remaining chlorophyll, which leads to improve the quality of the decaffeinated tea obtained. Contradictory to Cassel et al., (2010), Z. Chen et al., (2014) used SFE as a method to extract phenolic compounds (e.g. total phenols, flavonoids) from the tea. The RSM, Box- Behnken and Derringers desired function methodology design were used for the optimization extraction parameters. The study showed that the pressure and the co-solvents played a major role in the extraction process. The optimized conditions were as follows: 880 bar pressure, 50°C temperature, and 2.94 g/min flow rate of CO_2. The maximum of phenolics obtained was 131.24 mg GAE/100 mL, the total flavonoids was

194.60 mg QE/100 mL, the tannin content was 49.99 mg TAE/100 mL, and the total antioxidant activity was 262.23 μmol TEAC/100 mL. The most recent approach developed by Gadkari & Balaraman (2015) demonstrates the use of SFE for the decaffeination process in green tea and tests the solubility of caffeine with ethanol as a co-solvent at 50°C, 250 bar pressure, and 540 min extraction time resulted in a solubility range from 44.19 x 10^{-6} to 149.55 x 10^{-6}. As compared with the pure caffeine (61 times higher) the solubility of the extracted caffeine was found to be lower.

The temperature and pressure of the SFE system are generally the critical parameters. However, the density of the fluid and the solubility of the solute depend on the pressure applied to the system. The enhanced pressure beyond a certain limit will reduce the diffusivity of the solvent and results in decreased contact with the pores of the sample. Therefore, reducing the potential of solute dissolution creates a hindrance in the extraction process and can lead to negative extraction results. On the other hand, the temperature also confers more energy to the system, increases the diffusivity and the apparent volume of the solvent, and reduces the density and the solvent power. However, a decrease in the temperature increases the density and the solvation of the solutes. This particular effect is called a crossover effect, where the high temperatures result in lower yields, and lower temperatures provide higher extraction yields. Thus, an increase in kinetic energy resulting from an enhanced temperature is directly proportional to the rate of diffusion of CO_2 (Khaw, Parat, Shaw, & Falconer, 2017). The removal of solvent from the system also depends on several factors such as the solubility of the solute, the interactions of the solute-solid matrix, the localization of the solute in the matrix and its porosity. The success of the extraction depends on the selection of the conditions that are able to enhance the extraction

of the desirable compounds by regulating the solvation power, avoiding the influence of other materials, and reducing the co-extraction of other impurities (Pereira & Meireles, 2010).

The nature of the supercritical fluids like CO_2 is the rate-determining step of the SFE process. The bio-active compounds of interest such as polyphenols and alkaloids are less soluble in carbon dioxide used and hence increasing the pressure improves the solubility of the solutes. Modifiers are generally used to increase the solubility of polar compounds. SFE has an increased efficiency of extraction with the tenability of the solvent strength along with the preservation of organoleptic properties of the bio-active extracts. The method has the large number of variables for the optimization and a very strong interaction of the matrix and the bio-active compounds (Khaw et al., 2017). Table 4 provides a brief summary of the extraction process of SFE in the extraction of targeted bio-actives in tea over the years.

3.4.5 Pressured liquid extraction (PLE)

Pressurized liquid extraction process (PLE), one among several evolving processing extraction methods, makes the use of an organic solvent at an elevated temperature and pressure applied to a highly polar sample for the extraction of bio-active compounds (Zhao, Deng, Chen, & Li, 2013). Due to the high temperature used, the structural bonds in the bio-active compound weaken resulting in the rapid extraction of the selective bio-active compounds from the tea matrix (Piñeiro, Palma, & Barroso, 2004). The critical factors contributing to the extraction process include the high solubility of the bio-active compounds in the solvent and the high diffusion rate caused due to the weakening of the

bonds (Jacques, Dariva, de Oliveira, & Caramão, 2008). Accelerated solvent extraction (ASE®) technology, pressurized solvent extraction (PSE), pressurized hot-water extraction (PHWE) and sub-critical water extraction (SCWE), and/or superheated water extraction (SHWE) (when water is used as an extraction solvent), are different forms of PLE used for the extraction process (Mustafa & Turner, 2011).

The oxygen and light-sensitive compounds are protected due to the equipment setup making it an excellent alternative to traditional extraction methods. This extraction process has been thoroughly explained by comprehensive studies (Camel, 2001; Tura & Robards, 2002; Mustafa & Turner, 2011), where the researches have reported that the efficiency of the extraction depends on the natures of the matrix and the compound, as well as the location of the targeted bio-active compound inside the matrix (Pawliszyn, 2003; Mustafa & Turner, 2011). Pawliszyn (2003) assumed in his study that the solvent used forms a layer around the porous heterogeneous sample which enhances desorption of the bio-active compounds from the matrix site of the tea leaves. The bio-active compounds diffuse then into the organic solvent and finally get distributed into the extraction phase. They reach then the section where the phase is affected by convection and thereby the target bio-active compound is collected from the tea leaves. In this case, the solubility and the diffusion steps are the rate limiting steps in the process (Gogus & Ozel, 2004; Ong, Cheong, & Goh, 2006; Mustafa & Turner, 2011). The PLE not only had the highest recovery rate of target functional constituents but also possessed the maximum degree of accuracy due to automation (Zhao et al., 2013). PLE is normally used for the extraction and isolation of caffeine and catechins from tea. Furthermore, it was proved as the best extraction method for decaffeination without the extraction of other compounds like catechins.

In 2004, Piñeiro, Palma, & Barroso (2004) extracted catechins and epicatechins from tea leaves (non-fermented tea, fermented tea, and black tea) and grape seeds using different extraction methods (magnetic stirring, ultrasound-assisted extraction, and PLE), and pure solvents (water, methanol, ethanol, and ethyl acetate). When compared to the extractions based on their recoveries, PLE proved to have a highest recovery of 3.21% for catechin and 2.96% for epicatechin within 10 min of extraction. The stability of the catechins was unaffected at high temperature range (100-200°C) and pressure (100 atm). Methanol was the best pure solvent used for the extraction process. Thereby, the efficiency of extraction of catechins and epicatechins with PLE was found to be higher (95%).

Following this study, in 2005, Dawidowicz & Wianowska performed various experiments to investigate whether multiple extractions or a single step extraction was required for the complete recovery of the bio-active compounds from the plant material, as PLE was considered as the most effective way of preparation of the samples. A single step PLE was successfully used to save time for the tea sample with water as a solvent at 60 bar pressure, 100°C temperature, and for 10 min of PLE extraction. This process was considered as physio-chemically analogous to liquid-liquid extraction. The experiments were performed with 4 different samples (rutin in *Sambucus nigra L. flowers*, caffeine in green tea, black teas, and coffee beans), and the caffeine content was analyzed using HPLC. An efficient removal of the bio-active compounds from the matrix at high temperatures was recorded. Another experimental approach by Dawidowicz & Wianowska (2005a) consisted in the isolation and the removal of caffeine from green tea leaves with different methods of sample preparation (e.g. infusion, MAE, matrix solid phase dispersion, and PLE) using 0.5 g of sample, neutral glass-dispersion agent, 70°C temperature, 40 bar

pressure, 3 cycles, and during 10 min of extraction. The study showed that the PLE significantly reduced the efficiency of the isolation of caffeine in comparison with other methods of sample preparation due to the high pressure applied in the process. The study records that PLE was regarded as the best method for the extraction of caffeine from coffee but concludes that the PLE doesn't hold good for tea samples due to the difference in the properties of the matrices. The high temperature and pressure used in the process squeeze out the soft matrix in the tea leaves, thus making the diffusion of caffeine from the inside to the outside difficult and hindering the penetration of the solvent into the matrix. The results also suggested that higher number of extraction cycles, higher temperature, and other specified parameters were required for the isolation of caffeine from tea, which was found to be an expensive process when compared to other methods. However, Bermejo, Mendiola, Ibáñez, Reglero, & Fornari (2015) demonstrated to extract caffeine without extracting catechins using ethyl lactate as a solvent. They concluded that ethyl lactate was the best green solvent used to isolate caffeine from green coffee beans and green tea leaves. They were able to reduce the co-extraction of catechins, which are considered as highly functional bio-actives present in teas. The study concluded this based on the solubility of caffeine, which was higher in the mixtures of ethyl lactate and water (25:75%), at the optimum pressure and temperature applied. When water and ethyl lactate were used as separate extraction solvents, the yields were found to be 3.5 times and 1.5 times higher, respectively. The PLE was carried out for 20 min, at 100-200ºC temperature, in presence of 1 g of sea sand, and using different solvents (water, ethyl lactate, water + ethyl lactate (different ratios). The recovery of the key bio-active compounds was compared and studied using HPLC for the identification of the different catechin molecules and caffeine present

in 1 g of tea sample. This led to the recovery of caffeine in the range of 53-76%, and only the removal of 26-36% of catechins from the tea leaves. This study also claimed that the solvent used had a higher recovery potential than the work reported by Perva-Uzunalić et al., (2006), where other solvents were used.

In addition, Villanueva Bermejo et al., (2015) developed a method to extract lower amounts of caffeine and selective precipitation of catechins from green tea leaves, using green solvents (ethyl lactate and ethanol). The PLE was carried out using ethyl lactate and ethanol as solvents, at 100°C, 98 atm pressure, and 20 min extraction time. The influence of these factors was studied experimentally with regards to the catechins precipitation yield, the key bio-active compounds extracted, and the total phenolic content. The results showed that the decaffeination process was excellent when ethyl lactate was used as solvent. In fact, the precipitate had more than 1% of caffeine and 23% of catechins with high total phenolic content (590 mg of GA equivalence). The study concluded that the ethyl lactate served as the best solvent precipitating 2.3 times more than the amount obtained using ethanol. The combination of technologies proved to be the best one for the selective precipitation of catechins. The final reduction of caffeine content in the extract was calculated to be 93%.

Jacques et al. (2006) studied the chemical composition of mate leaves using different extraction techniques (PLE, maceration, sonication) and different solvents (*n*-hexane, toluene, dichloromethane, ethyl acetate, acetone, and methanol). The analysis of some compounds such as palmitic acid, phytol, stearic acid, squalene, and vitamin E showed no significant differences in the extraction methods, however, PLE (100°C, 102 atm pressure, methanol as solvent, 10 min extraction time, and 7.5 g of sample) resulted in minimized

extraction time and amount of solvent, and provided the highest mass yield in comparison to the other methods. The elevated temperature used in the method led to the extraction of more polar compounds, with methanol as the best extraction solvent. A factorial experimental design performed by Jacques, Dariva, de Oliveira, & Caramão (2008) investigated the influence of the various independent variables (time of extraction, amount of solvent used, polarity of the solvent, number of PLE cycles used, amount of solvent, flushing volume of the solvent and temperature used for extraction) on the extraction of mate tea leaves (*Ilex paraguariensis*) using PLE. Out of the seven parameters taken into account, the polarity of the solvent was found to have the highest effect on the extraction process followed by the amount of the sample used and the extraction temperature. The PLE was carried out at the following experimental conditions: methanol/hexanol as solvent, 10 min of extraction, 100°C temperature, 7.5 g of tea sample, and 100% of solvent flushing. The pressure was kept constant throughout the entire study at 1500 psi. Higher extraction efficiency was obtained when using the highest values for the solvent polarity, extraction temperature, amount of the sample, and solvent flushing. The optimum conditions for PLE in the entire study were as follow: for methanol (7.5 g of sample, 100 mL of solvent flushing, 10 min, 100°C, and 1 cycle), and for hexane (2.5 g of sample, 100 mL of solvent flushing, 10 min, 100°C, and 1 cycle). Quantification of the important bio-actives including caffeine, phytol, squalene, and vitamin E were characterized using GC-MS along with 37 other chemical compounds. Caffeine and palmitic acid were reported to be the more abundant among the other analyzed molecules in the study.

A review performed by Mustafa & Turner (2011) suggested how the PLE method (200°C, 35-200 atm, simple alcohols as solvent, 5-15 min of extraction time) was employed

for herbal plants and food in order to enrich the extracts in bio-active compounds such as phenolics, ligands, carotenoids, oils, lipids, essential oils, and nutraceuticals. The authors concluded that PLE was a very promising extraction method, compared to traditional ones, with many advantages that include the protection of the herbal plants against the oxidation and light. This review also confirmed the use of PLE to extract carotenoids from herbal teas other than phenols and caffeine.

Similarly, Zhao, Deng, Chen, & Li (2013) summarized the recent developments in herbal tea along with the phytochemical analysis of herbal teas in China. The process parameters for PLE fall in between the range of 500-3000 psi, 5-10 min, and 50-200°C. The photochemical analysis using PLE has a greater advantage of shortening the extraction time (5 min for the extraction of flavonoids, catechins, chlorogenic acid and epicatechin from Eagle tea (*Litsea coreana*) when it takes around 8 h with other methods). The authors also concluded that PLE had the highest recovery and the highest efficiency since it is highly automated. The accuracy of the process results was higher with a limitation of thermal degradation of the food product.

A more recent study conducted by Alkhateeb & Thurbide (2015) using simple micro PLE was tested for the removal of the caffeine from tea and pharmaceutical samples with minimum amount of sample and reduced amount of solvent. The 5-10 mg of tea samples was subjected to PLE at 275°C, 150 atm pressure, methanol as solvent, and for 20 s. The results obtained were compared with conventional PLE technique. The authors demonstrated that micro PLE was better and faster with lower amount of sample and solvent used, when compared with the conventional method. They also proved that it can provide rapid extraction results for difficult samples.

In addition to extraction of phytochemicals from tea, research work also focused on detecting pesticide residues. In 2008, Cho et al., tested the green tea samples for 14 types of pesticide residues (flufenoxuron, fenitrothion, chlorfluazuron, chlorpyrifos, hexythiazox, methidathion, chlorfenapyr, tebuconazole, EPN(O-ethyl-O-4 nitrophenyl phenulphosphotionate), bifenthrin, cyhalothrin, spirodiclofen, difenoconazole, and azoxystrobin) with different extraction methods such as PLE (100°C,102 atm, *n*-hexane, 5 min extraction time, two cycles, 1500 psi, and 60% flush volume), and liquid-liquid extraction. The study reported that bifenthrin was the only pesticide present in the tea samples and the research concluded that PLE could be used for the regular detection of pesticide residues present in the fruit and vegetable matrices as a faster and simpler method of extraction.

The temperature in PLE affects both the efficiency and the sensitivity of detection of the targeted bio-active compounds (Mustafa & Turner, 2011). Higher temperatures improve the efficiency of the extraction by the disruption of the bonds and helps to overcome the cohesive and adhesive interactions, thus lowering the activation energy required for the desorption process. It also decreases the surface tension by altering the wettability and the solubility of the sample (Mustafa & Turner, 2011). Increasing the temperature might affect extract additional bio-active compounds resulting in decreased selectivity.

Another important factor is the elevated pressure used in the process as it affects the boiling point of the solvent. In addition, the pressure exerted on the matrix results in the cell disruption, which enhances the mass transfer rate (Mustafa & Turner, 2011). The elevated pressure also helps in controlling the problems related to the bubbles found within

the matrix that hinder the solvent from reaching the bio-active compound and also boost the solubility and the desorption kinetics of the bio-active compounds (Mustafa & Turner, 2011). The results of PLE application under different processing conditions to extract specific bio-active compounds from various species of tea are shown in Table 5.

3.5. Innovative processing technologies: Advantages and drawbacks

Studies on the extraction of bio-active compounds like catechins, caffeine, and other flavonoids, and polyphenol compounds from tea have been recently carried out successfully using UAE and the extraction efficiency has been found to be relatively higher at lower temperatures. The most critical parameters affecting the extraction in UAE includes sonication power, frequency, solvent to solid ratio, temperature, and sonication time (Mason & Yiyun Zhao, 1994; Zeković et al., 2017). One of the limitations of using UAE, is when the sample is exposed to UAE for a longer period of time, it can significantly affect the process since it generates heat energy, which leads to the decomposition of the thermo-sensitive compounds. Thus, selecting an appropriate sonication time has an important role in the extraction process (Qazimi, Karapandzova, Stefkov, & Kulevanova, 2010). On the other hand, MAE is considered as a rapid alternative method, which couples microwave heating with chemical extraction techniques. MAE is most commonly employed for the extraction of polyphenols and flavonoids as well as a pre-treatment wherein the time and temperature play the most influential role in the process. The major advantage of using MAE would help in extracting healthy-functional constituents from tea by-products and results in higher extraction yield with shorter time, lower energy consumption, and higher extraction selectivity.

PEF serves as an effective method that is more specific for the extraction of intercellular compounds like polyphenols without the use of heat and pressure. The effectiveness of the process depends on major factors such as the intensity of electric field, pulse wave shape, selection of solvent, ratio of solute to solvent, duration of the pulse, and the temperature of the treatment. According to the studies presented by the authors, the PEF was clearly used as a method for the inactivation of the microorganisms present in the tea. The mechanism of electroporation also helped in the extraction of polyphenols to a greater extent with the optimized conditions. With respect to SFE, it was a more specific method mainly for the extraction of caffeine from tea. The critical parameters in SFE include the temperature and pressure, the flow rate of CO_2 and also the matrix composition of tea leaves. However, this method helped in the highest retention of the catechins in the cells rather than their extraction.

PLE is a process that can rapidly extract the targeted bio-active compounds, as there is a high chance of improved wetting of the molecules present inside the matrix by the organic solvent. The diffusion rate of the solute from the matrix is also increased (mass transfer) due to the breakdown of the bonds between the matrix and the bio-active compounds. The temperature and the pressure play an influential role in the extraction process. The higher temperature and pressure improve the solubility of the targeted bio-active compound, as there is a reduction in the viscosity of the organic solvent. The major drawback of the process is the initial expensive installation and setup. In addition, the method uses high temperature, thus making it unsuitable for the thermo-sensitive compounds. With all the conventional and traditional techniques used for the extraction process from tea, it is obvious that the novel processing technologies provide better results owing to the

technological advancements. However, each method involves different advantages, which are specific to the method and also have fewer limitations with respect to the process. The review on the whole had a broader view of the novel methods used in the extraction of bio-active compounds from tea. However, the study is not sufficient enough to draw any conclusion regarding the extraction process, as there are many variables such as the type of sample, the experimental conditions, the human error, and many other parameters playing a major role in the extraction process.

However, taking into account the review of all the methods, it is quite clear that each method of extraction is advantages in specific ways. Assuming that PEF as a method of extraction the major. PEF is a short, easy, and immediate extraction method, but pertaining to the fact that it can increase the extraction efficiency to an extent, it mainly helps to maintain bioactive increase the color, lesser processing, and increased storage. Most of the conditions used fall within the range of 20 to 40kV/cm field with a temperature range as low as 15-20°C. The pulses are given with a pulse width of 2 μs with 100 -200 μs pps and frequency as low as 120-125 Hz. The extraction efficiency of PEF falls only in the range of 27 to 32%. However, this can be a promising method if there is an assumption of using it in a large scale where the preservation and storage play as critical elements in the product formulations as it can help to preserve the color of the product and the bioactive without affecting the properties and does not involve the usage of organic solvents. Microwave-assisted extraction and Ultrasound-assisted extraction are both found to be necessary for the extraction of phytochemicals with higher temperatures and lower extraction time and latter lower temperatures and lower extraction time.

The PLE can be a method of extraction when the focus is given mainly for the extraction of caffeine and catechins from tea with a minimum extraction of 5 to 15 mins. The method, however, uses different organic solvents for the extraction process like water, ethanol, methanol, ethyl lactate, and simple alcohols and involve high-temperature extraction at 100 to 200°C and 3 to 20 MPa. It helps in faster extraction of bioactive like caffeine and catechins. Cost of reagents and pressure preparations are expensive to be used on a large scale as an industry. Similarly, SFE is regarded as a method for the extraction of phytochemicals, and the recovery and extraction rate is found to be more than 90%. This method of extraction is found to be better with the use of organic solvents. However, the extraction takes a very long time to a maximum from 540 min with a temperature range of 50 to 100°C. Overall, the best method of extraction with water as a solvent with minimum extraction time, higher efficiency of extraction can be subjective to two methods microwave-assisted and ultrasound-assisted extraction. On the other hand, if the preservation and storage are PEF is the best method of extraction and PLE for extraction of catechins and caffeine. SFE is comparatively best in comparison to UAE and MAE with maximum extraction of 90% but relatively takes a longer time of extraction with the usage of organic solvents. More extensive studies should be directed toward the extraction techniques, and also comparative studies with other novel extraction technologies need to be carried out from the quantity and quality viewpoints.

3.6. Conclusions and future directions

Over the two last decades, novel innovative processing technologies (e.g. UAE MAE, PEF, SCF, and PLE) are being used as an alternative technology for conventional extraction methods due to their high efficiency and the effectiveness in extracting bio-active compounds from various plant, vegetable, and agricultural residues. Several studies discussed in the present review have highlighted on the application of novel technologies on various types of teas and their by-products. The application of these technologies is better in performance over conventional solvent extraction techniques in terms of extraction time and temperature, amount of used solvents and the extraction efficiencies. Moreover, food-processing industries are taking sustainable initiatives to fully utilize the by-products that are traditionally considered to be an environmental issue. In this regard, the application of novel technologies for the extraction of bio-active compounds from tea by-products would not only provide a sustainable solution for tea industries but also generate value-added functional ingredients that have a commercial value. Additionally, novel-processing technologies might be used as tools to tailor foods with added or enhanced functional and nutritional values, which lowers the carbon footprint and substantially reduces the water volumes used in industrial heat transfer processes.

The biggest drawback for the application of novel technologies is the consumer acceptance, investments, and also the method reproducibility. During the extraction process, the food matrices are subjected to various combinations of pressure, time and temperature as the main parameters involved in the extraction technique. Improper application of process parameters can strongly initiate Maillard reactions, leading to the formation of carcinogenic substances. Hence, every food sample needs to be studied

uniquely and the process variables should be optimized. Also, the functionality of the bio-active compounds extracted using various novel techniques must be examined before the commercial approval.

In brief, the use of novel technologies ultimately produces higher-quality foods due to the reduced abuse of thermal treatments and chemical agents with higher safety attributes during the extended shelf life with a reasonable cost to be industrialized. Even though high investments are generally required to carry out-tailor made research by industries on these novel technologies, the results of fundamental research are very promising.

Table 1: The quality improvement of tea extracts and the retention enhancement of different types of bio-active compounds obtained from tea varieties under various UAE conditions

Sample	Extraction conditions	Extracted compounds	Key note (s)	Reference
Tea	60°C, 10 min, 20 kHz, water	Tea solids	- Higher extraction efficiency up to 40% after 10 min of sonication. - Improved extraction efficiency at lower temperatures.	(Mason & Zhao, 1994)
Tea infusions	60°C, 40 min, 40 kHz, 250 W, water	Polyphenols, amino acid and caffeine	- Better extraction yield of the chemical compounds aroma compounds and other glycosidic precursors at lower temperature. - Better sensory quality attributes of the UAE-extracted tea compared to the conventional extraction method.	(Xia et al., 2006)
Matte tea leaves	75°C, 180 min, 40 kHz, 90 W, hexane and ethanol	Caffeine, phytol, and palmitic and stearic acids	- No significant differences in quality of the extracts obtained from the different extraction methods.	(Assis Jacques et al., 2006)

Green tea leaves	28°C, 30 min, 25.1 kHz, water	Catechins	- UAE: an effective method for increasing the extraction yield of catechin from green teas at low temperatures. - The ultrasonic power applied was the most main parameter affecting on the catechins extraction.	(Koiwai & Masuzawa, 2007)
Green tea	90 min, water, acetone and ethanol	Catechins and caffeine	Significantly improved extraction yield.	(Saito et al., 2007)
Green tea, and Black tea	40°C, 10 min, 35 kHz, methanol, water, acetonitrile	Catechins (EGC, +C, EC, ECGC, and ECG), and caffeine	- Dynamic UAE: increasing the extraction efficiency, decreasing the extraction time and used solvent amount. - A significant reduction in the rate of oxidation and hydrolysis of the analysts.	(Xungang, Jibao, Zhengzhu & Zhang, 2007)
Tea	50°C, 20 s, 20 kHz, water	Tannic acid	- The best extraction solvent was methanol. - The highest extraction yield.	(Sonawane & Patil, 2008)
Tea seeds	30°C, 30 min, 24 kHz, 50 W, *n*-hexane	Oil	- A shorter time for the oil extraction with the minimal solvent usage. - A substantial increase in oil extraction yield (46.23-85.21%) with an increase of the ultrasonic power (10-50 W) and a decrease of the temperature.	(Shalmashi , 2009)
Green tea	45°C, 60 min, 37 kHz, 95 W, ethanol	Flavonoids	The high process repeatability with achieving the highest amount of extracted polyphenols.	(Naşcu-Briciu et al., 2011)
Yellow tea	38°C, 30 min, 20	Antioxidants (e.g., flavonoids,	The maximum extraction yield of polyphenols and methylxanthines from	(Horžić et al., 2012)

	kHz, 200 W, ethanol	non-flavonoids, Polyphenolics, and methylxanthines)	yellow tea using the ultrasound probe in presence of ethanol (75%) as solvent.	
Black tea, Green tea, Oolong tea and White tea	32°C, 21 min, methanol	42 volatile compounds, and caffeine	Better release of volatiles from the plant matrix at lower temperatures.	(Sereshti et al., 2013)
Green tea infusions	60°C, 15 min, water	Catechin (EGCG)	Increased extraction yield of EGCG by 15% with the highest oxidative stability.	(Lante & Friso, 2013)
Black tea, Green tea, Oolong tea and White tea	3 min, methanol and water	Theophylline, theobromine, and caffeine	- A simple, low-cost and eco-friendly procedure to isolate polar and hydrophilic molecular species from the aqueous solutions. - The high desorption of analysts with low volume of the organic solvent.	(Sereshti et al., 2014)
Green tea leaves	40°C, 120 min, ethanol	Caffeine, and catechins	- The high recovery of catechins in presence of ethyl acetate / dichloromethane. - More extraction efficiency than other methods based on the process time and the productivity rate. - The used low temperature decreased the process time and prevented the epimerization of catechins caused by extraction at high temperatures. - Better recovery of catechins from the green tea extracts by organic solvents.	(Choung et al., 2014)

Matte leaves	16°C, 47 kHz, water	Soluble matter	- The enhanced extraction yield (≈74%) at the reduced extraction times.	(Kotovicz et al., 2014)
Black tea	40°C, 1440 min, 25 kHz, 150 W, methanol and water	Polyphenols	- A higher quasi equilibrium concentrations in the liquid phase by the ultrasonic intensification process. - Increasing the amount of polyphenols extracted by 15%. - No help of the ultrasound in the replacement of the water amount in the solvent. - Ultrasound assisted in the extraction of prechosen and optimized the solvent amount. - Increasing the polyphenols content by 30-35%	(Both, Chemat, & Strube, 2014)
Tea (*Hedyotis diffusa* and *Hedyotis corymbosa*)	40°C, 15 min, 40 kHz, 185 W, water	Triterpenic acids (e.g., oleanolic, and ursolic acids)	USC-CO_2 (Ultrasound assisted supercritical carbon dioxide extraction) was higher than $SCCO_2$ due to the higher extraction yield (up to 15-16%), and lower extraction time (95 min), with the minimum of solvent.	(Wei & Yang, 2015)
Java tea	30 min, 20 kHz, 300 W	Bio-active compounds (phenolic, flavonoids)	A considerable yield (86-95%) in extracting bio-active compounds compared to the conventional Soxhlet method.	(Lam et al., 2016)
Vine tea (*Ampelopsis grossedentata*)	30°C, 31.98 min, 40 kHz, 200 W, Methanol	Flavonoids, dihydromyricetin, myricitrin, and myricetin	A considerable increase in extraction yield of bio-actives.	(Zhang et al., 2016)

Mountain tea (*Sideritis* spp.)	40°C, 30 min, 40 kHz, petroleum ether	Volatile compounds	- The quantity and quality increase bio-active compounds extracted. - Determination of the optimal method for analyzing volatiles from mountain tea and even other aromatic plants.	(Dimaki et al., 2017)
Green tea	65°C, 57 min, 28 kHz, 150 W, water	Caffeine and catechins	An increased extraction efficiency (85%), phenolics (96±6 mg gallic acid/g of DW), and antioxidant activity (EC_{50} value for DPPH inhibition = 66 mg/g) the bio-actives obtained from green tea.	(Ghasemzadeh-Mohammadi et al., 2017)
Herbal tea (*Coreopsis tinctoria*)	25°C, 30 min, 500 W, water	Saponins	- Extraction yield of saponins (33.4 g/kg).	(Luo et al., 2018)
Green tea leaves	80°C, 30 min, 500 W, water	Catechins	- Improved extraction efficiency of catechins from green tea in the presence of BGG-4 (betaine, glycerol and D (+) glucose). - More stability of catechins in DES (deep eutectic solvents) extracts compared to the other solvents used.	(Jeong et al., 2017)
Sage herbal by products of filter tea factory	75.4°C, 80 min, 40 kHz, 42.5 W, ethanol	Phenolics and flavonoids	- The most important extraction parameters were the temperature and the ethanol concentration. - More extraction yield of antioxidants from sage using the UAE method compared to traditional extraction methods.	(Zeković et al., 2017)

*1-Temperature(°C), 2-Extraction time(minutes), 3-Frequency(kHz), 4-Power(W), 5-Solvent

Table 2: A summary on the MAE conditions and advantages to extract bio-active compounds from different species of tea

Samples	Extraction conditions	Extracted compounds	Conclusions from the study	Reference
Green tea	Ethanol, 4 min, 700 W, 20:1, 90°C	Polyphenols, and caffeine	- Faster extraction with higher extraction yield than other conventional extraction methods.	(Pan, Niu, & Liu, 2003a)
Tea	Water, 65 min, 150 W, 100°C	Flavonoids TEC (tea epicatechins), TCD (total catechins derivatives), and TTP (total tea polyphenols)	- Maximum extraction yield within a short period of time.	(Sultana et al., 2008)
Green tea	Water, 60 min, 600 W, 20:1, 80°C	Polyphenols	- Suitable for producing tea extracts rich in antioxidants, flavanols and polyphenols, with the highest concentration of EGCG (epigallocatechin gallate) and antioxidant activity. - Shorter extraction time with a notable reduction in the energy consumption.	(Nkhili et al., 2009)
Black tea	Water, 600 W, 100:1	Polyphenols	Higher recovery rates of phenolic compounds compared to the normal brewing techniques without any negative effect on the tea's	(Spigno & De Faveri, 2009)

			antioxidant potential.	
Green tea, Oolong tea, and Black tea	Water, 2 min,1000 W, 20:1, 230°C	Phenolics (e.g., pyrogallol, catechol, dihydroconifery l alcohol, and vanillin)	- The high extraction yield of green tea with 24.6% pyrogallol. - The good extraction yield of oolong tea extract-with 10.3% dihydroconiferyl alcohol and 8.1% of vanillin. - A rapid extraction for tea phenols only within 2 min.	(Tsubaki, Sakamoto, & Azuma, 2010)
Black tea, and Green tea	Water, 450 W, 30:1, 70°C	Polyphenols	-Higher concentration of total polyphenols (26%) in green tea than that of black tea (16%) - MAE: an effective low-energy, and time-saving method for obtaining extracts rich in phenolic compounds with strong free-radical scavenging activities, from both teas.	(Dominiqu e Savio Nshimiyim ana, 2010)
Green tea	Water, 1 min, 400 W, 50:1	Catechins, and epicatechins	- A good recovery procedure for catechins (118%) and epicatechin (120%). - A simple, faster, and reliable technique for the catechin extraction from green tea.	(Li et al., 2010)

Green tea (decaffeinated)	Water, 3 min, 600 W, 20:1, 68°C	Polyphenols	- The extraction efficiency and polyphenols content were highly affected by the microwave irradiation time.	(Li & Jiang, 2010)
Tea	Ethanol, 10 min, 600 W, 12:1, 80°C	Polyphenols	- High extraction yield of polyphenols by 96.5%. - The extraction time was saved more than 8 and 5 times compared with HRE (Heat reflux extraction), UAE (Ultrasound assisted extraction). - Lower energy consumption and higher extraction selectivity compared to the other extraction methods studied.	(Wang, Qin, & Hu, 2010)
Tea	Ethanol, 4 min, 70 W, 100:1, 80°C	Caffeine	- High recovery rate of caffeine in the tea samples (88.2-99.3%). -The caffeine yield using DMAE (dynamic microwave-assisted extraction) (47 mg/g) was higher than SAME (static microwave-assisted extraction) (37 mg/g) with a reduced volume of organic solvent and reduced time required for the preparation.	(Wang et al., 2011)

Green tea, black tea, and Oolong tea	Water, ethanol, acetonitrile, 6 min, 600 W, 50:1, 80°C	8 catechins monomers, and caffeine	- Higher amounts caffeine and catechins (mainly EGCG) respectively in black and green teas - The increased extraction yield of catechins and caffeine with increasing the irradiation time by 6 min	(Rahim et al., 2014)
Tea camellia seed cake	Ethanol, 4 min, 400 W, 10:1, 60°C	Saponins	- Notable reduction of extraction time from 6 h to 4 min. - Enhanced extraction yield by 14% with a significant decrease (up to 50%) in the consumption of organic solvent.	(He et al., 2014)
Mulberry tea	Water, 11.41 min, 602.28 W, 80:1	1-deoxynojitimycin	MAE was more convenient than the extraction method by hot water immersion.	(Liu et al., 2014)
Tea	Ethanol, 3 min, 500 W, 100:1, 80°C	Polyphenols	The best method for the extraction of tea polyphenols.	(Bekdeşer et al., 2014)
Tea (*C. morifolium*)	Water, 5 min, 400 W, 20:1, 80°C	13 major bio-active compounds	- A reliable method to prepare samples for the extraction processes. - Chemical characteristics of different parts of the plant.	(Lam et al., 2016)

By product of filter tea factory- wild apple extract	Ethanol, 18.7 min, 600 W, 20:1	Polyphenols	- The ethanol concentration was the most influential parameter to extract phenols. - An attention on the reduction of the extraction time and the irradiation power to decrease/prevent the degradation of polyphenols.	(Pavlić et al., 2017)
Tea (Iranian green tea)	Ethanol, 7.8 min, 190 W, 40:1, 110°C	Caffeine and catechins	- The high extraction efficiency (95%) with a high total phenolic content (125±5 g of gallic acid/g DW). - The key role of temperature in the extraction of caffeine and catechins. - The best technique for the extraction of polyphenols with a yield of 90%.	(Ghasemzadeh-Mohammadi et al., 2017)
Sage herbal dust from filter tea factory	Ethanol, 18.7 min, 600 W, 40:1	Phenols and flavonoids	- A better recovery process for sage polyphenols compared to the conventional traditional methods. - Sage herbal dust from filter tea factory: A raw material for the extraction of polyphenolic compounds.	(Zeković et al., 2017)

	- The critical role of ethanol concentration in the efficiency of extraction process.

*1- Solvent, 2-Extraction time (minutes), 3-Power (W), 4-Solvent to solid ratio, 5-Temperature (°C)

Table 3 : Analysis of results of application of PEF in extracting different bio-active compounds from diverse varieties of teai

Sample	Extraction conditions*	Extracted compounds	Conclusions from the study	Reference
Green tea	38.4 kV/cm, 20°C, 160 µs, 667 pps, 2 µs	Polyphenols and free amino acids	- A promising technology to maintain the quality of the bio-active compounds and the color of green tea. - The inactivation of microorganisms (e.g., *Escherichia coli and Staphylococcus aureus*). - A synergistic effect in the reduction of the microorganisms by the low-temperature storage and antibacterial property of polyphenols extracted by the PEF method.	(Zhao et al., 2008)
Green tea	20-40 kV/cm, 121°C, 50-200 µs, 667 pps, 2µs	Polyphenols, catechins, and free amino acids	High retention of color and bio-active compounds.	(Wang et al., 2008)
Green tea infusions	38.4 kV/cm, 37°C, 200 µs, 667 Hz, 2 µs	NR	- A more shelf life period than 90 days (at 37 °C) for the infusions obtained by the PEF. - The increased growth of microorganisms under the storage at 25 and 37 °C. - No existence of any viable microorganisms immediately after the PEF treatment.	(Zhao, Yang, & Wang, 2009)
Green tea infusions	20 to 40 kV/cm, 5-15°C, 200 µs, 667 pps, 2 µs	Catechins, polyphenols, and free amino acids	- The increased content of amino acids (specifically theanine) by 7.5% at 40 kV/cm with the loss of volatiles (≈10%).	(Zhao, Yang, Wang, et al., 2009)

			- Efficient retention of the bio-active compounds and the color with the increased amino acid content.	
Tea	0.9 kV/cm, 5°C, 5 ×10^5 µs, 5 × 10^4 µs	Polyphenols	- A 27% maximum extraction yield for polyphenols. - Significant effects of the pulse and strength of electric field on the extraction yield.	(Zderic, Zondervan, & Meuldijk, 2013)
Black tea	20 kV/cm, 95.83 µs, 125 Hz, 2 µs	Total solids, polyphenols, and amino acids	- A 22.7% extraction yield for instant black tea powder. - Improving the tea solubility in cold water along with reducing the tea cream.	(Ye et al., 2014)
Unfermented Pu'er tea	18 kV/cm, 60 min (extraction time), 200 pps, 120 Hz, 0.3 µs	Polyphenolics, and theine	- Improving the taste, aroma and other sensory parameters in tea samples compared with the effect of natural aging on teas. - Effective in the artificial aging as it improved the content of tea extract and its taste. - A shorter and quicker method for aging of unfermented Pu'er tea. - A new method to enhance the tea quality and safety.	(Chen et al., 2016)
Tea	0.9 kV/cm, 10°C,3 x 10^6 µs	Polyphenols	- A 27% maximum extraction yield for polyphenols. - A direct and positive relationship between the time of pulse applied and the treatment time.	(Zderic & Zondervan, 2016)
Tea	1.1 kV/cm, 100 µs,	Polyphenols	The maximum extraction yield (32.5%) of polyphenols	(Zderic & Zondervan, 2017)

	$0.1*10^{-3}$s,50 pulses	with the PEF without destroying bio-active compounds compared to the conventional hot-brewing method.

*1-Field strength (kV/cm), 2-Temperature (°C), 3-Pulse duration (µs), 4-Pulses per second (pps) / Frequency (Hz), 5-Pulse width (µs)

Table 4 : Quality and quantity effects of SFE technique on various bio-active compounds obtained from tea varieties

Samples	Extraction conditions*	Extracted compounds	Conclusions from the study	Reference
Roasted Japanese tea	80˚C, 40.5 MPa, methanol, 10 min, 450-500 g/min	Mesitylene (1,3,5-trimethylbenzene), l-ethyl-3-methylbenzene, l-ethyl-4-methylbenzene, 2-propiophenone, tetrahydro-2-furanmethanol, dihydro-2-furanone, benzenedicarboxylic acid bis(2-methoxyethyl) ester, nonacosane, and caffeine	- A quick alternative method to the liquid solvent extraction. - Silylating agents was complexed with the sample and served as both polar modifier and derivatizing reagent. - A higher extraction yield and efficiency in SFDE (simultaneous supercritical fluid derivatization and extraction) method compared to the SFE one.	(Ward Hills et al., 1991)
Sassafras tea (unbrewed)	80˚C, 69.0 MPa, methanol, 15 min, 2 g/min	Safrole and allylbenzne	-A 96% and 101% recovery respectively for safrole and allylbenzenes. - A more accurate and better results within a short time period with the SFE in comparison to the steam distillation	(Heikes, 1994)
Mate tea	70°C, 25.5 MPa, organic solvents, 420 min, 0.9-1.2 g/min	Caffeine, theobromine, and theophylline	- The extractability rate of theophylline, theobromine and caffeine was 57, 68 and 94%, respectively - Higher selectivity of caffeine compared to theobromine, and theophylline towards CO_2. - A retrograde behavior for caffeine with the temperature was recorded while theobromine and theophylline had a normal behavior	(Saldaña et al., 1999)
Tea tree (*Melaleuca*	100°C, 7.4 MPa, hexzane	Monoterpenes	The sample matrix has a fundamental role mainly in the SFE process.	(Wong et al., 2001)

alternifolia Cheel) leaves	(rinse solvent), 10 min, 0.25 g/mL			
Mate tea leaves	70°C, 40 MPa, ethanol (co-solvent), 400 min, 5.7 g/min	Caffeine and methylxanthines	- A 98% extraction rate for caffeine - High efficiency of extraction process of methylxanthines by ethanol at low amounts - The applied temperature and pressure were critical for the extraction of bio-active compounds. - A short time period for the caffeine extraction using the SFE.	(Saldaña et al., 2002)
Tea seeds	60-80°C, 30-40 MPa, hexane, 20-30 min, 1 g/min	Tea seed oil	-The used modifier and pressure were critical parameters. - The best method for obtaining tea seed oil without using the organic solvent.	(Rajaei, Barzegar, & Yamini, 2005)
Korean tea	50°C, 40 MPa, water, 60 min, 468 g/min	Caffeine	- A 66% extraction rate for caffeine. - Extracting the catechins along with the caffeine at a higher temperature than 323 K.	(Kim, Kim, & Oh, 2007)
Green tea	60°C, 30 MPa, ethanol or water, 10 min, 12 g/min	Caffeine and catechins	- Obtaining the maximum removal (91.5%) of caffeine and the high retention of catechins (80.8%). - Critical parameters were: pressure, temperature, and ratio of CO_2 to tea	(Huang et al., 2007)
Tea	70°C, 30 MPa, ethanol, 120 min, 8.5 g/min	Caffeine and catechins	- A critical role for the type and concentration of co-solvent used in SFE process. - A significant decrease in the content of caffeine extracted using the SFE method (2.6%).	(Park et al., 2007)

			- A 37.8% reduction in ECGC by the SFE process.	
Green tea	70°C, 30 MPa, ethanol, 51 min, 1250 g/min	Volatile compounds and caffeine	- Higher caffeine and lower volatiles in tea extracts obtained by the SFE. - SFE is an efficient technique to decaffeinate green teas.	(Lee, Park, Kim, & Kim, 2007)
Tea	40°C, 40 MPa, water, 300 min, 468 g/min	Caffeine and EGCG	- A maximum extraction yield for caffeine (54%) and EGCG (21%) by the SFE. - Water as the best solvent for the selective extraction of the caffeine. - The selectivity was found to be 0.88 for water compared to 0.24 for ethanol.	(Kim, Kim, Kim, Oh, & Lee, 2008)
Mate tea	50°C, 15 MPa, methanol	Caffeine, theobromine, and polyphenolics	A suitable method only for the extraction of caffeine and theobromine and not for the other polyphenolics from tea.	(Cassel et al., 2010)
Green tea	63°C, 23 MPa, ethanol, 120 min, 8.5 g/min	Caffeine and catechins	- Extraction rate by SFE was 36.06% for caffeine and 40.61% for catechins. - The simultaneous extraction of chlorophyll caffeine.	(Park et al., 2012)
Tea	50°C, 30 MPa, no solvent, 10 min for static and 90 min for dynamic, 2000 g/min	Volatile compounds	Identification of 59 bio-active compounds using GC-MS in the essential oil of tea flowers.	(Chen et al., 2014)
Tea	50°C, 18.8 MPa, ethanol, 60 min, 2.94 g/min	Total phenols and flavonoids	- The most effective parameters: pressure and co-solvent used. - A high phenolic and flavonoid contents.	(Maran, Manikandan, Priya, & Gurumoorthi, 2015)

			- A high antioxidant activity for the obtained extracts.	
Green tea	50°C, 25 MPa, ethanol, 540 min	Caffeine	- The solubility ranged from 44.19-149.55 × 10^{-6} within a wide range of temperature and pressure. - Less solubility of the extracted caffeine compared to its pure form (61 times higher).	(Gadkari & Balaraman, 2015)

*1-Temperature (°C), 2-Pressure (MPa), 3-Solvent, 4-Time (min), 5-Flow rate

Table 5 : A list of the most important results and conditions of PLE application to extract bio-active compounds from different types of tea

Sample	Extraction conditions *	Extracted compounds	Conclusions from study	Reference
Tea leaves (e.g., non-fermented and fermented teas and black tea)	100-200°C, 10.1MPa (100atm), methanol, 10 min	Catechins and caffeine	- No significant effect of high temperature at the studied range on the stability of catechins. - Methanol: the best used pure solvent - Reducing the recovery level by 95% for catechins and epicatechin at 130 °C - The highest recovery rate with a relative standard deviation of 3.21% and 2.96% respectively for catechin and epicatechin.	(Piñeiro et al., 2004)
Sambucus nigra L. flowers, green tea, black teas, and	100°C, 6.07 MPa (60 atm), water, 10 min, 0.5 g	Rutin and caffeine	- The efficient and fast removal of bio-active compounds from the matrix at high temperatures.	(Dawidowicz & Wianowska, 2005a)

coffee beans			- A single-step PLE: a successful method used to save time instead of a multi-step PLE in various ratios of the solid to solvent.	
Green tea leaves	70°C, 4.05 MPa (40 atm), water, 10 min, 0.5 g	Caffeine	Squeezing the soft matrix in tea at combinations of high pressure and temperature makes it difficult to extract caffeine from tea.	(Dawidowicz & Wianowska, 2005b)
Mate leaves	100°C, 10.3 MPa (102 atm), methanol, 10 min, 7.5 g	Caffeine, palmitic acid, phytol, stearic acid, squalene, and vitamin E	- Substantial amounts of caffeine and palmitic acid in the obtained extracts - The minimal extraction time and used solvent, with the highest yield compared to the other methods (e.g. UAE, MAE) - The extraction of more polar compounds at elevated temperatures - Methanol as the best solvent used for extraction	(Assis Jacques et al., 2006)
Mate tea leaves	100°C, 10.3 MPa (102 atm), methanol, 10 min, 7.5 g, 100 °C, 102 atm, hexane, 10 min, 2.5 g	Caffeine, phytol, squalene, vitamin E, caffeine, palmitic acid, and 37 other chemical compounds	- The used solvent polarity, the sample amount, and extraction temperature had the highest effect on the quality and quantity parameters. - A significant difference in the extraction yield between methanol (13.83%) and hexane (1.67%).	(Jacques et al., 2008)
Green tea	100°C, 10.3 MPa (102	Flufenoxuron, fenitrothion,	- Bifenthrin was the only pesticide	(Cho et al., 2008)

	atm), *n*-hexane, 5 min	chlorfluazuron, chlorpyrifos, hexythiazox, methidathion, chlorfenapyr, tebuconazole, EPN (O-Ethyl-o-4 nitrophenyl phenulphosphotion ate), bifenthrin, cyhalothrin, spirodiclofen, difenoconazole, and azoxystrobin	identified in the tea samples. - A faster and simpler extraction method for bio-active compounds from tea samples.	
Green tea	200°C, 3.57 – 20.2 MPa (35-200 atm), simple alcohols, 5-15 min	Phenolic compounds, ligands, carotenoids, oils and lipids, essential oils, and other nutraceuticals	A promising method to extract carotenoids from tea	(Mustafa & Turner, 2011)
Herbal tea	50-200°C, 3.44 - 20.67 MPa (34-204 atm), organic solvents, 5-10 min	Flavonoids, catechins, chlorogenic acid, and epicatechin	- A quicker and more precision photochemical analysis for extracts obtained with the fast technique of PLE (5 min) compared with other extraction ones (8 h). - PLE: the best extraction method with high repeatability for the maximum yield at lower extraction time and solvent consumption.	(Zhao et al., 2013)
Green tea	99.85-199.85°C, 9.92 MPa (98 atm), ethyl lactate and water, 20 minutes,1 g	Caffeine and catechins	- A higher solubility of caffeine in mixtures of ethyl lactate and water at the optimum of pressure and temperature values. - The extraction yields using the combined	(Villanueva Bermejo et al., 2015)

			solvent of water-ethyl lactate were 3.5 and 1.5 times more than when water and ethyl lactate, respectively, were separately used. - A higher recovery rate for caffeine (53-76%) compared to that of catechins (26-36%).	
Green tea	100°C, 9.92 MPa (98 atm), ethyl lactate and ethanol, 20 min,1 g	Caffeine, catechins, and other phenolics	- Ethyl lactate as the best solvent for the decaffeination process. - The precipitates obtained by ethyl lactate solvent were 2.3 times more than those of ethanol. - A 93% reduction in caffeine content present in the extract - The percentage recovery of main catechins (EGCG) was in the range of 46-74%.	(Bermejo et al., 2015)
Green tea	275 °C, 15.19 MPa (150 atm), methanol, 20 s, 0.005-0.1 g	Caffeine	- Higher recovery of caffeine at higher temperatures. -The micro-PLE method: a fast extraction method with lower sample and solvent amounts compared with the conventional methods. - Having the large error values due to the sampler size.	(Alkhateeb & Thurbide, 2015)

*1-Temperature (°C), 2-Pressure (MPa/atm), 3-Solvent, 4-Time (min), 5-Sample size (g)

Chapter 4: Optimization and effect of various parameters of ultrasound assisted extraction in cold brewed black tea using OVAT analysis

Chapter 4: Optimization and effect of various parameters of ultrasound assisted extraction in cold brewed black tea using OVAT analysis

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4.1. Introduction

Tea (*Camellia sinensis)* is one of the most consumed beverages (McKay & Blumberg, 2002; Xiao, Zhang, Fan, & Han, 2017) with caffeine worldwide, which is rich in polyphenols and subsequent antioxidant activity. The significance of tea consumption is more than just considering it as a beverage but also to its health promoting effects. It has been reported that the major bio-active compounds in tea comprises of tea polyphenols (such as catechins, EGCG, tannins, etc.) which contribute to flavor, aroma, color and health benefits (Li et al., 2016; Zhao et al., 2014). In general, catechins account for about 60% of the total polyphenols present in the tea leaves (Lee, Hwang, Lee, & Choung, 2014; Van der Hooft et al., 2012). According to the manufacturing process, the tea is classified into three main categories as fermented black tea, unfermented green tea and partially fermented oolong tea. Among these, black tea is considered to be one of the primary sources of polyphenols and accounts for about 80% of the worlds tea production (Taguchi et al., 2015). Black tea being a rich source of antioxidant rich polyphenols, mainly theanins and tannins, confers health benefits such as preventing cardiovascular diseases, cancer and other pathological benefits (McKay & Blumberg, 2002; Tijburg, Mattern, Folts, Weisgerber, & Katan, 1997; Xiao et al., 2017). It is believed to react with the reactive oxygen species and reduce the oxidative stress in the human body (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Sano et al., 1995; Sesso, Gaziano, Buring, & Hennekens, 1999).

Brewing of tea with hot water tends to degrade this beneficial health promoting thermally liable compounds. It might also lead to the release of tannins which are responsible for the bitter astringency. In order to overcome these shortcomings, brewing

tea in cold water can help preserve these compounds and provide a flavorful black tea. But usage of cold brewing was limited due to slower extraction times. The traditional method of prolonged storage for cold brewing are associated with high cost for maintenance and excess energy. Hence, there is a need for alternative methods with increased yield and low-cost maintenance, with acceptable quality. Ultrasound-assisted extraction (UAE) is an alternative method used for controlled extraction of bio-active compounds. Ultrasonic waves create a cavitation resulting in the formation of bubbles and aid in efficient extraction of bio-actives (Majid, Nayik, & Nanda, 2015b; Vivek, Mishra, & Pradhan, 2019).

The ultrasonic waves are formed by the combination of low and high pressure called compression and rare fraction. The treatment with ultrasound is known to hold the organoleptic properties as well as the functional characteristics of food products (Tiwari, O'll Donnell, Muthukummarappan, & Cullen, 2008). Many researchers have used ultrasound for the different method of food applications for extraction of bio-actives (Bora, Handique, & Sit, 2017; Ertugay & Başlar, 2014; Lieu & Le, 2010; Nithila et al., 2014).

UAE can be used in combination with cold brewing of black tea in order to maximize the extraction efficiency and to minimize the extraction time. In this study, a classic univariate approach of OVAT (one-variable-at-a-time) procedure was used to evaluate the effect of ultra-sonication at different times (10, 20, 30, 40, 50, 60 mins), amplitudes (0%, 10%, 30%, 50%, 70%) and the solute to solvent ratios (1:25, 1:50, 1:75, 1:100) on improving the extraction of total phenolic content (TPC) and the radical scavenging activity from cold brewed black tea. All experiments were carried out at 4°C with pH of 5.4 and sample weight of 0.5 g. All the UAE treated samples along with controls

were analyzed for TPC and antioxidant activity (AC) using Folin-Ciocalteu and DPPH radical scavenging assay. Statistical analysis was done using ANOVA and the significant differences were calculated ($p<0.05$) using Tukey Honest significant difference (HSD).

4.2. Materials and methods

4.2.1. Reagents

Commercial black tea, obtained from Brenner®, Batavia, IL, USA was used as a raw material in this study for analysis. For analysis, methanol and sodium carbonate (Na_2CO_3) (Fischer Chemicals, Springfield Township, NJ, USA), DPPH (2, 2'-diphenyl1-picrylhydrazyl), Trolox (EMD Millipore, San Diego, CA, USA), Folin Ciocalteu reagent (Sigma Aldrich, St. Louis, MO, USA), Gallic acid (ChemImpex, Wood Dale, IL, USA), sodium hydroxide (Ricca Chemicals, Arlington, TX, USA) were used.

4.2.2. Conventional extraction using cold brewing

The conventional cold brewing method was followed with minor modifications **(Lin, Liu, & Mau, 2008)**. 0.5 g of black tea was mixed with 50 ml of double distilled water and temperature was set at 4ºC for 6 hours; then the sample was centrifuged (Model: Allegra X-30R, Beckman Coulter Inc., Chaska, MN, USA) at 3000 rpm for 3 minutes and filtered using a Whatman filter paper No.1 (125 mm) and the filtrate was stored at -40ºC for further analysis.

4.2.3. Ultrasound assisted extraction of cold brewed black tea using an ultrasonic probe

Ultrasound assisted extraction of cold brewed black tea was performed using Sonifier ® cell disrupter (Model: (Model: SLPe EDP 100-214-254, BRANSON Ultrasonics Corporation, Danbury, CT, USA) which is capable of operating at different amplitudes and sonication times. OVAT analysis is similar to a "Black Box Model". It involves changing one variable at a time while keeping the other parameters constant. It can be further explained by using a simple model (Figure 10) where the process has two inputs (X_1 and X_2) and an output or response (Y). Since, the process inside the box is unknown it is called the black box model.

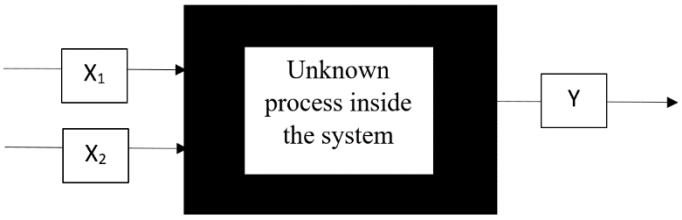

Figure 10: Unknown process inside the system with two parameter inputs (X_1 and X_2) and output Y

One of the best experimental design to determine the effect of the inputs in the system would be to hold one of the inputs fixed (X_1) and see the results of the experiment when the other input parameter (X_2) is free and varied. Then fix that parameter (X_2) at the best value for the output (Y). Next vary the other input parameter (X_1) and find out the best value for the other parameter (X_2) based on the output. This process of fixing parameters and varying one input parameters is carried out until we run out of input parameters. This method is widely used to find the effect of parameters on various process conditions and

output. Thus, OVAT is generally good until the true model inside the black box looks as soon in Figure 11.

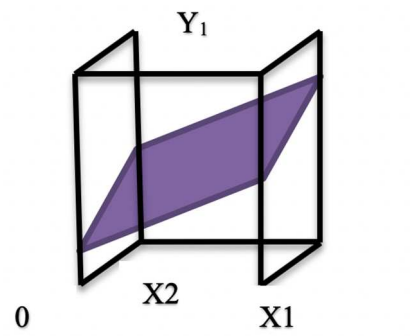

Figure 11: Illustrates a simple main effect model where $Y_1 = X_1 + X_2$

The following model is called the main effects model as it is flat in all the dimensions. No matter at which point one surface begins, increasing an input parameter should always have the same effect on the output response. However, the interactions between the inputs cannot be studied using a main effect model in OVAT model. This experimental setup was totally based on the main effect model where in the input parameters would include sonication time, amplitude and solvent volume for cold brewing of black tea.

From the literature, it was evident that the critical factors influencing the extraction process using ultrasound were temperature, amplitude, sonication time and the solvent volume used for the extraction process (Mason & Yiyun Zhao, 1994b; Zeković et al., 2017b). The frequency of the horn was maintained constant at 40 kHz and water was used as a solvent for ultrasound assisted extraction process. Table 6 shows the experimental layout of the design for OVAT analysis.

Table 6: Experimental design layout for OVAT analysis of parameters with respect to cold brewing of black tea.

Experiment	Runs	Amplitude (%)	Solvent volume(ml)	Sonication time(min)	Responses (TPC and %DPPH)
1	1	0	50	30	Best Amplitude(X_1)
	2	10	50	30	
	3	30	50	30	
	4	50	50	30	
	5	70	50	30	
2	6	X_1	25	30	Best Solvent volume(X_2)
	7	X_1	50	30	
	8	X_1	75	30	
	9	X_1	100	30	
3	10	X_1	X_2	10	Best Sonication time(X_3)
	11	X_1	X_2	20	
	12	X_1	X_2	30	
	13	X_1	X_2	40	
	14	X_1	X_2	50	
	15	X_1	X_2	60	
4	16	X_1	X_2	X_3	Maximum output of the responses

4.2.4. Analysis of water activity and moisture content

Black tea sample was analyzed for Initial moisture content using an instant moisture analyzer (Model: MB25, Ohaus Corp., Parsippany, NJ, USA) and it was measured in percentage moisture content on wet basis. The powdered black tea was stored at ambient temperature (21°C) in polyethylene bags throughout the study. The water activity was determined using a benchtop water activity meter (Model: Aqualab 3TE, Decagon Devices, Pullman, Washington, USA) with 0.5 grams of tea sample. The samples were analyzed in triplicate and the average moisture content was recorded.

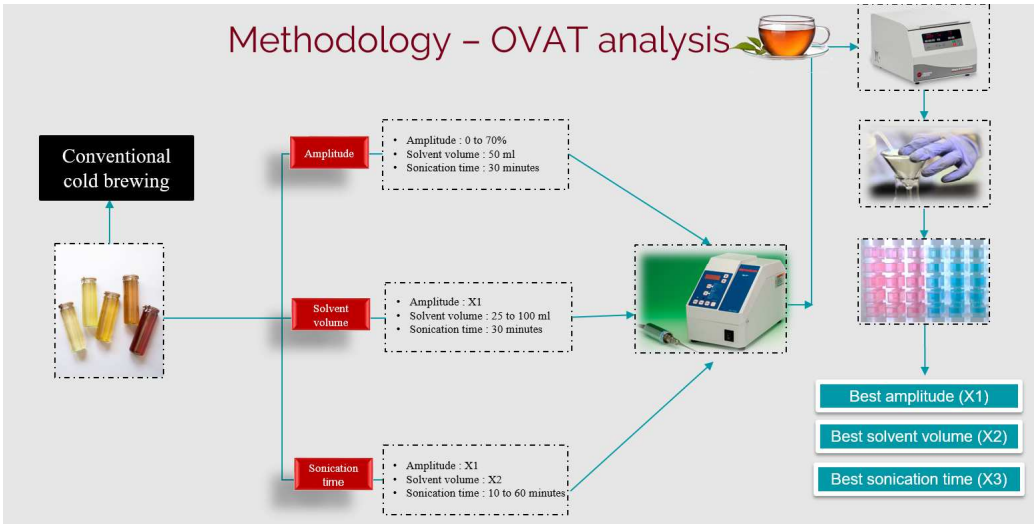

Figure 11a: Experimental representation of OVAT analysis

4.2.5. Analysis of Total phenolic content (TPC) of cold brewed black tea using Folin-Ciocalteau Assay

Folin-Ciocalteau Assay was used for determining the total phenolic content of cold brewed black tea (Folin & Ciocalteu, 1927; Singleton & Rossi, 1965; Singleton & Slinkard, 1977). This method is approved by AOAC for the analysis of phenol content in wines. It is also considered to be one of the most commonly used methods of determination of phenols. The total phenolics of a given sample is determined spectroscopically as described by (Budaraju, Mallikarjunan, Annor, Schoenfuss, & Raun, 2018; Dewanto, Xianzhong, Adom, & Liu, 2002). The standard curve was developed using Gallic acid (600 M). 500 µl of distilled water was mixed with 125 µl of sample or standard. Further, 125 µl of Folin-Ciocalteau reagent (FCR) and 1250 µl of 7% aqueous sodium bicarbonate solution was added to the mixture. Finally, distilled water was added to make up the final volume to 3000 µl. The reaction mixture was incubated in the dark for 60 minutes at ambient temperature. The absorbance was measured at 760 nm using a spectrophotometer (Model: UV-1800, Shimadzu Scientific Instruments Inc., Addison, IL, USA). The results were

reported as mg of Gallic acid equivalence (GAE). The experimental procedure were performed in quadruplicates and the average values were used for the data analysis.

4.2.6. Determination of antioxidant capacity of cold brewed black tea using DPPH radical scavenging activity

The electron transfer assay antioxidant DPPH assay was used to determine the antioxidant capacity of the cold brewed black tea, as explained by (Budaraju et al., 2018; Fogarasi, Kun, Tankó, Stefanovits-Bányai, & Hegyesné-Vecseri, 2015; Guo & Beta, 2013; Li, Shan, Sun, Corke, & Beta, 2005). The DPPH assay is the most commonly used method for different samples as it serves as a stable radical which can easily trap the free radicals (Gupta, 2015). The scavenging activity was represented as percentage of DPPH radical reacted and antioxidant activity was reported in percentage radical scavenging activity. A stock solution of 500 µM of Trolox was used as standard. 3.9 ml of 60 µM solution of DPPH was mixed with 100 µl of sample or standard to make a final volume of 4 ml. The mixture was shaken and left in dark environment at ambient temperature for 90 minutes for complete reaction to take place. The antioxidant capacity was calculated by measuring the absorbance of the sample and standard with DPPH at 515 nm using the spectrophotometer. The samples were analyzed in quadruplicates and the values were averaged for statistical approach and data analysis. Percentage DPPH activity was analyzed according to the following equation:

$$\%\text{DPPH Activity} = \left(1 - \frac{\text{A (sample)}}{\text{A(blank)}}\right) * 100 \quad (1)$$

Where A (sample) stands for absorbance of sample and A (blank) is the absorbance of control that contains only DPPH reagent.

4.3. Statistical Analysis

Two way-Analysis of Variance (ANOVA) was done using Minitab for each parameter: amplitude, solvent volume and sonication time separately. When an analysis was significant ($p \leq 0.05$), differences among the means were determined using Tukey-Kramer Honest Significant Difference (HSD) test. ANOVA and Tukey HSD tables can be found in Appendix 1. Kinetic modeling of data with respect to sonication time was done for TPC and %DPPH Antioxidant scavenging activity.

4.4. Results and discussion

4.4.1. Water activity and moisture content of black tea

The water activity of black tea sample was found to be 0.11± 0.01 at 21ºC. Moisture content can be referred to as the amount of water available in material or substance. Moisture content analysis plays a very important role in material quality and one of the essential parameters in quality control in most of industries. The moisture content of black tea was found to be 6.33 ± 0.22%. This value is in accordance with the literature that the moisture content in the black tea sample falls in the range of 4-7% (Ikeda, 2013).

4.4.2. Conventional cold brewing

The conventional cold brewing was done by steeping 0.5 g of sample in 50 ml water for a period of 6 hours at 4°C. The total phenolic content in the sample was found to be 19.50 ± 0.76 mg Gallic acid equivalent (GAE) per gram of the sample. This is in agreement with the study done by (Lantano, Rinaldi, Cavazza, Barbanti, & Corradini, 2015) where in the black tea was brewed for 12 hours at 4°C had a TPC content of 20.4 ±0.1 mg GAE per gram of sample. When the sample from the study was stored at 4°C for 12 hours (21.38±0.24 mg GAE/g of sample) for TPC and it was not significant change in the amount of total phenolics extracted. The percent antioxidant activity of the cold brewed tea was recorded to be 26.74 ± 0.36 (%DPPH). Contradictory to the results obtained from the black tea at 25°C (80%-90%) by Magammana, Rock, Wang, & Gray, 2019, it has been found that %DPPH scavenging activity was lower. However, there are other factors which influence the %DPPH activity which includes the amount of sample used, pH, type of solvent, extraction temperature and more importantly the sample variety under study.

Experimentally, this cold brewing of tea was found to be in correlation with respect to the amount of total phenolics obtained and showed a change in behavior with importance to the antioxidant activity. Moreover, DPPH is a very sensitive assay and it's subjected to changes even for minor modifications and the concentration of the DPPH reagent was lower and it might affect the range of antioxidant activity of the sample (Moharram & Youssef, 2016). In comparison with the hot brewing from the studies performed earlier the values ranged from 100-120 mg GAE/ g for TPC and 85-95 % for DPPH scavenging activity. These values are found to be similar to the works done by Bhuyan et al., 2013. It can be clearly seen that the cold brewed tea has lower TPC than the hot brewed black tea

and this can be closely attributed to the fact that cold water extracts lesser phenolics from black tea leaves than extraction at a higher temperature. This was one of the major reasons that might be the possible reason for longer extraction time from black tea at lower temperatures (Magammana et al., 2019). The tea sample used for this study was entirely different from the ones used for other research studies which also needs to be taken into account.

4.4.3. Effect of Amplitude

Amplitude refers to the maximum height of a wave (Medina-Torres, Ayora-Talavera, Espinosa-Andrews, Sánchez-Contreras, & Pacheco, 2017b). It is one of the most important parameters as it decides the intensity of ultrasound applied to the cold brewed tea sample. To determine the effect of amplitude on the extraction yield of TPC and %DPPH antioxidant scavenging activity, experiments were carried out at amplitude ranging from 10% to 70%. The UAE experiments were set as follows: Water as solvent, 0.5:50 as the solid to solvent ratio and extraction time of 30 minutes. The solvent volume and the sonication time were set constant based on preliminary study with cold brewed tea. Figure 12 and Table 15 (Appendix 1) shows the yield of TPC and percentage antioxidant activity of cold brewed tea subjected to various amplitudes. From the experiments, it was very evident that the TPC and the %DPPH activity increased with the increase in amplitude and had a positive correlation between TPC and %DPPH (Piluzza & Bullitta, 2011).

As shown in Figure 12, the extraction yield of TPC and the radical scavenging activity of DPPH initially remained constant. However, both TPC and %DPPH increased when the amplitude was increased from 50% (26.53±1.07 mg GAE/g; 25.05±0.21%) to 70% (36.38 mg GAE/g; 42.24±2.49%). Within the experimental conditions amplitude of

70% resulted in maximum phenolic content and antioxidant activity. These values were relatively higher than the values reported by (Xia, Shi, & Wan, 2006b). However, the sonication time was reduced from 40 minutes to 30 minutes to that of Xia et al (2006). On the other hand, Hamishehkar, Ilghami, & Ghanbarzadeh, (2015) noticed that increased amplitude of 92.68% increased the extraction of bio-active compounds. The total phenolic content obtained using ultrasound assisted extraction was higher in comparison to conventional extraction after 6 hours of extraction from cold brewed black tea. These comparisons show how important is the selection as well as the management of amplitude as a parameter in the ultrasound assisted extraction of cold brewed black tea.

Higher ultrasonic amplitude creates a stronger cavitation during the extraction and hence the mass transfer of the bio-actives from the cell to the surface occurs at a higher rate. However, at lower amplitudes, as the amount of frequency of the waves is lower and the cells tend to get partially disrupted (Anaya-Esparza, Ramos-Aguirre, Zamora-Gasga, Yahia, & Montalvo-González, 2018; Rubin et al., 2018). In other words, the lower amplitudes did break the cell walls but not enough to have an optimum mass transfer of the bio-actives from the cell to the surface. This is much evident from the amount of phenolics extracted after ultra-sonication treatment. On the other hand, the %DPPH antioxidant scavenging activity was found to be significantly different for 10% and 30%; 50% and 70% when compared with the control (0%). Increasing the amplitude from 30% to 50% suppressed the % antioxidant activity and the possible reason might be attributed to the fact that ultrasonication at lower intensities lead to extraction of other bio-active compounds and lesser amount of bio-active with antioxidant capacities. 70% amplitude was concluded as the best optimum based on the higher amount of both TPC and %DPPH activity. The

study is in agreement with the principle of application of amplitude as it plays a critical role in intensification of the extraction of bio-actives process due to the increase in the number of compression and rarefaction cycles of waves. Amplitude helps in higher extraction efficiency of the bio-actives (Al-Dhabi, Ponmurugan, & Maran Jeganathan, 2017a; Medina-Torres et al., 2017b) with antioxidant activity. However, it has to be noted that the ultrasonic probe system used has a system limitation of 70% amplitude as the maximum and hence the effect of amplitude beyond 70% was not studied.

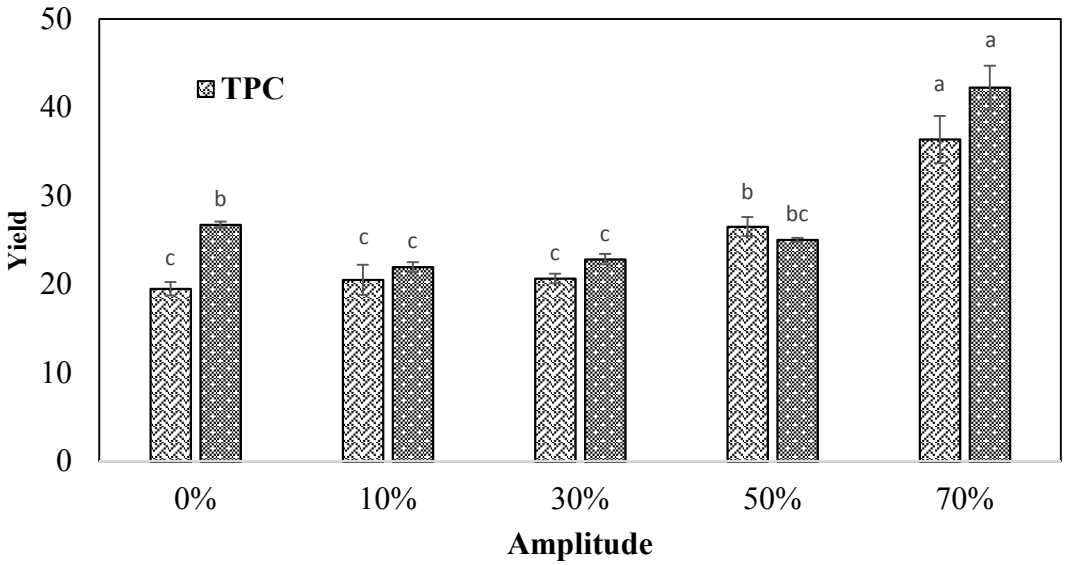

Figure 12: Effect of amplitude on cold brewed black tea. Error bars from the sample group having different letters are significantly different based on Tukeys HSD test. TPC is expressed in terms of mg of GAE/g and antioxidant scavenging activity is expressed in percentage.

4.4.4. Effect of solvent volume

The extraction efficiency depends on the solubility of bio-actives in the solvent used for extraction. The complex structure and chemical characteristics of the bio-actives compounds and the solvent makes it very difficult to predict the best solvent for extraction process (Chemat et al., 2017a). In this study, the experimental procedure used water as a solvent, since it was the major solvent used for cold brewing of tea in the industries. However, the bio-active compounds like polyphenols are extracted using organic solvents like methanol and ethanol. From literature (Dailey & Vuong, 2015), ethanol was found to be a good solvent but with a realistic perspective of scaling with respect to industrial basis, water was selected as solvent for cold brewing. To determine the effect of solid to solvent ratio w/v (solvent volume) on the extraction yield of TPC and the %DPPH activity, the experiments were carried out at solvent volume 25, 50, 75, 100. The UAE experiments were set as follows: Water as solvent, extraction time of 30 minutes and the constant temperature of 4ºC and 0.5 g of sample. 70% amplitude of UAE was applied to the samples to study the effect of solvent volume and Table 16 (Appendix 1) shows the yield of TPC and percentage antioxidant activity of cold brewed tea subjected to different solvent volumes. As shown in Figure 13 and Table 16 (Appendix 1), the extraction yield of TPC increased when the solvent volume was 75 ml (46.65 ±1.31 mg GAE/g) and started to decrease as the solvent volume increased. In addition, the radical scavenging activity was initially increased from 25 to 50 ml (40.63±0.69%) of solvent and decreased as the solvent volume increased from 75 to 100 ml (23.15±0.57%). Figure 13 also shows that the extracted phenolics are significantly more effective in a solid to solvent ratio of 1:150 however on the other hand, 1:100 ratio had significantly higher antioxidant activity. There

was a negative correlation between TPC and %DPPH activity which is contradictory to the literature study between TPC and %DPPH. The increase in the TPC with increase in the volume can be attributed to the fact that generally the amount of phenolics extracted increases with increase in solvent volume (Galvan d'Alessandro, Kriaa, Nikov, & Dimitrov, 2012). Lesser the volume the solution becomes saturated and the amount of phenolics extracted tends to be lesser when compared to the higher volumes. However, the antioxidant activity decreased due to effect of amplitude, which leads to the destruction of certain antioxidants and may also suggest that the amount of other phenolics which are not antioxidants were extracted at a higher rate. Moreover, higher amount of solvent means increased cost for other operations in the industry, such as concentration and filtration of the final product, as well as an increase in the waste generated (Medina-Torres et al., 2017b; Wong Paz, Muñiz Márquez, Martínez Ávila, Belmares Cerda, & Aguilar, 2015).

The possible reasons for the extraction process can be the formation of the bubbles due to the cavitation process as the ultrasound creates a significant pressure and break the water molecules into free radicals. In broad-spectrum, more solvent volume signifies effective dissolution of the target bio-active compounds leading to an increase in the extraction efficiency of the compound. However, it was found that there were no significant changes 50 to 75 ml. The possible reason is that the ultra-sonication process can promote the establishment of the dissolution equilibrium of the target compounds between the extraction solvent (water) and the cell wall of the tea samples and hence difficult for the recovery of the target compound (Chemat & Esveld, 2013; Liao, Qu, & Zheng, 2016; Park, Atobe, & Fuchigami, 2006). Since, the primary concern in the study was related to phenolics extracted than the antioxidant activity, 75 ml of solvent volume was considered

as the optimum condition. Therefore, the solid to liquid ratio of 1:150 was chosen for further optimization studies based on the maximum extraction of phenolic antioxidants.

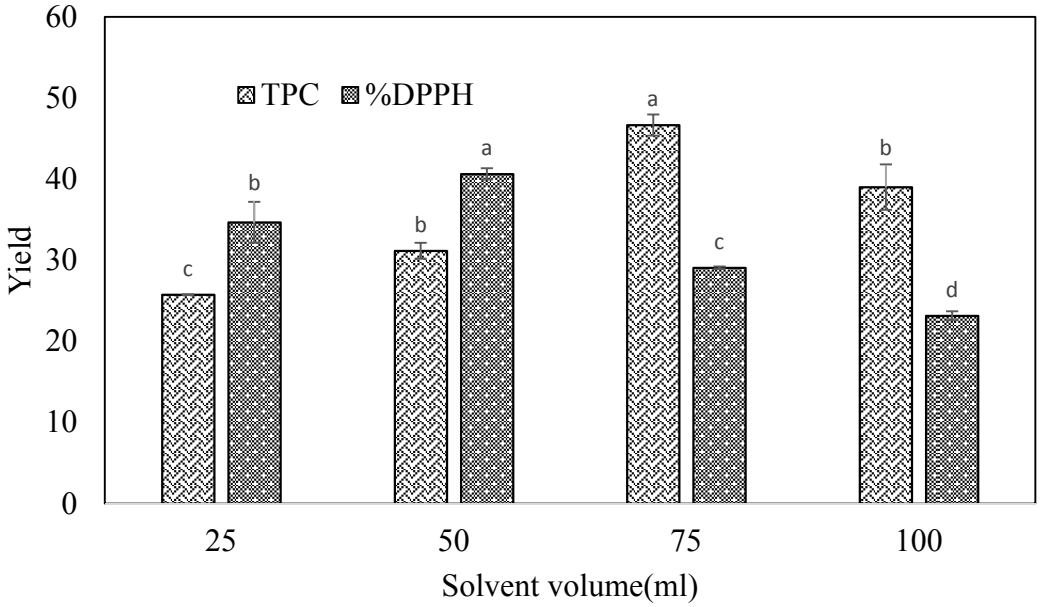

Figure 13: Effect of solvent volume on cold brewed black tea. Error bars from the sample group having different letters are significantly different based on Tukeys HSD test. TPC is expressed in terms of mg of GAE/g and antioxidant-scavenging activity is expressed in percentage.

4.4.5. Effect of sonication time

The sonication time is also a very important factor in the UAE process. Since, extraction time is the critical parameter for the major mass transfer phenomenon of bio-active compounds and thereby influencing the efficiency of extraction (Annegowda, Bhat, Min-Tze, Karim, & Mansor, 2012). The effect of sonication time on TPC and %DPPH activity was examined on cold brewed black tea. In order to obtain a maximum yield of TPC and %DPPH activity from cold brewed black tea at 4ºC ultrasound assisted extractions were performed at different time intervals (10, 20, 30, 40, 50 and 60 minutes). The operating conditions for extraction maintained during the study were 70% amplitude, 75

ml solvent volume and 0.5 grams of tea sample based on the previous results of the study. Water was used as a solvent and temperature of 4ºC was maintained constant throughout the study.

The effect of different extraction time on the yield of TPC and %DPPH activity is shown in Table 17 (Appendix 1). It can be seen from Figure 14 that amount of phenolics extracted increases significantly with increase in the sonication time; the relative response however reached a maximum at 50 min (57.63±1.31 mg GAE/g, 32.27±0.78%); after which it remained constant without further increase in the sonication time. This is in agreement with the study done by Annegowda et al., (2012) where the % inhibition values were found to be in correlation with the present study. One of the attributed reasons for lower extractability is that addition of water into the system reduces the antioxidant levels to a maximum extent (Annegowda et al., 2012; Paniwnyk, Cai, Albu, Mason, & Cole, 2009b; Parsons, 2015). This can be attributed to the fact that longer sonication time may lead to degradation of phenolic compounds due to oxidation and other environmental factors. The antioxidant activity remained fairly constant with a minor increase in activity from 10 minutes to 30 minutes however a significant increase was noticeable from 50 minutes and remained constant. The increase in sonication time didn't have much effect on the antioxidant activity of the black tea in comparison to the phenolic compounds extracted and the results are similar to the literature (Annegowda et al., 2012). It was initially observed that when the solvent was relatively fresh, the rate of extraction of phenolic compounds is higher and as the extraction time continues, the concentration of phenolic in the solvent gradually increases and the concentration gradient for phenolic compound between the tea leaves (biomass) decreases which ends up in slowing down the extraction

process and mass transfer. Moreover, the prolonged interval of sonication of tea leaves might lead to probable degradation due to the generation of free radicals.

The results further indicate that under ultrasound assisted extraction process, the diffusion of bio-active compounds like phenolic from the tea leaves to the solvent (water) might be improved under cold temperature conditions (4ºC) and the equilibrium for dissolution of these bio-active compounds can be established in a shorter period of time. But the phenolic compounds which exhibit antioxidant activity might be degraded due to prolonged exposure to the sonication process of ultrasound assisted extraction. A possible reason for the ultrasonic wave may disrupt rapidly, so there is a larger contact surface between the sample and solvent, which helps in improving the extraction yield of the target compound (Liao et al., 2016). The sonication time of 50 minutes resulted in maximum extraction of phenolic compound of 57.63±1.31 mg GAE/g of the sample and maximum antioxidant activity of 32.27±0.7%. Thus, the optimum parameters with respect UAE according to the OVAT analysis were recorded to be 70% amplitude, 75 ml solvent volume and 50 minutes of sonication time for maximum extraction of phenolics and maximum antioxidant activity for cold brewing of black tea.

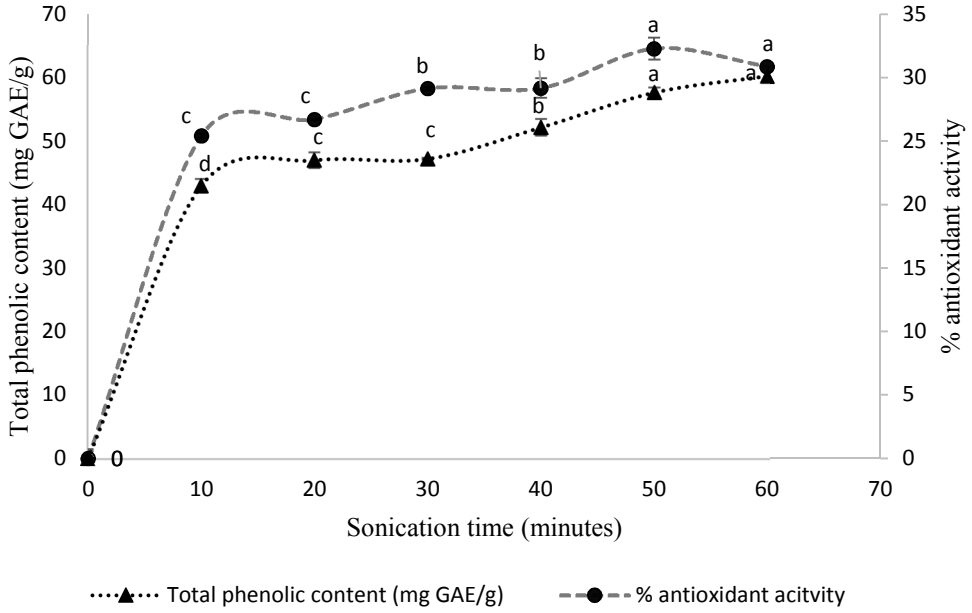

Figure 14: Trendline for the effect of sonication time on TPC and %DPPH scavenging activity. TPC is expressed in terms of mg of GAE/g and antioxidant scavenging activity is expressed in percentage.

4.4.6. Kinetic study of cold brewed black tea based on the sonication time

The experimental data obtained for sonication time was fitted for different kinetic models zero order, first order and pseudo second order to determine the rate of the reaction with respect to cold brewing. The predicted results from pseudo second order model was in good agreement with the experimental data having a coefficient of determination R^2 value of 0.9802 for TPC and 0.9938 for % antioxidant activity with respect to DPPH. In kinetic modelling of data, the pseudo second order equations can be expressed in the linear form as,

$$q_t = \frac{q_e^2 k_2 t}{1 + q_e k_2 t} \quad (2)$$

With boundary conditions where t = time and q_t is the amount of phenol extracted at time t (mg GAE/g of sample), q_e is the amount of phenol extracted at equilibrium (mg GAE/g

of sample) and k_2 is the rate constant of pseudo second order reaction kinetics. Table 18 & 20 (Appendix 1) shows the t/C values for pseudo second order modelling of Total phenolic content and percent antioxidant activity of DPPH. Also, from Figure 15 and 17 it is evident that the cold brewing reaction fits pseudo second order with R^2 value of 0.9802 for TPC and 0.9938 for % antioxidant activity with respect to DPPH. The second order rate constants (k_1) from the graphs was found to be $2x10^{-2}$ and $7x10^{-2}$ (g mg^{-1} min^{-1}) for TPC and antioxidant activity. The comparative study between the experimental data and the predicted values from pseudo second order equations is shown in Table 19 and 21 (Appendix 1). It is very evident that from Figure 16 and 18 that the experimental values are in accordance with the predicted values from the model for both total phenolic content and percentage radical scavenging activity of DPPH.

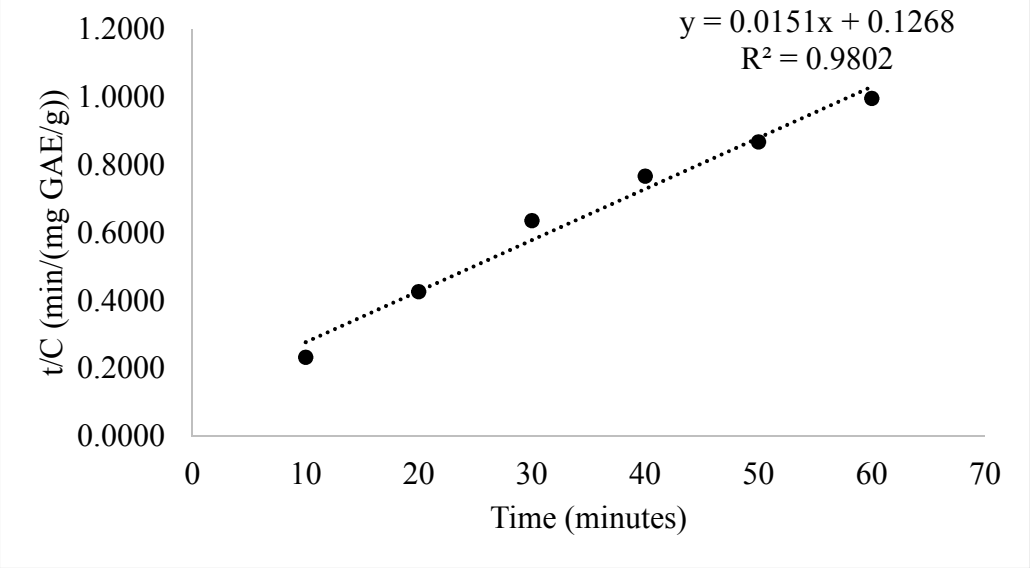

Figure 15: Scatter plot and t/C vs time for Total phenolic content

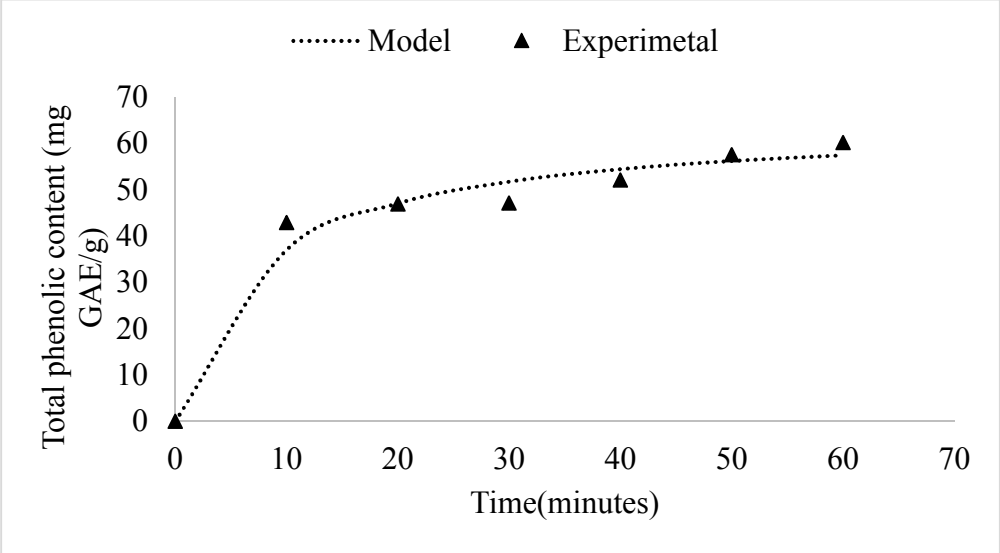

Figure 16: Comparison of experimental and predicted values for ultrasound assisted extraction of cold brewed black tea using pseudo second order model.

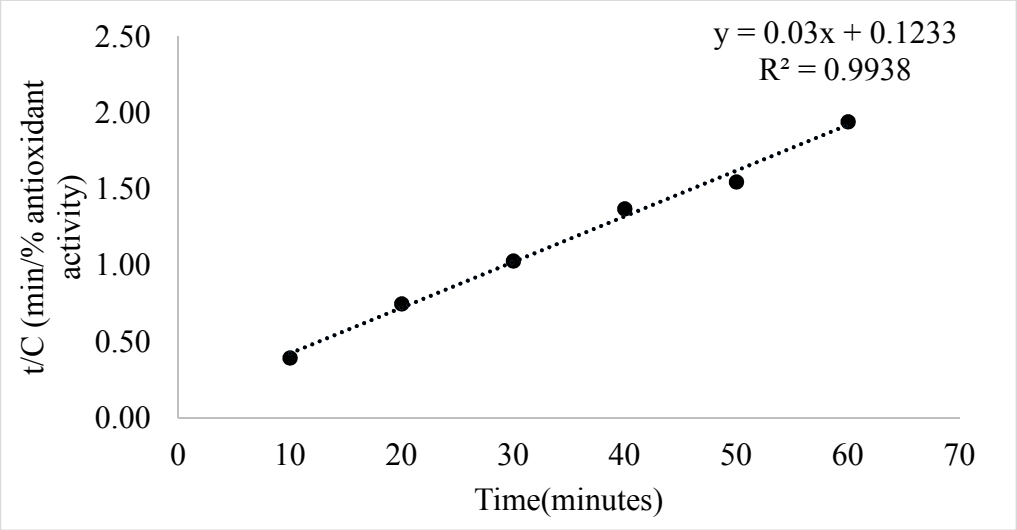

Figure 17: Scatter plot and t/C vs time for % antioxidant activity of DPPH

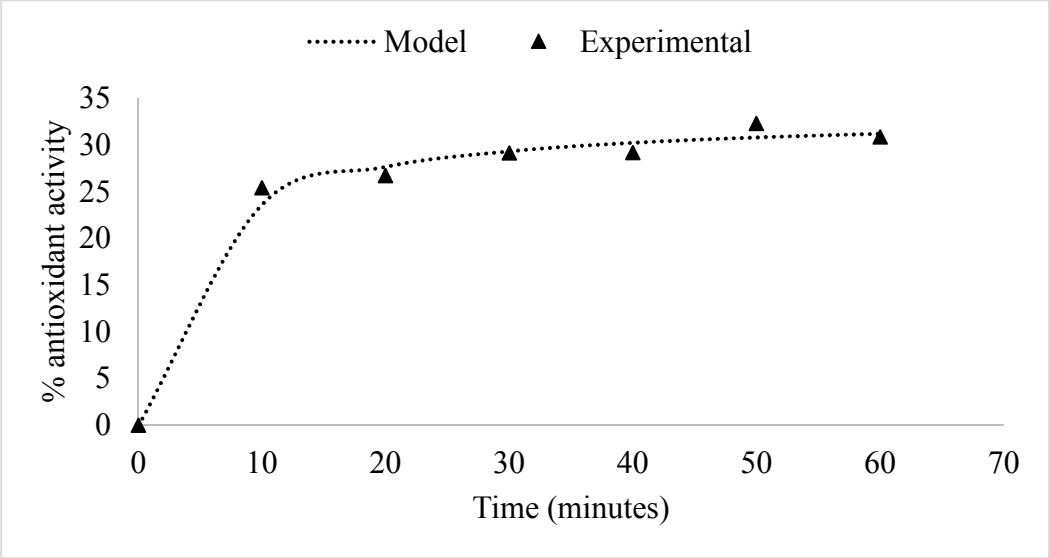

Figure 18: Comparison of experimental and predicted values for ultrasound assisted extraction of cold brewed black tea using pseudo second order model.

The kinetic study with respect to cold brewed black tea followed a pseudo second order which is in accordance with literature where hot brewing of black tea by (Fernando & Soysa, 2015) followed the same order. The rate constants for the polyphenols were stated to be $2.4 \times 10^{-2} \pm 0.7 \times 10^{-2}$ (g mg^{-1} min^{-1}) for hot brewing and it is similar to the values obtained in this study for phenolics (2×10^{-2} g mg^{-1} min^{-1}) thus proving the fact that the hot brewing occurs at the faster rate than cold brewing but use of sonication process can improve the extraction process to a maximum extent equivalent to hot brewing of black tea.

4.5. Conclusion

The present study clearly illustrated the importance of selecting process parameters for the cold brewing process of black tea using ultrasound assisted extraction process. This work also suggests the operating parameters with respect to ultrasound assisted extraction for maximum total phenolic content and % antioxidant activity using OVAT analysis. Using UAE, the optimum conditions for maximum total phenolic content and % antioxidant activity was obtained with 70% amplitude, 75 ml solvent volume and 50 min of sonication time at 4ºC according to OVAT analysis. The obtained results are very important as the difference in the most critical factors play a major role in efficiency of the process. The experiments under optimized condition provided a maximum TPC of 57.6±1.36 mg GAE/ g of tea and % antioxidant activity of 32.3±0.78%. The main advantage of the optimized condition is to minimize the sonication time to reduce the amount of energy spent due to ultra-sonication and also help in reducing the time taken for cold brewing of black tea. Currently, efforts are being made for usage of alternative technologies to develop rapid methods for food processing applications for maximum results. Usage of ultra-sonication process provides a better way to obtain maximum efficiency of the extraction process, shorter processing time period and increased rates of physical, chemical or physio chemical process in the system and also a change in the reaction pathways (Kasaai, 2013). Knowledge on the mechanism of ultrasound provides us an insight to examine some of the process parameters and how they affect the extraction efficiency to a larger extent. Results indicated that the proposed model helps to understand the effects of various process parameters with respect to ultra-sonication process in the cold brewing of black tea.

Chapter 5

Optimization of Ultrasound assisted extraction of cold brewed black tea using response surface methodology

Chapter 5: Optimization of Ultrasound assisted extraction of cold brewed black tea using response surface methodology

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Prepared for submission to the journal

5.1. Introduction

Tea is one of the widely consumed beverages worldwide and it has been indicated to have a wide range of health benefits. Many research studies have indicated that tea can reduce the risk of cancer and cardiovascular diseases (Bolling, Chen, & Blumberg, 2009; Yang, Lambert, & Sang, 2009; Zhang et al., 2012). These effects are attributed to the presence of bio-active compounds like catechins, purine alkaloids and theanins in tea (Khan & Mukhtar, 2007; X. Zhang et al., 2012). Tea comprises of many natural polyphenols (Lin, Chen, & Harnly, 2008) of which only few of the bio-active compounds have been identified and studied. The major bio-actives compounds in tea includes epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate. These have antioxidant, anticarcinogenic, antiomicrobial, and antiviral properties (Fraga, 2007; Song, Lee, & Seong, 2005). Thus, tea as a beverage serves as one of the most medicinal drink (Khokhar & Magnusdottir, 2002; Nadiah & Utra, 2016) with various medicinal properties.

Among various types of tea, the black tea is rich in theanins which is an amino acid that was first identified in leaves (Sakato, 1949) and represent almost 50% of the total amino acids present in the black tea. It is always associated with higher antioxidant activity than other teas (Pereira, Knor, Vellosa, & Beltrame, 2014). Theanins has many functions such as neuroprotection, anti-obesity and anti-tumor activity. In addition to theanins, tannins are present in abundance in black tea. The tannins are described to have both positive and a negative impact on the human body. These can serve as antioxidants and help to reduce the oxidative stress. But on the other hand, it is known that tannins are responsible for the inhibition of iron absorption (Hurrell, Reddy, & Cook, 1999) in our body. Thus, it is very important to minimize the extraction of tannins in brewing to a large

extent in spite of its beneficial antioxidant properties. Reducing the tannin content should not compromise the beneficial aspects of tea as they have other polyphenols that could provide the required antioxidant effects.

Black tea, a rich source of antioxidants, is known for its wide spectrum of secondary metabolites, which have been shown to exhibit health-promoting effects. Hot brewing of black tea leads to the degradation of these thermo-sensitive compounds and also results in astringency due to the release of bitter tasting tannins. Cold brewing preserves the flavors and slowly releases bitter compounds thus yielding better taste, but limited due to longer extraction times.

There are numerous methods for the extraction of polyphenolics from black tea such as supercritical carbon dioxide extraction, microwave assisted extraction, heat reflux, solvent extraction, ultrahigh pressure extraction and so on. All though there are reports on extraction of tea bio-actives, most of the reports and research work was based on optimization of hot brewed tea and there are only few studies carried out at room temperature or cold temperatures. However, ultrasound-assisted extraction (UAE) can be used in order to maximize the extraction efficiency and could be used in combination with cold brewing to minimize the extraction times. Therefore, we report here a detailed overview on the optimization of process parameters using UAE for the extraction of bio-active compounds from black tea under cold brewing conditions, which implies that the temperature was maintained constant at 4°C similar to the industrial process of cold brewing. The optimization was based on response surface methodology and other aim of the process optimization was to minimize the extraction of tannins.

In order to achieve higher extraction efficiency, it is very important to design optimal process conditions. RSM or response surface methodology is a collection of both mathematical and statistical methods which is used extensively for process optimization (Box & Wilson, 2018) in the production of enzymes, drugs and various extraction methods (Liu et al., 2010; Omwamba & Hu, 2009). In this study, the extraction of total phenolic, tannins and antioxidant scavenging activity of DPPH and ABTS were considered as the responses. The process optimization is not only based on the highest yield of phenolic compounds but also on the potent functions of the bio-active compounds. Nowadays, the choice of extraction techniques has to be used in-order to perform the extraction of the desired metabolite for a specific plant product and has to be a result of a compromise between the efficiency and reproducibility of the method of extraction with an ease of procedure. Along with this, consideration of cost, safety, quality, time and with variable degree of automation must be looked into for designing an optimal process.

5.2. Materials and methods

5.2.1. Chemicals and reagents

In this study, commercial black tea, obtained from Tea Co Brenner®, Batavia, IL, USA was used as a sample. Methanol and Sodium Carbonate (Na_2CO_3) was obtained from Fischer Chemicals, NJ, USA; DPPH (2,2'-diphenyl1-picrylhydrazyl), ABTS (2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid) and Trolox standards were purchased from EMD Millipore, San Diego, CA, USA. Folin Ciocalteu reagent from Sigma Aldrich, St. Louis, MO, USA, Gallic acid from ChemImpex, Wood Dale, IL, USA, potassium persulfate from Labchem, Zelienople, PA, USA and sodium hydroxide from Ricca

Chemicals, Arlington, TX, USA. For filtrations, Whatman filter paper No.1 (125mm) was used.

5.2.2. Sample preparation for Conventional extraction of cold brewed black tea

In this study, the cold brewing is performed with 0.5 g of black tea sample mixed with 50 ml of double distilled water and the tea was allowed to brew at a constant temperature of 4ºC for a duration of 6 hours. The cold brewed sample was centrifuged at 3000 rpm (Model: Allegra X-30R, Beckman Coulter Inc., Chaska, MN, USA) for 3 minutes to obtain a clear solution. It was then filtered with a 125mm Whatman filter paper No. 1 and the filtered tea was stored at -40ºC for further analysis.

5.2.3. Ultrasound assisted extraction of cold brewed black tea

Ultrasound assisted extraction of cold brewed black tea was performed using a Sonifier ® cell disrupter (Model: SLPe EDP 100-214-254, BRANSON Ultrasonics Corporation, Danbury, CT, USA) which is capable of operating at different amplitudes and sonication times. From the literature, it was evident that the critical factors influencing the extraction process using ultrasound were temperature, amplitude, sonication time and the solvent volume used for the extraction process. The effect of amplitude, solvent volume and the sonication time was studied using RSM. The experiments were carried out in a randomized order as suggested by design software (Design of experiments, Stat-ease Inc, Minneapolis, MN, USA). The experimental conditions were selected based on the results from OVAT analysis (Raghunath, Mallikarjunan, Schoenfuss, Roohinejad, & Gallaher 2019) (Table 6). The frequency of the horn was maintained constant at 40 kHz and water was used as a solvent for Ultrasound assisted extraction process. In general, conventional

cold brewing is performed at 4ºC in the brewing industries and hence temperature of extraction was kept constant at 4ºC throughout the UAE. After the extraction, the samples were subjected to centrifugation (Beckman Counter, Allegra X-30R Centrifuge, Chaska, MN, USA) at 3000rpm for 3 mins and the filtrate was collected after filtering it through Whatman filter paper No. 1 and stored at -40ºC for further analysis.

5.2.4. Analysis of water activity and moisture content

Black tea sample was analyzed for initial moisture content using an instant moisture analyzer (Model MB25, Ohaus Corp., Parsippany, NJ, USA) and it was measured in % moisture content, wet basis. The powdered black tea was kept in a polyethylene bags and stored at ambient temperature (21°C) throughout the study. The water activity was determined using a benchtop water activity analyzer (Model: Aqualab 3TE, Decagon Devices, Pullman, Washington, USA).

5.2.5. Analysis of Total phenolic content (TPC) with tannins of cold brewed black tea using Folin-Ciocalteau Assay

The phenolic content of the cold brewed black tea was measured using Folin-Ciocalteau Assay. Folin assay was formerly used for the determination of amino acid residues (Folin, O. & Ciocalteu, 1927), and then modified by (Singleton & Rossi, 1965) and (Singleton & Slinkard, 1977). The AOAC method identifies this method for the analysis of total phenol content in wines and is one of the most common methods used for the determination of total phenolics of a given sample spectroscopically as explained by (Budaraju et al., 2018; Dewanto et al., 2002) . Gallic acid (600M) was used to develop the

standard curve. Sample or standard (125 µl) was mixed with 500 µl of distilled water and 125 µl of Folin-Ciocalteau reagent (FCR) and 1250 µl of 7% aqueous sodium bicarbonate solution was added to the sample mixture. The final volume was made up to 3000 µl by the addition of distilled water. The reaction mixture was incubated in the dark for 1 hour at ambient temperature and the absorbance was measured at 760 nm using a spectrophotometer (UV-1800, Shimadzu Scientific Instruments Inc., Addison, IL, USA). The results were reported as mg of Gallic acid equivalence (GAE). All the experiments were performed and analyzed in quadruplicates and the average values were used for the data analysis.

5.2.6. Determination of antioxidant capacity of cold brewed black tea using DPPH radical scavenging activity

The electron transfer antioxidant DPPH assay was used to determine the antioxidant capacity of the cold brewed black tea, as explained by (Budaraju et al., 2018). The DPPH assay is the most commonly used method for different samples as it serves as a stable radical which can easily trap the free radicals (Fogarasi et al., 2015; Guo & Beta, 2013; Gupta, 2015; Li et al., 2005). In brief, an odd radical exhibits a very strong absorption band at a wavelength of 515 nm which loses its absorption once it is paired with a donated electron from a hydrogen or an antioxidant molecule. This scavenging activity was represented as percentage of DPPH radical activity. A standard curve was measured from a stock standard solution of 500 µM of trolox standard. 3900 µl of 60 µM solution of DPPH (freshly prepared every time at the time of analysis) was mixed with 100 µl of sample. The mixture with a final volume 4 ml was shaken and left in dark environment for 90 minutes

for complete reaction to take place. The antioxidant capacity was calculated by measuring the absorbance of the sample with DPPH at 515 nm using a spectrophotometer. The samples were analyzed in quadruplicates and the values were averaged for statistical approach and data analysis. Percentage DPPH activity was analyzed according to the following equation:

$$\%\text{DPPH} = \left(1 - \frac{A\,(sample)}{A(blank)}\right) * 100 \qquad (1)$$

Where A (sample) stands for absorbance of sample and A (blank) is the absorbance of control that contains only DPPH reagent.

5.2.7. Determination of antioxidant capacity of cold brewed black tea using ABTS assay

The antioxidant activity of cold brewed tea was also determined using ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging activity. The correlation between the amount of phenolics analyzed and the antioxidant activity was analyzed by using decolorization assay called Trolox equivalent antioxidant activity as described by (Dudonné, Vitrac, Coutiére, Woillez, & Mérillon, 2009). This assay involves reaction of ABTS cation radical with the phenolics present in the sample, which exhibit antioxidant activity. 7 mM of ABTS solution was mixed with equal volumes of 2.45 mM of potassium permanganate solution and the reaction mixture was kept in dark for 16 hours at room temperature. The reaction mixture was diluted with water until an absorbance of 0.70±0.02 was achieved at 734 nm. Then, 150 µl of the sample was mixed with 2850 µl of the ABTS radical solution and absorbance was taken after 1 hour of incubation at room temperature using the spectrophotometer. 0 to 500 µM concentrations of Trolox solution

were analyzed for the standard curve. The antioxidant activity was recorded as % ABTS antioxidant capacity. The assay was carried out in quadruplicates and the reagents for the analysis were freshly prepared each time at the time of analysis. Percentage ABTS activity was analyzed according to the following equation:

$$\%\text{ABTS} = \left(1 - \frac{A\,(sample)}{A(blank)}\right) * 100 \qquad (2)$$

Where A (sample) stands for absorbance of sample and A (blank) is the absorbance of control that contains only ABTS reagent.

5.2.8. Analysis of tannins by protein precipitation and Folin-Ciocalteau Assay

The tannin content of cold brewed black tea was measured using Folin-Ciocalteau assay, which was used by AOAC for the determination of phenolic in Wines, with a slight modification of protein precipitation (El-Din et al., (2015). This method gives the estimate of true tannins or total tannins present in the tea sample. The Tannins are well known to interact with the protein molecules and in this assay, casein, a milk protein, was used to precipitate the tannins from the tea sample along with the Folin-Ciocalteau reagent, which reacts with the phenols present in the sample. Gallic acid (0-100 µg) was used to measure the calibration curve and the results were expressed in Gallic acid equivalent (GAE). 1 g of casein was added to the sample solution and agitated for a period of 30 minutes for complete precipitation of the sample with the protein. The sample was centrifuged at 5000 rpm for 3 minutes and the supernatant was tested for phenolics. Initially, 0.5 ml of distilled water was added to 0.125 ml of the sample or the standard. To this reaction mixture 125 µl of Folin-Ciocalteau reagent and 1.25 ml of 7% sodium bicarbonate solution was added after 6 minutes and the final volume was adjusted to 3ml using distilled water. True tannins

present in the sample is calculated by subtracting non-tannin phenols (S2) from total phenols that included tannins (S1). The experiments were conducted and reported in quadruplicates and mean of the values were used for the analysis of the data. The total true tannins and Total phenolic content without the tannin content were used for the optimization of the process parameters.

5.2.9. Solvent

The solvent used in the UAE is mostly driven by the solubility of the target metabolite but can also depend on the physical parameters such as viscosity, surface tension and the vapor pressure of the solvent used. Since the solvent used in the experiments is mostly water it does affect the amount of bio-active compounds that are being extracted to an extent. But since the cold brewing is generally done with water and industrial scale the experiment was carried out by primarily using water as the solvent for extraction (Chemat et al., 2017b; Sanderson, 2004) . Hence, optimization is completely based on water as solvent by varying other parameters. The physical parameters as mentioned will interfere with the phenomenon of cavitation in the ultrasound, more particularly on the threshold phenomenon. The start of the cavitation process requires negative pressure during the rarefaction cycle in order to overcome the cohesive forces between the molecules in the liquid. Increase in the viscosity of the liquid, encourages an enhanced molecular interaction thereby increasing the cavitation threshold. Thus increase in viscosity increases the resistance if the sample movement to the waves produced by the ultra-sonication requiring higher amplitude to be used (Chemat et al., 2017b; Loupy et al., 2009).

5.2.10. Solvent Temperature

Temperature has a very strong interaction and has a great impact with the properties of the solvent. Increase in temperature, the sonication effects collapse due to the cavitation less violently and reduced effect of sonication to a maximum extent (Bendicho, 2009; Chemat et al., 2017b; Palma et al., 2013). Thus, lower temperatures favor the sonication effects and usually the temperature is controlled in-order to limit the increase or rise in temperature (Sališová, Toma, & Mason, 1997). More importantly, temperature plays a key role in the extraction of bio-active compounds in terms of extraction yield. The UAE is reported to have a beneficial effect at temperature range of 20 to 70°C along with the sonication when compared to the non-sonicated samples (Chemat et al., 2017b; Shirsath, Sonawane, & Gogate, 2012). This effect is caused to the increase in the number of bubbles formed and a larger solid solvent contact surface with enhanced solvent diffusion, desorption and diffusion of the bio-active compounds. Nevertheless, the temperature has a negative effect when boiling point of the solvent is reached and researchers generally suggest the lower temperatures like 30°C has the best effects (Esclapez, García-Pérez, Mulet, & Cárcel, 2011; Palma & Barroso, 2002; Zhang et al., 2008)

5.2.11. Ultra-sonication equipment

The ultra-sonication high power can be applied using two types of devices namely ultra-sonication bath and probe. Both these instruments are entirely based on the power transductor as a power source and the most commonly used ones are the piezo electric transducer. The bath has a stainless steel tank with one or more transducers and operate at a frequency of 40 kHz and sometimes equipped with temperature control. The ultrasonic probes are preferred for the extraction of bio-active compounds in general. The probe is

reported to be more powerful than bath due to the intensity of power being delivered on a smaller surface as in the tip of the ultra-sonication probe. The probe consists of a transducer bonded to the tip of the probe, which is immersed into the reactor with minimized energy loss over the period of time. There are different types of probes with diameters and tips for various purposes. The selection of a probe depends on the sample volume to be sonication and the intensity of the probe to the liquid media induces a negative impact in the reactor due to the temperature rise. But the process is taken care of by a double jacketed reactor or a water bath to maintain the temperature of the process consistent. The ultra-sonication equipment made today consists of stainless-steel reactor through which the sample or the fluid is pumped at a high pressure to conduct a mano-sonication process. The continuous reactor will be cooled and heated at the same time with a double walled mantle to conduct the mano-thermo-sonication process (Chemat et al., 2017b).

Table 7: Natural and coded values of independent variables of UAE used in response surface methodology

	Coded levels		
	-1	0	1
Variables	**Natural levels**		
Amplitude (%)	30	50	70
Solvent volume (ml)	25	50	75
Sonication time (mins)	30	45	60

Table 8: Experimentally obtained results for central composite design (CCD)- face centered design (FCD) for measured responses

Run	Amplitude (%)	Solvent Volume(ml)	Sonication time (minutes)	Total Phenolic content (mg GAE/g)	Total Tannin content (mg GAE/g)	DPPH Antioxidant scavenging activity (%)	ABTS Antioxidant scavenging activity (%)
1	50 (0)	25 (-1)	45 (0)	39.1	17.5	40.1	41.84
2	50 (0)	50 (0)	45 (0)	40.71	25.63	33.24	35.03
3	70 (1)	75 (1)	60 (1)	75.28	27.97	27.09	50.71
4	50 (0)	50 (0)	45 (0)	38.42	28.54	32.93	34.45
5	50 (0)	50 (0)	45 (0)	38.42	27.19	31.51	34.35
6	50 (0)	50 (0)	45 (0)	38.52	26.04	31.7	35.48
7	70 (1)	25 (-1)	60 (1)	44.16	30.63	33.6	39.48
8	70 (1)	25 (-1)	30 (-1)	72.59	6.46	37.15	61.58
9	50 (0)	50 (0)	60 (1)	47.58	30	29.5	28
10	30 (-1)	75 (1)	30 (-1)	49.5	8.75	15.89	41.26
11	50 (0)	50 (0)	45 (0)	41.75	25.1	32.53	35.29
12	30 (-1)	25 (-1)	30 (-1)	47.91	5.47	31.86	53.45
13	50 (0)	50 (0)	30 (-1)	46.65	15.52	27.76	28.28
14	30 (-1)	75 (1)	60 (1)	61.69	18.75	20.43	59.29
15	50 (0)	75 (1)	45 (0)	53.56	20.47	26.46	40.42
16	50 (0)	50 (0)	45 (0)	40.6	24.69	33.08	35.9
17	30 (-1)	25 (-1)	60 (1)	26.81	29.01	23.86	27.65
18	70 (1)	75 (1)	30 (-1)	58.25	21.56	20.03	28.74
19	30 (-1)	50 (0)	45 (0)	38.63	24.9	23.26	46.74
20	70 (1)	50 (0)	45 (0)	53.31	26.46	27.6	48.35

GAE, gallic acid equivalents; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiaziline-6-sulfonate

5.2. Experimental design and statistical analysis of responses

In this study, RSM was used to optimize the extraction of bio-active compounds from cold brewed black tea using ultrasonic cell disruption method. For this research, a three factored face centered central composite design (CCD) was developed using statistical design software (Design Expert, version 11, Stat-Ease Inc, Minneapolis, MN, USA). As shown in Table 7, three process factors namely Amplitude (%) as X_1, sonication time (minutes) as X_2 and solvent to solid ratio (V/W) as X_3, were analyzed at three different levels to investigate the effect the variables on the amount of phenolics, antioxidant activity and tannins extracted by face centered central composite design with 6 cube points. The highest and the lowest values of the parameters are coded as +1 and -1 with the mid value coded as 0.

The effect of amplitude (0, 10, 30, 50, and 70 %), solvent volume (25, 50, 75, and 100 ml) and the sonication time (10, 20, 30, 40, 50, and 60 min) was studied previously using one variable at a time (OVAT) analysis (Raghunath et al., 2019). The results from the previous study was summarized into intervals of 30 to 70% for amplitude, 25 to 75 ml of solvent volume and 30 to 60 mins of sonication time which produced a considerable change in the amount of bio-actives extracted. The levels from the OVAT study were used for the response surface modelling with highest and lowest values.

The Table **7** represents the matrix of the design with all the variables in both the non-coded and coded form. The results of the design were 21 experiments (Table 8). The Table 8 shows a quantitative analysis of measured data for the dependent variables namely, total phenolic content without tannin content, total tannin content, percentage antioxidant

activity for DPPH and % antioxidant activity of ABTS radical. The obtained results for the experimental data were fitted into the quadratic equations as shown in equation 3.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j, \quad (3)$$

In the above equation, the Y variable is called the dependent variable and the dependent variable is predicted using the independent variables in the process which are represented by X_1, X_2, and X_3 respectively. β_0 is called the constant coefficient and β_1, β_2, and β_3 corresponds to linear regression coefficients; β_{11}, β_{22}, and β_{33} corresponds to regression coefficients that are squared. β_{12}, β_{13}, and β_{23} corresponds to the interaction coefficients of the independent variables under consideration. The coefficients of responses were analyzed with ANOVA with 95% confidence interval.

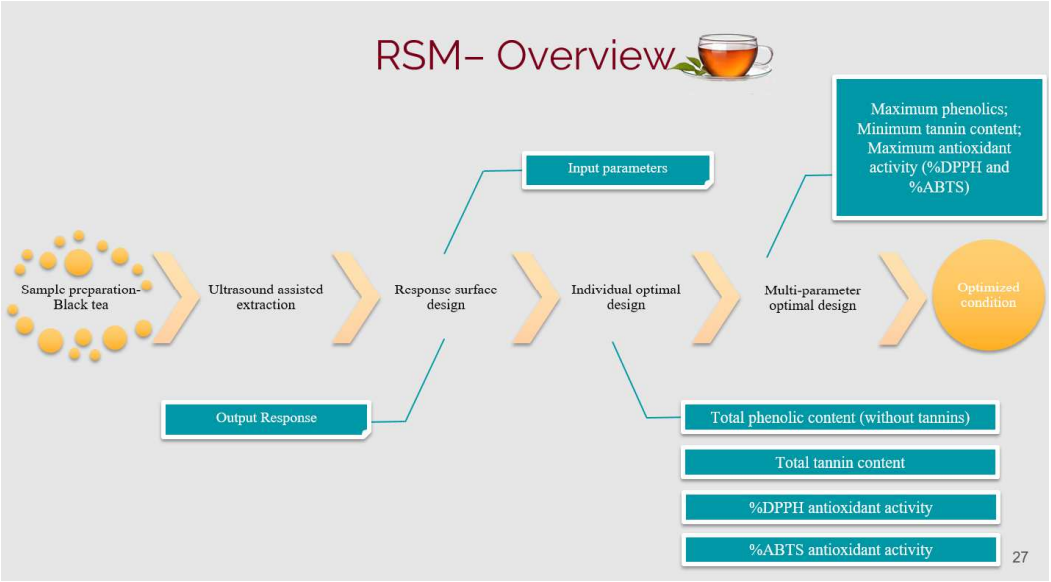


Figure 19: Overview of RSM experiments in the study

5.3. Validation of the optimized process

The validation of the optimized design was made with triplicate analysis of the optimized parameters in the study. The cold brewed black tea was subjected to ultra-sonication treatment under optimized conditions. The results obtained was compared with the theoretical values from the response surface design as well as with the control sample under study without the treatment of ultrasound.

5.4. Results and discussion

5.5.1. Water activity and moisture content of black tea

The water activity of black tea sample was found to be 0.11± 0.01 at 21ºC and the moisture content of black tea was found to be 6.33 ± 0.22 % on wet basis. The water activity of the sample is found to be low and hence the chances of microbial contamination can be assumed to be minimal. Moisture content analysis plays a crucial role in material quality and one of the essential parameters in quality control in various food industries. The value was found to be in correlation with the data published in the literature (Ikeda, 2013).

5.5.2. RSM model fitting

The effect of process conditions on four different parameters (TPC, TTC, DPPH, ABTS) were analyzed using central composite design. The results obtained for the parameters are shown in Table 8. ANOVA was applied in order to determine the coefficients of linear, quadratic and interaction terms for each response in Table 9. Influence of terms was described as statistically different ($p<0.05$) or insignificant ($p>0.05$). Coefficient of

determination (R^2) was first indicator of model adequacy (Table 10) and as well as ANOVA and other calculated statistical parameters explained significance of the models (Table 11). Relatively higher value of R^2 for all the four models or measured responses indicates that the second order polynomial equation is a good approximation of obtained results. Therefore, regression equations could be successfully applied as predictors in the investigated experimental domain. Predicted second order polynomial model for all the four investigated responses are presented in Table 9.

Table 9: Quadratic model equations for the investigated responses based on experimentation

Response	Quadratic Equation
TPC	$Y=151.26 - 0.3777X_1 - 1.4039X_2 - 3.5530X_3 - 0.0049X_1X_2 - 0.0010X_1X_3 + 0.0262X_2X_3 + 0.0106X_{11} + 0.0073X_{22} + 0.0240X_{33}$
TTC	$Y= -58.45 - 0.2490X_1 + 1.2082X_2 + 1.9667X_3 + 0.0048X_1X_2 - 0.0012X_1X_3 - 0.0104X_2X_3 + 0.0019X_{11} - 0.0094 X_{22} - 0.0095X_{33}$
%DPPH	$Y= 7.84 + 1.4859X_1 - 0.8727X_2 + 0.4616X_3 - 0.0010X_1X_2 + 0.0029X_1X_3 + 0.007X_2X_3 - 0.0141X_{11} + 0.0035X_{22} - 0.0108X_{33}$
%ABTS	$Y= 115.69 - 2.6177X_1 - 1.7068X_2 + 1.2991X_3 - 0.0102X_1X_2 + 0.0031X_1X_3 + 0.0293X_2X_3 + 0.0299X_{11} + 0.0088X_{22} - 0.0330X_{33}$

TPC, Total phenolic content; TTC, Total tannin content; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiaziline-6-sulfonate)

Table 10: Regression coefficients for various predicted second order polynomial model for different responses in study

Regression coefficient	Total phenolic content	Total tannin content	%DPPH	%ABTS
β_0	151.26	-58.45	7.84	115.69
β_1	-0.3777	-0.2490	1.4859	-2.6177
β_2	-1.4039	1.2082	-0.8727	-1.7068
β_3	-3.5530	1.9667	0.4616	1.2991

β_{12}	-0.0049	0.0048	-0.0010	-0.0102
β_{13}	-0.0010	-0.0012	0.0029	0.0031
β_{23}	0.0262	-0.0104	0.0077	0.0293
β_{11}	0.0106	0.0019	-0.0141	0.0299
β_{22}	0.0073	-0.0094	0.0035	0.0088
β_{33}	0.0240	-0.0095	-0.0108	-0.0330

DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiaziline-6-sulfonate)

5.5.4. Effect of UAE extraction factors on the extraction of total phenolics from cold brewed black tea

In this study, UAE was applied for maximum extraction of total phenolics from cold brewed black tea. In comparison to the classical methods of extraction, the application of UAE has its own benefits in terms of considerate reduction in the extraction time and increased extraction of bio-active compounds due to disruption of the cell wall structure and, therefore, accelerated diffusion through membranes. In combination with the water, as an extraction solvent, UAE satisfies the requirements of green processing and safe food production. According to the results on UAE of cold brewed black tea (Table 13) the highest extraction of total phenolic compounds without tannins (TPC) (74.629 mg GAE/g) was obtained by the application of ultrasonic amplitude of 70%, solvent volume of 75 ml and 60 minutes of sonication time, while the temperature was set at 4ºC. The lowest yield (26.81 mg GAE/g) was obtained by setting the extraction parameter to 30% amplitude, 25 ml solvent volume and 60 minutes of sonication time. The results obtained show that this method of extraction is much more efficient than the classical method of brewing, by using water as solvent. The UAE of Total phenolic from cold brewed black tea for 1/2 hour was 4.0 times higher than extraction by conventional brewing method for 6 hours. Therefore, for cold brewing of black tea with high concentration of phenolic compounds (without tannin content), UAE should be applied. In addition, the use of ultrasound is advisable for

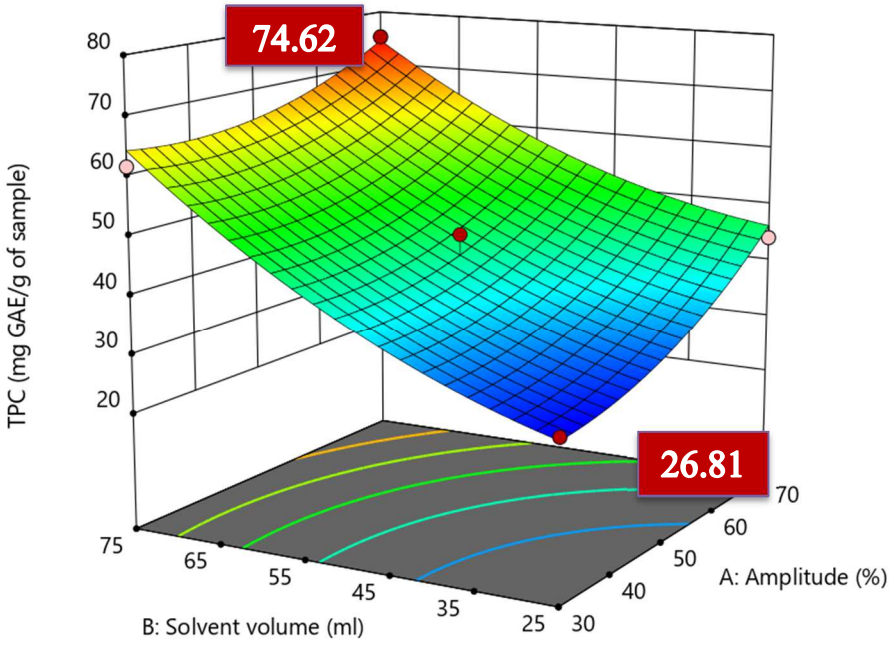
extracting thermo sensitive compounds (Adinath, Singh, Navin Chandra, & Jai, 2016). The higher amount of phenolics extracted is in accordance with the work carried out by (Mason & Yiyun Zhao, 1994a) which showed a 40% increase in the extraction efficiency of tea solids after 10 minutes of sonication. Both, Chemat, & Strube, 2014 showed an increase of 30-35% in polyphenols content with UAE at 40ºC. A similar study on the extraction of polyphenols from yellow tea was done with the help of ultrasonic probe and showed a maximum yield of phenolics at 30 minutes of sonication and agrees well to the current study in achieving maximum phenolics at 30 minutes of sonication time (Horžić, Jambrak, Belščak-Cvitanović, Komes, & Lelas, 2012b). A comparative study done by (Choung et al., 2014) from green tea leaves also proved that UAE was found to be more effective in accordance with time and productivity at lower temperature (25°C).

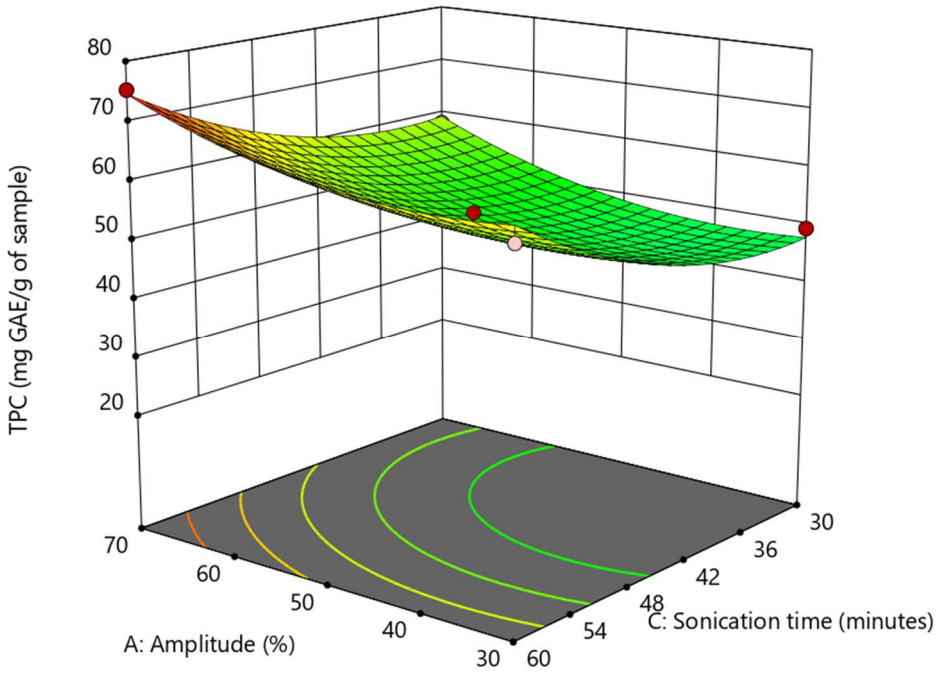
To determine the optimal levels of independent variables for the total phenolic extraction from cold brewed tea, RSM was applied and the response surface plots were created. According to the results obtained and further mathematical analysis, extraction of Total phenolic from black tea was described by the following equation as

$$\text{TPC (without tannins)} = 151.26 - 0.3777X_1 - 1.4039X_2 - 3.5530X_3 - 0.0049X_1X_2 - 0.0010X_1X_3 + 0.0262X_2X_3 + 0.0106X_{11} + 0.0073X_{22} + 0.0240X_{33} \quad (4)$$

The most dominant and highly significant factor that affects the extraction of total phenolics from cold brewed black tea was found to be amplitude and solvent volume which is in accordance with (Medina-Torres et al., 2017a). This implies that with an increase in amplitude, there is an increased compression and rarefaction of waves resulting in higher extraction of phenolic from tea leaves (Al-Dhabi, Ponmurugan, & Maran Jeganathan, 2017b).

The results further indicate that the increase in sonication time with increase in amplitude increased the amount of phenols. The reason behind this can be explained by the effect of amplitude as the intensity of the waves and sonication time as the interaction of the sonication. Thus, increased levels of both sonication time and amplitude leads to improved extraction opportunities. In addition, increase in sonication time and solvent volume also increased the phenolic content. However, it can be noted that there is only a slight difference of 7% in the total phenolics extracted when the both the sonication time and the solvent volume decreased. Increasing sonication time didn't produce a substantial increase in the phenolic content and might not be cost effective.




TPC (mg GAE/g of sample)
80
70
60
50
40
30
20
70
60
50
40
30
A: Amplitude (%)
30
36
42
48
54
60
C: Sonication time (minutes)

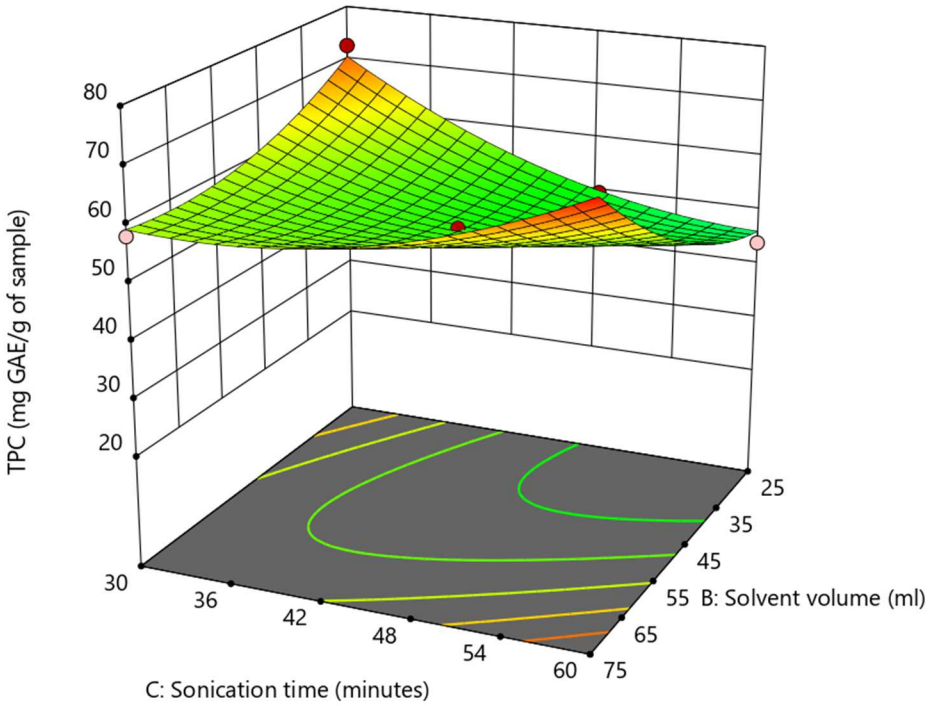


Figure 190: Three dimensional plot (a) showing the mutual effect of amplitude and solvent volume; three dimensional plot (sonication time: 60 min, temperature: 4°C) (b) showing mutual effect of amplitude and sonication time (solvent volume: 75ml, temperature: 4°C), and the three dimensional plot (c) showing the mutual effect of sonication time and solvent volume on total phenolic content (without tannins) extracted from cold brewed black tea using ultrasound assisted extraction (amplitude:70%, temperature:4°C).

5.5.5 Effect of UAE extraction factors on the extraction of total tannin content from cold brewed black tea

Similar to the extraction of total phenolic content, the data obtained for total tannin content (Table 8) were fitted into a second order polynomial equation and the equation is given by

Total Tannin Content = - 58.45 - 0.2490X_1 + 1.2082X_2 + 1.9667X_3 + 0.0048 X_1X_2 - 0.0012X_1- 0.0104X_2X_3 + 0.0019X_{11} - 0.0094 X_{22} - 0.0095X_{33} (5)

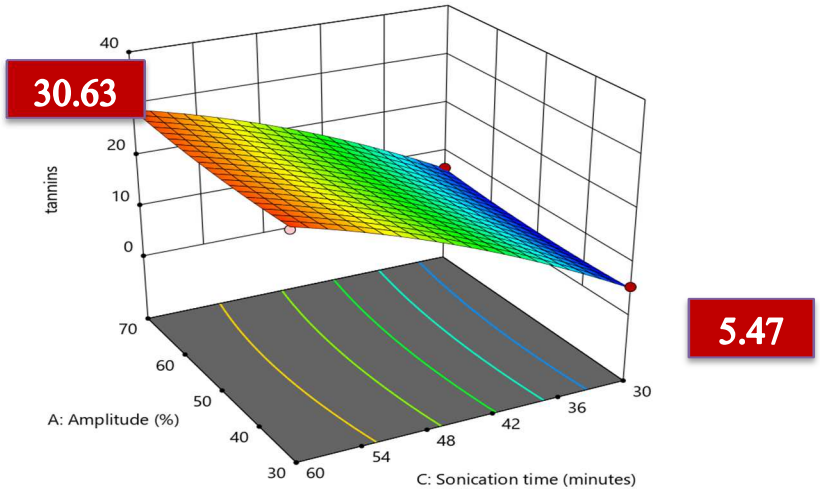
The tannin content varied from 5.47 mg GAE/g to 30.63 mg GAE/g for cold brewed black tea which is in accordance with the literature (Annegowda, Anwar, Mordi,

Ramanathan, & Mansor, 2010). Both the lowest and the highest tannin content was obtained under the conditions as: 30% amplitude, 25ml solvent volume, 30 minutes of sonication time and 70% amplitude, 25ml solvent volume and 60 minutes of sonication time, respectively. The Fisher's F test for the model had a high F value of about (43.12) and low p value (<0.0000) indicating that the model is a good fit for the data obtained. In the given model, the lack of fit (0.2741) was not significant in relation to the pure error. This gives the indication that the model stands true for all the predictions under all the combinations of independent variables. The R^2 values for the predicted model also suggest a satisfactory correlation between the actual and the predicted values as it was close to 1.

The 3D response surface of this model helped to understand that all the three independent variables like amplitude, solvent volume and sonication time has shown to have an effect on the extraction of tannins. The optimum operating parameters for minimum total tannin content was selected based on the desirability as 42.96% amplitude, 25 ml solvent volume and 30 minutes of sonication time.

The major aim of the study was to minimize the tannin content from extraction due to inhibition in iron absorption (Delimont et al., 2017). This is very important as the black tea lead to a significant reduction in iron absorption (Kim & Miller, 2018). However, it has to be noted that application of ultra-sonication minimized the total tannins extracted (83%) to a larger extent but cannot completely avoid the extraction of tannins from the tea leaves. With minimum total tannins of 4.89 mg GAE/g per run, the total phenolic content, %DPPH and %ABTS activity for the optimal process parameters were predicted using the model equations as shown in Table 13.

The decrease in sonication time, amplitude and solvent volume will lead to minimized tannin extraction (4.89 mg GAE/g) and TPC at this optimum condition was found to be 70% extractable (52.193 mg GAE/g). This optimized condition also showed higher amount of %DPPH and %ABTS antioxidant activity. Tannins are extracted at higher temperatures in hot brewing and due to the external physical force, ultrasound also results in higher tannin extractions (Mason & Yiyun Zhao, 1994a). Many of the optimization studies is based on increasing the extractability of tannins due to its antioxidant activities. Nevertheless, this study focused on reducing the extraction of tannins to a large extent by optimization due to their inhibition in iron absorption.



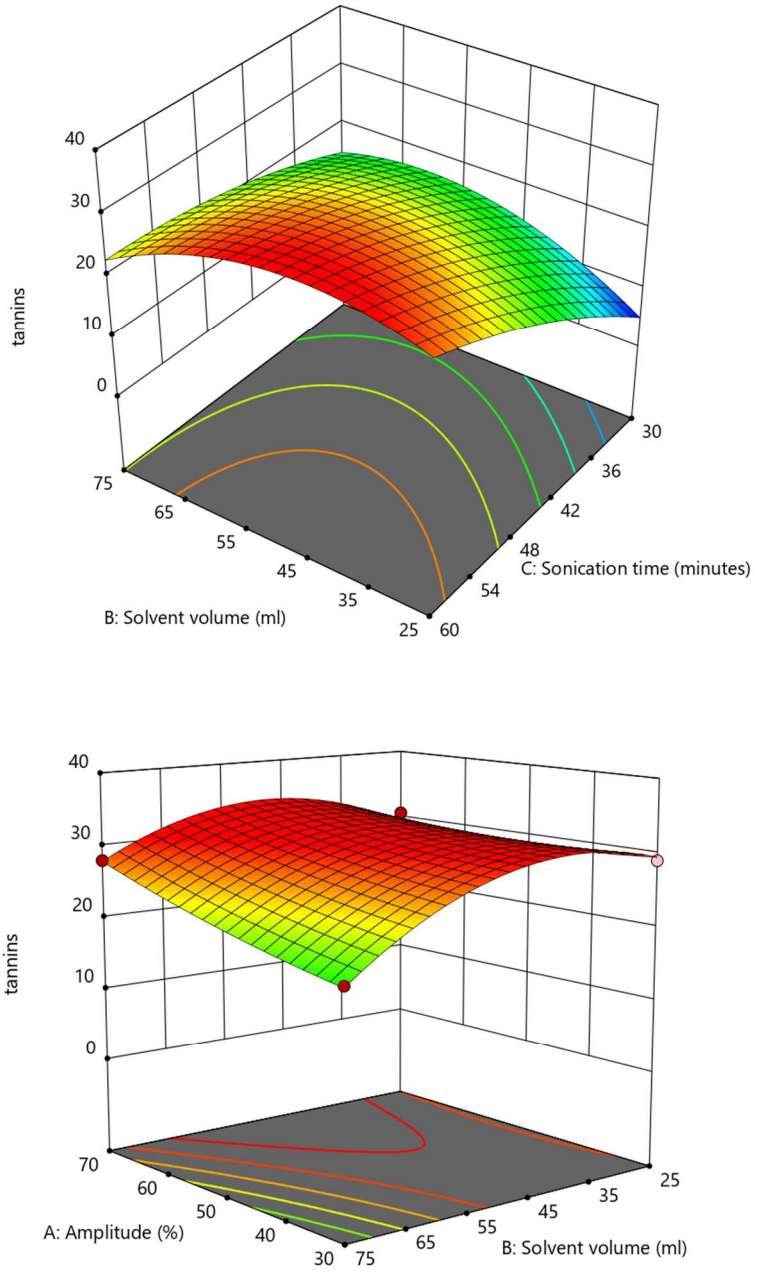


Figure 201: Three dimensional plot (a) showing the mutual effect of amplitude and solvent volume; three dimensional plot (solvent volume: 25ml, temperature: 4°C) (b) showing mutual effect of amplitude and sonication time (amplitude: 52%, temperature: 4°C), and the three dimensional plot (c) showing the mutual effect of sonication time and solvent volume on total tannins content extracted from cold brewed black tea using ultrasound assisted extraction (sonication time: 60 min, temperature: 4°C).

Table 11: ANOVA for fitted models

Source	Sum of squares	Degree of freedom	Mean of square	F value	*p* value
Total phenolic content					
Model	3695.94	9	299.55	51.85	<0.0001
Residual	57.78	10	5.78		
Lack of fit	47.08	5	9.42	4.40	0.0648
Pure error	10.69	5	2.14		
Total	2753.72	19			
Coefficient of determination (R^2)	0.9601				
Total Tannin content					
Model	1104.76	9	122.75	43.12	<0.0001
Residual	28.47	10	2.85		
Lack of fit	18.17	5	3.63	1.76	0.2741
Pure error	10.30	5	2.06		
Total	1133.22	19			
Coefficient of determination (R^2)	0.9523				
DPPH- % antioxidant scavenging activity					
Model	677.99	9	75.33	46.83	<0.0001
Residual	16.09	10	1.61		
Lack of fit	13.40	5	2.68	4.98	0.0513
Pure error	2.69	5	0.5380		
Total	649.08	19			
Coefficient of determination (R^2)	0.9560				
ABTS- % Radical scavenging activity					
Model	1953.98	9	217.11	391.87	<0.0001
Residual	5.54	10	0.5540		
Lack of fit	3.73	5	0.7463	2.06	0.2228
Pure error	1.81	5	0.3617		
Total	1959.52	19			
Coefficient of determination (R^2)	0.9946				

TPC, Total phenolic content; TTC, Total tannin content; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiaziline-6-sulfonate)
P<0.01 and statistically significant

Table 12: Predicted optimized condition values of individual investigated responses for cold brewing of black tea based on maximum phenolics and antioxidant activity with minimum tannins

Optimized conditions	
Amplitude (%)	69.892
Solvent volume (ml)	25
Sonication time (mins)	30
Predicted values	
TPC (mg GAE/g)	70.404
TTC (mg GAE/g)	6.32
DPPH (% antioxidant scavenging activity)	37.129
ABTS (% antioxidant scavenging activity)	61.581

TPC, Total phenolic content; TTC, Total tannin content; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiaziline-6-sulfonate)

5.5.6. Effect of UAE extraction factors on the antioxidant capacity %DPPH from cold brewed black tea

In this study, the radical scavenging activity of DPPH ranged from 15.89% to 40.1% and is known to range between 20 to 45% for black teas depending on the temperature and amplitude applied (Bakht et al., 2018). From the research done by (Bakht et al., 2018; Chen, Wang, Zhang, & Huang, 2012) on the effect of ultrasound on black tea, it was evident that both temperature and amplitude played a significant role in enhancing or reducing the scavenging activity of DPPH and activity dropped down at 50ºC to a larger extent. The reason reported was that some of the thermos-sensitive compounds was destroyed at that temperature and led to a decrease in antioxidant activity (Bakht et al., 2018; Chen et al., 2012). This helped to understand the balance the lower temperature effects that lead to enhanced extraction of bio-actives.

The results of the study for DPPH radical scavenging activity indicated that the processing parameters had an impact on the processing parameters. The quadratic equation for %DPPH scavenging activity is given by,

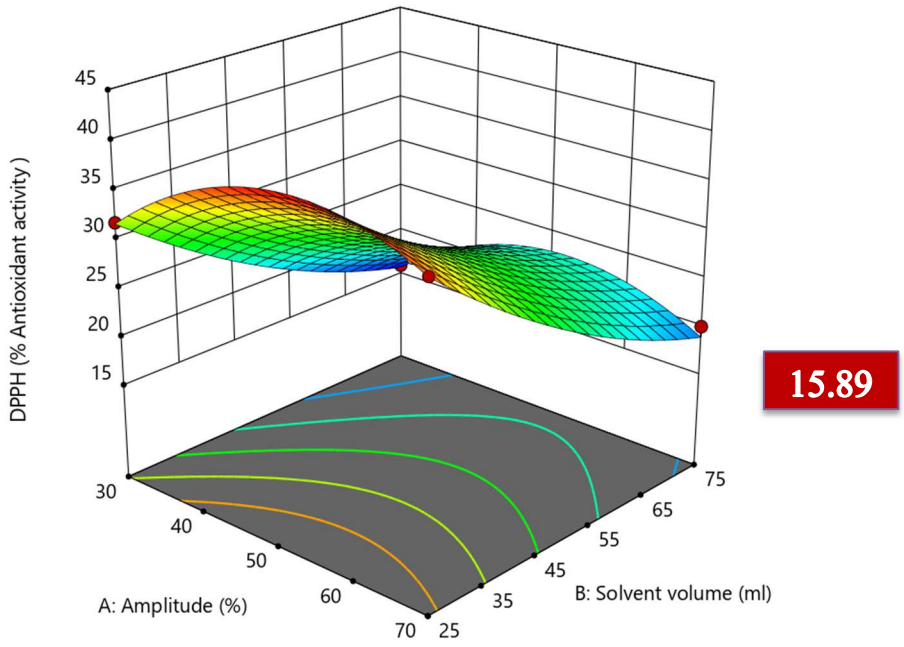
$$\%DPPH = 7.84 + 1.4859X_1 - 0.8727X_2 + 0.4616X_3 - 0.0010X_1X_2 + 0.0029X_1X_3 + 0.007X_2X_3 - 0.0141X_{11} + 0.0035X_{22} - 0.0108X_{33} \quad (6)$$

The lowest value of radical scavenging activity was obtained under the following process parameters: 30% amplitude, 75 ml solvent volume and 30 minutes of sonication time. On the other hand, the highest radical scavenging activity of DPPH was obtained under 50% amplitude, 25 ml solvent volume and 45 minutes of sonication time, which were different from the conditions for highest total phenolic content and %ABTS scavenging activity. The coefficients of regression and ANOVA analysis are reported in Tables 10 and 11 respectively. According to the ANOVA analysis, the linear terms of amplitude and sonication time and interaction terms between the amplitude and solvent volume as well as the quadratic term of amplitude were positive.

Further analysis suggests that the increase in solvent volume, amplitude and sonication time till the mid-way increased the %DPPH activity and then decreased. The possible reason for the increase might be due to increased extraction of bio-active compounds due to increased disruption of cells (tea leaves) with increased sonication time and amplitude. The decrease noted in the study might lead to two possible reasons one is the effect of destruction of various compounds due to continuous extraction or extraction of compounds which do not exhibit antioxidant activity. However, increased sonication time with lower solvent volume resulted in higher %DPPH activity. Thus, in order to obtain a maximum % antioxidant scavenging activity by DPPH (40.31 %) the optimum conditions

were found to be 55% amplitude, 25 ml solvent volume, 30 min sonication time. The associated values of TTC, TPC, %ABTS for the optimum conditions were calculated and represented in Table 13.

Similar to work conducted by (Altemimi, Choudhary, Watson, & Lightfoot, 2015) indicated that highest DPPH antioxidant scavenging activity of 64.18% was observed at 37 kHz frequency with 50% amplitude and 30 minutes of sonication time which is close to optimized conditions of this study. Thus, the results obtained from this study for radical scavenging activity of DPPH was in agreement with parallel research work (Wang et al., 2013) on Sparganii rhizoma.



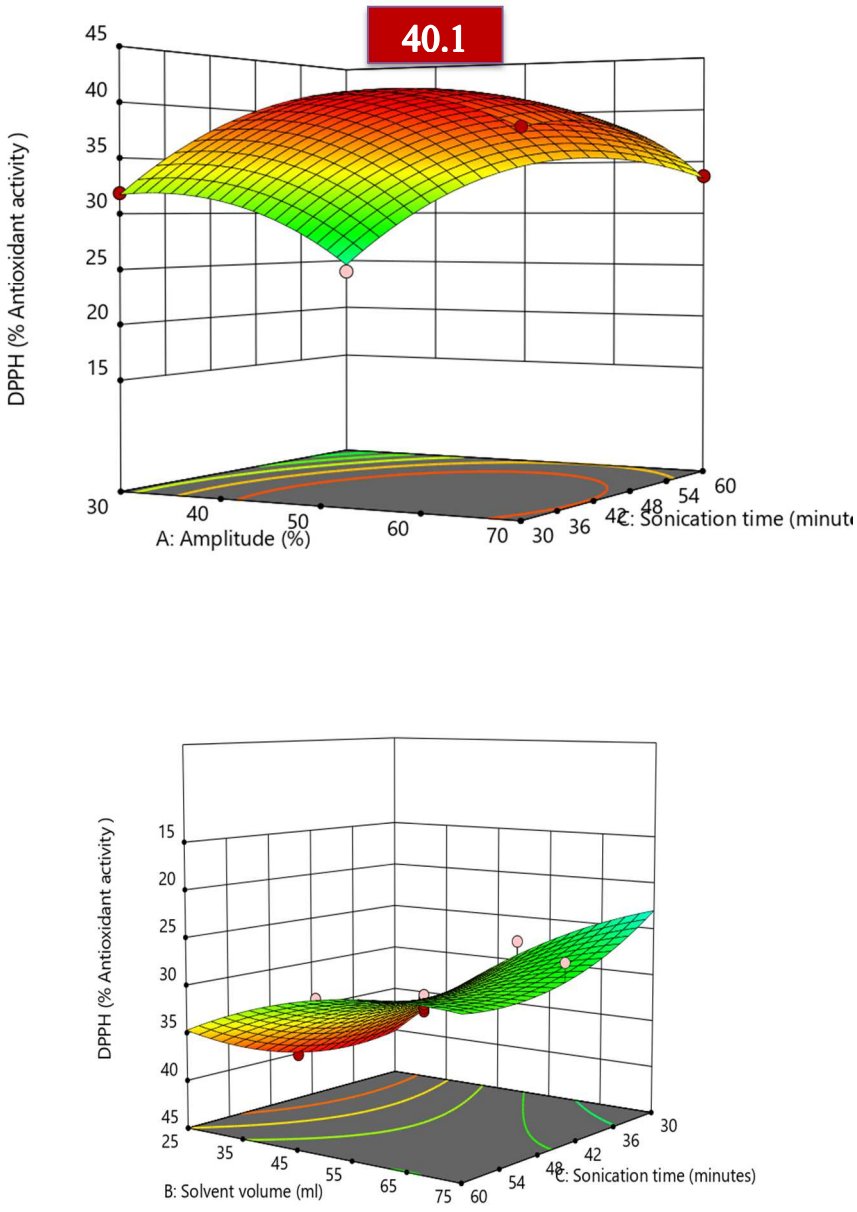


Figure 212: Three-dimensional plot (a) showing the mutual effect of amplitude and solvent volume; three-dimensional plot (sonication time: 30 min, temperature: 4°C) (b) showing mutual effect of amplitude and sonication time, and the three-dimensional plot (solvent volume: 25ml, temperature: 4°C) (c) showing the mutual effect of solvent volume and sonication time on antioxidant activity (DPPH) extracted from cold brewed black tea using ultrasound assisted extraction (amplitude: 60%, temperature : 4°C).

5.5.7 Effect of UAE extraction factors on the antioxidant capacity of %ABTS from cold brewed black tea

The results of processing parameters on the %ABTS scavenging activity is reported in Table 8. The highest antioxidant activity of 61.58% was obtained under the following process parameters: 70% amplitude, 25 ml of solvent volume and 30 minutes while the lowest antioxidant activity was observed at 50% amplitude, 50 ml solvent volume and 60 minutes of sonication time. The second order polynomial equation for %ABTS antioxidant capacity is given by

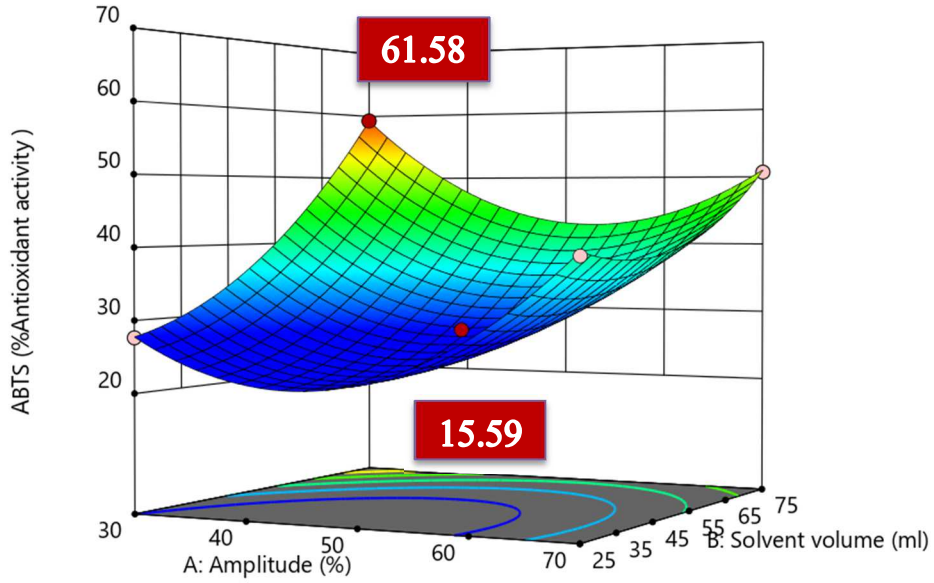
$$\% \text{ ABTS} = 115.69 - 2.6177X_1 - 1.7068X_2 + 1.2991X_3 - 0.0102X_1X_2 + 0.0031X_1X_3 + 0.0293X_2X_3 + 0.0299X_{11} + 0.0088X_{22} - 0.0330X_{33}. \quad (7)$$

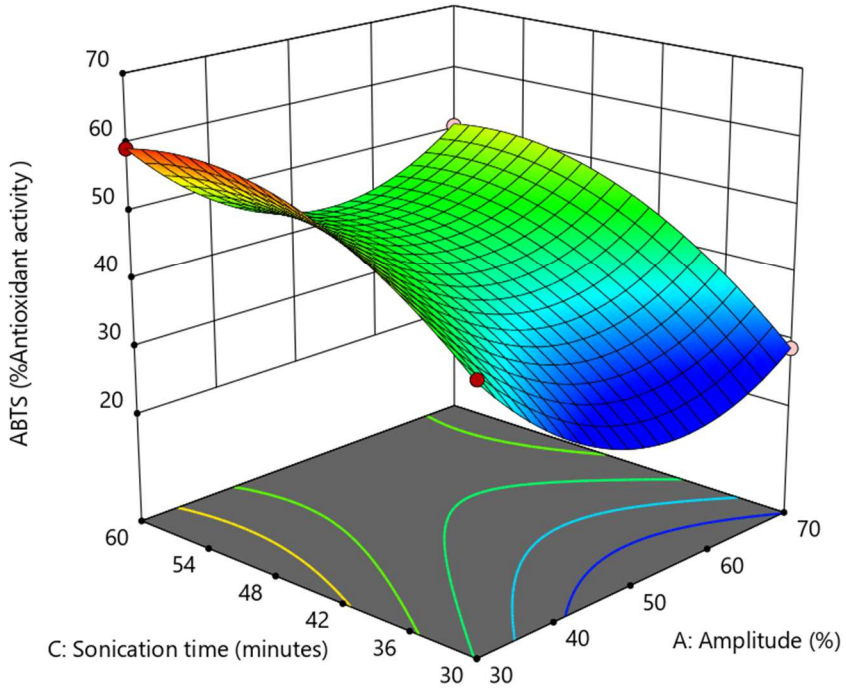
From the response surface model, decrease in sonication time and solvent volume increases the %ABTS activity. Similar to the work done by (Annegowda et al., 2010) shows a significant decrease in %ABTS activity with increased sonication time. The probable reason for decreased activity during prolonged sonication might be the cause of decreased area of diffusion with aligns with this study (Annegowda et al., 2010; Szydłowska-Czerniak & Tułodziecka, 2014).

According to the results from Table 13, the optimum conditions for maximum %ABTS radical scavenging activity were found to be 70% amplitude, 25 ml solvent volume and 30 minutes sonication time. This optimum condition ensures higher TPC owing to the fact the decrease in solvent volume and sonication time can affect the TPC content as discussed earlier and a minimum TTC of 6.28 mg GAE/g and %DPPH of 40.31% and 61.581% for ABTS.

The values obtained for %ABTS and %DPPH activities are different due to the fact the DPPH assay is more sensitive to environmental conditions and it is subject to differ

with solvents used, pH and other parameters (Pisoschi & Negulescu, 2012; Prior, Wu, & Schaich, 2005b). On the other hand, the ABTS radical is very stable and it is not variable to solvents used as well as other environmental conditions (Gupta, 2015).





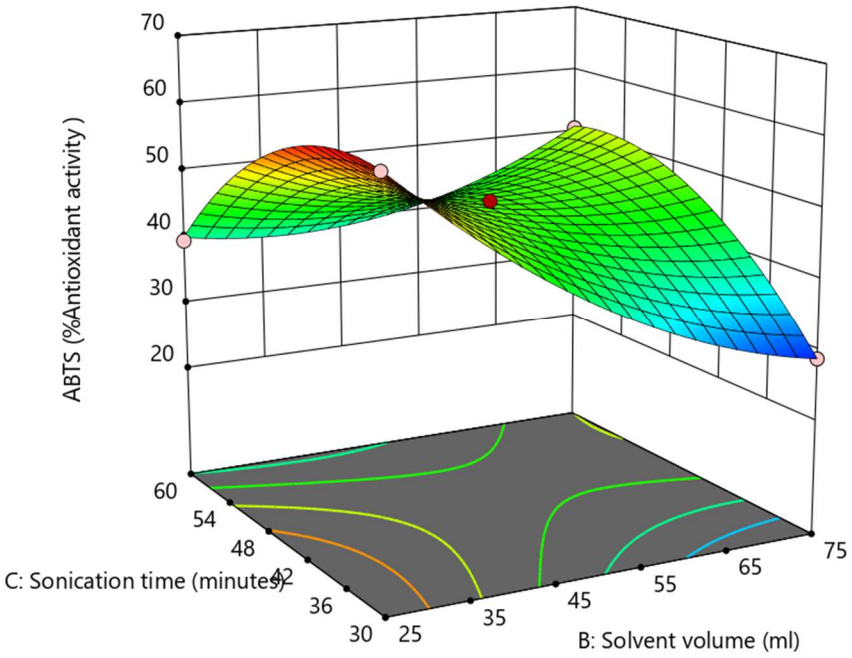


Figure 223: Three dimensional plot (a) showing the mutual effect of amplitude and solvent volume (sonication time: 42.6 min, temperature: 4°C); three dimensional plot (b) showing mutual effect of amplitude and sonication time (solvent volume: 75ml, temperature : 4°C), and the three dimensional plot (c) showing the mutual effect of sonication time and solvent volume on antioxidant activity (ABTS) extracted from cold brewed black tea using ultrasound assisted extraction (amplitude: 70%, temperature: 4°C).

Table 13: Predicted optimum conditions for individual responses

Optimized conditions – Maximum phenolic content	
Amplitude (%)	70
Solvent volume (ml)	75
Sonication time (mins)	60
Predicted values	
TPC (mg GAE/g)	74.62
TTC (mg GAE/g)	28.33
DPPH (% antioxidant scavenging activity)	27.40
ABTS (% antioxidant scavenging activity)	50.92
Optimized conditions- Minimum tannin content	
Amplitude (%)	42.96
Solvent volume (ml)	25
Sonication time (min)	30
Predicted values	
TPC (mg GAE/g)	52.19
TTC (mg GAE/g)	4.89
DPPH (% antioxidant scavenging activity)	38.59
ABTS (% antioxidant scavenging activity)	45.54
Optimized conditions- Maximum Antioxidant scavenging activity (%DPPH)	
Amplitude (%)	54.91
Solvent volume (ml)	25.12
Sonication time (min)	30
Predicted values	
TPC (mg GAE/g)	58.19
TTC (mg GAE/g)	5.28
DPPH (% antioxidant scavenging activity)	40.31
ABTS (% antioxidant scavenging activity)	47.18
Optimized conditions- Maximum Antioxidant scavenging activity (%ABTS)	
Amplitude (%)	70
Solvent volume (ml)	25
Sonication time (min)	30
Predicted values	
TPC (mg GAE/g)	70.23
TTC (mg GAE/g)	6.28
DPPH (% antioxidant scavenging activity)	37.38
ABTS (% antioxidant scavenging activity)	61.58

TPC, Total phenolic content; TTC, Total tannin content; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiaziline-6-sulfonate)

Table 14: Desirability of the optimized models

Optimized models	Desirability
Model 1 – Maximum TPC	0.987
Model 2 – Minimum TTC	1
Model 3 – Maximum DPPH activity	1
Model 4 – Maximum ABTS activity	1
Model 5 – Combined model	0.949

TPC, Total phenolic content; TTC, Total tannin content; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2,2′-azinobis (3-ethylbenzothiaziline-6-sulfonate)

5.5.8. Optimization of the process parameters for cold brewed black tea and validation of the response surface model

Multi-parameter optimization of the UAE for cold brewed black tea was the main goal of the research study. The estimated conditions and predicted values of the responses are presented in the **Table 12**. The multi-parameter optimized condition for maximum extraction of TPC, %DPPH and %ABTS and minimum extraction of TTC, simultaneously, were found to be 69.9% amplitude, 25 ml solvent volume and 30 minutes of sonication time. The predicted values for the process responses are as follows: 70.4 mg GAE/g, 6.32 mg GAE/g, 37.12%, 61.581% for TPC, TTC, %DPPH, and %ABTS respectively. The desirability of the optimized condition was 0.949.

The response surface model represented that dependent variables were affected by the independent variables for ultrasound assisted extraction for cold brewed black tea. Validation study was done in order to verify the results of the theoretically determined models under the optimum conditions specified. T-test was used to determine the difference between the experimental and theoretical values. The test proves that results are in good agreement with predicted values.

The validation of the model was conducted at 70%, 25 ml and 30 mins of amplitude, solvent volume and sonication time, respectively. With these optimized conditions, the predicted responses for the yield was 70.4 mg GAE/g, 6.32 mg GAE/g, 37.12%, 61.58% for TPC, TTC, %DPPH, and %ABTS, respectively. The experimental values for the optimized process conditions for individual responses and multiparameter responses are summarized in Table 26 and 27(Appendix 1). Based on the comparison, the experimental values were in agreement with the predicted values and thus validating the response surface model.

5.5. Conclusion and future trends of ultra-sonication

Utilization of ultrasound technology for the extraction of bio-actives in food has evolved. This newer system for cold brewing of teas in the market will provide net advantages which includes increased yield and selectivity, reduced extraction time and extract with quality and safety with easy integration in industry and eco-friendly.

RSM was successfully applied to optimize the conditions of the ultrasound assisted cold brewing of black tea. The results obtained shows that a second order polynomial model described the extraction process effectively. This study summarizes the effect of various process parameters of UAE for cold brewing of black tea. The optimized condition for maximum extraction of bio-actives for cold brewing black tea was found to be 69.9% amplitude, 25 ml solvent volume and 30 minutes of sonication time. In conclusion, this research also helped to understand the critical parameters based on the responses required. The presented results can stand as a bridge for designing novel techniques for accelerated extraction process for cold brewed black tea.

In order to ensure safety, sustainability and eco-friendly methods, it is very important to design an equipment for industrial application with maximum process extraction and reduced energy consumption. Both the types of ultra-sonication devices are used industrially but the choice of systems is based on the required potential or efficiency, the choice of the matrix (sample) and the application for which it is desired (Chemat et al., 2017b). The major factor influencing an industrial set up will be the quantity of the sample or the product to be treated and the ultra-sonication probe are usually restricted to a smaller volume. This often being a case, one of the solutions used industrially is usage of continuous system that will handle a larger amount of volume with a restriction in the volume of the reactor and then concentrating the ultra-sonication power to maximum to the restricted volume. A large number of companies have been already using the ultra-sonication technology and in the industrial basis most of the compounds extracted are directly used as in a liquor industry or can be used as a food and cosmetic additives. Thus, from this study, we can propose to use the model in an industrial scale using continuous system for the ultra-sonication process. The take away from the research was that the usage of ultra-sonication for the cold brewing of black tea with maximum advantage of bio-actives can potentially reduce the brewing time from 6 hours to maximum of 30 minutes. This will in turn help to save energy to a large extent and at the same time help to increase the production/day and benefit the company economically. However, ultra-sonication is sometimes regarded as expensive but owing to a one-time investment in the equipment will help to save both energy and increase the profit and production for a company.

Chapter 6 :Concluding remarks and next steps

An optimal design for cold brewed black tea was successfully developed using response surface methodology. This study demonstrates that amplitude, sonication time and solvent volume are the critical parameters into consideration to optimize the extraction yield of various responses. An increased amplitude with decreased solvent volume and sonication time resulted in maximum extraction of phenolic content with maximum antioxidant activity and minimum tannin content. Successful validation study of cold brewing black tea indicates that the ultrasound can be used as an alternative method for extraction with minimal extraction time. The beverage industry can use this technology to produce cold brewed black tea production more gallons per day. However, the experimental design needs to be scaled up to be used in an industry.

Research is currently being considered as a comparative analysis, which suggests that RSM is better method of optimization than OVAT analysis. The optimization design with RSM shows an increase of four times the amount of total phenolics extracted whereas the OVAT model shows only an increase of 2.9 times with respect to the conventional cold brewing methods. Thus, the study proves that RSM is always an advanced and better method of analysis for optimal designs. Additional steps for the research include investigating the individual profile of the phenolics extracted and optimization based on the compound required. The flavor profile of conventional vs ultrasound assisted cold brewing of black tea may help in understanding the compounds extracted at different levels of process parameters.

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Chapter 8: Appendix

Appendix 1. Analysis of total phenolic content and % antioxidant scavenging activity of cold brewed black tea

Table 15: Yield of TPC and % Antioxidant scavenging activity as a function of amplitude

Amplitude (%)	Total phenolic content (mg GAE/g)	% ANTIOXIDANT activity (DPPH)
0	19.50 ± 0.76^{c}	26.74 ± 0.36^{b}
10	20.52 ± 1.70^{c}	21.95 ± 0.55^{c}
30	20.66 ± 0.54^{c}	22.85 ± 0.61^{c}
50	26.53 ± 1.07^{b}	25.05 ± 0.21^{bc}
70	36.38 ± 2.67^{a}	42.24 ± 2.49^{a}

Lowercase letters in each row indicates significant difference among samples (p-value≤0.05)

Table 16: Yield of TPC and % Antioxidant scavenging activity as a function of solvent volume

Solvent volume(ml)	Total phenolic content (mg GAE/g)	% Antioxidant activity (DPPH)
25	25.76 ± 0.05^{c}	34.66 ± 2.52^{b}
50	31.16 ± 0.97^{b}	40.63 ± 0.69^{a}
75	46.65 ± 1.31^{a}	29.07 ± 0.15^{c}
100	39.01 ± 2.79^{b}	23.15 ± 0.57^{d}

Lowercase letters in each row indicates significant difference among samples (p-value≤0.05)

Table 17: Yield of TPC and % Antioxidant scavenging activity as a function of sonication time

Sonication time (minutes)	Total phenolic content (mg GAE/g)	% Antioxidant activity (DPPH)
10	42.95 ± 1.53^{d}	25.40 ± 0.62^{c}
20	46.97 ± 1.04^{c}	26.70 ± 0.17^{c}
30	47.19 ± 1.23^{c}	29.14 ± 0.03^{b}
40	52.15 ± 0.16^{b}	29.16 ± 0.15^{b}
50	57.63 ± 1.31^{a}	32.27 ± 0.78^{a}
60	60.23 ± 0.75^{a}	30.86 ± 0.89^{a}

Lowercase letters in each row indicates significant difference among samples (p-value≤0.05)

Table 18: Pseudo second order modelling data for total phenolic content

Sonication time (minutes)	Total phenolic content (mg GAE/g) –Experimental value	Total phenolic content (mg GAE/g)- Model value
10	42.95	36.03
20	46.97	46.71
30	47.19	51.83
40	52.15	54.83
50	57.63	56.81
60	60.23	58.20

Table 19: Comparison of Experimental and predicted values using pseudo second order model for Total phenolic content for cold brewed black tea

Sonication time (minutes)	Total phenolic content (mg GAE/g)	t/C (min/ (mg GAE/g))
10	42.95 ± 1.53^{d}	0.2328
20	46.97 ± 1.04^{c}	0.4258
30	47.19 ± 1.23^{c}	0.6357
40	52.15 ± 0.16^{b}	0.7670
50	57.63 ± 1.31^{a}	0.8676
60	60.23 ± 0.75^{a}	0.9962

Lowercase letters in each row indicates significant difference among samples (p-value≤0.05)

Table 20: Pseudo second order modelling data for % antioxidant activity

Sonication time (minutes)	Total phenolic content (mg GAE/g)	t/c (min/ (mg GAE/g))
10	25.40 ± 0.62^{c}	0.3937
20	26.70 ± 0.17^{c}	0.7491
30	29.14 ± 0.03^{b}	1.0295
40	29.16 ± 0.15^{b}	1.3717
50	32.27 ± 0.78^{a}	1.5494
60	30.86 ± 0.89^{a}	1.9443

Table 21: Comparison of experimental and predicted values using pseudo second order model for % antioxidant activity for cold brewed black tea

Sonication time (minutes)	% antioxidant activity– Experimental value	% antioxidant activity- Predicted value
10	25.40	23.63
20	26.70	27.66
30	29.14	29.33
40	29.16	30.24
50	32.27	30.81
60	30.86	31.21

Table 22: Raw data for total phenolic content

Sample	Parameter	Dry sample weight(g)	Volume(ml)	Dilution factor	Abs(760nm)	GAE concentration (µ.g/ml)	Total phenolic content µg GAE / g dry weight of sample)	Total phenolic content mg GAE / g dry weight of sample)	Average	SD	SE
70%a		0.5	50	10	0.201	39.435	39434.78	39.43			
70%b		0.5	50	10	0.178	34.435	34434.78	34.43	36.39	2.67	1.54
70%c		0.5	50	10	0.182	35.304	35304.35	35.3			
50%a		0.5	50	10	0.145	27.261	27260.87	27.26			
50%b		0.5	50	10	0.136	25.304	25304.35	25.3	26.54	1.07	0.62
50%c		0.5	50	10	0.144	27.043	27043.48	27.04			
30%a		0.5	50	10	0.115	20.739	20739.13	20.74			
30%b	Amplitude (%)	0.5	50	10	0.117	21.174	21173.91	21.17	20.67	0.55	0.32
30%c		0.5	50	10	0.112	20.087	20086.96	20.09			
10%a		0.5	50	10	0.123	22.478	22478.26	22.48			
10%b		0.5	50	10	0.11	19.652	19652.17	19.65	20.52	1.7	0.98
10%c		0.5	50	10	0.109	19.435	19434.78	19.43			
cb1		0.5	50	10	0.109	19.435	19434.78	19.43			
cb2		0.5	50	10	0.113	20.304	20304.35	20.3	19.51	0.76	0.44
cb3		0.5	50	10	0.106	18.783	18782.61	18.78			
25a		0.5	25	10	0.257	51.609	25804.35	25.8			
25b		0.5	25	10	0.256	51.391	25695.65	25.7	25.77	0.06	0.04
25c		0.5	25	10	0.257	51.609	25804.35	25.8			
50a		0.5	50	10	0.186	36.174	36173.91	36.17			
50b		0.5	50	10	0.177	34.217	34217.39	34.22			
50c	Solvent volume(ml)	0.5	50	10	0.181	35.087	35086.96	35.09	35.16	0.98	0.57
75a		0.5	75	10	0.167	32.043	48065.22	48.07			
75b		0.5	75	10	0.159	30.304	45456.52	45.46	46.65	1.32	0.76
75c		0.5	75	10	0.162	30.957	46434.78	46.43			
100a		0.5	100	10	0.102	17.913	35826.09	35.83			
100b		0.5	100	10	0.114	20.522	41043.48	41.04	39.01	2.8	1.61
100c		0.5	100	10	0.112	20.087	40173.91	40.17			
10a		0.5	75	10	0.155	29.435	44152.17	44.15			
10b		0.5	75	10	0.146	27.478	41217.39	41.22	42.96	1.54	0.89
10c		0.5	75	10	0.153	29	43500	43.5			
20a		0.5	75	10	0.165	31.609	47413.04	47.41			
20b		0.5	75	10	0.16	30.522	45782.61	45.78	46.98	1.05	0.61
20c		0.5	75	10	0.166	31.826	47739.13	47.74			
30a		0.5	75	10	0.167	32.043	48065.22	48.07			
30b	Sonication time(min)	0.5	75	10	0.16	30.522	45782.61	45.78	47.2	1.23	0.71
30c		0.5	75	10	0.166	31.826	47739.13	47.74			
40a		0.5	75	10	0.198	38.783	58173.91	58.17			
40b		0.5	75	10	0.18	34.87	52304.35	52.3	54.15	3.49	2.01
40c		0.5	75	10	0.179	34.652	51978.26	51.98			
50a		0.5	75	10	0.192	37.478	56217.39	56.22			
50b		0.5	75	10	0.2	39.217	58826.09	58.83	57.63	1.32	0.76
50c		0.5	75	10	0.197	38.565	57847.83	57.85			
60a		0.5	75	10	0.203	39.87	59804.35	59.8			
60b		0.5	75	10	0.203	39.87	59804.35	59.8	60.24	0.75	0.43
60c		0.5	75	10	0.207	40.739	61108.7	61.11			

Table 23 : Raw data for antioxidant scavenging activity (%DPPH)

Sample	Parameter	Sample weight(g)	Volume (ml)	Dilution Factor	Absorbance (515nm)	% Inhibition	Average	SD	SE
70%a		0.5	50	10	0.544	41.44	40.85	2.49	1.43
70%b		0.5	50	10	0.555	40.26			
70%c		0.5	50	10	0.51	45.04			
50%a		0.5	50	10	0.697	24.97	25.13	0.21	0.12
50%b		0.5	50	10	0.694	25.3			
50%c		0.5	50	10	0.697	24.9			
30%a	Amplitude	0.5	50	10	0.71	23.57	24.59	0.62	0.35
30%b	(%)	0.5	50	10	0.72	22.5			
30%c		0.5	50	10	0.72	22.5			
10%a		0.5	50	10	0.725	21.96	22.23	0.54	0.31
10%b		0.5	50	10	0.72	22.5			
10%c		0.5	50	10	0.724	21.4			
cb1		0.5	50	10	0.677	27.13	26.91	0.36	0.21
cb2		0.5	50	10	0.681	26.7			
cb3		0.5	50	10	0.68	26.4			
25a	Solvent volume	0.5	25	10	0.63	32.19	34.71	2.53	1.46
25b	(ml)	0.5	25	10	0.583	37.24			
25c		0.5	25	10	0.605	34.56			
50a		0.5	50	10	0.544	41.44	40.85	0.7	0.4
50b		0.5	50	10	0.555	40.26			
50c		0.5	50	10	0.555	40.2			
75a		0.5	75	10	0.686	26.16	27.66	1.66	0.96
75b		0.5	75	10	0.658	29.17			
75c		0.5	75	10	0.66	28.9			
100a		0.5	100	10	0.71	23.57	23.04	0.57	0.33
100b		0.5	100	10	0.72	22.5			
100c		0.5	100	10	0.71	23.4			
10a		0.5	75	10	0.709	23.68	24.76	1.2	0.69
10b		0.5	75	10	0.689	25.83			
10c		0.5	75	10	0.69	25.7			
20a		0.5	75	10	0.719	22.6	24.76	2.4	1.38
20b		0.5	75	10	0.679	26.91			
20c		0.5	75	10	0.682	26.6			
30a		0.5	75	10	0.686	26.16	27.66	1.71	0.99
30b	Sonication time(min)	0.5	75	10	0.658	29.17			
30c		0.5	75	10	0.659	29.1			
40a		0.5	75	10	0.677	27.13	28.04	1.06	0.61
40b		0.5	75	10	0.66	28.96			
40c		0.5	75	10	0.659	29			
50a		0.5	75	10	0.628	32.4	31.92	0.55	0.32
50b		0.5	75	10	0.637	31.43			
50c		0.5	75	10	0.637	31.43			
60a		0.5	75	10	0.703	24.33	27.34	3.99	2.3
60b		0.5	75	10	0.647	30.36			
60c		0.5	75	10	0.633	31.89			

Table 24: Model comparison with the control for TPC and antioxidant activity

Sample	TPC	%DPPH
Cold brew control	19.51± 0.76	26.74±0.36
OVAT model	57.6± 1.31	32.3± 0.78
RSM Model	79.4±1.85	38.15±1.28

OVAT-One variable at a time; RSM- Response surface methodology

Table 25: Comparison of optimized conditions using OVAT analysis and Response surface methodology model

Model	Amplitude (%)	Solvent Volume(ml)	Sonication time (minutes)	TPC (mg GAE/g)	% antioxidant activity
OVAT model	70	75	50	57.6±1.31	32.3±0.78
RSM model	69.892	25	30	79.4±1.85	38.15±1.28

OVAT-One variable at a time; RSM- Response surface methodology

Table 26: Validation study of individual response optimization model

Individual optimum models	Amplitude (%)	Solvent Volume(ml)	Sonication time (minutes)	Experimental value	Predicted value
Maximum TPC (mg GAE/g)	70	75	50	75.07±0.80	74.62
Minimum TTC (mg GAE/g)	43	25	30	5.13±0.70	4.89
Maximum %DPPH	55	25	30	40±1.51	40.31
Maximum %ABTS	70	25	30	62.97±1.66	61.58

Table 27: Validation study of optimized model - RSM

Optimized conditions		
Amplitude (%)		69.892
Solvent volume (ml)		25
Sonication time (mins)		30
	Experimental value	**Predicted values**
TPC (mg GAE/g)	72.20±0.17	70.404
TTC (mg GAE/g)	6.77±0.40	6.32
DPPH (% antioxidant scavenging activity)	37.67±0.31	37.129
ABTS (% antioxidant scavenging activity)	62.97±1.66	61.581