EFFECTS OF TEA (*CAMELLIA SINENSIS*) ON ANTIOXIDANT POTENTIAL AND FERMENTATION CHARACTERISTICS OF YOGURT

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FACULTY OF SCIENCE
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ABSTRACT

The present study investigated the effects of green, white and black tea (*Camellia sinensis*; 2% w/v) on the fermentation of milk and antioxidant potential of yogurt. Each tea (water extract) was added into milk-starter culture mixture and incubation was carried out at 42°C until pH was reduced to 4.5. The yogurts were then refrigerated (4°C) for up to 21 days and samples were analysed for antioxidant potential (diphenyl picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP) and ferrous ion chelating (FIC) assays), pH, titratable acid and viable yogurt bacteria counts. Tea yogurts had higher antioxidant potentials ($p < 0.05$) than plain yogurt with green tea yogurt (GTY) having the highest FRAP (2.49 - 2.98 mmol Fe^{2+} E/L) and black tea yogurt (BTY) having the highest FIC (87.50 - 89.87 %) activity throughout the storage period. Both GTY (90.07 - 96.74%) and white tea yogurt (WTY; 89.83 - 96.39%) showed the highest DPPH radical scavenging activity throughout the storage period. The presence of green and black tea water extracts prolonged the milk fermentation time (>270 minutes and 240 minutes respectively) to pH 4.5 compared to control (180 minutes). The pH of tea yogurts during refrigerated storage (pH 4.33 - 4.53) was similar to control (pH 4.28 - 4.41) but greater acid production was observed in all tea yogurts (0.78 - 0.99% lactic acid equivalent; LAE) compared to plain yogurts (0.70 - 0.91% LAE). Highest acid content was found in WTY yogurt at the end of fermentation (0.89 ± 0.02 % LAE) and GTY at the end of the storage period (0.99 ± 0.03 % LAE). All yogurts maintained high viable counts of yogurt bacteria throughout the storage period with higher *Lactobacillus spp.* counts for tea yogurts (6.27 - 7.03 log CFU/ml) compared to plain yogurt (6.08 - 6.54 log CFU/ml). *Streptococcus thermophilus* counts increased in all yogurts during the first week of storage. LCMS analysis revealed the absence of several phenolic compounds in yogurts, despite their presence in tea water extracts, as well as the presence of new phenolic compounds,

suggesting possible tea polyphenol metabolism by yogurt bacteria. Tea can be used to enhance the antioxidant properties and sustain viable yogurt bacteria during refrigerated storage.

ABSTRAK

Kajian ini dijalankan untuk menyiasat kesan penambahan teh hijau, teh putih dan teh hitam (*Camellia sinensis*; 2% w/v) ke atas potensi antioksida dan penapaian susu. Setiap jenis ekstrak teh ditambah kepada campuran susu-kultur pemula bakteria diikuti dengan pengeraman pada suhu 42°C sehingga pH turun ke 4.5. Dadih kemudiannya disimpan pada suhu 4°C selama 21 hari dan sampel dadih dianalisis untuk potensi antioksida (teknik "DPPH radical scavenging", FRAP dan FIC), pH, kandungan asid tertitrat (TA) dan bilangan bakteria hidup. Kesemua dadih yang mengandungi teh menunjukkan potensi antioksida yang lebih tinggi berbanding dadih biasa dengan dadih teh hijau menunjukkan nilai FRAP yang paling tinggi (2.49 - 2.98 mmol Fe^{2+} E/L) manakala dadih teh hitam menunjukkan nilai FIC yang paling tinggi (87.50 - 89.87 %) sepanjang tempoh simpanan. Kedua-dua dadih teh hijau (90.07 - 96.74%) dan dadih teh putih (89.83 - 96.39%) menunjukkan nilai DPPH paling tinggi sepanjang tempoh simpanan. Kehadiran ekstrak teh hijau dan teh hitam menangguhkan tempoh penapaian susu (>270 dan 240 minit masing-masing) untuk menurun ke pH 4.5 berbanding kawalan (180 minit). Sepanjang tempoh simpanan (4°C), nilai pH dadih yang mengandungi teh (pH 4.33 - 4.53) hampir sama dengan kawalan (pH 4.28 - 4.41) tetapi kandungan asid tertitrat yang lebih tinggi diperhatikan pada kesemua dadih teh (0.78 - 0.99% senilai asid laktik; LAE) berbanding dadih kawalan (0.70 - 0.91% LAE). Kandungan asid paling tinggi diperhatikan dengan dadih teh putih pada hujung proses penapaian (0.89 ± 0.02 % LAE) dan dadih teh hijau pada hujung tempoh simpanan (0.99 ± 0.03 % LAE). Kesemua dadih menunjukkan kuantiti bakteria hidup yang tinggi sepanjang tempoh simpanan dengan nilai kultur *Lactobacillus spp.* yang lebih tinggi (6.27 - 7.03 log CFU/ml) pada dadih teh berbanding dadih kawalan (6.08 - 6.54 log CFU/ml). Kuantiti bakteria hidup kultur *Streptococcus thermophilus* bagi kesemua dadih didapati meningkat pada minggu pertama tempoh simpanan. Analisis LCMS

menunjukkan kehilangan beberapa sebatian fenolik dalam dadih walaupun pada awalnya dikesan dalam ekstrak teh dan juga kehadiran beberapa sebatian penolik baru menyarankan kemungkinan metabolisme sebatian fenolik dalam teh oleh kultur bakteria hidup di dalam dadih. Hasil ujikaji ini menyokong penambahan ekstrak teh ke dalam susu untuk menghasilkan dadih berkandungan antioksida yang tinggi tanpa merencatkan pertumbuhan kultur hidup dalam dadih sepanjang tempoh simpanan dingin.

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M. Premalatha

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LIST OF SYMBOLS AND ABBREVIATIONS

etc	=	et cetera
α	=	alpha
β	=	beta
NaOH	=	Sodium hydroxide
HCl	=	Hydrochloric acid
$FeSO_4.7H_2O$	=	iron (II) sulphate heptahydrate
$FeSO_4.xH_2O$	=	iron (II) sulphate hydrate
TA	=	Titratable acidity
GAE	=	Gallic acid equivalent
LCMS	=	Liquid chromatography mass spectrometry
TPTZ	=	2,4,6-tris (2-pyridyl)s-triazine
DPPH	=	2,2-Diphenyl-1-picrylhydrazyl
FRAP	=	Ferric reducing antioxidant potential
FIC	=	Ferrous ion chelating
TPC	=	Total phenolic content
MW	=	Molecular weight
m/z	=	Mass to charge ratio
CFU	=	Colony forming unit
GTE	=	Green tea extract
WTE	=	White tea extract
BTE	=	Black tea extract
PY	=	Plain yogurt
GTY	=	Green tea yogurt
WTY	=	White tea yogurt

BTY	=	Black tea yogurt
R_t	=	Retention time
ROS	=	Reactive oxygen species
CVD	=	Cardiovascular disease
BHT	=	Butylatedhydroxytoluene
BHA	=	Butylatedhydroxyanisole
PPO	=	Polyphenol oxidase
LAB	=	Lactic acid bacteria
v/v	=	Volume per volume
w/v	=	Weight per volume
µg	=	microgram
ml	=	Mililiter
µl	=	Microliter
M	=	Molar
mM	=	Milimolar
° C	=	Degree Celcius
nm	=	nanometer
ANOVA	=	Analysis of variance

CHAPTER 1.0:

INTRODUCTION

Free radicals such as reactive oxygen species (ROS) are continually produced in our body as a by-product of many metabolic processes. Under normal conditions, the body has its own antioxidant defence system comprising of several enzymes such as catalase, superoxide dismutase and glutathione peroxidase to detoxify these free radicals (Scheibmeir *et al.*, 2005). Dietary antioxidants such as vitamins C, E and A also play a crucial role in fighting these free radicals (Urso & Clarkson, 2003). However, when there is an over-production of these free-radicals leading to an imbalance between the generation and elimination of free radicals in the body, a situation known as oxidative stress occurs. This in turn results in oxidative damage to cellular components and biomolecules, thus marks the onset of many degenerative diseases related to aging such as CVD, diabetes, cancer and neurodegenerative diseases (Aruoma, 1998).

Since antioxidants are vital for their role to delay or inhibit oxidation of cellular components (Halliwell *et al.*, 1992), adequate intake of these compounds in the diet will be beneficial to protect against oxidative damages to the cell. However, the use of synthetic antioxidants such as butylatedhydroxytoluene (BHT) and butylatedhydroxyanisole (BHA) are still under evaluation in many countries due to their potential health hazard (Wang *et al.*, 2009). In this regards, extracts of many medicinal plants or herbs, rich in phenolic compounds are increasingly used either as additive in food or consumed directly as functional food as a natural source of antioxidant (Wong *et al.*, 2006).

Yogurt is a coagulated milk product obtained from fermentation process carried out by the combined activity of two lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus delbereuckii* subsp *bulgaricus* (Abu-Tarboush, 1996).Yogurt is traditionally consumed as a health food due to its nutritional properties (Adolfsson *et al.*, 2004) and its health benefits can be further enhanced by incorporating probiotic

strains of lactic acid bacteria (Shah, 2007). Regular consumption of yogurt with live cultures and probiotic strains is said to be effective in reducing serum cholesterol levels, lactose digestion in case of lactose intolerance, bowel syndromes, gut infections and inflammation, diarrhoea and colon cancer (Lourens-Hattingh & Viljoen, 2001; Adolfsson *et al.*, 2004).

Yogurts also contains bioactive peptides, protein fragments released upon proteolysis by the microbial strains (Gobetti *et al.*, 2002) which can improve heart health, bone health, immune defence, digestive system health and effective in body weight management (Korhonen, 2009). In addition, the antioxidant activity encrypted within these fragments enforces their role as a functional ingredient (Sanlidere Aloglu & Oner, 2011), thus increasing the popularity of yogurt as a functional food. In view of these peptides derived antioxidant activities, several studies have looked into the manipulative ways whereby inclusion of extra ingredients such as herbs and spices (Amirdivani & Baba, 2011; Shori & Baba, 2011) as well as fruits (Zainoldin & Baba, 2010; Karaaslan *et al*., 2011) can further enhance nutritional and therapeutic values of yogurt.

Tea (*Camellia sinensis*) is a common beverage being consumed worldwide. Tea is a rich source of phenolic compounds, namely flavanols (e.g. catechins, quarcetin, kaemperol and myrecitin) and phenolic acids (e.g. gallic acid), hence making it a potent source of antioxidants (Dufresne & Farnworth, 2001). The methods used in commercial production of tea give rise to many different types of tea in market. Tea has varying chemical compositions attributed to the processing steps (Almajano *et al.*, 2008). There are three kinds of tea, namely the unfermented green and white tea, the partially fermented oolong tea and the completely fermented black tea (Sharangi, 2009). The difference in the catechin contents in turn affects the antioxidant properties of the

different tea types. For example when fermentation is involved, catechins are oxidised or condensed to larger polyphenols such as theaflavins and thearubigins.

The abundance of tea polyphenols, mainly catechins, in green tea has great medicinal and health benefits (Graham, 1992). They are effective against various forms of cancers, in which the polyphenols are able to regulate various stages of the disease progression, including the cancer cell growth, survival and metastasis (Dufresne & Farnworth, 2001). The polyphenols are also effective in reduction of cholesterol, hypertension and CVD, prevention of diabetes, liver disease, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, treatment of arthritis and respiratory diseases, suppress aging, improve oral health and digestion as well as enhancement of the immune system (Sharangi, 2009).

The catechins and tannins also exhibit antimicrobial effects on a broad range of pathogenic bacteria. They inhibit food bound bacteria (Taguri *et al.*, 2004) but not intestinal lactic acid bacteria (Gramza & Korczak, 2005; Hara, 1998) including the yogurt starter microorganisms (Jaziri *et al.*, 2009). This criterion allows tea extracts to be incorporated into yogurts not only as a supplement to fortify the antioxidant potential of yogurt, but also to protect the fermented milk product from pathogenic or undesirable bacteria.

Since yogurt bacteria can be affected by the inclusion of tea (Najgebauer-Lejko *et al.*, 2011) it is important to establish the differences in the types of tea used on microbial growth and the subsequent effects of microbial metabolism on the changes of key chemical components in tea. Thus, the objectives of this study were to:

(a) Compare the total phenolic content and antioxidant activity of green tea, white tea and black tea.

(b) Evaluate the changes in total phenolic content, antioxidant potential, acid production and viability of yogurt bacteria and probiotics due to addition of tea and the stability during refrigerated storage.

(c) Identify the major phenolic compounds present in tea extracts and changes in the composition of these phenolic compounds in yogurt.

CHAPTER 2.0:

LITERATURE REVIEW

2.1. Yogurt

2.1.1. Introduction and history of yogurt

Yogurt is a fermented milk product and is defined as "a coagulated milk product obtained by lactic acid fermentation through the action of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* from milk" (Mareschi & Cueff, 1989). The name "yogurt" was probably adapted from the word "jugurt", a Turkish word first used in the 8th century (Rasic & Kurmann, 1978). To date, there are almost 400 generic names for yogurts or similar fermented milk products manufactured worldwide (Kurmann *et al.*, 1992).

The ancient Turkish people who lived as nomads were considered the first to make yogurt. Since the early days, the consumption of yogurt is regarded closely to health benefits and longevity of human life (Metchnikoff, 2004). The first production of yogurt on commercial basis by Danone in 1922 has initiated the dramatic increase in yogurt production on large scale in later years (Trachoo, 2002). Yogurt became a much acceptable product worldwide after 1950s following the introduction of new varieties such as fruit flavoured, sweetened and low fat yogurts in the market (Marshall, 1987; Tamime & Robinson, 1999). The worldwide per capita consumption of yogurt has increased since 1960s (IDF, 1982a, 1992).

Tamime and Robinson (1999) and Batish *et al.* (2004) have classified the different types of yogurts on the basis of chemical composition, physical properties, flavours and post-fermentation processing as listed below:

(a) Types based on chemical composition

- Yogurt can be categorized according to fat content and thus called full fat yogurt (> 3.0 % fat), medium fat yogurt (0.5 % to 3.2 % fat) and low fat yogurt (< 0.5 % fat).

(b) Types based on physical nature of product

- Taking into consideration the steps in the fermentation process and the resulting texture of the coagulum, yogurt can be divided into set, stirred and fluid types. In the manufacture of set yogurt, the fermentation process proceeds in a retail container and results in a semi solid or gel like texture of the coagulum. In the production of stirred yogurt, the coagulum is broken by means of agitation following the fermentation of milk in bulk. The product is then pumped through a screen, followed by cooling and packaging. Fluid yogurt or also known as drinking yogurt is manufactured in a similar way as stirred yogurt, but this type of yogurt has lower viscosity. Milk base with low fats and total solids are used in the manufacture of fluid yogurt.

(c) Types based on flavours

- Yogurt can be divided into three categories based on flavour, namely natural or plain yogurt, fruit yogurt and flavoured yogurt. Natural yogurt refers to the traditionally manufactured yogurt with the characteristic sharp acidic taste. Fruit yogurt is manufactured by incorporation of fruits and sweetening agents into natural yogurt. The common fruits widely used in the manufacture of yogurts are apricot, blackberries, blackcurrants, peaches, pineapples, raspberries and strawberries. Carbohydrates such as glucose, fructose, sucrose and maltose present in varying levels in each of the fruit types contribute to their different levels of sweetness. In the manufacture of flavoured yogurt, synthetic flavouring and colouring compounds are added into yogurt in place of fruit ingredients.

(d) Types based on post-fermentation processing

- Based on this criterion, yogurts can be divided into four types, namely pasteurized yogurt, concentrated yogurt, frozen yogurt and dried yogurt. Pasteurized yogurt is

obtained when yogurt is heat treated following the fermentation process to inactivate the starter culture and their enzymes as well as other contaminating microorganisms such as yeast and moulds. This will help to extend to much longer the shelf-life rather than the 3 to 4 weeks under refrigerated storage conditions without post fermentation heat treatment. Concentrated or strained yogurt is obtained when yogurt is concentrated using cloth bag, mechanical separators, ultrafiltration or product formulation techniques to increase the solid content to around 24%. The product also has a higher content of lactic acid. Frozen yogurt is obtained when yogurt is deep-frozen to at least -20°C. It is similar to ice cream in physical state but possess the sharp acidic flavour of yogurt. High level of sugar and stabilizers are added into it in order to maintain the consistency of the coagulum during freezing. Dried yogurt refers to yogurt manufactured in powder form. Since it is concentrated before drying, it has a high total solid content between 90 to 94 %. It is manufactured considering stability aspect during storage and can be readily utilised.

2.1.2. Manufacture of yogurt

Yogurt is produced using milk as the raw material. Mammalian milk mainly consists of water, fat, protein, lactose and minerals. Milks from different mammalian species worldwide can be used to make yogurt. The variation in the chemical composition of milks from different mammalian species is shown in Table 2.1. Other factors such as breed, age of animal, season of year, environmental temperature, stage of lactation and nutrition status may also affect the chemical composition of milk (Tamime *et al.*, 2011).

Table 2.1: Chemical composition (g/100g) of milk from different mammalian species

Species	Water	Fat	Protein	Lactose	Ash
Buffalo	82.1	8.0	4.2	4.9	0.8
Camel	87.1	4.2	3.7	4.1	0.9
Cow	87.4	3.9	3.3	4.7	0.7
Goat	87.0	4.5	3.3	4.6	0.6
Horse	88.8	1.9	2.6	6.2	0.5
Sheep	81.6	7.5	5.6	4.4	0.9
Yak	82.7	4.8	3.3	4.7	0.7

(Adapted from Lentner, 1981; Jenness, 1988; Holland *et al.*, 1991)

The differences in the chemical composition of the individual milks can influence the organoleptic and rheological property of the final yogurt product and hence its quality (Tamime & Robinson, 1999). A high percentage of fat such as those in buffalo and sheep milks, can give rise to a rich and creamy textured yogurt with great mouth feel property (Tamime & Robinson, 1999). The viscosity and texture of yogurt is also affected by the protein content of milk. The protein profile of goat's milk shows slightly lower casein content as compared to cow's milk, with extremely low levels of α_{s1}-casein, elevated levels of α_{s2}-casein and β-casein as well as higher extend of casein micelle dispersion (Remeuf & Lenoir, 1986; Vegarud *et al.*, 1999). These properties result in a soft coagulum formation which gives rise to an almost semi-liquid fermented milk product with an unsatisfactory mouth feel property (Martin-Diana *et al.*, 2003; Tamime *et al.*, 2011).

In the traditional production of yogurt, milk was boiled to 2/3 of original volume to concentrate them, followed by inoculation with yogurt from previous day and incubation at room temperature overnight. The slow acidification of milk due to low incubation temperature not only delayed the production process, but also promotes undesirable side effects such as whey syneresis, that could affect the quality of yogurt (Tamime & Robinson, 1999). In order to ensure acceptable quality of yogurt, standard

common processing steps are practiced for yogurt manufacture on commercial basis (Lee & Lucey, 2010; Figure 2.1).

The fat and solids non fat (SNF) content in milk is adjusted to meet the legal standards of the country as well as to produce a final yogurt product with the desired physical property and flavour. The minimum legal standards for SNF content in yogurt for many countries ranges from 8.2 to 8.6g per 100g of yogurt, but nevertheless most commercial yogurts made it between 14 to 15g per 100g of yogurt. Increasing the SNF content in yogurt increased the viscosity of the end product (Tamime & Robinson, 1999). Harwalkar and Kalab (1986) reported that a higher total solid content resulted in shorter casein particles chains in yogurt, with lesser susceptibility to syneresis. The SNF content in yogurts could be increased by the incorporation of full cream or skimmed milk powder, buttermilk powder, whey powder or whey protein concentrates and casein powder into milk. In addition, techniques such as concentration by vacuum evaporation and membrane processing such as reverse osmosis and ultrafiltration could also increase the total solids content of milk (Tamime & Robinson, 1999; Lee & Lucey, 2010).

Since the yogurt coagulum is often subjected to stirring in the fermentation tank and post fermentation heat treatment, the viscosity of the product may be altered, and in extreme cases, may show whey separation. Thus, stabilizers, mostly natural gums, such as pectin and gelatine, modified gums such as xanthan and dextran or synthetic gums such as polyvinyl derivatives, are often added to the milk base to promote and maintain the desirable yogurt properties such as texture, viscosity, appearance and mouth feel.

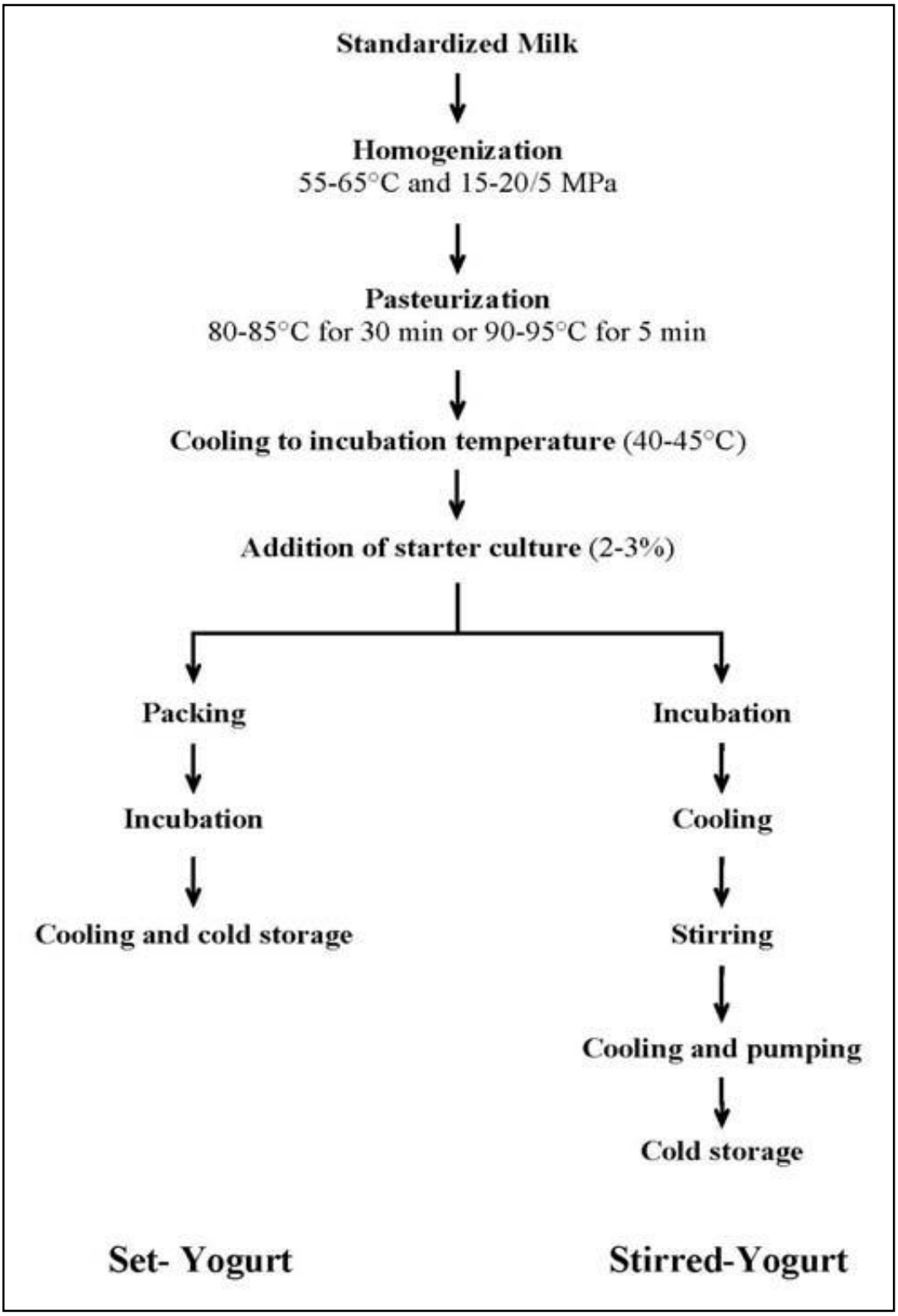

Figure 2.1: Main processing steps in the manufacture of set and stirred yogurt (Lee & Lucey, 2010)

Sweetening agents are sometimes added, normally in the production of fruit or flavoured yogurt, to reduce the sharp acidic taste of yogurt. However, incorporation of sweetening agents should be done only when sugar tolerant starter culture strains are employed in the yogurt, since high sugar concentrations may inhibit the growth of starter culture (Tamime & Robinson, 1999).

Homogenisation is a crucial step in yogurt manufacture since it breaks down the fat into smaller globules and prevents it from clustering and rising to the surface. Homogenisation also results in increased protein-protein interaction and casein-fat globule membrane interaction, in which casein and whey proteins build a new lining on the surface of fat globules. This interaction could promote hydrophilicity and water binding capacity in yogurts, which attributes to decreased syneresis in the final product. In addition, homogenisation may also serve as means of homogenous mixing for yogurts fortified with powdered ingredients. The positive attributes of homogenisation could only be achieved provided the correct temperature and pressure are applied. Thus, a pressure of 15 to 20MPa and temperature between 55 to 65°C is commonly applied during homogenisation (Tamime & Robinson, 1999; Vedamuthu, 1991; Walstra, 1998; Tamime & Deeth, 1980).

Heat treatment at a temperature of 85°C for 30 minutes or between 90 to 95°C for 5 minutes is commonly practiced in the yogurt manufacture. At this temperature, most undesirable microorganisms associated with raw milk will be destroyed hence, reducing competition for the yogurt starter culture. In addition, heat treatment aids in removing dissolved oxygen in the milk, thus generating a micro-aerophilic environment that facilitate the growth of the starter culture (Tamime & Robinson, 1999; Tamime & Deeth, 1980; Lee & Lucey, 2010). Heat treatment also alters the protein composition of the milk, namely the casein and whey protein interaction, which in turn, improves the texture of the yogurt. Casein fractions of protein in milk have the tendency to associate

with calcium and clump, resulting in micelle formation (Tamime & Robinson, 1999). On the other hand, whey proteins, mainly β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), do not clump together or react with calcium in their native state. However, heat treatment above 80°C results in denaturation of whey proteins, which in turn promotes β-Lg and κ-casein interaction. This favourable interaction not only results in a bigger micelle size, but also attributes to a much stable gel consistency with reduced syneresis (Tamime & Deeth, 1980).

2.1.3. Microbiology and biochemistry of yogurt production

Yogurt is a product obtained from lactic acid fermentation of milk, carried out by the combined activity of 2 lactic acid bacteria, namely, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. During fermentation, these bacteria convert lactose in the milk into lactic acid which results in acidification and gelation of milk (Lee & Lucey, 2010). The incorporation of other beneficial lactic acid bacteria into yogurt as optional additions or adjuncts is also allowed, but *S.thermophilus* and *L. delbrueckii* subsp. *bulgaricus* remain as the essential microbes for yogurt production (Batish *et al*., 2004).

S.thermophilus are spherical cells, lesser than 1μm in diameter and appear in pairs or long chains. Their fermentation ability of sugars is restricted only to few types, which include lactose, sucrose, glucose and sometimes galactose (Hardie, 1986). They produce mainly L (+)-lactic acid from fermentation. On the other hand, *L. delbrueckii* subsp. *bulgaricus* are rod shaped cells with rounded ends and appear singly or in short chains with size ranges between 0.5-0.8 × 2-9 μm. Their fermentation ability of sugars is also limited to few types, which are glucose, lactose, fructose and occasionally galactose or

mannose (Kandler & Weiss, 1986). They produce mainly D (-)-lactic acid from fermentation.

Both *S.thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are thermophilic strains, the former being able to grow between a temperature range of 20 to 50 °C and the latter having a growth temperature between 22 to 60 °C. The optimum growth temperature for both strains are between 40 to 45 °C, which is the common temperature applied for milk fermentation. Both strains are homofermentative in nature, thus produce lactic acid as the predominant end product of sugar fermentation (Rasic & Kurmann, 1978; Tamime & Robinson, 1999).

Both *S.thermophilus* and *L. delbrueckii* subsp. *bulgaricus* display an associative growth pattern in mixed yogurt culture, with constant changes in the ratio between the two strains (Radke-Mitchell & Sandine, 1984). Marshall (1987) has described the relationship between both bacteria as protocooperative rather than symbiotic growth, owing to the fact that each bacteria species is able to grow independently in pure culture. A higher rate of acid development occurred in mixed yogurt culture containing both the strains compared to their single strain cultures (Pette and Lolkema, 1950a). In addition, the total proteolysis (Rajagopal & Sandine, 1990) and acetaldehyde production (Hamdan *et al.*, 1971) in mixed yogurt culture were far more than the sum of values obtained when each strain was employed individually in pure culture.

The proteolytic activity by lactic acid bacteria can be an essential aspect for the nutrition and growth of the bacteria, but not for the organoleptic properties. This is because proteolysis of the exogenous nitrogen source in the medium will provide the bacteria with the supply of amino acids required for growth (Zourari *et al.*, 1992). Although both *S.thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are proteolytic strains, the latter has a higher proteolytic activity. Rajagopal and Sandine (1990) studied

the proteolytic activity of both yogurt bacteria by detecting the free tyrosine and tryptophane liberated into reaction medium via spectrophotometric method and reported that the different strains of *L. delbrueckii* subsp. *bulgaricus* could release 61 to 144.6µg of tyrosine/ml of milk while strains of *S.thermophilus* could release only 2.4 to 14.8µg of tyrosine/ml of milk. The absence of extracellular proteolytic activity and the low free amino acid and peptide content of milk sets a limitation to the growth of *S.thermophilus* in pure culture (Zourari *et al.*, 1992).

In a mixed culture, yogurt fermentation proceeds in two stages. During the first stage, *L. delbrueckii* subsp. *bulgaricus* carries out proteolysis on the milk caseins and liberates essential amino acids and peptides to stimulate the growth of *S.thermophilus* (Sandine & Elliker, 1970; Radke-Mitchell & Sandine, 1984). Since the lactobacilli are microaerophilic in nature, they grow slowly during this stage (Vedamuthu, 1991). The streptococci grow very quickly making use of the amino acids liberated by the lactobacilli, and in turn produce lactic acid which reduces the pH of the milk to an optimum level that is suitable for the growth of the lactobacilli (Lourens-Hattingh & Viljoen, 2001). In addition, the streptococci produce a stimulatory factor similar to formic acid to stimulate the growth of the lactobacilli (Galesloot *et al.*, 1968). A study by Driessen *et al.* (1982) showed that the large amount of carbon dioxide produced by *S.thermophilus* during fermentation as a result of its urease activity (Tinson *et al.*, 1982b) could also serve as stimulatory factor to promote the growth of the lactobacilli, since heat treatment of milk prior to fermentation would have removed the dissolved carbon dioxide in milk. At the end of the first stage, the accumulation of lactic acid in the medium slows the growth of *S.thermophilus* and marks the beginning of the second stage of fermentation, predominated by *L. delbrueckii* subsp. *bulgaricus*, owing to the fact that the lactobacilli are more acid tolerant than the streptococci (Rasic & Kurmann, 1978).

Streptococci and lactobacilli are mainly employed in yogurt manufacture for milk acidification, synthesis of aromatic compounds and development of a coagulum with desired texture and viscosity (Zourari *et al.*, 1992). Both *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* carry out the homolactic fermentation of milk via Embden-Meyerhof-Parnas (EMP) pathway resulting in lactic acid as the major end-product. The EMP pathway for glucose utilization and Leloir pathway for galactose utilization in both strains are illustrated in Figure 2.2.

The lactic acid produced during fermentation decreases the pH of the milk progressively, leading to solubilisation of colloidal calcium phosphate, the structure responsible for the stability of casein micelles in milk. As the pH of the milk drops towards the isoelectric point of casein (pH 4.6), the negative charges on casein are progressively neutralized, resulting in a decreased electrostatic repulsion between charged groups which earlier had contributed to the stability of the casein micelle. In contrast, casein-casein interaction now increases, due to increase in hydrophobic and electrostatic charge interactions which eventually lead to aggregation of casein particles and formation of a three-dimensional gel-like structure (Lucey, 2004; Horne, 1998).

Although the characteristic acidic and sharp flavour of yogurt is a direct consequence of lactic acid production, other compounds from the metabolic activity of both bacterial strains were equally important contributing factors towards the aroma and flavour of yogurt. Acetaldehyde was regarded to be the major flavour component (Pette & Lolkema, 1950c) which can be further categorized (Tamime and Robinson, 1999) into 4 main groups, namely non-volatile acids, volatile acids, carbonyl compounds (including acetaldehyde, acetone and diacetyl) as well as miscellaneous compounds obtained from thermal degradation of lipids, protein and lactose during heat treatment of milk prior to fermentation.

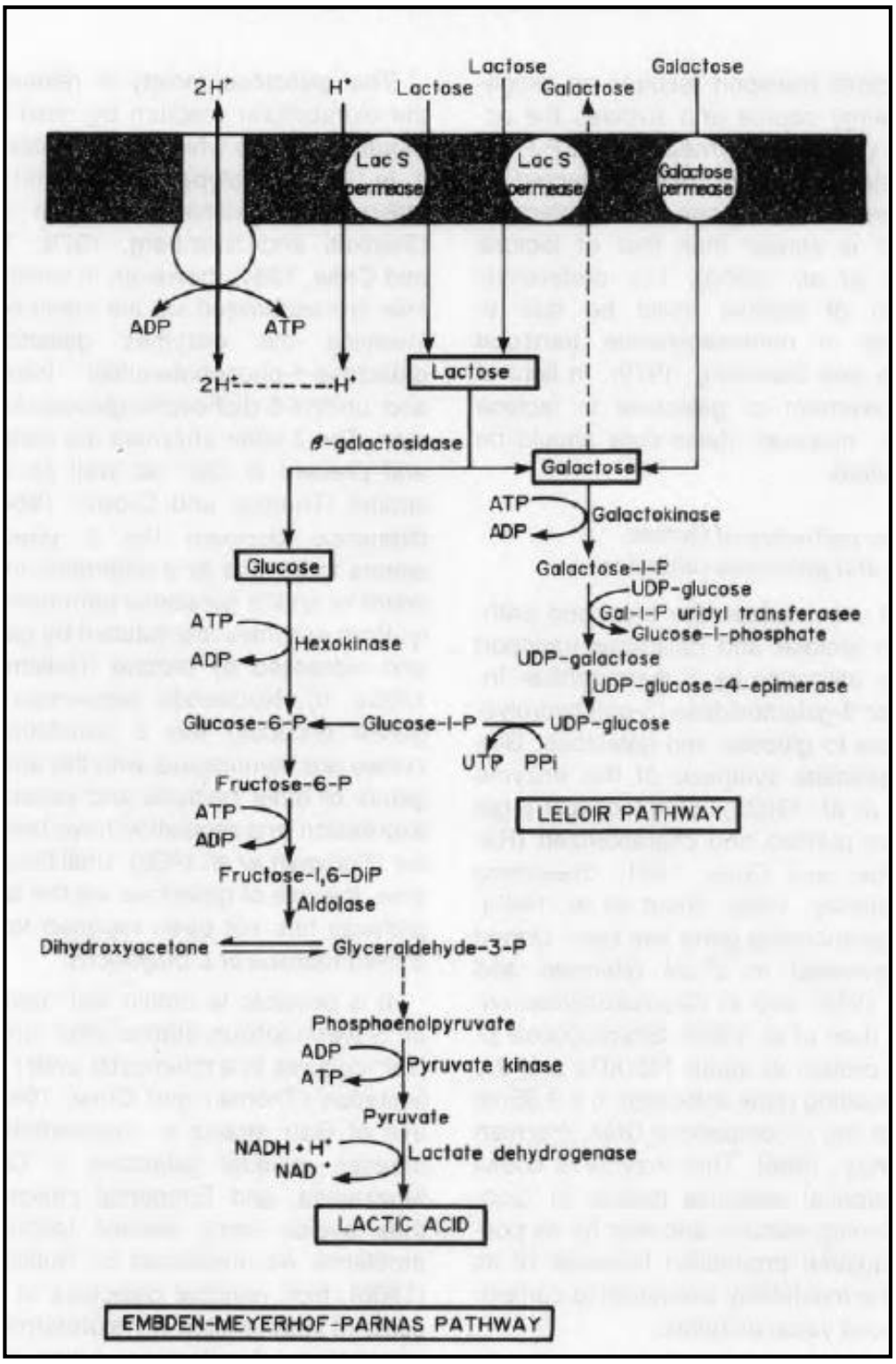

Figure 2.2: EMP pathway for lactose utilization and Leloir pathway for galactose utilization in both strains of yogurt bacteria (*Adapted from Hutkins & Morris, 1987; Thompson, 1987 & De Vos, 1990*)

Although there are several pathways possible for acetaldehyde production, the activity of enzyme threonine aldolase which catalyses the cleavage of threonine to acetaldehyde and glycine appeared to be the most significant pathway, accounting to the fact that this enzyme could be found in both yogurt bacteria strains (Sandine & Elliker, 1970; Wilkins *et al.*, 1986a). The activity of threonine aldolase in streptococci decreased with increase in growth temperature close to yogurt fermentation temperature (Lees & Jago, 1976b; Wilkins *et al.*, 1986b), and hence acetaldehyde is produced predominantly by *L. delbrueckii* subsp. *bulgaricus* during yogurt manufacture in a mixed culture (Zourari *et al.*, 1992).

2.1.4. Probiotics in yogurt

Probiotics can be defined as mono or mixed culture of viable microorganisms, which when present in sufficient number could potentially benefit the host by altering the properties of indigenous microflora in the host. This group of microorganisms colonizes the gastrointestinal (GI) tract and alters the balance of the microbiota in the tract, thus improving the health status of the host (Fuller, 1992; Havenaar & Huis In't Veld, 1992). Most of the probiotic bacteria are lactic acid bacteria from the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium* and *Enterococcus*, with members of the genera *Lactobacillus* and *Bifidobacterium* being commonly used in production of functional food, especially in the dairy industry (Shah, 2007).

Probiotic culture, if they are to be incorporated as dietary adjunct and exert health benefits, should possess certain criteria. These criteria are summarized in the followings (Martin & Chou, 1992; Gililand, 1989; Hoier, 1992; Saarela *et al.*, 2000; O'Grady & Gibson, 2005):

(1) Preferably from human origin

(2) Non-pathogenic, non-toxic, do not carry any antibiotic resistance gene that could be transferred and no history of adverse side-effects

(3) Able to adhere to gut cells and resistant to bile salts and acid secretion in gut in order to survive the GI tract

(4) Possess antimicrobial activity and able to exert antagonistic activity against pathogens commonly found in food or in the intestine such as *Helicobacter pylori* and *Salmonella sp.*

(5) Possess anticarcinogenic and antimutagenic activities

(6) Produce good organoleptic properties in products, inclusive of sensory and mouth feel properties

(7) Remain stable and viable during processing and storage

Probiotic strains are generally used as dietary adjuncts in dairy industry, due to their slow growth in milk and prolonged fermentation time (Shah, 2004). Since yogurt is a healthy dairy product attributed to the fermentation metabolites produced by the traditional yogurt bacteria (Hoeir, 1992), it was proposed that yogurt can be used as a probiotic carrier not only to shorten the fermentation time but also to add up on its nutritional-physiological value (Shah, 2000, Lourens-Hattingh &Viljoen, 2001; Shah, 2007). The shortening of the fermentation time is made possible due to the higher proteolytic activity of the traditional yogurt bacteria in comparison to the probiotic strains (Shihata & Shah, 2000). In addition, the use of yogurt to deliver probiotic strains is further rationalized by the fact that the traditional yogurt bacteria, *S.thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are not probiotic strains since they are not indigenous microflora of the GI tract and do not have the ability to survive in the GI tract since not resistant towards acid and bile salts (Gililand, 1979).

Although there is no general agreement on the level of viable probiotic bacteria that should be present in a product, Rybka and Kailasapathy (1995), Kurman and Rasic

(1991) and Shah (2007) have suggested a minimum value of 10^6 - 10^8 cfu/mL of product (Lourens-Hattingh &Viljoen, 2001) in order to exhibit its health benefits. Such high values are required to take into account the possibility of these probiotic microorganisms to decline in number during processing, storage and transit along the GI tract (Shah & Vasiljevic, 2008).

2.1.5. Health benefits of yogurt

The main reason for fermenting dairy products is to improve the shelf life of milk. The fermentation of dairy products however has evolved to become a practice to improve the health. It was reported that the consumption of yogurt from goat's milk was found to cure Emperor Francis I of France from severe diarrhoea. The scientific rationale of yogurt consumption to improve health was attributed to the longevity of Bulgarian peasants to their habit of consuming large amounts of fermented milk, called "yahourt" (Tamime & Robinson, 1999). The auto-intoxication theory put forward by Metchnikoff suggests that the bacteria present in yogurt could displace the toxin producing bacteria in the intestine thus controlling infections by enteric pathogens, which in turn resulted in prolonged life (Metchnikoff, 2004).

Over the years, researches in sciences have documented many reports on health promoting properties and therapeutical applications of yogurts containing live cultures and probiotics. Health benefits and therapeutic values of yogurt include:

(a) <u>Improves nutritional value of food</u>

The nutrient content of yogurt is closely associated to the nutrient composition of the milk from which it is produced. However, factors such as stages of milk processing prior to and during fermentation, the bacterial strains employed in fermentation, the type of milk solids added, the temperature and

duration of fermentation process as well as storage conditions of the yogurts could result in variation of the nutrient composition of yogurt in comparison to the milk source it was derived from (Adolfsson *et al.*, 2004). Yogurt bacteria and probiotics are able to synthesise folic acid and the amount of folate produced is strain dependent. Crittenden *et al.* (2003) reported that *S. thermophilus* and *Bifidobacteria* could produce folate thus enhancing the folate content of yogurts while *Lactobacilli* on the other hand, utilize folate in milk causing depletion.

Yogurts are better protein source than milk since the protein content in yogurts is generally higher than milk due to fortification with nonfat dry milk during processing and concentration (Adolfsson *et al.*, 2004). In addition, proteolysis of milk proteins by yogurt bacteria results in predigestion of the milk protein prior to ingestion which improves the digestibility of the protein in yogurts compared to that of milk (Rasic & Kurmann, 1978; Shahani & Chandan, 1979). Finer coagulation of casein as a result of heat treatment of milk prior to fermentation as well as acid production during fermentation also improves the protein digestibility of yogurt compared to milk. Besides that, essential amino acids are found abundant in the casein and whey protein fractions of yogurt (Bissonnette & Jeejeebhoy, 1994).

Proteolysis attributed to microbial activity of the starter cultures also gives rise to the production of bioactive peptides (Gobetti *et al.*, 2002; Gobetti *et al.*, 2007). These peptides can be defined as specific fragments of proteins that remain inactive when present in the parent protein but upon enzymatic or microbial hydrolysis and release have biological actions beneficial to health (Kitts & Weiler, 2003) such as modulating weight management, immunostimulatory effects, digestive, heart and bone health as well as memory power and stress management (Korhonen, 2009). For instance milk fermented with *L.delbrueckii* ssp.

bulgaricus, *S.thermophilus* and *L.lactis* biovar *diacetylactis* produce bioactive peptides containing Ser-Lys-Val-Tyr-Pro sequence (ACE-inhibitory peptide) with hypotensive properties (Ashar & Chand, 2004).

The concentration of conjugated linoleic acid (CLA), a long-chain biohydrogenated derivative of linoleic acid was found to be higher in yogurt in comparison to milk (Shantha *et al.*, 1995). CLA was found to display anti-carcinogenic (Kemp *et al.*, 2003), anti-inflammatory and anti-artherosclerotic activities (Vasiljevic & Shah, 2007).

(b) Potential for prevention of osteoporosis

Yogurt is a good source of calcium which is vital for bone formation and mineralization (Adolfsson *et al.*, 2004). The calcium in yogurt is easily ionized due to the low pH and in turn, improves intestinal calcium uptake (Bronner & Pansu, 1999). The enhanced bone mineralization in rats fed with yogurt compared to rats fed with diet containing calcium carbonate could also relate to greater bioavailability of calcium in yogurt (Kaup *et al.*, 1987). Since the risk of bone loss and osteoporosis was found to be greater in postmenopausal women and often related to low calcium intake (Ervin & Kennedy-Stephenson, 2002), the consumption of yogurt is beneficial in minimizing the risk of osteoporosis.

(c) Reducing lactose intolerance

Lactose maldigestion is a common disorder observed in almost half of the world's adult population, with high prevalence in the Asian region (Fernandes *et al.*, 1987). Individuals with lactose maldigestion have low levels of intestinal β-galactosidase activity, thus resulting in insufficient lactose digestion in the small

intestine. The undigested lactose is fermented by the microflora population in the colon, resulting in the release of short chain fatty acids such as butyrate, acetate and propionate (Adolfsson *et al.*, 2004). These fatty acid by-products then combine with electrolytes and increase osmotic water flow into the lumen of the colon, inducing diarrhoea. The fermentation of lactose in the colon also produces gases such as methane, carbon dioxide and hydrogen that are released as flatus. Individuals with lactose maldigestion often suffer from symptoms known as lactose intolerance such as bloating, abdominal cramps, flatulence and diarrhoea upon ingestion of milk (Adolfsson *et al.*, 2004).

Fermented milk products could be accepted better than unfermented milk products by individuals with lactose intolerance. This is because lactose in the milk is utilized by the bacterial culture for growth during fermentation, resulting in partial hydrolysis of lactose that reduces lactose content of fermented milks. In addition, the endogeneous β-galactosidase activity of the bacteria was found to persist in the gastrointestinal tract, thus continuing lactose hydrolysis in the intestine which improves lactose tolerance (Vasiljevic & Shah, 2008; Kim & Gilliland, 1983). The higher viscosity of fermented milks delays gastric emptying and increase transit time through the GI tract, thus improves lactose absorption (Vasiljevic & Shah, 2008).

(d) Control of intestinal infections

Most of the probiotic bacteria incorporated in yogurts have antimicrobial properties. *L. acidophilus* and *Bifidobacterium* have suppresive effects on many food borne pathogens such as *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Clastrodium perfringens* (Gilliland & Speck, 1977a; Hughes & Hoover, 1991; Lim *et al.*, 1993; Shah, 1999). Lourens-Hattingh and

Viljoen (2001) have summarised the mechanisms in which the probiotic bacteria could inhibit the pathogens into the following 3means:

(i) Production of antimicrobial compounds

(ii) Competing with the pathogens for adhesion sites and nutrients in the gastrointestinal tract

(iii) Modulation of immune system

Lactic and acetic acids, hydrogen peroxide, bacteriocins, low molecular weight peptides and antifungal peptides are common antimicrobial compounds produced by the probiotic bacteria. Organic acids produced by the probiotic bacteria reduces the pH in the GI tract, thus creates a bacteriocidal effect, especially towards pathogenic gram negative bacteria (Vasiljevic & Shah, 2008). The bacteriocins produced by the probiotic culture could exhibit narrow spectrum by acting against the same species or broad spectrum by acting across the genera (Cotter *et al.*, 2005). Rossland *et al.* (2005) have reported that the presence of pathogens stimulated the production of antimicrobial compounds by the probiotic culture.

The number of cells secreting Immunoglobulin A (IgA) and the production of IgA was found to rise upon oral administration of yogurt containing *L.acidophilus* and *L.casei* to mice in a dose-dependent manner (Perdigon *et al.*, 1995). Secretory IgA is a component of the gut associated lymphoid tissue responsible for mucosal immune response in the GI tract. Secretory IgA prevents colonization and mucosal penetration of pathogenic bacteria in the gut. The peritoneal macrophages of mice fed with fermented milk containing *L.acidophilus* and *L.casei* was also found to display higher phagocytic activity (Perdigon *et al.*, 1988).

(e) Inhibition of *Helicobacter pylori*

Helicobacter pylori are opportunistic pathogens often present in the stomach without any early symptoms. Infection by the bacteria progressively leads to chronic gastritis, peptic ulcers and higher risks of gastric malignancies (Plummer *et al.*, 2004). Although *H.pylori* infection could be treated successfully using the combination of two antibiotics and a proton pump inhibitor, this approach is often not a desirable choice due to the high cost of treatment and adverse side effects associated with the treatment, which include antibiotic associated diarrhoea and development of antibiotic resistance by intestinal pathogens (Malfertheiner *et al.*, 2002).

The bacterial load and inflammation in *H.pylori* infected patients were found to reduce upon consumption of probiotic cultures. *H.pylori* infection can be suppressed successfully by *L.casei* strain *Shirota* (Sgouras *et al.*, 2004; Cats *et al.*, 2003), *L. johnsonii* La1 and *L.gasseri* OLL2716 (Felley *et al.*, 2001), and a combination of *B.animalis* Bb12 and *L.acidophilus* La5 (Wang *et al.*, 2004). In most of the studies, these probiotic strains were incorporated into fermented milks and yogurts. However, Vasiljevic and Shah (2008) have concluded that the consumption of probiotic strains alone could not combat *H.pylori* infection; rather, the approach should be combined together with the antibiotic treatment to reduce the side effects associated with the treatment.

(f) Potential of preventing hypercholesterolaemia

An elevated level of serum cholesterol , often associated with consumption of diet rich in saturated fats, increases the risk of coronary heart disease. The consumption of fermented milk containing probiotic cultures have been shown to reduce serum cholesterol level (Mann & Spoerry, 1974; Hepner *et al.*, 1979;

Ouwehand *et al.*, 2002) and low density lipoprotein (Xiao *et al.*, 2003) but increase high density lipoprotein (Kawase *et al.*, 1999; Kiebling *et al.*, 2002). However, the hypocholesterolemic effect of probiotic yogurt were not seen in other studies (Masssey, 1984; Pulusani & Rao, 1983; Rossouw *et al.*, 1981).

Several possible mechanisms on how the probiotic strains may cause the hypocholesterolemic effect have been postulated. Begley *et al.* (2006) proposed that the enzyme bile salt hydrolase (BSH) present within the probiotic bacteria is responsible for deconjugation of bile salts. The low pH as a result of lactic acid production in yogurts stimulates co-precipitation of cholesterol with the deconjugated bile salts which is then removed from the body through the faecal route (Marshall, 1996; Kailasapathy and Rybka, 1997). Gilliland and Speck (1977b) had earlier suggested that as the probiotic strains deconjugate the bile salts into free acids which can be easily eliminated from the GI tract, new bile acids will be synthesised in the liver from cholesterol to compensate the loss, thus reducing the total cholesterol level in the body. More human clinical trials are required to establish the potential of probiotic yogurts to reduce cholesterol level as well as to develop a better understanding on the mechanism in which the probiotic strains could possibly reduce the cholesterol level.

(g) <u>Prevention and reduction of diarrhoea symptoms</u>

Administration of antibiotics for treatment often results in significant reduction of indigenous microflora present in the gut and unfavourable increase of *Clostrodium difficile*, an indigenous pathogen often present in low counts in the gut of healthy individual. The elevated toxin level produced by the increasing pathogen results in antibiotic associated diarrhoea. (Shah, 2007; Vasiljevic & Shah, 2008).

Consumption of probiotic yogurts can reduce symptoms of antibiotic associated diarrhoea, traveller's diarrhoea and rotavirus associated infantile diarrhoea (Shah, 2006b). For instance a reduction in the duration of diarrhoea in children suffering from rotavirus associated diarrhoea can be reduced upon consumption of a beverage containing probiotic strains, *L.rhamnosus* GG, *Bifidobacterium animalis* Bb-12, *L. reuteri* and *L. acidophilus* (Gundalini *et al.*, 2000). A decrease of 52%, 8% and 34% in the occurrence of antibiotic associated diarrhoea, traveller's diarrhoea and other types of acute diarrhoea respectively was also observed upon probiotic administration (Sazawal *et al.,* 2006).

The mechanism by which the probiotic dairy products act against diarrhoea is not well understood but several mechanisms have been proposed. Freites *et al.* (2003) suggested that the probiotic strains could secrete some soluble factors capable of modifying the glycosylation state of the epithelial cell receptors in the gut, thus competitively eliminate the rotavirus that causes diarrhoea. Alternatively, probiotic strains could improve the function of the mucosal barrier in the gut by protecting the cytoskeleton proteins in the epithelial cells from disruption by the pathogens (Resta-Lenert and Barrett, 2003).

(h) Antimutagenic and anticarcinogenic activity

Endogenous mutagens produced by the body and exogenous mutagens present in the environment and food could cause damage to the DNA and potentially induce mutation responsible for ageing and various degenerative diseases, tumour formation and progression of cancer. Probiotic cultures capability to supress mutation and cancer progression have been shown in animal models as well as bacterial and mammalian cell cultures. Butyric acid, an organic acid produced by probiotic bacteria as antimutagens was found to exhibit a broad

spectrum of antimutagenic activity (Lankaputhra and Shah, 1998). The study also revealed higher antimutagenic activity by live cultures compared to killed cell cultures thus emphasizing the importance of consuming live probiotic cultures in diet. Probiotic strains could also supress the proliferation of tumour cells as demonstrated in the lower tumour cell counts in rats implanted with tumour cells and fed with milk fermented with *L.acidophilus*, compared to a control group which was not fed with the fermented milk (Shahani *et al.*, 1983).

Microbial enzymes such as β-glucuronidase, azoreductase and nitroreductase from the endogenous gut microflora could convert procarcinogens to carcinogens, thus increasing the risk of cancer (Goldin & Gorbach, 1984). However, the levels of these enzymes decreased upon the administration of some strains of *L.acidophilus* and *Bifidobacterium sp.*, a situation advantageous towards a decrease in the conversion of procarcinogens to carcinogens as well as tumour development (Yoon *et al.*, 2000). The reduction in the enzyme activities could also be attributed to the short chain fatty acids produced by *L.acidophilus*, *Bifidobacterium sp.*, *L.plantarum* and *L.rhamnosus* (Cenci *et al.*, 2002). Ohashi *et al.* (2000) have reported reduced risk of bladder cancer and urinary mutagen excretion in Japanese population following the habitual consumption of fermented milk containing *L.casei* strain *Shirota*.

(i) Prevention of urogenital infections

A healthy human vagina consists of various indigenous microflora, from which lactobacilli seem to be the dominant species. The lactobacilli colonizing the vagina are believed to confer protective effects by the production of lactic acid, hydrogen peroxide as well as bacteriocins that inhibit other pathogenic microorganisms. When the balance of the indigenous microflora is disrupted, the

chances of contradicting infections such as bacterial vaginosis increase too (Sieber & Dietz, 1998). The potential of yogurt containing live *L.acidophilus* culture in the treatment of bacterial vaginosis have been widely recognised (Hilton *et al.*, 1992; Shalev *et al.*, 1996). The ingested *L.acidophilus* may have displaced *Candida sp.* in the GI tract, and from there colonize the urogenital tract by competitive inhibition of *Candida sp.*, thus decreasing the occurrence of the infection (Drutz, 1992).

2.1.6. Yogurt as functional food

Functional food can be defined as food similar in appearance to conventional food, designed to be consumed on daily basis as part of a normal diet but also impart additional health benefits beyond providing the basic nutrition (Roberfroid, 1999). Table 2.2 summarizes the different types of functional food available and their description.

Table 2.2: Types of functional food

Type of functional food	Description
Fortified products	Increasing the content of existing nutrients
Enriched products	Adding new nutrients or components normally not found in a particular food
Altered products	Replace existing components with beneficial components
Enhanced commodities	Changes in the raw commodities that have altered nutrient composition

(*Adapted from Spence, 2006*)

Yogurt is as a functional food by virtue of the presence of live bacteria. Continuous studies have looked into ways to enrich yogurt with natural ingredients that have proven health claims. These enriched products are anticipated to boost yogurt's antioxidant potentials and provide additional protections to consumers against common disorders such as hypertension and diabetes mellitus. Some of the studies include:

(a) Supplementation of yogurt with spices and herbs

Amirdivani and Baba (2011) studied the effects of supplementing yogurt with three different spices, namely peppermint, dill and basil and found that these yogurts had higher antioxidant potential and ACE-inhibitory activity, thus a good candidate to reduce the risk of hypertension.

Shori and Baba (2011) studied the effects of incorporating cinnamon into camel-milk and cow-milk derived bio-yogurts. The total phenolic content of the bio-yogurts increased upon cinnamon supplementation in comparison to plain yogurt, hence their antioxidant potential. In addition, both bio-yogurts showed enhanced *in vitro* inhibition activity on α-amylase and α-glucosidase enzymes, thus potential in management of type-2 diabetes mellitus.

(b) Supplementation of yogurt with fish oil

Estrada *et al.* (2011) fortified strawberry yogurt with salmon fish oil, since it is a potent source of polyunsaturated fatty acid (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). PUFA was found, on regular consumption basis, to be beneficial in treatment of cardiovascular disease. However, the fish oil was microencapsulated before addition into the yogurt as an attempt to reduce the oxidization of the fatty acids during storage that may lead to rancidity and unpleasant odour and taste.

(c) Supplementation of yogurt with fruit

Euterpe oleracea (Acai) fruit juice is a rich source of anthocyanin and phenolic compounds, thus conferring strong antioxidant properties. The colouring function of this juice in yogurt was found to be stable at low pH, thus

making it a good substitute for chemical flavouring and colouring agents (Coisson *et al.*, 2005).

Karaaslan *et al.* (2011) studied the effects of supplementing yogurt with acidified ethanol extracts of four different types of grapes and callus. The main advantage of callus over fresh fruit extracts include continuous production of natural compounds and the ability to manipulate the biosynthesis of phenolic compounds and antocyanins at gene level. The remarkable increase in antioxidant potential by red grape supplemented yogurt and callus supplemented yogurt makes callus a potent source of antioxidant and efficient in treatment of cancer and other degenerative diseases. This could be partially explained by the additional bioactive phenolic compounds, as shown via gas chromatography, in callus supplemented yogurt.

(d) Supplementation of yogurt with chitosan

Chitoson is a potential anti-diabetic agent as it could improve glucose tolerance and insulin secretion as well as reduces triglyceride levels in the blood. Seo *et al.* (2009) studied the effects of adding nano-powdered chitosan to cholesterol reduced yogurt in an attempt to produce health promoting nutraceutical foods. It was concluded that nano-powdered chitosan could be supplemented at the level of 0.3 to 0.5% (v/v) into yogurt to confer the health benefit without adverse effects on the physiological and sensory properties of yogurt.

(e) Supplementation of yogurt with yam

The main active functional compound in yam, allantoin and diosgenin, have been associated with some health benefits. The former was able to cure inflammation and ulcers in the body while the latter was reported to inhibit

cholesterol absorption in the body. Kim *et al.* (2011) studied the effects of yam supplementation on physiochemical and sensory properties of yogurt. The study showed that yam supplemented yogurt was a good nutraceutical product without adverse effects on the physiochemical and organoleptic properties of yogurt.

(e) Supplementation of yogurt with tea

Tea polyphenols are potent source of antioxidant with the capacity to act against various degenerative diseases. Najgebauer-Lejko *et al.* (2011) studied the effects of green tea and Pu-erh tea addition on antioxidant potential and viability of yogurt microflora. From their study, tea was found to enhance the growth of the yogurt starter culture as well as the antioxidant potential of yogurts.

2.2. Tea

2.2.1. Introduction

Tea (*Camellia sinensis*) has its origin from China. Its popularity as beverage is attributed to its characteristic flavour, unique aroma and also various health benefits associated with it. Tea is widely cultivated in more than 30 countries around the world (Graham, 1992) with China, India, Japan, Sri Lanka, Indonesia and Central African countries dominating 80% of world tea production (Sang *et al.*, 2011). The cultivation of tea generally requires the tropical or subtropical climate with high humidity, sufficient rainfall, good drainage and slightly acidic soil (Graham, 1999). India is the largest producer of teas while China and Japan are the largest consumers of tea (Dubeau *et al.*, 2011). The consumption of tea which began almost 5000 years ago in China aims to eliminate toxins, improve blood flow and improve resistance to disease (Balentine *et al.*, 1997; Cai, 1979; Yan *et al.*, 1998).

Tea can be divided into three types based on the method of processing the leaves, namely the non-fermented green and white tea, partially fermented oolong tea and

fermented black tea (Horzic *et al.*, 2009). The diversity in the methods of processing have given rise to many other unique type of teas in market including Pu-erh tea, scented teas, flavoured teas, organic teas, decaffeinated teas and herbal teas (Reeves *et al.*, 1987; Karori *et al.*, 2007).

In the manufacture of green tea, the plucked tea leaves are immediately withered and subjected to steaming (Japan) or pan-frying (China) prior to rolling and drying to inactivate the endogenous polyphenol oxidase (PPO) enzyme as well as native microflora in the leaves (Gondoin *et al.*, 2010; Kim *et al.*, 2011). This prevents the polyphenols, mainly catechins, in tea from oxidation thus, retaining a large amount of polyphenols in green tea leaves. In the processing of black tea, steaming or pan-frying steps are omitted but the leaves are crushed or bruised to disrupt cellular compartmentalization and bring the phenolic compounds in the leaves into close approximation with the enzyme PPO, thus allowing the rolled leaves to be fully fermented by the active PPO enzyme (Rusak *et al.*, 2008). As a result of the fermentation, a large portion of the catechins are condensed into larger polyphenols, known as theaflavins and thearubigins which contribute to the characteristic reddish-black colour, reduced astringency and bitterness as well as elimination of the leafy and grassy flavour in the black tea (Kim *et al.*, 2011).

Oolong tea on the other hand, is produced in a similar way as black tea, but at shorter fermentation time. As such, the characteristic of oolong tea lies in between green and black teas (Del Rio *et al.*, 2004). White tea is derived from very young tea leaves or buds which are covered with tiny, silvery hairs, plucked just before the buds are fully opened. It is harvested only once in a year during early spring. It is not withered, crushed or rolled unlike green, black and oolong teas, but steamed and air-dried immediately after plucking, to prevent PPO catalysed oxidation of the phenolic compounds making it the least processed form of tea varieties. Thus, it retains a large

amount of catechins and has a very refreshing light and delicate taste (Rusak *et al.*, 2008). Figure 2.3 summarizes the methods of processing tea leaves and the resulting tea products obtained.

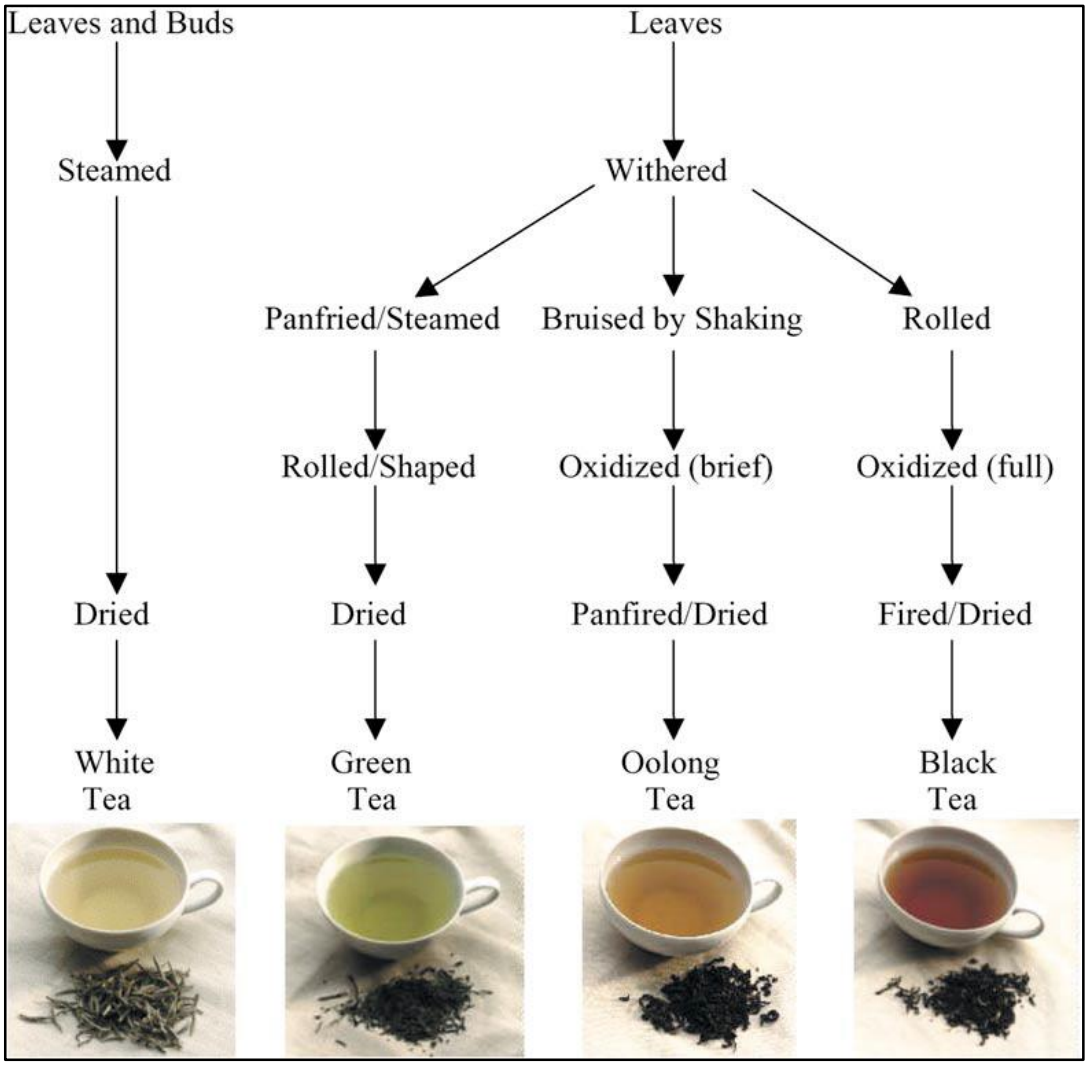

Figure 2.3: Methods of processing tea (*Adapted from Santana-Rios et al., 2001*)

The patterns of tea production and consumption worldwide shows 78%, 20% and 2% for black, green and oolong tea respectively. Black tea is commonly consumed in Western countries, green tea in Asian countries while oolong tea is predominantly consumed in Southern China (Khan & Mukhtar, 2007).

2.2.2. Composition of tea

The plant polyphenols in tea can be divided into flavonoid, phenolic acid, tannins, stilbanes and lignans (Ignat *et al.*, 2011). Of the 6 classes of flavonoid (anthocyanin, flavanol, flavonol, flavanone, flavone, isoflavone) commonly found in plants tea is reported to be a rich source of flavanols and flavonols (Wang *et al.*, 2000).

Catechins are the primary flavanols in tea that contribute 30-42% of the dry weight in green tea leaves (Khan & Mukhtar, 2007; Balentine *et al.*, 1997). These colourless, water-soluble compounds are responsible for the bitterness and astringency taste of fresh green tea infusions. Catechins are characterized by the di- or tri-hydroxyl group substitution of the B ring and meta-5, 7-dihydroxy substitution of the A ring. There are 6 major forms of catechins found in fresh tea leaves namely (−)epicatechin (EC), (−)epicatechin-3-gallate (ECG), (−)epigallocatechin (EGC), (−)epigallocatechin-3-gallate (EGCG), (+)catechin and (+)gallocatechin (GC) (Figure 2.4). Among all the catechin derivatives found in tea leaves, EGCG was reported to be the most abundant form of catechin, contributing about 50-80% of total catechins found in green tea leaves (Sang *et al.*, 2011; Chu & Juneja, 1997; Khan & Mukhtar, 2007). The catechin content in black tea is reduced to 10-12% due to fermentation process (Dufresne & Farnworth, 2001).

In the production of black tea, almost 75% of catechins present in the green tea leaves are oxidized and polymerised into theaflavins and thearubigins (Figure 2.5) by the action of PPO enzyme during fermentation process. Thus, both theaflavins and thearubigins are the major form of polyphenols unique to black tea and together contribute to the characteristic colour, strength, body and mouth feel properties of black tea infusions. Theaflavins account for about 3-6% of dry weight while thearubigins account for about 12-18% of dry weight in black tea leaves (Khan & Mukhtar, 2007). Theaflavin is a bright red-orange coloured pigment and could be present in four major

forms, namely theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate and theaflavin 3, 3'-digallate as well as other minor derivatives such as theaflavic acid and theaflavates (Harbowy & Balentine, 1997; Sang *et al.*, 2004). Thearubigins are red-brown or dark-brown polymers (Harbowy & Balentine, 1997) with high molecular weight, ranging from 700-40000 Da (Sanderson *et al.*, 1972). They are water-soluble and acidic in nature (Khan & Mukhtar, 2007). However, information on the structure and chemical nature of thearubigins are still scarce.

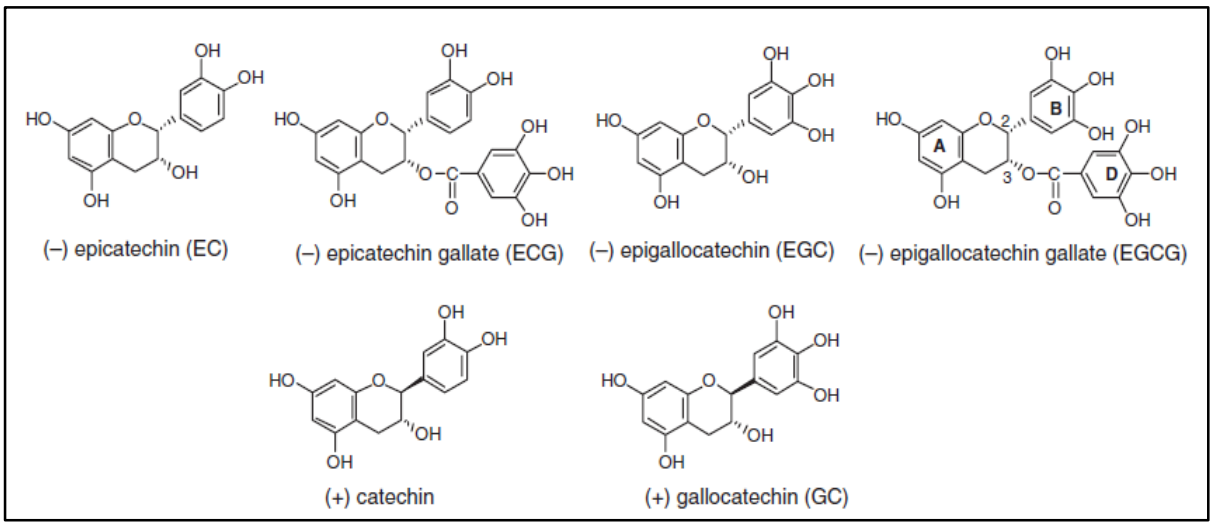

Figure 2.4: Major form of catechins found in fresh tea leaves *(Adapted from Zaveri, 2006)*

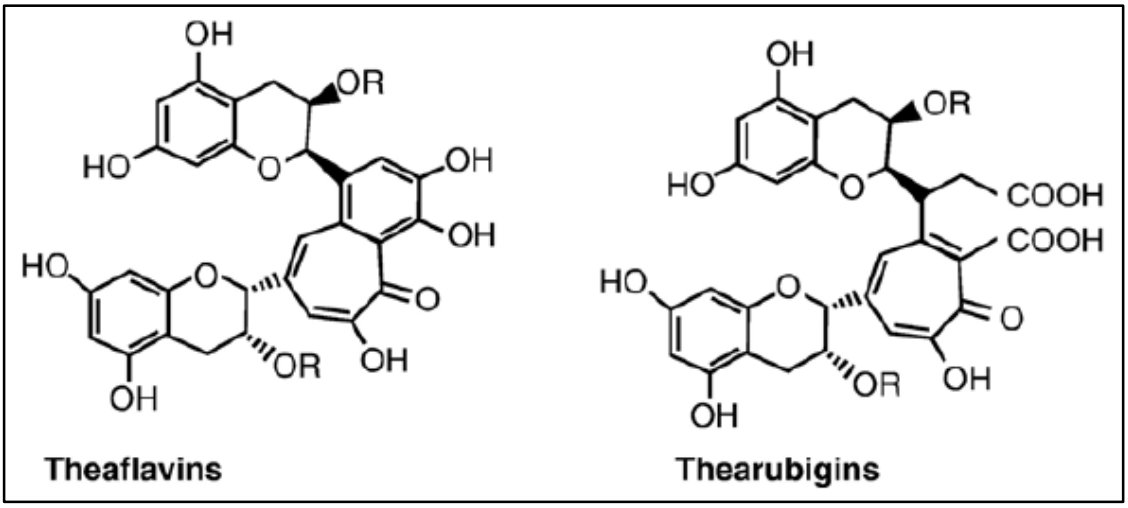

Figure 2.5: The major forms of catechins in black tea leaves, R refers to galloyl group *(Adapted from Katiyar et al., 2007)*

Flavonols present in tea contribute to almost 5-10% of dry weight in green tea and 6-8% of dry weight in black teas (Dufresne & Farnworth, 2001). The major flavonols include quarcetin, kaemperol and myrecitin (Figure 2.6). Although they may exist in both forms, glycosides and aglycones, the former are the major form in tea infusions, probably due to poor solubility of the aglycones in water. The sugar moieties often found attached to flavonols include glucose, rhamnose, galactose, arabinose and fructose (Balentine *et al.*, 1997; Engelhardt *et al.*, 1992).

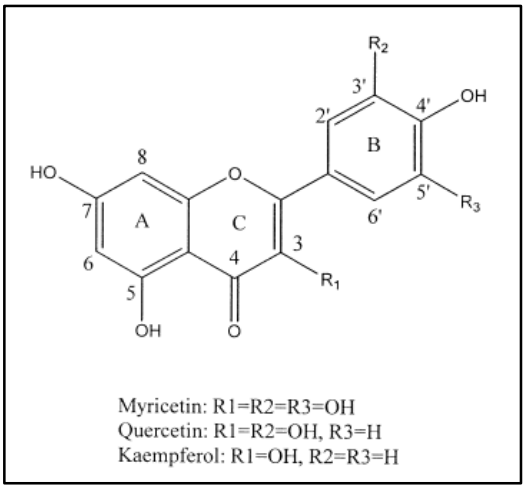

Figure 2.6: Structure of flavonols present in tea *(Adapted from Wang et al., 2000)*

Other constituents of tea leaves include phenolic acids such as gallic acid, caffeic acid and quinic acid, collectively present about 2% in green tea and 10-12% in black tea. The gallic acid content increases in black tea due to de-esterification of 3-galloylcatechin derivatives by enzymatic action during fermentation (Harbowy and Balentine, 1997). Methyxanthines such as caffeine, theobromine and theophylline are also found, contributing about 7-9% and 8-11% of dry weight in green tea and black tea respectively (Dufresne & Farnworth, 2001). The insignificant difference in caffeine content between green and black tea indicated that caffeine remain stable and unchanged during fermentation (Sang *et al.*, 2011).

Of the 17% nitrogeneous materials found in tea, protein contributes about 6% while amino acids and nucleic acids contribute approximately 8% of the dry weight.

Theanine is the predominant form of amino acid in both green and black teas contributing about 3% of dry weight and was found to be unique to tea. Minerals make up about 10-15% of dry weight and consist mainly of potassium, calcium, magnesium and aluminium. Tea also contains many volatile compounds that make up only a small proportion of the dry weight, lesser than 0.1%. Linalool is the most abundant and may be responsible for flavour (Sang *et al.*, 2011; Dufresne & Farnworth, 2001). The chemical constituents of tea leaves as reported by many studies may differ due to differences in tea types, age of tea leaves, climate, geochemical backgrounds as well as methods of tea processing and extraction.

2.2.3. Health benefits of tea

The remarkable rise in the popularity of tea over the days could be attributed to the various health promoting properties. In most cases, the polyphenols found in tea have been reported to be the active ingredient conferring these health benefits and these are presented in the following:

(a) Potent source of antioxidant

The catechins found in tea, especially EGCG, elicit better antioxidant activity in comparison to vitamins C and E, tocopherol and carotene (Sharangi, 2009). The antioxidant nature of the tea catechins in descending strength was reported as EGCG > ECG > EGC > EC > Catechin (Luczaj & Skrzydlewska, 2005).

The strong antioxidant activity is attributed to the presence of dihydroxy or trihydroxy structure in catechins, which not only allows delocalization of electrons that quench free radicals, but also chelate metal ions thus preventing generation of free radicals (Khan & Mukhtar, 2007). The catechins could also

promote the activity of some detoxifying enzymes with antioxidative nature such as gluthathione peroxidase (Sharangi, 2009) as well as inhibit enzymes that could promote free radical generation such as xanthine oxidase (Luczaj & Skrzydlewska, 2005). Other studies (Miller *et al.*, 1996; Leung *et al.*, 2001) however showed that theaflavins in black tea have greater antioxidant activity than EGCG, accounting to the presence of more hydroxyl groups responsible for the radical scavenging activity. Among the theaflavins, the antioxidant activity decreases in the manner of theaflavin 3, 3'-gallate > theaflavin-3-gallate = theaflavin-3'-gallate > theaflavin.

The antioxidative nature of tea polyphenols enables them to combat oxidative damage on lipids, proteins and DNA by reactive oxygen species (ROS), thus preventing various degenerative diseases. In a clinical study by Freese *et al.* (1999), 20 healthy women consuming a diet rich with linoleic acid showed diminished levels of plasma malondialdehyde upon consumption of encapsulated green tea extract for four weeks. Plasma malondialdehyde level is an indicator of lipid peroxidation which reflects oxidative damage in the body.

(b) <u>Protection against various forms of cancers</u>

The reputation of tea as significant anti-cancer agent centres on its ability to supress carcinogenesis at 3 critical stages, namely cancer initiation, promotion and progression (Dufresne & Farnworth, 2001). The predominant chemopreventive agent in most cases was EGCG which played its role mostly by modulation of multiple cellular signalling pathways (Shankar *et al.*, 2007).

Phase I enzymes in the body, such as cytochrome P450, are responsible to convert inactive procarcinogens to carcinogens that cause DNA mutation and induce tumour formation. Phenolic compounds in tea are able to constrain

cancer initiation by either neutralizing the procarcinogens before they can be activated by Phase I enzymes, inhibit phase I enzymes thus preventing activation of procarcinogens, or activating phase II enzymes resulting in conjugation of active carcinogens followed by their inactivation and excretion out of the body (Lampe, 1999; Gordon, 1996; Lin *et al.*, 1999).

The mitotic signal transducers responsible for cancer cell proliferation could be inhibited by EGCG and theaflavin-3-3'-digallate, thus suppressing the potential of cancer promotion and progression (Lin *et al.*, 1999). In addition, Naasani *et al.* (1998) have reported that telomerase activity of cancer cells were effectively suppressed by EGCG, thus restraining the life span of cancer cells. Telomerase is an enzyme absent from normal somatic cells, but found in cancer cells and crucial for proliferation of the cells. Jankun *et al.* (1997) have reported that the activity of urokinase enzyme responsible for growth and metastasis of cancer cells was inhibited by EGCG by intervening with the potential of the enzyme to recognize its substrates.

The polyphenols in tea have been reported to be effective against several types of cancers including skin cancer (Katiyar *et al.*, 2007), lung cancer (Cao *et al.*, 1996; Landau *et al.*, 1998; Kubik *et al.*, 2004), breast cancer (Leong *et al.*, 2008; Lambert & Yang, 2003 a,b), pancreatic cancer (Lyn-Cook *et al.*, 1999, Lambert & Yang, 2003 a,b), prostate cancer (Lyn-Cook *et al.*, 1999), liver cancer (Landau *et al.*, 1998; Sueoka *et al.*, 2001) and GI tract cancer (Ju *et al.*, 2005).

(c) <u>Reduction of serum cholesterol levels, hypertension and CVD</u>

Hypercholesterolemia is a clinical condition characterized by elevated levels of cholesterol circulating in the blood. In such case, the chances for low-density

lipoproteins (LDL) to deposit at lesion sites of arterial wall increase. When endogenous antioxidants in the body are depleted, the deposited LDL is subjected to oxidation which induces modifications in the lipoproteins. This in turn stimulates inflammatory responses in which macrophages and monocytes accumulate in abundance at the lesion site, forming lipid-laden foam cells and atherosclerotic plaques. The plaques protrude from the inner surface of the artery, restricting the blood flow leading to further complications such as hypertension, arthrosclerosis, thrombosis, stroke and heart attack (Dufresne & Farnworth, 2001)

Significant reduction in serum and liver cholesterol level, atherogenic index and liver weight was reported in hypercholesterolemic diet induced rats after green tea administration (Yang & Koo, 1997). The reduction in serum cholesterol levels was attributed to precipitation of cholesterol followed by faecal elimination (Tijburg *et al.*,1997; Chopra & Thurnham; 1999) or inhibition of squalane epoxide, a rate-limiting enzyme in cholesterol biosynthesis (Abe *et al.*, 2000). Davies *et al.* (2003) reported reduction in LDL and total cholesterol by 11.1% and 6.5% respectively in adults with mild hypercholesterolemia upon consumption of five servings of black tea per day.

In an animal model study using apo E deficit mouse, the development of atherosclerosis was impeded by quarcetin found in both green and black teas and theaflavin found mainly in black tea (Loke *et al.*, 2010). Negishi *et al.* (2004) reported reduction of blood pressure in spontaneously hypertensive rats upon administration of green tea extract. Thus both studies support earlier observation that the consumption of more than 10 cups of green tea a day could reduce the risk of death from CVD (Nakachi *et al.* 1998; Sueoka *et al.*, 2001).

(d) Prevention and control of diabetes

Type II diabetes is a common metabolic disorder characterized by intolerance to glucose and lipid metabolism by peripheral tissues, due to insulin resistance and insufficient insulin secretion by the pancreatic β-cells (Khan & Mukhtar, 2007). Iso *et al.* (2006) showed that drinking more than 6 cups of green tea per day could reduce the risk of developing diabetes. In addition, administration of green tea extracts to fructose-fed rats by Wu *et al.* (2004b) was found to decrease the occurrence of insulin resistance, hyperglycaemia and other metabolic disorders.

Several possible mechanisms underlying the anti-diabetic effects of tea have been proposed. Wu *et al.* (2004a) suggested the positive effects of green tea consumption to EGCG was associated with the enhancement of insulin sensitivity and glucose uptake by adipocytes. This is in contrast to tea inhibition of glucose transporters in the intestinal epithelium, thus the anti-diabetic effect (Shimizu, 1999). The inhibition of starch digesting enzyme, amylase, by tea polyphenols is considered instrumental in suppressing the surge in serum glucose level after ingestion of a carbohydrate rich meal (Hara & Honda, 1990).

(e) Prevention and control of obesity

An imbalance between energy intake and energy expenditure often results in obesity, a common metabolic disorder (Sharangi, 2009). In lean and obese Zucker rats, administration of purified EGCG was found to reduce body weight or prevent increase in body weight significantly (Kao *et al.*, 2000). Murase *et al.* (2006) have reported reduction of diet-induced obesity in mice with intake of tea catechin coupled with frequent exercise. In other studies, Venables *et al.* (2008)

and Takashima *et al.* (2004) reported increase in fat oxidation during exercise, usually between 3 to 7%, following consumption of green tea catechins.

(f) Treatment of allergic responses and arthritis

In allergic responses, the occurrence of inflammation, dermatis, mastocytosis and asthma is considered as a consequence from the release of histamine by mast cells. Alexis *et al.* (1999) have reported inhibition of histamine release by 90% in a rat cell culture by EGCG. In addition, a concentration dependant inhibition of histamine release by quarcetin was also observed.

Rheumatoid arthritis is a clinical condition associated with the inflammation and damage of joints. A significant reduction in inflammatory mediators, neutral endopeptidase activity, IgG and type II collagen-specific IgG levels by green tea polyphenols were observed in arthritic joints of mice, thus reduction in the incidence of arthritis (Haqqi *et al.*, 1999).

(g) Improvement of oral health

The ability of tea plant to extract fluoride ions from soil makes it a good source of fluoride which could prevent dental decay by binding to enamel particles on tooth surface (Sharangi, 2009). Hamilton-Miller (2001) suggested the possibility that the decline in the severity of dental caries is as a consequence of regular tea drinking habit. In addition, Sakanaka *et al.* (1990) and Inoue *et al.* (1996) have reported that EGCG and ECG in tea could inhibit the growth of cariogenic bacteria on tooth surface.

(h) Treatment of skin disorders

The antiseptic property of tannins and flavonoids in tea allows it to be used for treatment of itching and inflammation of insect bites (Sharangi, 2009). Alexis *et al.* (1999) have reported that the enzyme 5α-reductase present in the skin was inhibited by EGCG and ECG, thus permitting treatment of androgen dependent dermatologic disorders such as acne.

(i) Protection against cataract

In rabbits which the eye lens were exposed to UVA to induce photo-oxidative stress, green tea polyphenols were found to enter the eyes and inactivate the enzyme catalase found in the lens, thus protect against cataract (Zigman *et al.*, 1999). Thiagarajan *et al.* (2001) have reported suppression in progression of eye lens cataract by green and black teas, in rats with selenite-induced cataract. Grunberger and Frenkel (1997) have patented a product formulation containing caffeic acid esters, components commonly found in tea and coffee, for inhibition of cataract.

(j) Neurological and physiological benefits

Theanine, the unique amino acid of tea, could act as a neurotransmitter by modulating levels of brain serotonin and dopamine, reducing stress, and enhancing alertness, memory power and learning ability (Juneja *et al.*, 1999; Unno *et al.*, 1999). Since neurodegenerative diseases such as Parkinson's and Alzheimer's disease are often related to ageing and increased risk of oxidative damages, tea polyphenols would be able to confer protection against such diseases due to their antioxidative nature. Mandel *et al.* (2008) have emphasized the role of EGCG as a neuro-protective agent based on many cellular and animal model studies. Choi *et al.* (2002) have reported a reduction in the loss of

dopaminergic neurons in the substantia niagra which is otherwise common in the progression of Parkinson's disease, upon oral administration of EGCG.

(k) Antibacterial and antiviral properties

Tea elicit antibacterial effects against a wide range of bacteria including *Vibrio cholerea*, *Salmonella typhi*, *Campilobacter jejuni*, *Campilobacter coli*, *Helicobacter pylori*, *Shigella*, *Salmonella*, *Clostrodium* and *Pseudomonas* (Yamamoto *et al.*, 1997; Diker & Hascelik, 1994; Alexis *et al.*, 1999; Maity *et al.*, 1998; Toda *et al.*,1991;Diker *et al.*,1991). Tea could modulate the intestinal microflora by eliminating the enterobacteria which produce harmful substances and in turn, increase the levels of beneficial lactobacilli and bifidobacteria which produce organic acid that confer antimicrobial property in the gut (Weisburger, 1999).

Rotavirus propagation in monkey cell culture and influenza A virus in animal cell culture were strongly supressed by tea polyphenols. In addition, the human immunodeficiency virus (HIV) propagation was also inhibited by tea polyphenols, namely EGCG and ECG, by inhibiting the enzyme reverse transcriptase which is otherwise responsible for the propagation and establishment of the virus in the host (Yamamoto *et al.*, 1997).

2.3. Oxidative damage and antioxidants

2.3.1. Free radicals and oxidative stress

Free radicals can be characterized by the presence of at least one unpaired electron in the outermost shell which confers high reactivity to these particles (Gutteridge & Mitchell, 1999; Kuhn, 2003). Free radicals that consist of oxygen are referred to as reactive oxygen species (ROS) (McDermott, 2000) examples of which include

superoxide anion radical (O_2•−), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2). Hydroxyl radicals are considered to be the most reactive form of ROS (Droge, 2002).

ROS can be generated in the body during cellular respiration via oxidative phosphorylation, during inflammatory responses by leucocytes namely phagocytic cells such as macrophages and neutrophils, during physical activities as a result of increased rate of oxidative phosphorylation as well as by exogenous oxidants such as cigarette smoke, air pollutants and UV radiation (Temple, 2000; Urso & Clarkson, 2003). The generation of ROS is also closely related to participation of redox-active metals such as iron and copper. Under stress conditions, the superoxide anion radical is able to release Fe (II) from iron-containing molecules, which then participates in Fenton reactions and generate abundant of the reactive hydroxyl radicals (Valko *et al.*, 2005). However, under normal circumstances, the body has several defence systems to combat these free radicals before they can harm the cells. The defence systems include:

(a) endogenous enzymes such as catalase, superoxide dismutase and glutathione reductase

(b) endogenous factors such as glutathione and coenzyme Q

(c) dietary antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol),vitamin A (β-carotene and other carotenoids) and selenium.

These defence systems ensure that the free radicals which are generated are continuously quenched to maintain the body in a steady-state (Temple, 2000). Lack of dietary antioxidants, overexposure to environmental oxidants, low levels of antioxidative enzymes as well as tissue injury could lead to overproduction of free radicals than the ability of the body to neutralize them, thus disrupting the steady state balance of the body leading to accumulation of free radicals. These free radicals will then attack adjacent cellular components, especially lipids, initiating a chain reaction known as lipid peroxidation which in turn generates more free radicals in the body that

may harm other cellular components such as proteins and DNA. Under this condition, the body is known to be under oxidative stress and is vulnerable to the pathogenesis of many degenerative diseases which include cardiovascular diseases such as hypertension, atherosclerosis and coronary heart disease, insulin and non-insulin dependent diabetes mellitus, autoimmune conditions such as rheumatoid arthritis, eye diseases such as retinal damage and cataract, cancer, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and schizophrenia (Temple, 2000; Scheibmeir, 2005). The risk of degenerative diseases in the body increase with age due to accumulation of free radicals in the body.

2.3.2. Antioxidants

Antioxidants can be defined as substances that when present together with an oxidizable substrate even at low concentrations, could prolong or supress the oxidation of the substrate significantly (Halliwell, 1997). Niki *et al.* (1995) have classified the mechanism of antioxidant activities into three groups:

(a) preventive antioxidants which act as first line of defence to supress the formation of free radicals

(b) scavenging antioxidants which act as second line of defence to remove free radicals before they can cause oxidative damages to the cell

(c) repair and *de novo* antioxidants which act as third line of defence to repair the damages done by free radicals and restore the function of the cell.

Although the body is well equipped with natural defence system comprising of various enzymes with antioxidative nature, dietary intake of antioxidants is thought to further strengthen the endogenous antioxidant defence system (Benzie, 2003). Many synthetic antioxidants have been commercialized especially to be in use as food stabilizers such as Butylatedhydroxyanisole (BHA), Butylatedhydroxytoluene (BHT),

and tert-Butylhydroquinone (TBHQ) (Diaz *et al.*, 1997). However, the safety aspect of their use still remains questionable due to their possibility of promoting cancer (Ito *et al.*, 1986; Botterweck *et al.*, 2000).

During the past two decades, plants namely fruits, vegetables and spices have been extensively studied as a potent source of natural antioxidant. Such claims have been attributed to the phytochemicals, namely phenolic compounds found in plants that are able to scavenge free radicals by acting as reducing agents, hydrogen donors, singlet oxygen quenchers or metal ion chelaters (Rice-Evans *et al.*, 1996; Babbar *et al.*, 2011). Since antioxidant activities of different plant species are collective readings of various bioactive ingredients present in the plants (Barreira *et al.*, 2008), several parameters need to be used to assess the antioxidant properties. These parameters include total polyphenols, total flavonoids, radical scavenging activity, ferrous ion chelating ability, ferric reducing antioxidant power and β-carotene bleaching capacity (Krishnaiah *et al.*, 2011).

CHAPTER 3.0:

MATERIALS AND METHODS

3.1. Materials

3.1.1. Milk

Pasteurized full cream milk (Dutch Lady, Malaysia) was used for making yogurt. The milk was purchased from a local hypermarket and was at least 2 weeks from the date of expiry.

3.1.2. Tea

Three types of tea leaves used in this study were Long Jing green tea and Shou Mei white tea (China origin; Purple Cane Enterprise, Malaysia) and black tea (Malaysia origin; Lipton, Malaysia) purchased from local hypermarket. The tea leaves were ground to pass through 1mm screen and were kept in dry air tight containers and stored at room temperature away from direct sunlight.

3.1.3. Starter culture

Starter culture was prepared according to the method described by Amirdivani and Baba (2011) with slight modification. Pasteurized full cream milk (1L) was pre-heated to 41°C. A sachet of yogurt and probiotic bacteria mix consisting of *Lactobacillus acidophilus* LA-5, *Bifidobacterium* Bb-12, *Lactobacillus casei* LC-01 and *Streptococcus thermophilus* Th-4 (Herbal Science Sdn. Bhd., Malaysia) was added to the milk. The contents of a probiotic capsule containing approximately 75g of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Bifidobacterium infantis* and *Bifidobacterium longum* (Advanced MultiBlend PROBIOtix, Bio-Life, Malaysia) was also added to the milk. The milk bacteria mixture was stirred well and incubated at 41°C for 12 hours. The yogurt formed was refrigerated (4 °C) and used as starter culture within 7 days.

3.1.4. Chemicals and reagents

3.1.4.1. Chemicals used in total phenolic content (TPC) assay

Gallic acid, sodium carbonate and 2M Folin-Ciocalteu's phenol reagent were purchased from Sigma-Aldrich Chemical Co., USA.

3.1.4.2. Chemicals used in DPPH radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was supplied by Sigma-Aldrich Chemical Co., USA.

3.1.4.3. Chemicals used in FRAP assay

2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was purchased from Sigma-Aldrich Chemical Co., USA. Iron (II) sulphate heptahydrate, glacial acetic acid and sodium acetate anhydrous were products of Friendemann Schmidt, Australia. Iron (III) chloride anhydrous was purchased from John Kollin Chemicals, UK.

3.1.4.4. Chemicals used in FIC assay

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4" (Ferrozine) and iron (II) sulphate hydrate was obtained from Sigma-Aldrich Chemical Co., USA.

3.1.4.5. Chemicals used in determination of titratable acid

Phenolphtalein indicator was purchased from Friendemann Schmidt, Australia

3.1.4.6. Chemicals used in microbiology analysis

Buffered peptone water, MRS agar and M17 agar were purchased from Oxoid, UK. Lactose monohydrate was a product of Systerm, Malaysia.

3.1.4.7. Miscellaneous reagents

Hydrochloric acid and sodium hydroxide pellets were products of Friendemann Schmidt, Australia. Ethanol and methanol were purchased from Systerm, Malaysia.

3.2. Instruments

The following instruments available in the laboratory were used to carry out the experiments: Water bath (Memmert), pH meter (Hanna Instruments, Model HI 8424), autoclave (Tommy, Model SS-325), oven (Memmert), centrifuge (Eppendorf 5804R), homogenizer (Polytron, Model PT-MR 2100), incubator (Revco Ultima), weighing machine (Adventurer Ohaus), vertical laminar flow cabinet (Labcaire), spectrophotometer (Genesys 10UV), ice maker (Scotsman AF200), 4°C refrigerator (Panasonic, Model NR-B651G), – 20 °C refrigerator (Sanyo Ultra Low, Model MDFU 4086S) and electric hot plate (Thermolyne).

3.3. Apparatus

The following apparatus were used to carry out the experiments in the laboratory: Beaker (50ml, 100ml, 250ml, 500ml, 1000ml, 2000ml), spatula, measuring cylinder (10ml, 50ml, 100ml, 1000ml), Eppendorf pipette (1-5ml, 10-100µl,100-1000µl), stirring rod, dropping pipette, Erlenmeyer flask (50ml), volumetric flask (25ml, 50ml, 100ml, 250ml, 500ml, 1000ml), test tube, test tube rack, plastic centrifuge tubes with caps (50ml), thermometer, aluminium foil, air tight containers (to store tea leaves), disposable plastic containers (to store yogurt), Schott bottle (100ml, 250ml, 500ml,1000ml), burette, glass pipette (5ml, 10ml), pi-pump, filter funnel, distilled water

bottle, disposable pipette tips (1-5ml, 10-100µl, 100-1000µl), Bunsen burner, hockey stick or spreader, disposable petri dish (size 90mm × 15mm) and plastic cuvette (1.5ml)

Sterilization of apparatus used for yogurt preparation, where necessary, was achieved by means of autoclaving (121 °C for 15 minutes) prior to use.

3.4. Methods

3.4.1. Preparation of yogurt

Plain yogurt and three types of tea-yogurt were prepared according to the method described by Jaziri *et al.* (2009) with slight modifications. Pasteurized full cream milk (100 ml) was warmed to 85°C. Treated milk was mixed with 2% (w/v) of green, white or black teas (2 g/100 ml) corresponding to the strength of a "normal cup of tea" (Yam *et al.*, 1997). The teas were allowed to infuse into the milk for 10 minutes followed by filtration through fine tea strainer to remove visible particles. The resulting tea-milk infusions (90 ml) were aliquoted into disposable plastic containers placed in an incubator (45°C) followed by the addition of starter culture (10 ml) into the milk-tea infusion. Plain yogurt was prepared in the same manner as previously described using milk instead of milk-tea infusate. All inoculated milk and milk-tea infusates were incubated in a water-bath at 42°C until the pH values reached 4.5. The yogurts were then refrigerated (4°C) up to 21 days.

3.4.2. Sampling of yogurts for analysis

Samples of each yogurt type were removed from the fridge the following day (day 1) and on days 7, 14 and 21 of storage. All samples were analysed for changes in total

phenolic content, antioxidant activities (DPPH radical scavenging activity, ferric reducing antioxidant potential (FRAP) and ferrous ion chelating (FIC) ability) as well as changes in fermentation characteristics determined by changes in pH, titratable acid and viable cell counts (VCC) of selected yogurt microflora during storage. In addition, changes in the phenolic contents of yogurt during pre- and post-fermentation stages were also analysed by liquid chromatography mass spectrometry (LCMS) method.

3.4.3. Preparation of yogurt water extract

3.4.3.1. Preparation of reagents for yogurt water extraction

HCl (0.1 M) was prepared by mixing 37% fuming HCl (8.33 ml) in distilled water (100 ml) and the volume was made up to 1000 ml in a volumetric flask. NaOH (0.1 M) was prepared by dissolving NaOH pellets (4g) in distilled water (100 ml) and the volume was made up to 1000 ml in a volumetric flask.

3.4.3.2. Preparation of yogurt water extracts

Water extraction of yogurt was carried out as described by Amirdivani and Baba (2011). Plain- and tea-yogurts (10 g) were weighed into plastic centrifuge tubes. The yogurts were then homogenised (Polytron, at highest setting for 10 seconds) with sterile distilled water (2.5 ml). The pH of the yogurts was determined using a pH meter and the yogurts were subsequently acidified to pH 4.0 by adding HCl (0.1 M). The acidified yogurts were then incubated for 10 minutes in a water bath (45 °C) followed by centrifugation (5000g, 4 °C, 10 minutes). The pH of the resulting supernatant was then adjusted to 7.0 using NaOH (0.1 M) followed by another step of centrifugation (5000g, 4 °C, 10 minutes). The clear supernatant obtained was stored in a freezer (-20 °C) and used for analysis within 1-2 weeks of preparation.

3.4.4. Preparation of tea water extracts

The strength of the tea infusions used for analysis was 2% (w/v). Boiled hot water (85°C; 100 ml) was poured into a beaker containing tea (2 g). The beaker was covered using aluminium foil and the tea was brewed for 10 minutes. The brewed tea was filtered using fine tea strainer and the filtrate was cooled to ambient temperature. The tea infusates (tea water extracts) were then centrifuged (5000g, 4°C, 10 minutes) and the harvested supernatants were refrigerated (4°C) and used for analysis within 1-2 weeks of preparation.

3.4.5. Determination of total phenolic content (TPC)

3.4.5.1. Preparation of chemical reagents for determination of TPC

Methanol solution (50% v/v) was prepared by diluting methanol (5 ml) with distilled water (5 ml). The preparation of stock gallic acid (0.5 mg/ml) was carried out as follows: a solution of gallic acid (1 mg/ml) was first prepared by dissolving gallic acid powder (10 mg) in 50% methanol solution (10 ml). The resulting gallic acid solution (500 µl) was further diluted with 50% methanol solution (500 µl) to prepare the stock gallic acid solution (0.5mg/ml). Sodium carbonate solution (20% w/v) was prepared by dissolving sodium carbonate powder (20 g) in distilled water (100 ml).

3.4.5.2. Preparation of gallic acid calibration curve

Stock gallic acid solution (0.5 mg/ml) was diluted with suitable volume of 50% methanol solution as described in the table below to obtain the following concentrations; 50, 100, 150, 200, 250, 300, 350 µg/ml.

Table 3.1: Preparation of various concentrations of standard gallic acid solutions from stock gallic acid solution (0.5 mg/ml)

Gallic acid concentration (µg/ml)	Volume of stock gallic acid (µl)	Volume of 50% methanol solution (µl)
0	0	100
50	10	90
100	20	80
150	30	70
200	40	60
250	50	50
300	60	40
350	70	30

TPC assay was carried out on the standards and a graph of absorbance at 765 nm against the concentration of the standards was plotted.

3.4.5.3. TPC assay

TPC assay was carried out according to the method described by Najgebauer-Lejko *et al.* (2011). Samples of yogurt water extracts, tea extracts or standard solutions of gallic acid (100 µl) were mixed with distilled water (7.9 ml) and 2M Folin-Ciocalteu reagent (0.5 ml). After thorough mixing, the mixture was allowed to stand at room temperature for 5 minutes. Sodium carbonate solution (1.5 ml, 20% w/v) was added into the mixture and following a brief mixing the mixture was left standing in the dark for 2 hours at room temperature. Absorbance at 765 nm was measured against distilled water as blank using a spectrophotometer (Genesys 10UV). The results were converted into total phenolic content using the gallic acid calibration curve and expressed as µg gallic acid equivalent per millilitre (µg GAE/ml).

3.4.6. Determination of antioxidant activity by measurement of DPPH radical scavenging activity (DPPH assay)

3.4.6.1. Preparation of chemical reagents for measurement of DPPH assay

For the preparation of DPPH reagent (0.07 mM/L), a stock DPPH solution (1 mM/L) was first prepared by dissolving DPPH powder (39 mg) in methanol (100 ml). The resulting solution (7 ml) was further diluted using methanol in a 100 ml volumetric flask. The DPPH reagent was freshly prepared each time it was required for analysis and used within 2 hours of preparation.

3.4.6.2. DPPH radical scavenging assay

The DPPH radical scavenging assay was carried out according to the procedure described by Najgebauer-Lejko *et al.* (2011). Samples of yogurt water extracts or tea extracts (100 µl) were mixed with DPPH reagent (3.9 ml, 0.07mM/L) followed by incubation in the dark at room temperature for 2 hours. Absorbance at 515 nm was measured against methanol blank using a spectrophotometer (Genesys 10UV). The radical scavenging activity was calculated as below:

$$\text{Scavenging activity (\%)} = 1 - [\text{Abs}_{\text{sample @ 515nm}} / \text{Abs}_{\text{control@515 nm}}] \times 100\%$$

Control was a mixture of methanol (100 µl) and DPPH reagent (3.9 ml) only, without addition of any samples.

3.4.7. Determination of antioxidant activity by measurement of ferric reducing antioxidant potential (FRAP)

3.4.7.1. Preparation of chemical reagents for measurement of FRAP

For the preparation of stock iron (II) sulphate heptahydrate solution ($FeSO_4.7H_2O$, 10 mM), iron (II) sulphate heptahydrate powder (139 mg) was dissolved in distilled water (50 ml). HCl (30 mM) was prepared by diluting the stock 0.1M HCl solution (300 ml) with distilled water to make up the volume to 1000 ml in a volumetric flask.

TPTZ reagent (8 mM) was prepared by dissolving TPTZ powder (63 mg) in 30 mM HCl (25 ml) solution. Iron (III) chloride ($FeCl_3$, 20 mM) solution was prepared by dissolving iron (III) chloride powder (162 mg) in 36% fuming HCl (approximately 10 ml) and the volume was made up to 50 ml in a volumetric flask by adding distilled water.

For the preparation of acetic acid (0.281 M), a stock solution of acetic acid (1 M) was first prepared by diluting glacial acetic acid (57.2 ml) with distilled water to 1000 ml in a volumetric flask. The resulting 1 M acetic acid solution (281 ml) was further diluted with distilled water to make up a volume of 1000 ml in a volumetric flask to produce 0.281 M acetic acid solution. For the preparation of acetate buffer (300 mM, pH 3.6), sodium acetate powder (1.593 g) was dissolved in 0.281 M acetic acid (approximately 900 ml) followed by adjustment of pH to 3.6 using 0.1M HCl . The final volume of the buffer was made up to 1000 ml by the addition of 0.281 M acetic acid in a volumetric flask.

A working FRAP reagent was prepared by mixing 300 mM acetate buffer, 8 mM TPTZ reagent and 20 mM $FeCl_3$ solutions in a 10:1:1 ratio. For preparation of FRAP reagent (100 ml), 300 mM acetate buffer (83.3 ml), 8 mM TPTZ reagent (8.3 ml) and 20 mM $FeCl_3$ solution (8.3 ml) were mixed together. The FRAP reagent was freshly prepared before use each time and warmed at 37 °C in a water bath for 10 minutes prior to use.

3.4.7.2. Preparation of iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$) calibration curve

The standard solutions for FRAP calibration curve was prepared and subjected to FRAP assay each time samples were analysed for FRAP. Stock $FeSO_4.7H_2O$ solution

(10 mM) was diluted with appropriate volumes of distilled water as described in Table 4 to obtain the following concentrations; 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mM.

Table 3.2: Preparation of various concentrations of $FeSO_4.7H_2O$ solution from stock $FeSO_4.7H_2O$ solution (10 mM)

Concentration of $FeSO_4.7H_2O$ solution (mM)	Volume of stock $FeSO_4.7H_2O$ solution (µl)	Volume of distilled water (µl)
0	0	400
0.3	12	388
0.4	16	384
0.5	20	380
0.6	24	376
0.7	28	372
0.8	32	368
0.9	36	364
1.0	40	360

3.4.7.3. FRAP assay

The FRAP assay was carried out following the procedures described by Benzie and Strain (1996). Freshly prepared working FRAP reagent (3.6 ml) was added into a test tube containing yogurt water extract, tea extracts or standard $FeSO_4.7H_2O$ solutions (400 µl) and the contents were thoroughly mixed. The mixtures were then incubated at 37°C in a water bath for 10 minutes. Absorbance at 593 nm was measured against distilled water blank using a spectrophotometer (Genesys 10UV). The results were calculated from the $FeSO_4.7H_2O$ calibration curve and expressed as mmol Fe^{2+} equivalent/ L (mmol Fe^{2+} E/ L).

3.4.8. Determination of antioxidant activity by measurement of ferrous ion chelating (FIC) ability (FIC assay)

3.4.8.1. Preparation of chemical reagents for FIC assay

For the preparation of iron (II) sulphate hydrate solution ($FeSO_4.xH_2O$, 2mM), a stock $FeSO_4.xH_2O$ solution (10mM) was first prepared by dissolving $FeSO_4.xH_2O$ powder (76 mg) in approximately 20-25 drops of concentrated sulphuric acid and the final volume was made up to 50 ml in a volumetric flask by adding distilled water. The resulting 10 mM $FeSO_4.xH_2O$ solution (10 ml) was further diluted with distilled water to make up a final volume of 50 ml in a volumetric flask.

Ferrozine (5 mM) was prepared by dissolving ferrozine powder (62 mg) in distilled water (25 ml).

3.4.8.2. FIC assay

FIC assay was carried out following the procedures described by Chan *et al.* (2007). The $FeSO_4.xH_2O$ solution (2 mM) and ferrozine solution (5 mM) were both diluted 20 times prior to their use in the assay. Diluted $FeSO_4.xH_2O$ solution (1 ml) was added to samples of yogurt water extract or tea extracts (1 ml) and mixed well. Diluted ferrozine solution (1 ml) was then added to the mixture followed by incubation for 10 minutes at room temperature. Absorbance (Abs) at 562 nm was measured against distilled water blank using a spectrophotometer. The FIC ability of samples was

calculated as below:

$$\text{FIC ability (\%)} = 1 - [Abs_{\text{sample @562 nm}} / Abs_{\text{control @562 nm}}] \times 100\%$$

Control was a mixture of $FeSO_4.xH_2O$ solution (1 ml), ferrozine solution (1 ml) and distilled water (1 ml).

3.4.9. LCMS analysis of phenolic compounds in tea extracts and yogurt water extracts

The samples of yogurt water extracts on day 1 of fermentation and tea extracts were analysed for key phenolic compounds using LCMS. A full scan of samples with MS/MS data collection was performed using AB Sciex 3200Q Trap LCMS/MS system. Analysis of the compounds was carried out using the negative ionization mode (*m/z* M - H^+). Chromatographic separation of the compounds in the samples was carried out using a Phenomenex Aqua C18 column (50 mm × 2 mm × 5 µM). Samples (20 µl) were filtered with nylon membrane (0.22 µm) and injected into the column.

For the separation, a gradient run programme was carried out using a gradient of mobile phase A (water with 0.1% formic acid and 5 mM ammonium formate) and mobile phase B (acetonitrile with 0.1% formic acid and 5 mM ammonium formate). The total run time of the programme was 15 minutes and the gradient profile was set as follows: 10% A to 90% B from 0.01 min to 8.0 min, held for 3 min and back to 10% A in 0.1 min, followed by re-equilibration for 5 min. Identities of the compounds were obtained by matching the molecular ion fragments (*m/z*) obtained from the analysis with literature data as well as using the advanced chemometrics mass fragmentations predictive software.

3.4.10. Determination of pH and titratable acid (TA)

For the pH measurement, the yogurt samples were initially homogenized (Polytron, at a maximum setting for 10 seconds) in distilled water (1:1 ratio) followed by pH reading (Kailasapathy, 2006). The pH meter (Hanna Instruments, Model HI 8424) was calibrated using pH 4.0 and 7.0 buffer solutions prior to use.

For the determination of TA, the yogurt samples were first homogenized with distilled water (1:9 ratio) in an Erlenmeyer flask. Several (3-5) drops of phenolphthalein indicator (0.1% w/v ethanol) were added into the flask. The yogurt mixture was then

titrated using NaOH (0.1 N) with consistent swirling until the development of a faint pink colour was observed. The volume of NaOH (0.1 N) used for the neutralization of the acid in yogurt was recorded. The amount of titrable acid (TTA) produced in the yogurt during fermentation was calculated (Sadler & Murphy, 1998) as below:

$$\% \text{ of lactic acid equivalent} = V_{NaOH} \times \text{Dilution factor (10)} \times 0.1N \times 0.009 \times 100\%$$

V_{NaOH} = Volume of NaOH used to neutaralize the acid;

0.1N = Normality of NaOH;

0.009 = conversion factor, 1ml NaOH (0.1N) neutralizes 0.009g of lactic acid

3.4.11. Determination of microbial viable cell counts

3.4.11.1. Preparation of culture media and chemical reagents for determination of microbial viable cell counts

Buffered peptone water (2% w/v) was prepared by dissolving peptone water powder (20 g) in distilled water (500 ml) and the volume was made up to 1000 ml in a volumetric flask. The peptone water solution (9 ml) was then distributed into plastic centrifuge tubes.

Lactose monohydrate solution (10% w/v) was prepared by dissolving lactose monohydrate powder (5 g) in distilled water (approximately 30 ml) and heated gently to dissolve. The volume was made up to 50 ml in a volumetric flask.

MRS agar was prepared by dissolving MRS agar powder (62 g) in distilled water (1000 ml). The solution was boiled gently to dissolve the agar powder with continuous stirring to prevent sedimentation and burning of the agar powder.

M17 agar was prepared by dissolving M17 agar powder (48.25 g) in distilled water (950 ml). The solution was boiled gently to dissolve the agar powder with continuous stirring to prevent sedimentation and burning of the agar powder.

The lactose monohydrate solution (10% w/v), MRS agar, M17 agar and the centrifuge tubes containing buffered peptone water were all sterilized by autoclaving at 121 °C for 15 minutes. The molten MRS and M17 agars were placed in an oven (50 °C) after sterilization to maintain the liquidity of the agar while waiting to be plated.

3.4.11.2. Preparation of yogurt samples for microbial analysis

The laminar flow cabinet was switched on before carrying out the microbial bench work and the surface of the working place was cleaned with ethanol. All procedures were aseptically carried out inside the laminar flow cabinet. Yogurt samples (1 ml) were decimally diluted using sterile buffered peptone water (9 ml). The mixture was thoroughly shaken for uniform distribution and several other diluents (10^{-1} to 10^{-6}) were prepared using the serial dilution method.

3.4.11.3. Enumeration of *Streptococcus thermophilus* by spread plate method

The enumeration of *Streptococcus thermophilus* was carried out using M17 agar as described by Shori and Baba (2012). The sterile M17 molten agar (950 ml) was cooled to 45°C prior to the addition of sterilized lactose monohydrate solution (50 ml, 10% w/v). The agar mixture (15 ml) was then dispensed into petri dishes and allowed to solidify at room temperature. The appropriately diluted yogurt sample (0.1 ml, see section 3.4.11.2) was transferred onto the surface of the agar using sterile disposable pipette tips and the sample was spread evenly on the surface of the agar using a sterile spreader. The petri dish was then covered with its lid, sealed with parafilm and placed in

inverted position in an incubator (37 °C) for 24 hours. The viable cells on the agar surface after 24 hours were calculated as below:

$$\text{CFU/ml} = \frac{\text{Number of colonies formed} \times \text{Dilution factor of sample}}{\text{Volume of yogurt sample aliquot (0.1 ml)}}$$

The viable cell counts (VCC) were then expressed as $\log_{10}$CFU/ml.

3.4.11.4. Enumeration of *Lactobacillus sp.* by pour plate method

The enumeration of *Lactobacillus sp.* was carried out using MRS agar as described by Shori and Baba (2012). The sterile MRS agar (1000 ml) was cooled to 45°C and dispensed into petri dishes in a volume sufficient to cover the surface of the plate (approximately 10 ml). The appropriately diluted yogurt sample (1 ml, see section 3.4.11.2) was transferred into the molten agar in the petri dishes using sterile disposable pipette tips. The petri dishes containing the agar-sample mixture was gently tilted and swirled for homogeneous mixing. The agar was then allowed to solidify at room temperature.

Once the agar had solidified, a second layer of molten MRS agar (10 ml) was poured onto the surface of the solidified agar to create an anaerobic condition. This method was adopted since the lab lacks the facility of anaerobic jar or a 10% carbon dioxide incubator. The second layer of molten agar was then allowed to solidify at room temperature. The petri dishes were covered with lids, sealed with parafilm and placed in inverted position in an incubator (37 °C) for 72 hours. The viable cells on the agar surface after 72 hours were calculated as below:

$$\text{CFU/ml} = \frac{\text{Number of colonies formed} \times \text{Dilution factor of sample}}{\text{Volume of yogurt sample aliquot (1.0 ml)}}$$

The viable cell counts were then expressed as $\log_{10}$CFU/ml.

3.4.12. Statistical analysis

All experiments were performed in three separate batches and in duplicates. Results for each analysis were expressed as mean ± standard deviation. All data were subjected to a two way analysis of variance (ANOVA) and the significance of differences between means was determined on the basis of Duncan test at significance level of $p<0.05$. The statistical analysis was carried out using IBM SPSS Statistics version 20.0 software.

CHAPTER 4.0:

RESULTS

4.1. Total phenolic content of tea extracts and yogurts

The total phenolic content (TPC) in both tea extracts and yogurt water extracts were determined from the regression equation of a calibration curve of known concentrations of standard gallic acid solution (i.e. gallic acid equivalents (GAE), Appendix 1). The TPC in water extracts of tea, and yogurt extracts before fermentation and during refrigerated storage are summarized in Table 4.1, Table 4.2 and Figure 4.1 respectively.

Table 4.1: TPC of tea extracts

Extract	TPC (µg GAE/ ml)
GTE	3220.15 ± 37.80^{a}
WTE	2811.26 ± 44.74^{b}
BTE	2504.59 ± 24.48^{c}

The TPC values of tea extracts, in gallic acid equivalent (GAE) are based on the infusion of 2% (w/v) dried ground tea leaves typically used in a cup of tea.
Values are means of three replicates ± standard deviation.
GTE= Green tea extract; WTE=White tea extract; BTE= Black tea extract.
Means with superscripts having different letters are significantly ($p < 0.05$) different.

Green tea extract (GTE) showed the highest phenolic content (3220.15 ± 37.80 µg GAE/ml) followed by white tea extract (WTE) and black tea extract (BTE) (2811.26 ± 44.74 µg GAE/ml and 2504.59 ± 24.48 µg GAE/ml respectively). The TPC of each yogurt (Y) type was significantly different from each other during refrigerated storage, with GTY > WTY > BTY > PY. A reduction in TPC for all tea-milk mixture (Table 4.2) in comparison to their respective tea extracts (Table 4.1) was noticed prior to bacterial fermentation. A reduction by 2740.67 µg GAE/ml, 2444.37 µg GAE/ml and 2226.22 µg GAE/ml was observed for green tea-milk mixture, white tea-milk mixture and black tea-milk mixture respectively.

A small increase in TPC was observed for all yogurts following fermentation (Table 4.2) particularly for PY and GTY (increments of 10.56 µg GAE/ml and 46.86 µg GAE/ml respectively; $p<0.05$). Reduction in TPC ($p < 0.05$) was observed for all tea

yogurts by the second week of storage, i.e. 85.93, 56.3, 37.22 µg GAE/ml for GTY, WTY and BTY respectively.

Table 4.2: TPC in milk + tea mixture before fermentation and in yogurts during refrigerated storage

Storage day	TPC (µg GAE/ml)			
	PY	GTY	WTY	BTY
Before fermentation	118 ± 3.33^{aA}	479.48 ± 8.34^{aB}	366.89 ± 6.19^{abC}	278.37 ± 20.16^{abD}
Post fermentation: Day 1	128.56 ± 1.47^{bA}	526.34 ± 16.02^{bB}	394.12 ± 17.56^{bC}	294.67 ± 11.82^{bD}
Day 7	126.70 ± 4.72^{bA}	467.08 ± 19.44^{aB}	367.26 ± 21.60^{abC}	268.93 ± 18.95^{abD}
Day 14	122.82 ± 3.06^{abA}	440.41 ± 2.74^{cB}	337.82 ± 15.38^{cC}	257.45 ± 17.78^{aD}
Day 21	135.04 ± 5.94^{cA}	465.23 ± 6.31^{aB}	345.60 ± 18.86^{acC}	269.30 ± 13.83^{abD}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
Before fermentation, refers to mixture of milk and tea prior to initiation of fermentation by starter culture. For PY (control), sample before fermentation contains milk and starter culture (10% v/v) only.
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

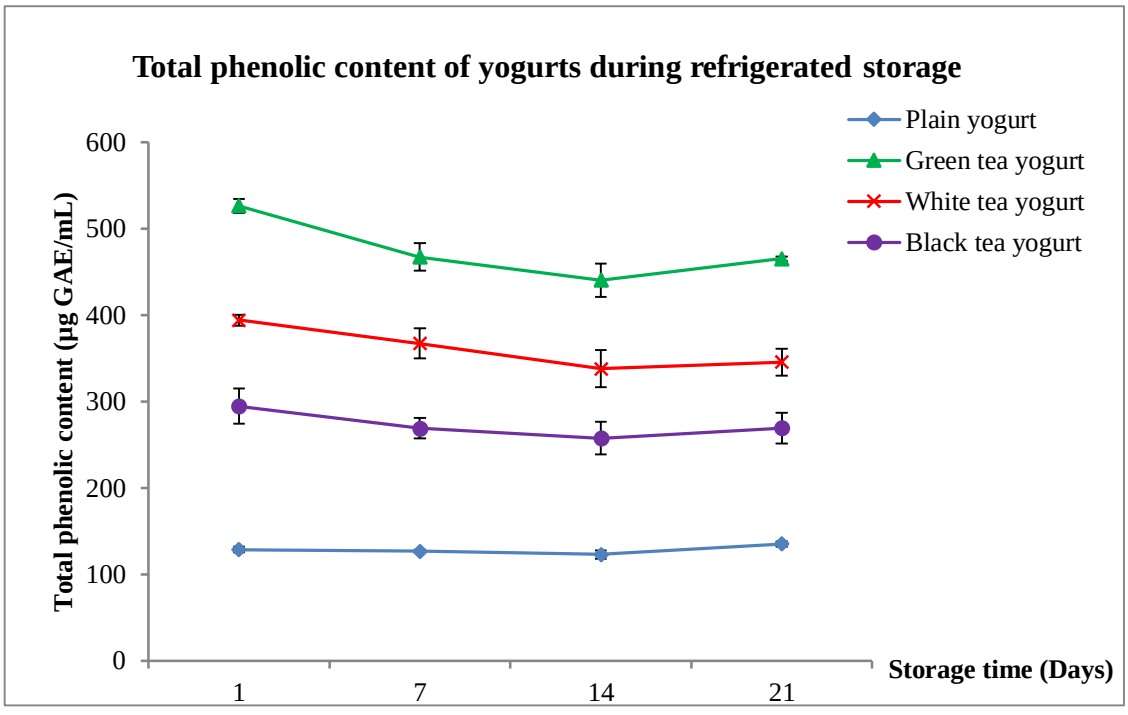

Figure 4.1: TPC in yogurts during 21 days of refrigerated storage at 4°C.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$.

4.2. Antioxidant potential of tea extracts and yogurts

4.2.1. DPPH radical scavenging activity

All tea extracts showed high DPPH radical scavenging activity (Table 4.3). Milk showed very small DPPH radical scavenging activity (5.84%) but the addition of tea extracts increased this value to a range between 87-92% (Table 4.4) in the order of GTY > WTY > BTY > PY. Fermentation of milk had no effects on antioxidant capacity in PY but the presence of tea extracts appeared to increase DPPH radical scavenging activity by about 6%. The highest radical scavenging activity was recorded on day 1 of storage for all yogurts. However, by the end of week two of storage, a reduction in radical scavenging activities by 2.44 %, 6.61 %, 6.64 % and 12.57 % for PY, GTY, WTY and BTY respectively were observed (Table 4.4; Figure 4.2).

Table 4.3: DPPH radical scavenging activity of tea extracts

Extract	DPPH radical scavenging activity (%)
GTE	98.53 ± 0.06^{a}
WTE	98.11 ± 0.17^{b}
BTE	97.71 ± 0.17^{c}

The DPPH radical scavenging values of tea extracts are based on the infusion of 2% (w/v) dried ground tea leaves typically used in a cup of tea.
Values are means of three replicates ± standard deviation.
GTE= Green tea extract; WTE=White tea extract; BTE= Black tea extract.
Means with superscripts having different letters are significantly ($p < 0.05$) different.

Table 4.4: DPPH radical scavenging activity of milk + tea mixture before fermentation and of yogurts during refrigerated storage

Storage day	DPPH radical scavenging activity (%)			
	PY	GTY	WTY	BTY
Before fermentation	5.84 ± 0.17^{ab}	91.77 ± 0.09^{a}	90.96 ± 0.19^{a}	87.88 ± 0.34^{ac}
Post fermentation: Day 1	5.50 ± 0.44^{abA}	96.74 ± 0.62^{bB}	96.39 ± 0.50^{bB}	95.37 ± 0.18^{bC}
Day 7	6.88 ± 1.56^{aA}	96.22 ± 0.5^{bB}	95.99 ± 0.44^{bB}	89.26 ± 2.35^{cC}
Day 14	3.06 ± 1.53^{cA}	90.13 ± 0.09^{cB}	89.75 ± 0.34^{cB}	82.80 ± 1.28^{dC}
Day 21	5.01 ± 1.32^{bA}	90.07 ± 0.38^{cB}	89.83 ± 0.23^{cB}	85.99 ± 1.38^{aC}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
Before fermentation, refers to mixture of milk and tea prior to initiation of fermentation by starter culture.
For PY(control), sample before fermentation contains milk and starter culture (10% v/v) only.
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

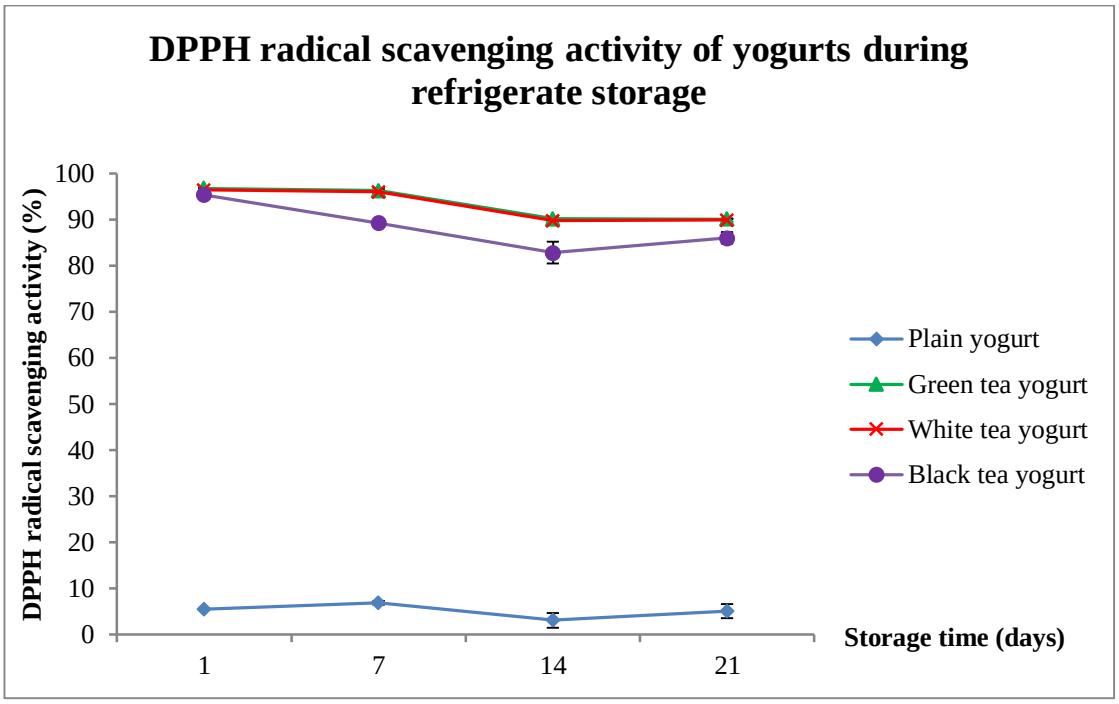

Figure 4.2: DPPH radical scavenging activity of yogurts during 21 days of refrigerated storage at 4°C.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$.

4.2.2. Ferric reducing antioxidant potential (FRAP)

The ferric reducing antioxidant potential (FRAP) of both tea extracts and yogurt water extracts were determined from the regression equation of known concentrations of $FeSO_4.7H_2O$ solution (Appendix 2). The FRAP of the tea extracts are summarized in Table 4.5 whereas the FRAP of the treatments before and after fermentation and during refrigerated storage are summarized in Table 4.6 and presented in Figure 4.3.

Table 4.5: FRAP of tea extracts

Extract	FRAP (mmol Fe^{2+} E/ L)
GTE	27.15 ± 1.58^{a}
WTE	22.47 ± 1.51^{ab}
BTE	18.35 ± 1.16^{b}

The FRAP values (mmol Fe^{2+} E/ L) of tea extracts are based on the infusion of 2% (w/v) dried ground tea leaves typically used in a cup of tea.
Values are means of three replicates ± standard deviation.
GTE= Green tea extract; WTE=White tea extract; BTE= Black tea extract.
Means with superscripts having different letters are significantly ($p < 0.05$) different

GTE (27.15 ± 1.58 mmol Fe^{2+} E/ L) had the highest FRAP value, followed by WTE and BTE (22.47 ± 1.51 mmol Fe^{2+} E/ L and 18.35 ± 1.16 mmol Fe^{2+} E/ L respectively). Although the amount of tea added into milk was the same as in tea extract, all tea yogurt extracts showed a much reduced FRAP value compared to their respective tea extracts (Table 4.6) The decrease in FRAP values during fermentation of milk was highest in GTY (0.73%) followed by PY (0.42%), BTY (0.29%) and WTY (0.26%) in comparison with FRAP values of milk + tea mixture before fermentation. FRAP values decreased for all yogurts during refrigeration to the lowest values by day 14 of storage.

Table 4.6: FRAP of milk + tea mixture before fermentation and of yogurts during refrigerated storage

Storage day	FRAP (mmol Fe^{2+} E/ L)			
	PY	GTY	WTY	BTY
Before fermentation	0.69 ± 0.22^{aA}	3.71 ± 0.48^{aB}	2.85 ± 0.13^{aC}	2.14 ± 0.38^{aD}
Post fermentation: Day 1	0.27 ± 0.03^{bA}	2.98 ± 0.21^{bB}	2.59 ± 0.22^{aC}	1.85 ± 0.25^{abD}
Day 7	0.21 ± 0.04^{bA}	2.67 ± 0.20^{bcB}	2.28 ± 0.10^{bC}	1.59 ± 0.12^{bcD}
Day 14	0.17 ± 0.05^{bA}	2.25 ± 0.12^{cB}	2.07 ± 0.10^{bC}	1.39 ± 0.06^{cD}
Day 21	0.20 ± 0.04^{bA}	2.49 ± 0.08^{bcB}	2.12 ± 0.09^{bC}	1.48 ± 0.09^{cD}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
Before fermentation refers to milk added with tea without fermentation by starter culture.
For PY (control), sample before fermentation contains only milk and starter culture (10% v/v) only.
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

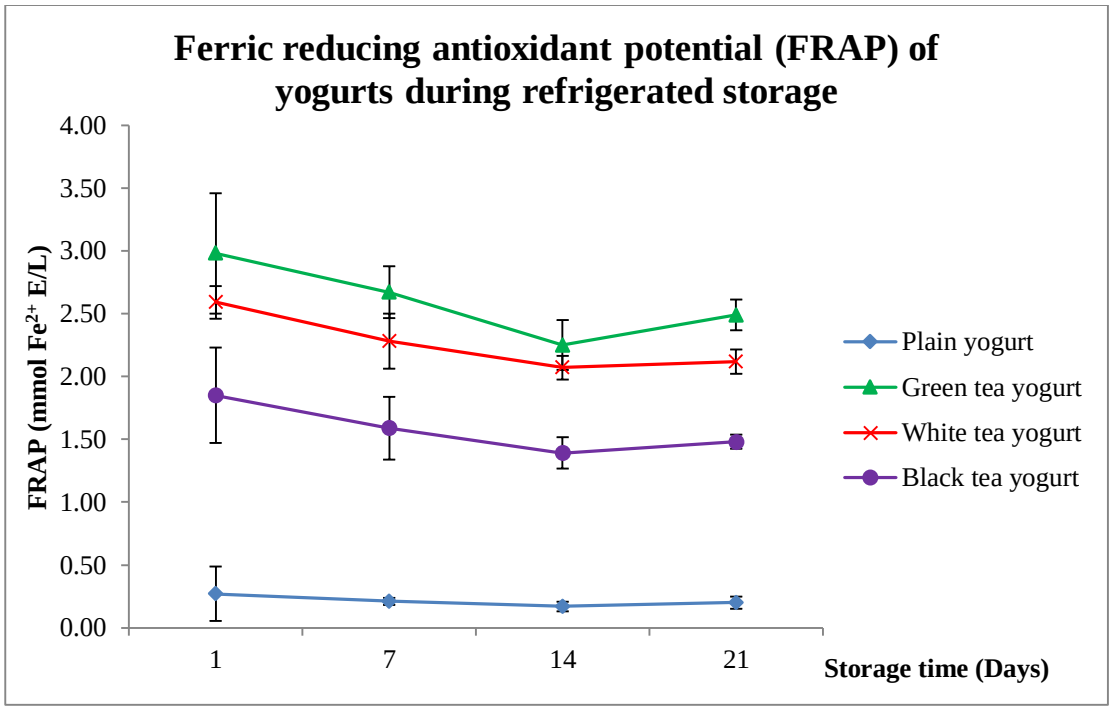

Figure 4.3: FRAP of yogurts during 21 days of refrigerated storage at 4°C.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$.

4.2.3 Ferrous ion chelating (FIC) ability

Table 4.7: FIC ability of tea extracts

Extract	FIC ability (%)
GTE	84.4 ± 0.34[a]
WTE	84.07 ± 0.31[a]
BTE	84.83 ± 0.16[a]

The FIC values of tea extracts are based on 2% (w/v), similar to the amount of tea added into milk.
Values are means of three replicates ± standard deviation.
GTE= Green tea extract; WTE=White tea extract; BTE= Black tea extract.
Means with superscripts having same letters indicates insignificant differences ($p < 0.05$).

All tea extracts showed similar FIC ability (~84%). The FIC ability of tea extracts and tea + milk mixture (85-88%) were higher than milk alone (66%; Table 4.8). The FIC ability of tea yogurts were not additive because the values ranged 82-90% following fermentation, which were not different compared to tea + milk mixture. However, FIC ability of PY was markedly reduced following the fermentation of milk (i.e. from 66% to ~50%). The FIC ability of all tea yogurts during refrigerated storage remained practically unchanged whereas PY underwent gradual reduction in FIC ability to the lowest values (43.80 ± 1.91%) by the end of first week of refrigerated storage (Figure 4.4).

Table 4.8: FIC ability of milk + tea before fermentation and of yogurts during refrigerated storage

Storage day	FIC ability (%)			
	PY	GTY	WTY	BTY
Before fermentation	66.09 ± 1.03^{aA}	86.56 ± 1.03^{aB}	85.30 ± 1.04^{aC}	88.49 ± 0.54^{aD}
Post fermentation: Day 1	47.67 ± 0.92^{bA}	83.70 ± 0.51^{bB}	82.44 ± 0.48^{bC}	87.50 ± 0.33^{bD}
Day 7	43.80 ± 1.91^{cA}	83.54 ± 0.74^{bB}	82.41 ± 1.80^{bB}	87.48 ± 0.50^{bC}
Day 14	47.12 ± 1.91^{bA}	85.23 ± 0.80^{cB}	84.93 ± 0.43^{aB}	89.07 ± 0.21^{aC}
Day 21	50.74 ± 1.57^{dA}	86.71 ± 0.16^{aB}	85.78 ± 0.14^{aB}	89.87 ± 0.45^{cC}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
Before fermentation, refers to mixture of milk and tea prior to initiation of fermentation by starter culture. For PY (control), sample before fermentation contains milk and starter culture (10% v/v) only.
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

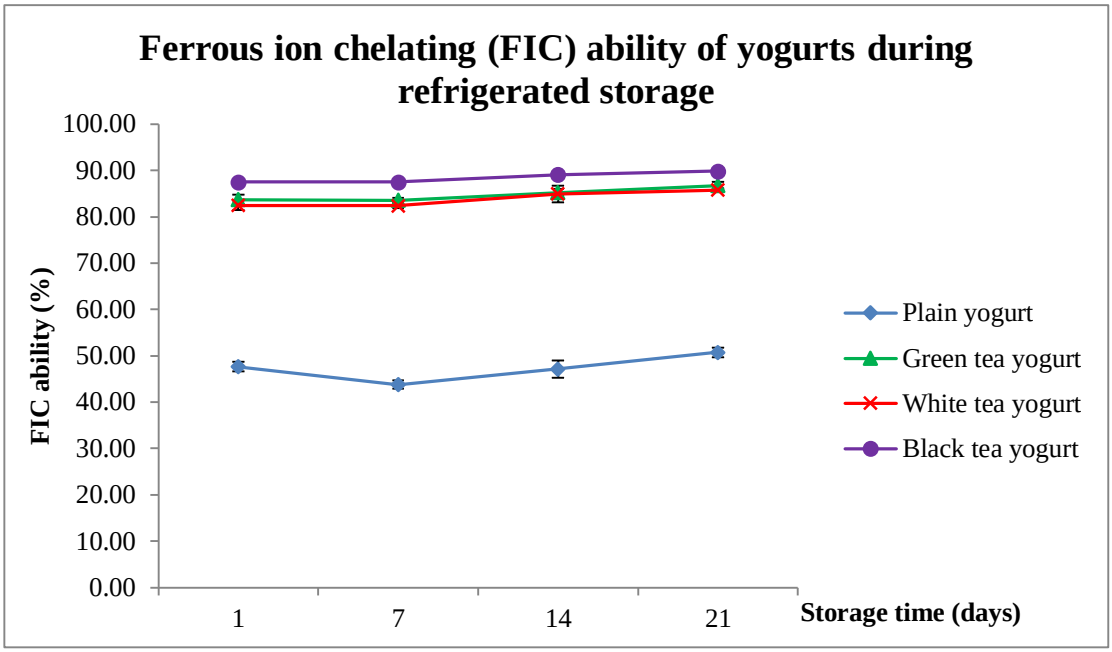

Figure 4.4: FIC ability of yogurts during 21 days of refrigerated storage at 4°C. Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$.

4.3. Phenolic compounds in tea extracts and yogurt water extracts by LCMS

The components in tea or yogurt were separated into various fractions and these were ionised prior to identification based on their mass to charge ratio (m/z) (Pitt, 2009). The LCMS profiles and the phenolic compounds identified in tea extracts are shown in Figures 4.5 - 4.7 and Tables 4.9 - 4.11 respectively. The phenolic compounds particularly epigallocatechin (EGC), gallocatechin (GC), chlorogenic acid, quarcetin-3-glucoside, myricitin-3-O-glucoside, procyanidin B1, strictinin, theaflavin-3-O-gallate and kaempferol-rhamnose-hexose-rhamnose showed the most variation in quantities in tea water extracts analysed. Some of the phenolic compounds present in the tea extracts such as quinic acid, catechin, 5-caffeoylquinic acid, epicatechin gallate (ECG), kaempferol-3-O-glucoside, epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), myricitin-3-O-glucoside and dicaffeoquinic acid conjugate were not identified in the tea yogurts. In addition new compounds such as quinic acid conjugate and other

compounds of unknown identity which were earlier absent in the tea extracts were discovered following fermentation.

Figures 4.8 - 4.11 show the LCMS profiles of four yogurt extracts. Identified compounds from these profiles are presented in Tables 4.12 – 4.15. A summary on the comparison of the presence and/or absence of phenolic compounds in tea and tea-yogurts extracts are shown in Table 4.16.

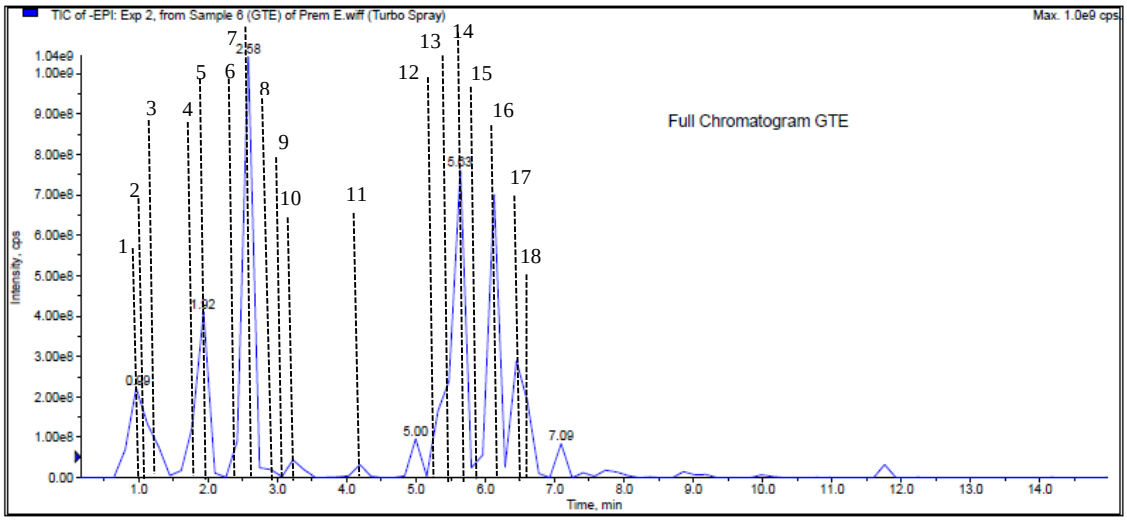

Figure 4.5: Full chromatogram of GTE obtained from liquid chromatography

[a] Position of dotted lines with numbers represent retention time in which elutants were subjected to fragmentation via mass spectrometry for identification of phenolic compounds.

[b] Identity of the phenolic compounds present within each retention time are summarized in Table 4.9.

Table 4.9: Identification of phenolic compounds present in GTE via LCMS

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
1	0.97	192	191	93, 85	Quinic acid
2	1.13	344	343	191, 93, 85	5-caffeoylquinic acid
3	1.29	306	305	219, 137, 125, 109	Gallocatechin (GC)
4	1.78	290	289	245, 203, 123, 109	Catechin
5	1.94	578	577	407, 289, 245, 161, 125	Procyanidin B1
6	2.42	338	337	191, 173, 163, 119, 93	p-coumaroylquinic acid
7	2.58	458	457	169, 125	Epigallocatechin gallate (EGCG)
8	2.74	170	169	125, 67	Gallic acid
9	3.07	305	304	167, 137, 125	Epigallocatechin (EGC)

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
10	3.23	594	593	383, 353	Dicaffeoquinic acid conjugate
11	4.19	458	457	169, 125	Gallocatechin gallate
12	5.32	564	563	383, 353	Arabinosyl-glucosyl apigenin
13	5.48	480	479	316, 287, 271	Myricitin-3-O-glucoside or galactoside
14	5.64	442	441	289, 169, 125	Epicatechin gallate (ECG)
15	5.80	290	289	245, 123, 109	Epicatechin (EC)
16	6.12	756	755	285	Kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
17	6.45	740	739	285	Kaempferol-rhamnose-hexose-rhamnose
18	6.61	448	447	284, 255, 227	Kaempferol-3-O-glucoside

(Table 4.9 continued)

[a] R_t – retention time; MW – molecular weight; MS – *m/z* (mass to charge ratio)
[b] Position and retention time are based on the GTE chromatogram in Figure 4.5.
[c] Only major MS/MS fragments for each phenolic compound are shown in the table. For detailed mass spectra of each identified phenolic compound, refer to section 3.1 of Appendix.

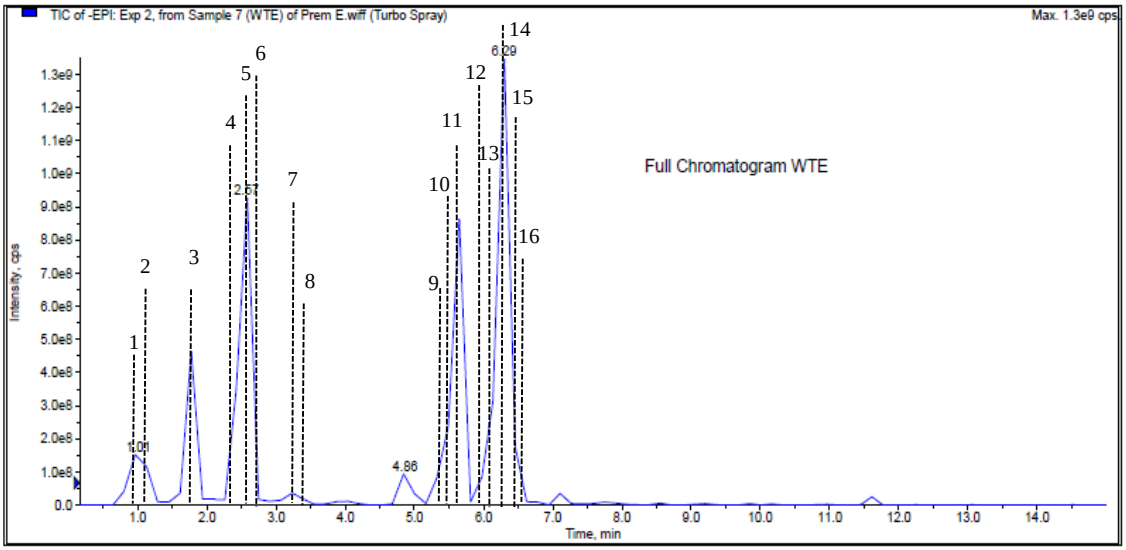

Figure 4.6: Full chromatogram of WTE obtained from liquid chromatography

[a] Position of dotted lines with numbers represent retention time in which elutants were subjected to fragmentation via mass spectrometry for identification of phenolic compounds.
[b] Identity of the phenolic compounds present within each retention time are summarized in Table 4.10.

Table 4.10: Identification of phenolic compounds present in WTE via LCMS

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
1	0.97	192	191	93, 85	Quinic acid
2	1.13	344	343	191, 93, 85	5-caffeoylquinic acid
3	1.78	634	633	301, 275	Strictinin
4	2.42	290	289	245, 203, 123, 109	Catechin
5	2.59	458	457	169, 125	Epigallocatechin gallate
6	2.75	170	169	125, 67	Gallic acid
7	3.23	594	593	383, 353	Dicaffeoquinic acid conjugate
8	3.39	458	457	305, 219, 169, 125	Gallocatechin gallate
9	5.33	564	563	383, 353	Arabinosyl-glucosyl apigenin
10	5.49	480	479	316, 287, 271	Myricitin-3-O-glucoside or galactoside
11	5.65	442	441	289, 169, 125	Epicatechin gallate
12	5.81	290	289	245, 123, 109	Epicatechin
13	6.14	610	609	300, 271	Quercetin-rhamnosylgalactoside or rutinoside
14	6.30	756	755	285	Kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
15	6.46	448	447	284, 255, 227	Kaempferol-3-O-glucoside
16	6.62	594	593	285, 255, 293	Kaempferol-3-rutinoside

[a] R_t – retention time; MW – molecular weight; MS – *m/z* (mass to charge ratio)
[b] Position and retention time are based on the WTE chromatogram in Figure 4.6.
[c] Only major MS/MS fragments for each phenolic compound are shown in the table. For detailed mass spectra of each identified phenolic compound, refer to section 3.2 of Appendix.

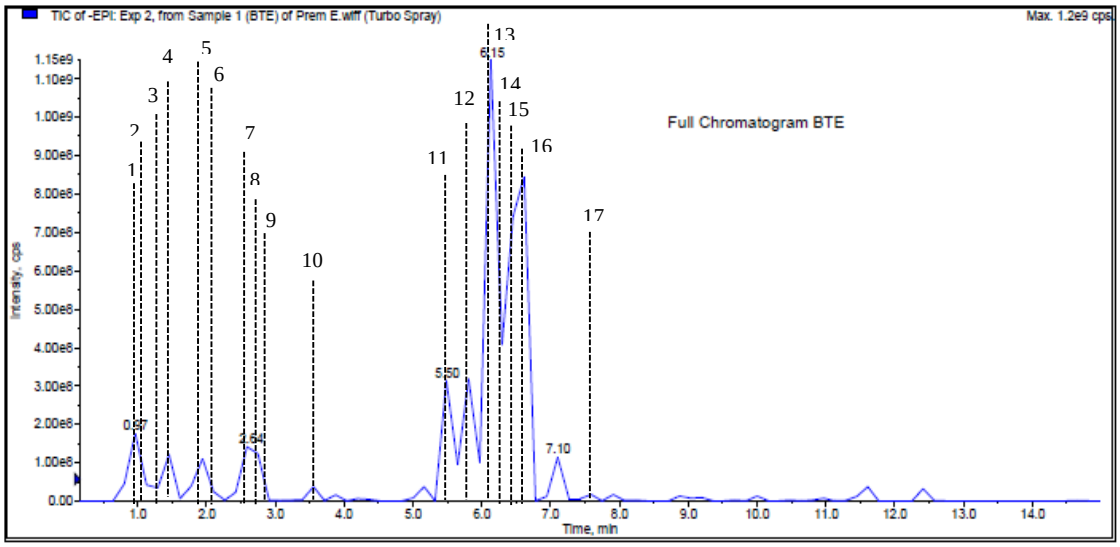

Figure 4.7: Full chromatogram of BTE obtained from liquid chromatography

[a] Position of dotted lines with numbers represent retention time in which elutants were subjected to fragmentation via mass spectrometry for identification of phenolic compounds.

[b] Identity of the phenolic compounds present within each retention time are summarized in Table 4.11.

Table 4.11: Identification of phenolic compounds present in BTE via LCMS

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
1	0.97	192	191	93, 85	Quinic acid
2	1.13	344	343	191, 93,85	5-caffeoylquinic acid (Quinic acid conjugate)
3	1.29	354	353	191, 135	Chlorogenic acid
4	1.45	760	759	607, 589, 425, 169	Prodelphinidin A-2 3'-O-gallate
5	1.78	290	289	245, 203, 123, 109	Catechin
6	2.10	338	337	191, 173, 119, 93	p-coumaroylquinic acid
7	2.59	290	289	245, 123, 109	Epicatechin
8	2.75	458	457	169, 125	Epigallocatechin gallate
9	2.91	170	169	125, 67	Gallic acid
10	3.55	594	593	383, 353	Dicaffeoquinic acid conjugate
11	5.49	564	563	383, 353	Arabinosyl-glucosyl apigenin
12	5.81	442	441	289, 169, 125	Epicatechin gallate
13	6.13	610	609	300, 271, 255	Quercetin-rhamnosylgalactoside or rutinoside
14	6.29	464	463	300, 271, 255	Quercetin-3-glucoside or galactoside
15	6.46	594	593	285, 255	Kaempferol-3-rutinoside

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
16	6.62	448	447	300, 284, 255, 227	Kaempferol-3-O-glucoside
17	7.59	716	715	563, 545, 389, 281, 269, 253, 241, 169	Theaflavin-3-O-gallate

(Table 4.11 continued)

[a] R_t – retention time; MW – molecular weight; MS – *m/z* (mass to charge ratio)
[b] Position and retention time are based on the BTE chromatogram in figure 4.7.
[c] Only major MS/MS fragments for each phenolic compound are shown in the table. For detailed mass spectra of each identified phenolic compound, refer to section 3.3 of Appendix.

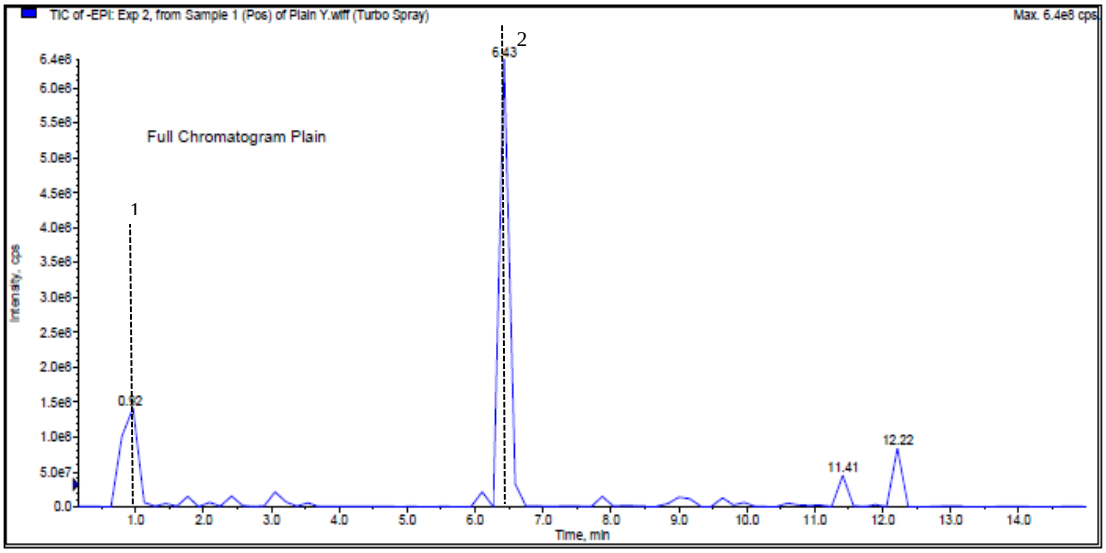

Figure 4.8: Full chromatogram of PY obtained from liquid chromatography

[a] Position of dotted lines with numbers represent retention time in which elutants were subjected to fragmentation via mass spectrometry for identification of phenolic compounds.
[b] Identity of the phenolic compounds present within each retention time are summarized in Table 4.12.

Table 4.12: Identification of phenolic compounds present in PY via LCMS

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
1	0.96	432	431	89	Unknown
2	6.43	723	724	677, 451, 225	Unknown

[a] R_t – retention time; MW – molecular weight; MS – *m/z* (mass to charge ratio)
[b] Position and retention time are based on the PY chromatogram in figure 4.8.
[c] Only major MS/MS fragments for each phenolic compound are shown in the table. For detailed mass spectra of each identified phenolic compound, refer to section 3.4 of Appendix.

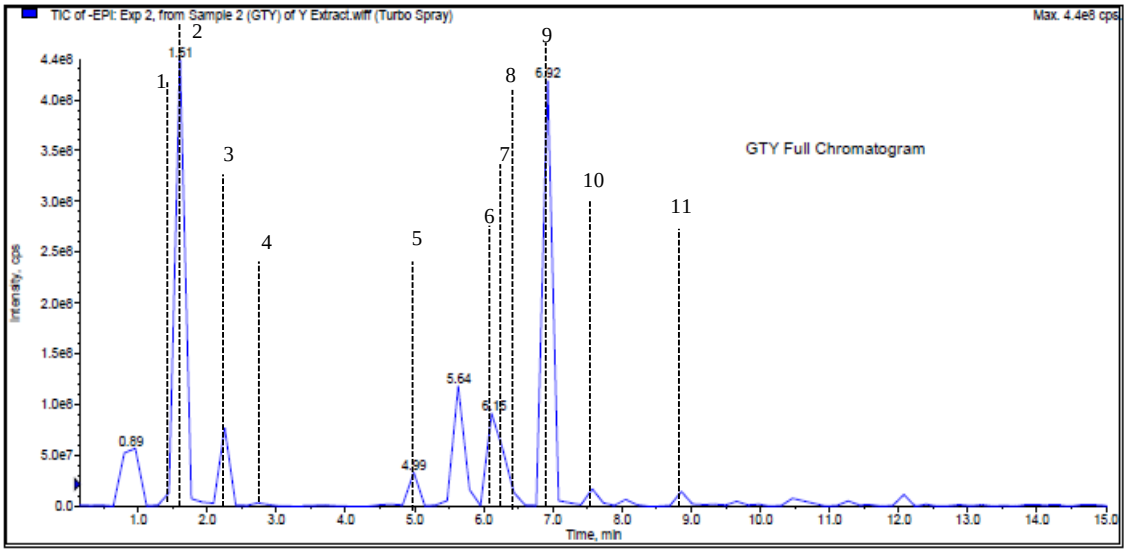

Figure 4.9: Full chromatogram of GTY obtained from liquid chromatography

[a] Position of dotted lines with numbers represent retention time in which elutants were subjected to fragmentation via mass spectrometry for identification of phenolic compounds.

[b] Identity of the phenolic compounds present within each retention time are summarized in Table 4.13.

Table 4.13: Identification of phenolic compounds present in GTY via LCMS

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
1	1.45	338	337	191, 163, 119	p-coumaroylquinic acid
2	1.61	901	900	883, 865	Unknown
3	2.26	290	289	245, 203, 123, 109	Epicatechin
4	2.74	338	337	191, 173	Quinic acid conjugate
5	4.99	564	563	383, 353	Arabinosyl-glucosyl apigenin
6	6.12	756	755	285	Kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
7	6.28	740	739	285	Kaempferol-rhamnose-hexose-rhamnose
8	6.44	594	593	285, 255	Kaempferol-3-rutinoside
9	6.92	724	723	677	Unknown
10	7.57	769	768	619, 195	Unknown
11	8.86	332	331	215, 185	Unknown

[a] R_t – retention time; MW – molecular weight; MS – *m/z* (mass to charge ratio)

[b] Position and retention time are based on the GTY chromatogram in figure 4.9.

[c] Only major MS/MS fragments for each phenolic compound are shown in the table. For detailed mass spectra of each identified phenolic compound, refer to section 3.5 of Appendix.

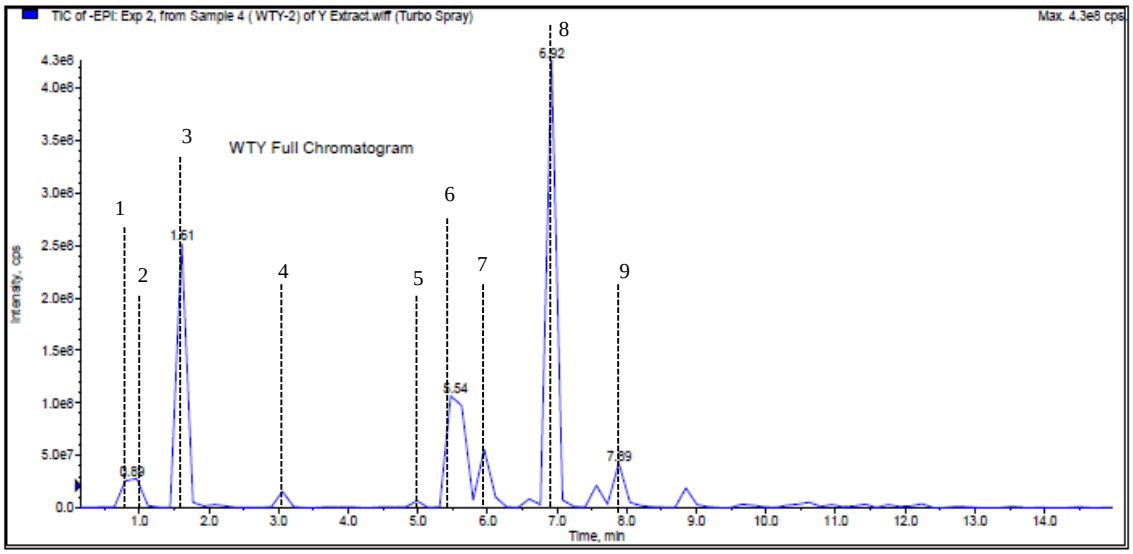

Figure 4.10: Full chromatogram of WTY obtained from liquid chromatography

[a] Position of dotted lines with numbers represent retention time in which elutants were subjected to fragmentation via mass spectrometry for identification of phenolic compounds.

[b] Identity of the phenolic compounds present within each retention time are summarized in Table 4.14.

Table 4.14: Identification of phenolic compounds present in WTY via LCMS

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
1	0.80	378	377	161	Unknown
2	1.12	170	169	125, 67	Gallic acid
3	1.61	901	900	883, 865, 726	Unknown
4	3.06	720	719	546, 528, 318	Unknown
5	4.99	564	563	383, 353, 297	Arabinosyl-glucosyl apigenin
6	5.47	452	451	225	Unknown
7	5.96	756	755	285	Kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
8	6.92	724	723	677, 451, 225	Unknown
9	7.89	997	996	439	Unknown

[a] R_t – retention time; MW – molecular weight; MS – *m/z* (mass to charge ratio)

[b] Position and retention time are based on the WTY chromatogram in figure 4.10.

[c] Only major MS/MS fragments for each phenolic compound are shown in the table. For detailed mass spectra of each identified phenolic compound, refer to section 3.6 of Appendix.

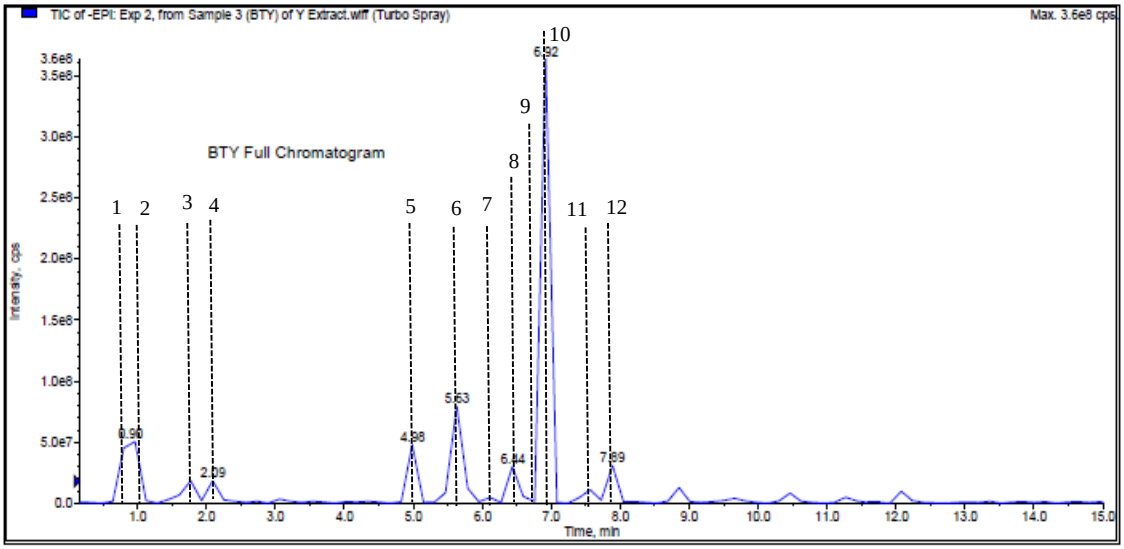

Figure 4.11: Full chromatogram of BTY obtained from liquid chromatography

[a] Position of dotted lines with numbers represent retention time in which elutants were subjected to fragmentation via mass spectrometry for identification of phenolic compounds.

[b] Identity of the phenolic compounds present within each retention time are summarized in Table 4.15.

Table 4.15: Identification of phenolic compounds present in BTY via LCMS

Position	R_t (min)	MW	MS	MS/MS fragmentation	Phenolic compound
1	0.80	378	377	161	Unknown
2	1.13	170	169	125, 67	Gallic acid
3	1.77	901	900	883, 865, 708	Unknown
4	2.09	338	337	191, 173, 93	p-coumaroylquinic acid
5	4.99	564	563	383, 353	Arabinosyl-glucosyl apigenin
6	5.63	498	497	451, 225	Unknown
7	6.11	610	609	300, 271, 255	Quercetin-rhamnosylgalactoside or rutinoside
8	6.44	594	593	285, 255	Kaempferol-3-rutinoside
9	6.60	590	589	257	Unknown
10	6.92	724	723	677, 451, 225	Unknown
11	7.57	769	768	619, 195	Unknown
12	7.89	997	996	975, 833, 683, 439	Unknown

[a] R_t – retention time; MW – molecular weight; MS – *m/z* (mass to charge ratio)

[b] Position and retention time are based on the BTY chromatogram in figure 4.11.

[c] Only major MS/MS fragments for each phenolic compound are shown in the table. For detailed mass spectra of each identified phenolic compound, refer to section 3.7 of Appendix.

Table 4.16: Summary of phenolic compounds present in tea extracts and yogurt extracts

Compound	MW	GTE	WTE	BTE	PY	GTY	WTY	BTY
PHENOLIC ACIDS:								
Gallic acid	170	√	√	√			√	√
Quinic acid	192	√	√	√				
Quinic acid conjugate	338					√		
p-coumaroylquinic acid	338	√		√		√		√
5-caffeoylquinic acid	344	√	√	√				
Chlorogenic acid	354			√				
Dicaffeoquinic acid conjugate	594	√	√	√				
FLAVONOIDS:								
1. <u>Flavanols:</u> Catechin	290	√	√	√				
Epicatechin (EC)	290	√	√	√		√		
Epigallocatechin (EGC)	305	√						
Gallocatechin (GC)	306	√						
Epicatechin gallate (ECG)	442	√	√	√				
Epigallocatechin gallate (EGCG)	458	√	√	√				
Gallocatechin gallate (GCG)	458	√	√					
Theaflavin-3-O-gallate	716			√				
Procyanidin B1	578	√						
2. <u>Flavonols:</u> Kaempferol-3-O-glucoside	448	√	√	√				
Quarcetin-3-glucoside	464			√				
Myricitin-3-O-glucoside	480	√	√					
Kaempferol-3-rutinoside	594		√	√		√		√
Quarcetin-rhamnosylgalactoside or rutinoside	610		√	√				√
Kaempferol-rhamnose-hexose-rhamnose	740	√				√		
Kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside	756	√	√			√	√	
3. <u>Flavones:</u> Arabinosyl-glucosyl apigenin	564	√	√	√		√	√	√
TANNINS:								
Strictinin	634		√					
Prodelphinidin A-2 3'-O-gallate	760			√				

UNKNOWN COMPOUNDS:								
Unknown	332					√		
Unknown	378						√	√
Unknown	432				√			
Unknown	452						√	
Unknown	498							√
Unknown	590							√
Unknown	720						√	
Unknown	724				√	√	√	√
Unknown	769					√		√
Unknown	901					√	√	√
Unknown	997						√	√

The symbol "√" indicates the presence of the compound in the respective extracts.
MW – Molecular weight of the compound

4.4. Changes in the acidity of yogurts

4.4.1. Changes in pH during the course of fermentation

The initial pH readings for all tea yogurts (BTY (5.93 ± 0.03), GTY (6.04 ± 0.01) and WTY (6.02 ± 0.03)) were lower ($p < 0.05$) than PY (6.10 ± 0.01). The changes in pH values of yogurts for every 30 minutes throughout the fermentation process are summarized in Table 4.17 and presented in Figure 4.12.

Table 4.17: pH profile of yogurts during the fermentation process

Time (min)	pH of yogurts			
	PY	GTY	WTY	BTY
0	6.10 ± 0.01^{aA}	6.04 ± 0.01^{aB}	6.02 ± 0.03^{aB}	5.93 ± 0.03^{aC}
30	5.92 ± 0.01^{bA}	5.93 ± 0.02^{bA}	5.92 ± 0.03^{bA}	5.83 ± 0.04^{bB}
60	5.60 ± 0.01^{cA}	5.73 ± 0.05^{cB}	5.69 ± 0.05^{cB}	5.62 ± 0.05^{cA}
90	5.22 ± 0.03^{dA}	5.48 ± 0.05^{dB}	5.38 ± 0.04^{dC}	5.40 ± 0.03^{dC}
120	4.92 ± 0.02^{eA}	5.20 ± 0.03^{eB}	5.05 ± 0.05^{eC}	5.13 ± 0.04^{eD}
150	4.72 ± 0.01^{fA}	4.98 ± 0^{fB}	4.83 ± 0.03^{fC}	4.92 ± 0.05^{fD}
180	4.58 ± 0.01^{gA}	4.83 ± 0.02^{gB}	4.66 ± 0.03^{gC}	4.75 ± 0.03^{gD}
210	4.46 ± 0.02^{hA}	4.72 ± 0.02^{hB}	4.56 ± 0.04^{hC}	4.64 ± 0.02^{hD}
240	4.42 ± 0.03^{iA}	4.64 ± 0.04^{iB}	4.45 ± 0.02^{iA}	4.54 ± 0.00^{iC}
270	4.36 ± 0.03^{jA}	4.55 ± 0.02^{jB}	4.37 ± 0.02^{jA}	4.49 ± 0.04^{jC}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

The pH of each yogurt type decreased significantly for every 30 minutes interval throughout the fermentation period. The pH of each tea yogurt was significantly different from each other as well as with PY after 2 hours of incubation with GTY having the highest pH (5.20 ± 0.03) among all four yogurts. The earliest yogurt to reach pH 4.5 was PY (180 minutes), followed by WTY, BTY and GTY (210, 270 and >270 minutes respectively).

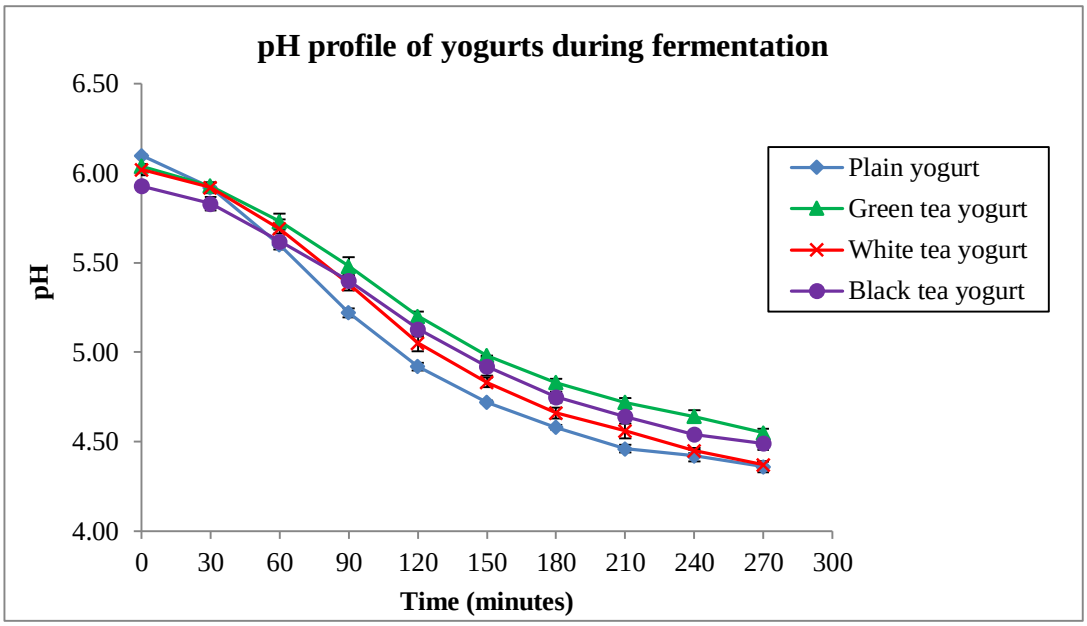

Figure 4.12: Changes in pH during fermentation of milk in the presence or absence of tea.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$. Plain yogurt (control) refers to yogurt without incorporation of tea (milk + starter culture only).

4.4.2. Changes in pH during refrigerated storage

The changes in pH values of all yogurt types during storage is summarized in Table 4.18 and presented in Figure 4.13. The pH of each yogurt type was significantly different from each other, with tea yogurts always having higher pH values compared to PY during the 21 days of refrigerated storage. The pH values of all yogurts throughout the storage period was in the order of PY < WTY < BTY < GTY. For each of the yogurt

types, a significant reduction in pH was observed during the first week of storage followed by a significant increase in pH during the second week of storage. Minimal changes in pH occurred during the third week of storage.

Table 4.18: Changes in pH of yogurts during refrigerated storage

Storage day	pH of yogurts			
	PY	GTY	WTY	BTY
1	4.41 ± 0.02^{aA}	4.50 ± 0.02^{aB}	4.43 ± 0.02^{aC}	4.45 ± 0.01^{aD}
7	4.28 ± 0.02^{bA}	4.46 ± 0.04^{bB}	4.33 ± 0.03^{bC}	4.38 ± 0.01^{bD}
14	4.36 ± 0.05^{cA}	4.52 ± 0.04^{aB}	4.41 ± 0.04^{aC}	4.47 ± 0.03^{aD}
21	4.35 ± 0.04^{cA}	4.53 ± 0.05^{aB}	4.41 ± 0.05^{aC}	4.46 ± 0.04^{aD}

Values are means of three replicates ± standard deviation.
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
PY (control) refers to yogurt without incorporation of tea (milk + starter culture only).

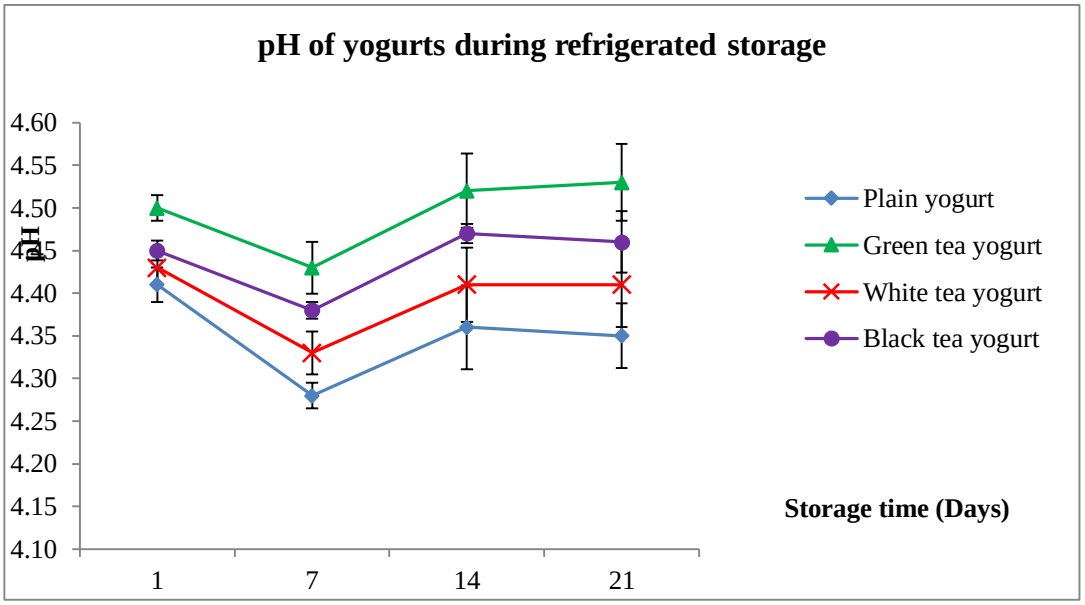

Figure 4.13: Changes in pH values of yogurts during 21 days of refrigerated storage at 4°C.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$. Plain yogurt (control) refers to yogurt without incorporation of tea (milk + starter culture only).

4.4.3. Changes in titratable acidity (TA) during fermentation of milk

The changes in TA of yogurts during fermentation of milk is summarized in Table 4.19 and presented in Figure 4.14. Table 4.20 summarizes the gradient values of the slope calculated from the graph in Figure 4.14 and hence the rate of titratable acid production during the course of fermentation. The TA of all three tea yogurts were higher than PY at all times during the fermentation. An increase in TA ($p<0.05$) was observed for PY between 0-120 minutes and 150-240 minutes, which were similar for WTY (between 0-90 minutes and 120-150 minutes) and BTY (between 0-60 minutes and 90-150 minutes) but slightly delayed for GTY (between 60-150 minutes and 180-210 minutes).

Table 4.19: Titratable acidity (TA) profile of yogurts during the fermentation process

Time (min)	TA of yogurts (% lactic acid equivalent)			
	PY	GTY	WTY	BTY
0	0.17 ± 0.02^{aA}	0.41 ± 0.04^{aB}	0.36 ± 0.05^{aBC}	0.35 ± 0.02^{aC}
30	0.31 ± 0.01^{bA}	0.48 ± 0.03^{bB}	0.43 ± 0.02^{bC}	0.41 ± 0.00^{bC}
60	0.37 ± 0.02^{cA}	0.49 ± 0.01^{bB}	0.55 ± 0.02^{cC}	0.52 ± 0.02^{cBC}
90	0.51 ± 0.02^{dA}	0.61 ± 0.02^{cB}	0.67 ± 0.03^{dC}	0.55 ± 0.01^{cA}
120	0.65 ± 0.03^{eA}	0.66 ± 0.04^{dA}	0.72 ± 0.05^{dA}	0.66 ± 0.02^{dA}
150	0.67 ± 0.02^{eA}	0.70 ± 0.02^{eB}	0.78 ± 0.02^{eC}	0.73 ± 0.04^{eD}
180	0.71 ± 0.01^{fA}	0.74 ± 0.03^{eA}	0.83 ± 0.04^{efB}	0.74 ± 0.03^{eA}
210	0.76 ± 0.01^{gA}	0.83 ± 0.03^{fB}	0.89 ± 0.02^{fgC}	0.76 ± 0.02^{eA}
240	0.81 ± 0.00^{hA}	0.86 ± 0.01^{fgBC}	0.89 ± 0.02^{fgB}	0.83 ± 0.03^{fAC}
270	0.83 ± 0.03^{hA}	0.87 ± 0.01^{gAB}	0.91 ± 0.04^{gB}	0.84 ± 0.03^{fA}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
PY (control) refers to yogurt without incorporation of tea (milk + starter culture only).
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

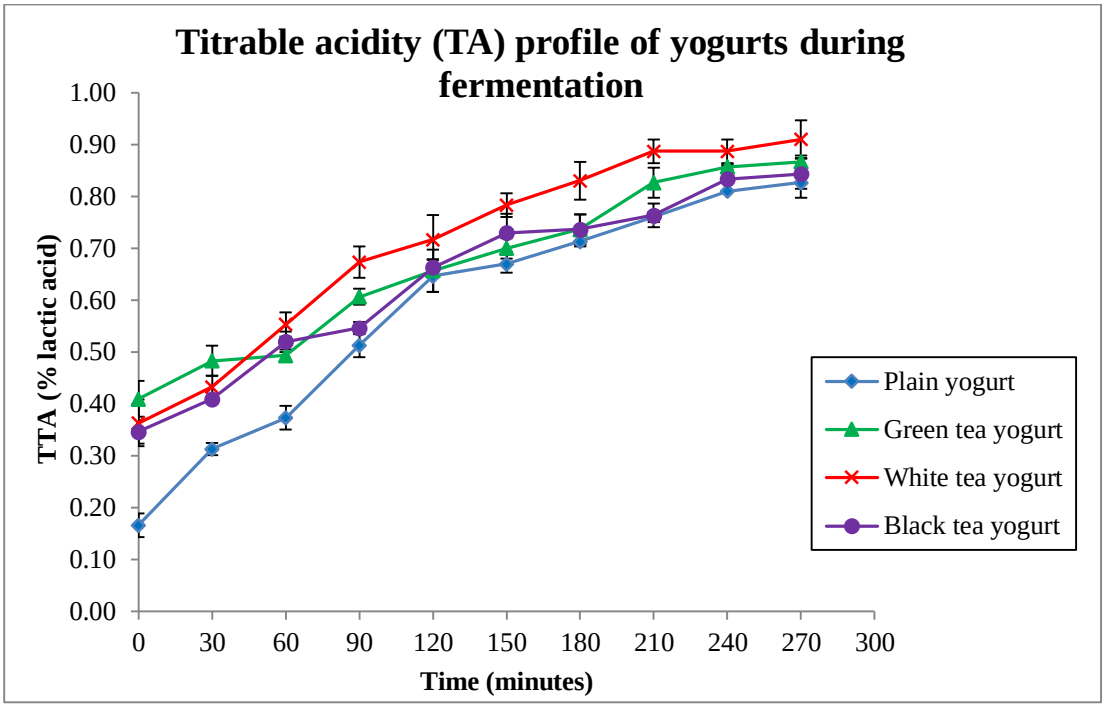

Figure 4.14: Changes in titratable acidity (TA; % lactic acid equivalent) during fermentation of milk in the presence or absence of tea.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$. Plain yogurt (control) refers to yogurt without incorporation of tea (milk + starter culture only).

Table 4.20: The rate of titratable acid (TA) production during fermentation of milk

Time interval (min)	Rate of TA production (% lactic acid equivalent/min)			
	PY	GTY	WTY	BTY
0-30	4.67×10^{-3}	2.33×10^{-3}	2.33×10^{-3}	2.0×10^{-3}
30-60	2.0×10^{-3}	0.33×10^{-3}	4.0×10^{-3}	3.67×10^{-3}
60-90	4.67×10^{-3}	4.0×10^{-3}	4.0×10^{-3}	1.0×10^{-3}
90-120	4.67×10^{-3}	1.67×10^{-3}	1.67×10^{-3}	3.67×10^{-3}
120-150	0.67×10^{-3}	1.33×10^{-3}	2.0×10^{-3}	1.0×10^{-3}
150-180	1.33×10^{-3}	1.33×10^{-3}	1.67×10^{-3}	0.33×10^{-3}
180-210	1.67×10^{-3}	3.0×10^{-3}	2.0×10^{-3}	0.67×10^{-3}
210-240	1.67×10^{-3}	1.0×10^{-3}	0.00	2.33×10^{-3}
240-270	0.67×10^{-3}	0.33×10^{-3}	0.67×10^{-3}	0.33×10^{-3}

Rate was calculated from the gradient of the slope at each time interval.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
PY (control) refers to yogurt without incorporation of tea (milk + starter culture only).

The rate of titratable acid (TA) production for all yogurts could be divided into 2 phases i.e. a marked increase within the first two hours of fermentation followed by a gradual increase during the remaining period of incubation. The rate of acid production was the highest for PY compared to all tea yogurts during the first 30 minutes of fermentation (Table 4.20). The rate of increase in acid production by PY was almost linear especially between 60-120 minutes of fermentation and between 150-240 minutes. A similar pattern in the rate of titratable acid production was also observed for WTY with a linear increase in rate of acid production between 30-90 minutes and 90-210 minutes of fermentation. However, lower rate of acid production occurred for GTY and BTY during certain time intervals i.e. between minutes 30-60 for GTY and between minutes 60-90 and 150-210 for BTY.

4.4.4. Changes in titratable acidity (TA) during refrigerated storage

The changes in TA values for all yogurt types during storage is summarized in Table 4.21 and presented in Figure 4.15. All tea yogurts had higher TA than in PY on day 1 of storage. The TA production for all yogurts was found to increase during refrigerated storage. This occurred ($p<0.05$) during days 1-7 of storage for PY, between days 7-21 for GTY, between days 7-14 for WTY and throughout the 21 days of storage period for BTY.

Table 4.21: Changes in titratable acidity (TA) of yogurts during refrigerated storage

Storage day	TA of yogurts (% lactic acid equivalent)			
	PY	GTY	WTY	BTY
1	0.70 ± 0.02^{aA}	0.84 ± 0.03^{aB}	0.85 ± 0.01^{aB}	0.78 ± 0.03^{aC}
7	0.85 ± 0.01^{bA}	0.87 ± 0.01^{aA}	0.88 ± 0.04^{aA}	0.85 ± 0.02^{bA}
14	0.88 ± 0.02^{bcA}	0.95 ± 0.05^{bA}	0.94 ± 0.04^{bA}	0.89 ± 0.01^{cA}
21	0.91 ± 0.02^{cA}	0.99 ± 0.03^{cB}	0.96 ± 0.04^{bAB}	0.91 ± 0.01^{dA}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
PY (control) refers to yogurt without incorporation of tea (milk + starter culture only).
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

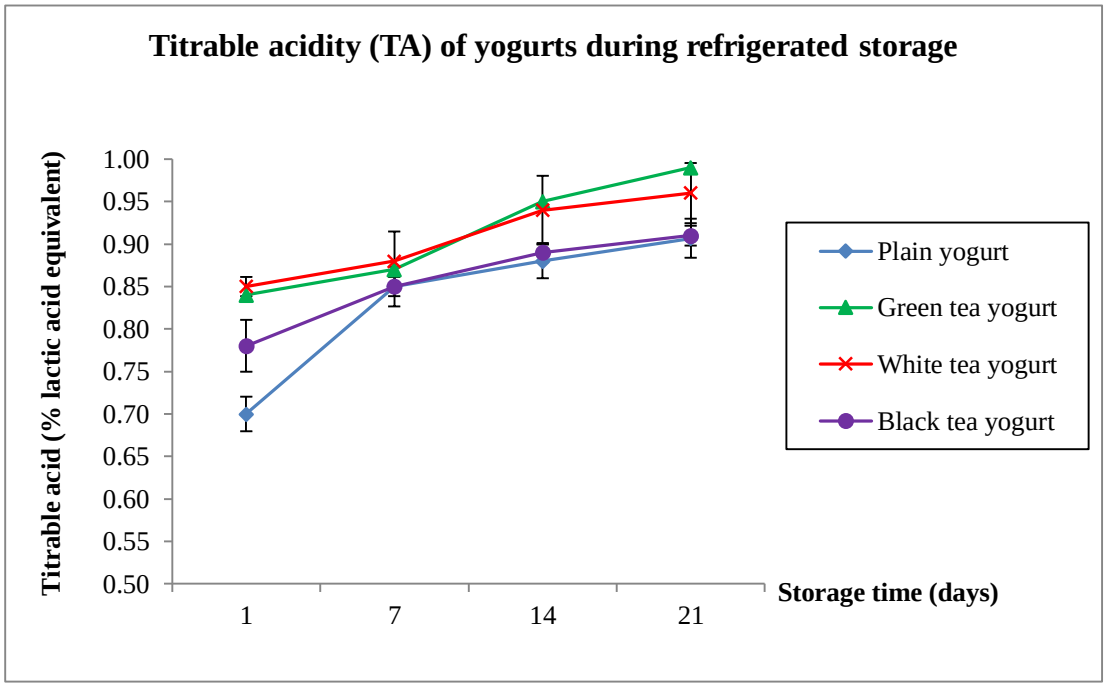

Figure 4.15: Changes in titratable acidity (TA) of yogurts during 21 days of refrigerated storage at 4°C.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$. Plain yogurt (control) refers to yogurt without incorporation of tea (milk + starter culture only).

4.5. Viability of yogurt starter culture

4.5.1. Viable cell counts of *Streptococcus thermophilus* during refrigerated storage

The changes in *S.thermophilus* counts in all yogurts during storage is summarized in Table 4.22 and presented in Figure 4.16. All yogurts maintained high *S.thermophilus* counts, ranging between 10^8-10^9 CFU/ml throughout the refrigerated storage period. *S.thermophilus* counts were different ($p<0.05$) between each yogurt type with PY always showing the highest counts followed by WTY, GTY and BTY. There was a tendency for a slight increase in viable *S.thermophilus* counts at the end of the first week of storage. The increase was highest for WTY (0.11 log CFU/ml) followed by GTY, BTY and PY (0.10 log CFU/ml, 0.09 log CFU/ml and 0.07 log CFU/ml) respectively. However, these values were found to reduce by 0.18, 0.14, 0.15 and 0.17 log CFU/ml for PY, GTY, WTY and BTY respectively by the end of the third week of storage.

Table 4.22: Viable cell counts of *S.thermophilus* in yogurts during refrigerated storage

Storage day	*Streptococcus thermophilus* counts (log CFU/ml)			
	PY	**GTY**	**WTY**	**BTY**
1	9.40 ± 0.02^{aA}	9.14 ± 0.03^{acB}	9.22 ± 0.03^{aC}	9.06 ± 0.04^{acD}
7	9.47 ± 0.02^{bA}	9.24 ± 0.03^{bB}	9.33 ± 0.05^{bC}	9.15 ± 0.04^{bD}
14	9.39 ± 0.03^{aA}	9.17 ± 0.04^{aB}	9.27 ± 0.04^{cC}	9.08 ± 0.04^{abD}
21	9.29 ± 0.03^{cA}	9.10 ± 0.05^{cB}	9.18 ± 0.03^{dC}	8.98 ± 0.04^{cD}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
PY (control) refers to yogurt without incorporation of tea (milk + starter culture only).
[abcd] Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
[ABCD] Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

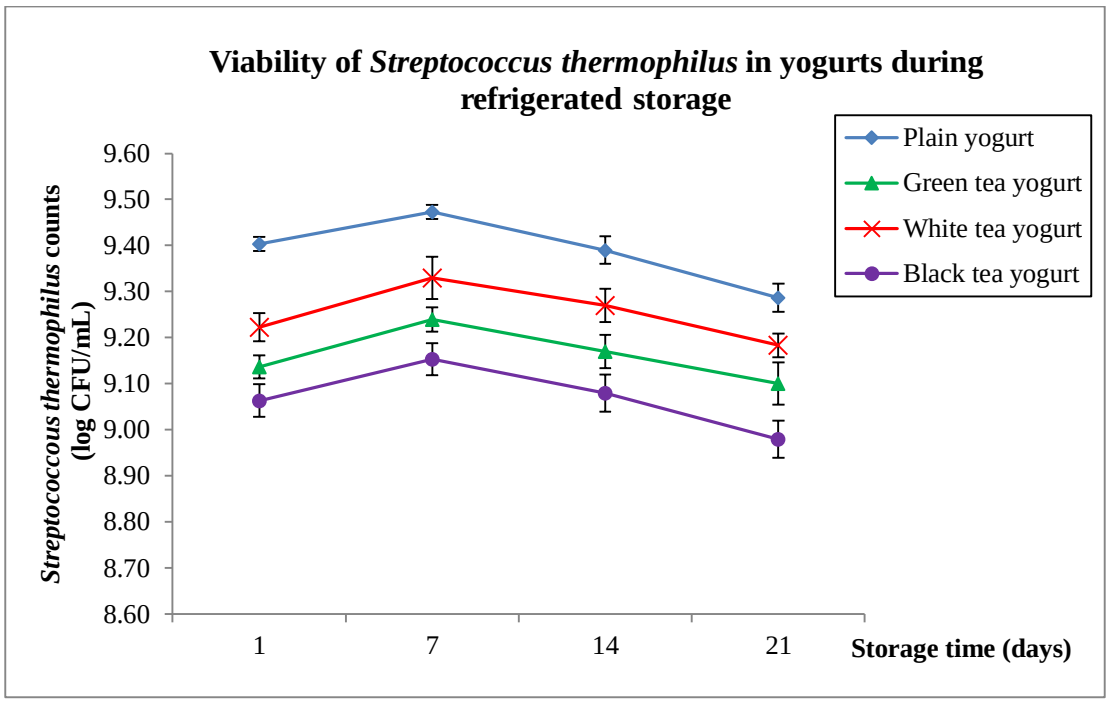

Figure 4.16: Changes in viable cell counts of *Streptococcus thermophilus* in yogurts in the presence and absence of tea during 21 days of refrigerated storage at 4°C.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$. Plain yogurt (control) refers to yogurt without incorporation of tea (milk + starter culture only).

4.5.2. Viable cell counts of *Lactobacillus* spp during refrigerated storage

All yogurts maintained a high level of viable *Lactobacilllus* spp counts, ranging between 10^6-10^7 CFU/ml throughout the post-fermentation storage period (Table 4.23 and Figure 4.17). The lactobacilli counts were significantly different between each yogurt type with WTY always showing the highest CFU values followed by GTY, BTY and PY.

The reduction in viable lactobacilli during the first week of storage was insignificant for each yogurt type. Viable lactobacilli in yogurts decreased ($p<0.05$) from day 7 to day 21 of refrigerated storage by 0.38 log CFU/ml, 0.41 log CFU/ml, 0.50 log CFU/ml and 0.35 log CFU/ml for PY, GTY, WTY and BTY respectively.

Table 4.23: Viable cell counts of *Lactobacillus* spp in yogurts during refrigerated storage

Storage day	*Lactobacillus* spp counts (log CFU/ml)			
	PY	GTY	WTY	BTY
1	6.54 ± 0.05^{aA}	6.83 ± 0.08^{acB}	7.03 ± 0.05^{aC}	6.67 ± 0.04^{aD}
7	6.46 ± 0.05^{aA}	6.75 ± 0.03^{aB}	6.98 ± 0.07^{aC}	$6.62 \pm 0.^{07abD}$
14	6.30 ± 0.04^{bA}	6.64 ± 0.05^{bB}	6.78 ± 0.05^{bC}	6.48 ± 0.10^{bD}
21	6.08 ± 0.04^{cA}	6.34 ± 0.03^{cB}	6.48 ± 0.02^{cC}	6.27 ± 0.02^{cD}

Values are means of three replicates ± standard deviation.
PY=Plain yogurt; GTY= Green tea yogurt; WTY= White tea yogurt; BTY= Black tea yogurt.
PY (control) refers to yogurt without incorporation of tea (milk + starter culture only).
^{abcd} Means with superscripts having different letters in the same column are significantly ($p < 0.05$) different.
^{ABCD} Means with superscripts having different letters in the same row are significantly ($p < 0.05$) different.

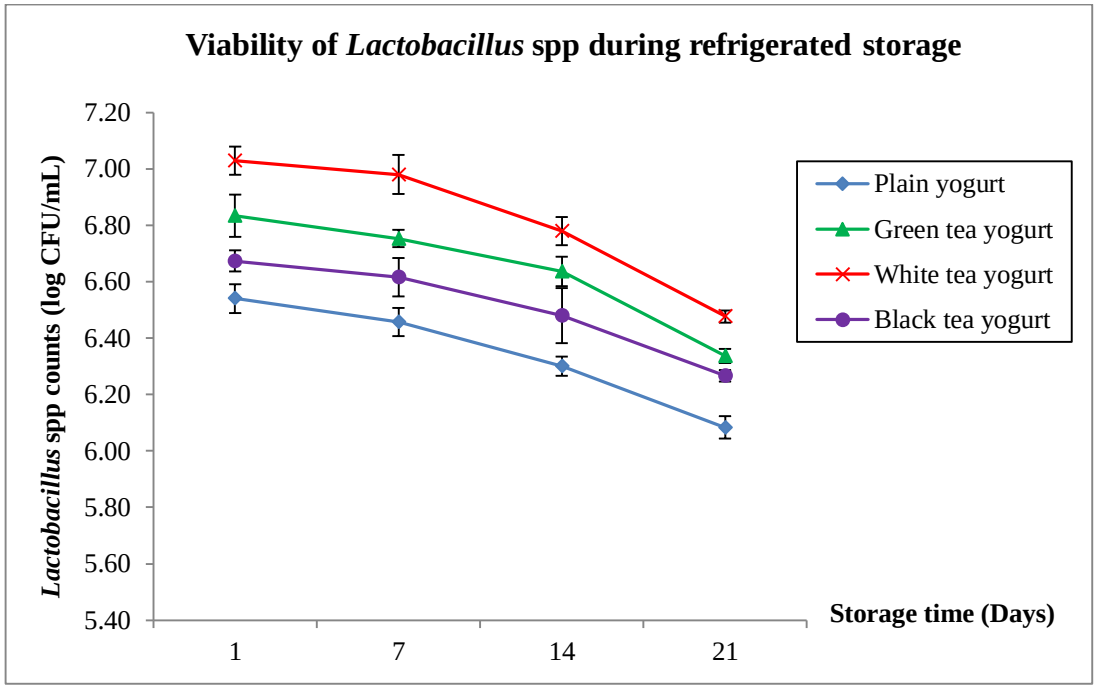

Figure 4.17: Changes in viable cell counts of *Lactobacillus* spp in yogurts in the presence and absence of tea during 21 days of refrigerated storage at 4°C.
Error bars represent a pooled standard deviation of the mean (n=3). The level of significance was preset at $p < 0.05$. Plain yogurt (control) refers to yogurt without incorporation of tea (milk + starter culture only).

CHAPTER 5.0:

DISSCUSSION

5.1. Effects of addition of tea on phenolic content of yogurts

The phenolic content of plant material correlated well with their antioxidant activity (Velioglu *et al.* 1998), and thus its application in routine determination of total phenolic content (TPC) in the evaluation of plant antioxidant potential. However, interpretation of data relating TPC and antioxidant need to be made with caution because variations in TPC in plants are caused by factors such as environmental conditions, genetic factors, degree of maturity, degree of polymerization of the phenolic compounds, type of solvent used for extraction, extraction time and temperature as well as interaction of the phenolic compound with other food constituents (Marathe *et al.*, 2011). The TPC of tea extracts and yogurt water extracts were determined using Folin-Ciocalteu's reagent which consist of phosphomolybdic acid and phosphotungstic acid. The assay is based on electron transfer, in which the polyphenol donates an electron to the reagent, thus reducing both the acids into molybdenum and tungsten oxides respectively, resulting in the development of a deep blue colour which could be monitored as absorbance at 765 nm. The intensity of the blue colour is proportional to the number of the aromatic phenolic group present in the extract (Singleton & Rossi, 1965). Among the tea extracts, the highest TPC value of GTE could be attributed to the highest content of flavonoids (Table 4.16) in the tea leaves (Komes *et al.*, 2007). Since white tea consist of young tea leaves or buds covered with tiny silvery hairs, the presence of trichome (cell wall covered with lipophilic cuticle) could influence the migration kinetics of catechins, hence reducing the extraction efficiency of the polyphenols compared to green tea under the same conditions (Rusak *et al.*, 2008). In addition, the polyphenol oxidases and peroxidases enzymes responsible for oxidation of phenolic compounds in white tea leaves do not undergo inactivation process unlike green tea leaves. These enzymes could remain active during storage resulting in a gradual oxidation of the phenolic compounds thus explaining the lower TPC value for

WTE compared to GTE (Jiang, 2009). The lowest TPC value of BTE (Table 4.1) was probably due to oxidative degradation and polymerization of phenolic compounds during fermentation in the manufacturing process of black tea. This is in line with a reduction by 74%, 91%, 51% and 62% for EGCG, EGC, EC and ECG respectively as well as 38% for total flavonol glycosides observed during the 80% fermentation of green tea to black tea (Kim *et al.*, 2011).

The reduction in TPC of all tea-milk mixture and tea-yogurts (Table 4.2) in comparison to their respective tea extracts could be attributed to milk-polyphenol interaction as reported by previous studies (Dubeau *et al.*, 2010; Ryan & Petit, 2010; Kartsova & Alekseeva, 2008; Sharma *et al.*, 2008). Milk-polyphenol interaction is common in proline rich milk proteins (casein) due to the strong affinity of the proline groups towards the hydroxyl groups present in the phenolic compounds (Arts *et al.*, 2002). The binding capacity between the tea polyphenols and protein increases as the number of hydroxyl (OH) group increase in the phenolic compound in the order of C ~ EC > EGC > EGCG (Hasni *et al.*, 2011). In the present study, a relatively high decline in TPC was noticed for both GTY and WTY upon fermentation, by 83.7% and 86% respectively, in comparison to TPC of their respective tea extracts probably due to the abundant presence of catechin and epicatechin in both green tea and white tea.

The highest reduction in TPC by 88.2% in the present study was observed with BTY. The milk-polyphenol interaction is size dependent in which bigger phenolic compound possess stronger binding capacity with milk protein. As such, black tea, having bigger phenolic compounds such as theaflavin and thearubigin as a result of the oxidative fermentation during post-harvest processing is more likely to have stronger milk-polyphenol interaction (Dubeau *et al.*, 2010). Although the milk-polyphenol interaction leads to precipitation of the phenolic compound that may decrease the antioxidant potential, Yuksel *et al.* (2010) have suggested this hydrophobic binding as a

useful attribute since the cross linkage between flavonoids and milk proteins could be beneficial in producing novel dairy products with desired texture.

Proteolysis in milk released amino acids with phenolic side chains such as tyrosine, which contribute to the increase in TPC (Shah, 2000a; Korhonen, 2009; Gobetti *et al.*, 2007). This was demonstrated by an increase in TPC on day 1 of storage following fermentation (Table 4.2). In addition, microbial metabolism of phenolic compounds in the yogurt as well as production of new phenolic acids during post-acidification may result in an increase of phenolic groups as the ring structure is broken down (Blum, 1998). The higher TPC of all tea yogurts compared to PY throughout the storage period may be explained by the presence of indigenous phytochemicals in the yogurts, namely the tea flavonoids. PY do not contain tea extracts, hence the considerable amount of TPC for PY may be attributed to phenolic compounds naturally present in milk derived probably from the feed given to the ruminant, amino acid catabolism as well as smaller peptides released from milk protein proteolysis by yogurt bacteria (O'Connell & Fox, 2001).

Reduction in TPC by 4.5%, 16.3%, 14.3% and 12.6% for PY, GTY, WTY and BTY respectively in the following two weeks of storage was similar to the results reported by Karaaslan *et al.* (2011) and can be explained by degradation of phenolic compounds e.g. virgin olive oil (Okogeri & Tasioula-Margari, 2002) and broccoli (Lemoine *et al.*, 2007) during storage. A degradation of green tea catechins and black tea theaflavins as high as 50% could occur during one month of storage in soft drinks containing acidic preservatives (Lun-Su *et al.*, 2003). The degradation of polymeric phenolic compounds into monomeric phenolic acids via hydrolytic and oxidative cleavage may have contributed to the reduction in pH of milk-polyphenol system during the first week of storage as observed in this study (Table 4.18). The slight increment in TPC of all yogurts during the last week of storage could be related to the interaction of

the monomeric phenolics from the breakdown, with milk protein molecules leading to polymerization and formation of new phenolic compounds (Wegrzyn *et al.*, 2008). Some considerations must be made to take into account the tendency for an over-estimation of phenolic compounds in the sample, since the Folin-Ciocalteu spectrophotometric method is non-specific and may also measure any other components that may interfere with the reaction (Ainsworth & Gillespie, 2007).

5.2. Effects of tea on antioxidant potential of yogurts

Phenolic compounds could act as antioxidants via their ability to donate hydrogen or electron resulting in the termination of a chain reaction or by chelating transition metal ions hence, terminating the Fenton reaction (Rice-Evans *et al.*, 1997). An antioxidant compound that directly scavenge free radical is termed as primary antioxidant while compounds that prevent the formation of free radical via Fenton reaction is known as secondary antioxidant (Chan *et al.*, 2010). Both DPPH and FRAP measures the primary antioxidant properties of the sample while FIC assay measures the secondary antioxidant property of the sample (Deetae *et al.*, 2012).

The ability to scavenge free radical is one of the important mechanisms in which antioxidants inhibit lipid peroxidation, which is a chain reaction responsible for generating free radicals continuously in the body (Cheung *et al.*, 2003). DPPH (1,1-diphenyl, 2-picryl hydrazyl) is a stable organic free radical with a deep purple colour. DPPH radical scavenging activity is based on a hydrogen transfer reaction between the antioxidant component in the sample and the free radical. In the presence of antioxidant compounds which act as hydrogen donor, DPPH is reduced leading to decolourization of the reagent into pale yellow colour which could be monitored as absorbance at 517 nm (Deng *et al.*, 2011). The higher the degree of decolourization, the lower the

absorbance value, hence the greater is the antioxidant potential of the sample. DPPH radical scavenging assay was used in this study since it is relatively simple, cost effective, and sensitive to detect active components in low concentrations (Sang *et al.*, 2002). Besides, certain side reactions of polyphenols such as metal ion chelation and enzyme inhibition do not influence the radical scavenging activity, hence provide a reliable measure of the antioxidant activity (Babbar *et al.*, 2011).

The ferric reducing antioxidant potential (FRAP) is based on electron transfer and measures the ability of an antioxidant sample to reduce ferric-tripiridyltriazine (Fe^{3+}-TPTZ complex) to the ferrous form (Fe^{2+}) at low pH, leading to the formation of an intense blue colour which could be monitored as absorbance at 593nm. Since excess of Fe^{3+} is used, the rate limiting factor for the reaction and the intensity of the blue colour formed depends entirely on the reducing ability of the sample (Benzie & Strain, 1996). Transition metal ions such as copper (Cu^{2+}) and ferrous (Fe^{2+}) ions are highly reactive and readily participate in Fenton reaction, thus generating hydroxyl radicals that promote lipid peroxidation leading to oxidative damages *in vivo* at different levels (Halliwell, 1991). A high ferrous ion chelating (FIC) ability will reduce the amount of Fe^{2+} ions in the body and inhibit the generation of reactive oxygen species (ROS) as well as lipid peroxidation. In this assay, the antioxidant compound being a chelating agent will compete with ferrozine to form complexes with ferrous ion (Fe^{2+}), resulting in a decrease in the intensity of the purple colour of the complex which can be monitored as absorbance at 562nm. The lesser the intensity of the purple colour, the lower is the absorbance, hence the higher is the chelating ability or antioxidant potential of the sample.

The order of DPPH radical scavenging activity (Table 4.2) and FRAP (Table 4.5) for all tea extracts in the present study relate well with each tea total phenolic content (TPC; Table 4.1). The presence of tea catechins and some low molecular polyphenols

contributed to the high antioxidant potential of the tea extracts (Zhu *et al.*, 2002). The differences in scavenging activity and Fe^{3+} ions reducing ability between the individual tea types may be ascribed to the nature of the different phenolics present in varying amount in each of the tea extract, thus influencing their hydrogen or electron transfer ability (Manian *et al.*, 2008). The reduction in DPPH radical scavenging activity (Table 4.4) and FRAP (Table 4.6) of all tea-milk mixture prior to fermentation and tea yogurts after fermentation in comparison to their respective tea extracts was similar to that observed for TPC. This could be related to milk-polyphenol interaction as described earlier, which could reduce their antioxidant capacity *in vitro* (Arts *et al.*, 2001). Increasing the size of polyphenols is related to decreasing antioxidant potential (Dubeau *et al.*, 2010). This could explain the lower DPPH radical scavenging activity and FRAP observed with BTY in the present study. In addition, the polymerization of phenolic compounds, in this case catechins into larger theaflavin and thearubigin during black tea processing (Lu and Foo, 2000) may result in stearic hindrance that affect the ability of the antioxidant active site to approach the Fe^{3+}-TPTZ complex in FRAP assay resulting in a lower FRAP value for BTY.

The increase in DPPH radical scavenging activity of all tea yogurts on day 1 of storage may be attributed to the metabolic activity of the yogurt bacteria that remain viable and active during refrigerated storage which lead to the alteration of some of the phenolic compounds in the yogurts and their antioxidant activity (Blum, 1998). Acid production as a result of lactic acid fermentation could affect racemisation of molecules (Wotton Beard *et al.*, 2011) which alters the biological activity and antioxidant potential. Besides, the structural changes could also lead to different behaviour of the metabolites in different assays. In this regard the increased antioxidant activity in fermented milk products occured as a result of the proteolysis by LAB and the subsequent release of bioactive peptides (Pihlanto *et al.* 2006). For instance κ-casein-

peptides with DPPH radical scavenging activity (Kudoh *et al.,* 2001) were produced after milk fermentation by *Lactobacillus delbreuckii* subsp *bulgaricus.* The reduction in DPPH radical scavenging activity and FRAP of yogurts in the following weeks of storage may be attributed to the further degradation of phenolic compounds by the yogurt bacteria.

The reduction in DPPH radical scavenging activity and FRAP of tea yogurts by the second week of storage is associated with the reduction observed in TPC values of tea yogurts. However, for PY, despite significant reduction in DPPH radical scavenging activity by the second week of storage, the fluctuations in values of FRAP throughout the storage period remain insignificant. Such differences in the results shown by both antioxidant assays could be as a consequence of the ability of DPPH to measure antioxidant capability of certain components which cannot be measured by FRAP (Najgebauer-Lejko *et al.*, 2011). A comparable observation to this was lactoferrin in milk serum which is the major component that possess radical scavenging activity but cannot be measured by FRAP method (Smet *et al.*, 2008).

The high DPPH radical scavenging activity (greater than 90%) shown by all tea extracts and tea yogurts suggest the presence of synergistic interactions between individual polyphenols in each tea type or between polyphenols and non-phenolic components present in the tea that contribute to distinct antioxidant activity. However, this possibility need to be further investigated since the ability of some non-phenolic compound to dissolve in water may have also contributed to the enhanced radical scavenging activity (Othman *et al.*, 2007).

In contrast to the results of TPC, DPPH radical scavenging activity and FRAP assays, FIC values was highest in BTY throughout the storage period whereas GTY and WTY were not different to each other from day 7 of storage onwards (Table 4.8).

Similar observation has been reported by Chan *et al.* (2007 & 2010) and Ho *et al.* (2010). Since metal chelating capacity is related to the availability of binding site for metal ions provided by the antioxidant ligand, the presence of bigger phenolic compounds such as theaflavin in black tea could probably offer more binding sites for ferrous ions, thus increasing their chelating ability in comparison to GTY and WTY (Graf *et al.*, 1984). Alternatively, the higher FIC ability of BTY could be attributed to the presence of some other bioactive compounds besides polyphenols, for instance the presence of a specific group with adjacent carbonyl and hydroxyl functions as in theaflavins (Marathe *et al.*, 201; Lin *et al.*, 2008).

It can be concluded that compounds with high FRAP or DPPH radical scavenging activity may not necessarily show high FIC ability. Compounds with high FIC ability will be able to prevent compounds with high FRAP from accumulating metal ions (Fe^{2+}) that could lead to oxidative damage (Cao *et al.*, 1997). GTY and WTY exhibit better radical scavenging avtivity and Fe^{3+} reducing ability, but slightly lower FIC ability in comparison to BTY. Since total antioxidant capacity of a food sample may be as a result of multiple reaction mechanisms, a single antioxidant assay may not reflect the total antioxidant capacity (Du *et al.*, 2009). Hence, this explains the relevance of assessing the antioxidant potential of tea yogurts in this study using three different methods.

The high antioxidant activity observed in all tea yogurts in comparison to PY may be a direct consequence of the polyphenols present in tea leaves. However, considerable amount of antioxidant activities shown by PY in all three assays indicate the presence of compounds that have antioxidant property. Low molecular weight peptide fractions isolated from yogurt was found to contain compounds which possess high reducing power and ability to chelate metal ions like Fe^{2+} while high molecular

weight fractions showed high DPPH radical scavenging activity (Sabeena Farvin *et al.*, 2010).

5.3. LCMS analysis of phenolic compounds in tea extracts and tea-yogurts

The phenolic composition of GTE and WTE are remarkably similar with some exceptions to flavonol glycoside compositions (Table 4.16) as reported in earlier studies (Gondoin *et al.*, 2010). Flavonol glycosides such as kaempferol-3-rutinoside and quarcetin-rhamnosylgalactoside or rutinoside found in WTE were absent in GTE. In addition, catechin derivatives such as gallocatechin (GC) and epigallocatechin (EGC) were not detected in WTE but were present in GTE. Despite having many physiological roles in plant survival including UV-B protection, disease resistance and defence against predation (Harborne & Williams, 2000), the contents of these flavonoids compounds in plants do reduce as plant matures, thus explaining the differences in phenolic compound observed between GTE and WTE (Song *et al.*, 2012).

Gallo-flavanols have high oxidative potential and tendency to dimerise readily into theaflavins and thearubigins (Karori *et al.*, 2007; Wang *et al.*, 2000) compared to catechol-flavanols. Thus, the absence of gallocatechin (GC), epigallocatechin (EGC) and gallocatechin gallate (GCG) and the subsequent presence of theaflavin-3-O-gallate observed in BTE could be direct consequences of oxidative fermentation during the manufacturing process. Similarly, the absence of several flavonol glycosides such as myrecitin-3-O-glucoside, kaempferol-3-O-galactosyl-rhamnosyl-glucoside and kaempferol-rhamnose-hexose-rhamnose in black tea but which are present in green tea can also be attributed to oxidative fermentation (Wang and Helliwell (2001), Kim e*t al.*, (2011)).

Fermentation of milk by yogurt bacteria yields interesting observations with regard to formation and disappearance of polyphenolic compounds. Most of the catechin and its derivatives present in the tea extracts were not detected in the tea yogurts. In addition several flavonol glycosides such as myrecitin-3-O-glucoside, kaempferol-3-rutinoside and kaempferol-3-O-glucoside were detected in WTE but not in WTY. New compounds were also detected in tea yogurts, for instance kaempferol-3-rutinoside and quinic acid conjugate which were absent in GTE but present in GTY. The two unidentified peaks found in PY may be compounds derived from metabolism of milk protein by yogurt bacteria since one of the peaks (MW=723) was detected in all tea yogurts.

The differences in phenolic compounds between tea extracts and their respective tea yogurts as well as the presence of several new peaks in each tea yogurt which were previously absent in the respective tea extracts suggest the possibility of phenolic compounds being metabolized by the yogurt microflora. The ability to metabolize phenolic compounds have been identified in several *Lactobacilli sp.* inclusive of *L. brevis* (Curiel *et al.*, 2010), *L. plantarum* (Tabasco *et al.*, 2011) and *L. hilgardii* (Alberto *et al.*, 2001) and the activities of enzymes tannase, phenolic acid decarboxylase and benzyl alcohol dehydrogenase were considered responsible (Rodríguez *et al.*, 2009). Serological (London, 1976) and 16S rRNA (Stackebrandt *et al.*, 1983) studies showed that all LAB with exception to *Bifidobacteria* are phylogenetically related and share some common pathways of carbohydrate metabolism with differences in the mode of breaking the carbon skeleton (Kandler, 1983). Thus it is highly likely that LAB in the present study may confer the similar ability to metabolize phenolic compounds found in tea. However, these possibilities need to be verified further by DNA sequencing (to detect the presence of above mentioned enzymes in yogurt bacteria). Some considerations may also be given to the gradual degradation of

polymeric compounds (Wegrzyn *et al.*, 2008) or milk-polyphenol interaction (Hasni *et al.*, 2011) as explained earlier.

5.4. Effects of tea on the acidification of yogurt

The lower pH (Table 4.17) and higher titratable acid (TA) (Table 4.19) for tea yogurts at the initial stage of fermentation (0 min) than those in PY may be attributed to the presence of naturally occurring phenolic acids such as gallic acid and quinic acid in all tea leaves (Dufresne & Farnworth, 2001). Fermentation process during the manufacture of black tea leaves increased phenolic acid content (Harbowy & Balentine, 1997) and thus may explain the lowest pH value of BTY among the three tea yogurts.

The reduction in pH (Figure 4.12) and increase in TA (Figure 4.14) in all yogurts throughout the course of fermentation was as a result of accumulation of lactic acid and other organic acids such as butyric acid, citric acid, acetic acid, acetaldehyde and formic acid (Ostlie *et al.*, 2003). As fermentation progressed, pH of PY was much lower than that of tea yogurts whereas TA of tea yogurts were higher than PY. This could be explained by the fact that the former (pH) is a measure of free H^+ ions concentration in the milk sample whereas the latter (TA) reflects the total amount of H^+ ions present in the milk sample with exception to those bound to alkaline ions (Shori & Baba, 2011). Since organic acids are generally weak acids with low degree of dissociation in aqueous solution the measurement of TA would be a much reliable approach to determine the actual amount of acid produced, hence the fermentation potential of the microbes (Geidem *et al.*, 2007).

The highest rate of TA production by PY compared to all tea yogurts during the first 30 minutes of fermentation (Table 4.19) indicate the presence of a lag period in the growth of the yogurt bacteria by tea. Some component in tea could have negative effects

on yogurt bacteria because phenolic compounds in olive do act as natural defence against phytopathogenic bacteria (Medina *et al.*, 2009; Servili *et al,* 2011). The steady and linear increase in TA production at two distinct phases observed for PY (between 60-120 minutes and 150-240 minutes) and WTY (between 30-90 minutes and 90-210 minutes) marks the typical example of associative growth pattern characterized by mixed culture of yogurt bacteria.

Radke-Mitchell and Sandine (1984) and Shah (2000b) showed that the early stage of fermentation is predominated by *S. thermophilus* which grow rapidly making use of the amino acids liberated by the proteolysis carried out by lactobacilli and in turn produces lactic acid which lowers the pH of the medium to 5.5. The favourable acidic environment and growth factors such as formic acid and carbon dioxide produced by the *S.thermophilus* will then stimulate the growth of *Lactobacillus delbrueckii* subsp *bulgaricus* which dominates the second stage of fermentation below pH 5.0, producing lactic acid and acetaldehyde which contribute to the characteristic sharp and acidic flavour of yogurt. GTY showed a very slow rate of acid production at the beginning of fermentation (Table 4.20). A slow rate of acid production was also observed with BTY especially during minutes 120-210 of fermentation. The irregular pattern of acid production and the prolonged fermentation time to reach the range of pH 4.5- 4.6 observed with GTY and BTY suggest that some components present in tea leaves may have inhibitory effect towards the growth and metabolism of the yogurt bacteria. The present findings however appear to contradict that of Najgebauer-Lejko *et al.* (2011) which reported that tea supported growth of yogurt starter culture whereas Jaziri *et al.* (2009) reported that tea neither supported nor inhibited the activity of the yogurt bacteria. Prolonged fermentation time of yogurts upon supplementation of plant extract rich in phenolic compounds suggest an increase in buffering capacity of yogurt (Michael *et al*., 2010), which resisted pH changes despite accumulation of organic acid.

While this pH buffering effects may be advantageous to the proliferation of yogurt bacteria, a longer fermentation time may result in greater extent of proteolysis in yogurts that in turn contribute to greater syneresis in the final product (Gassem & Frank, 1991), which is undesirable.

The significant reduction in pH of all yogurts during the first week of storage is an indication of post-acidification attributed to the metabolic activity of viable yogurt bacteria (Kneifel *et al.*, 1993; Beal *et al.*, 1999). However, subsequent increase in pH of all yogurts in the following weeks of storage could be as a result of accumulation of ammonia which was contributed by the urease activity of *S.thermophilus.* Urease breaks down the milk urea into carbon dioxide and ammonia which decreases the rate of acidification and in turn leads to the alkalinisation of the medium (Zourari *et al.*, 1992; Salaun *et al.*, 2005). Milk protein breakdown by the action of indigenous proteolytic enzymes in milk such as plasmin, which are heat stable and relatively active during cold storage (Kelly *et al.*, 2006) may also result in products with alkaline nature.

5.5. Effects of tea on the viability of yogurt starter culture

Regular ingestion of yogurt containing a high number of viable lactic acid bacteria at the time of consumption will be a desirable characteristic of the product to confer health benefits to consumers. To achieve this, several countries have established a minimum value of lactic acid bacteria to be present in fermented milk products during shelf life which ranges from 10^6 to 10^8 CFU g^{-1} (IDF, 1988). The viability of yogurt microflora during storage is dependent on several factors which include incubation and storage time, final fermentation pH, strain association, incubation temperature during fermentation, type and amount of inoculum used, nutrient accessibility in the medium and presence of undesirable microorganisms (Beal *et al.*, 1999; Cais-Sokolinska and

Pikul, 2004). In the present study, the viable counts of yogurt bacteria in PY and in all tea yogurts (*S. thermophilus* > 10^9 CFU/ml, *Lactobacillus spp.* > 10^6 CFU/ml throughout the storage period) were within the minimum levels suggested by the International Dairy Federation. The higher counts of *S. thermophilus* than *Lactobacillus spp.* by more than 1 log order was in accordance with several previous studies (Birollo *et al.*, 2000; Dave and Shah, 1996; Zare *et al.*, 2011; Najgebauer-Lejko *et al.*, 2011; Vinderola *et al.*, 2000). Since aerobic condition favours growth of *S. thermophilus*, the use of plastic containers rather than glass containers for storage of yogurt could have resulted in the higher counts of *Streptococci* compared to *Lactobacilli* as plastic containers permit greater oxygen permeation during refrigerated storage (Ranadheera *et al.*, 2012). In addition the higher viable counts could also be due to the use of starter culture containing dominant *S. thermophilus* compared to the *Lactobacilli* (Birollo *et al.*, 2000). The higher counts of *S. thermophilus* compared to *Lactobacillus spp.* would be a desirable trait in commercial production of low-acid mild yogurt, since lactobacilli are more acid tolerant compared to streptococci and often result in greater post-acidification during storage (Barrantes *et al.*, 1996).

The absence of any contaminating microorganisms such as yeast, moulds or coliform bacteria in all yogurts throughout the storage period indicate that the tea yogurts prepared do not contain possible contamination attributable to the addition of tea infusate. Accumulation of organic acids and other antimicrobial components such as bacteriocins in the yogurts as a result of yogurt bacteria metabolism are known to prevent the growth of contaminating microorganisms above mentioned (Vandenbergh, 1993; Caplice and Fizgerald, 1999). In addition, the release of aromatic compounds from *S. thermophilus* (Sikes and Hilton, 1987) and *L.delbruckeii* subsp. *bulgaricus* (Abdel-Bar *et al.*, 1987) metabolism have inhibitory effects against many contaminating microorganisms. The antimicrobial activity of tea polyphenols against a wide range of

food-borne pathogens (Chou *et al.*, 1999; Hamilton-Miller, 1995; Yam *et al.*, 1997) could also contribute to the absence of contaminants in tea yogurts.

Polyphenols with its high antioxidant activities could have also improved viability of the bacteria in tea yogurts by acting as radical scavengers (Figueiredo *et al.*, 2008). The significant increase in viability of *S. thermophilus* during the first week of storage prior to a decrease was in accordance to several previous studies (Birollo *et al.*, 2000; Najgebauer-Lejko *et al.* 2011). The increase in *S. thermophilus* counts was supported by the decrease in pH and increase in TA during the first week of storage for all yogurts, showing that the increasing number of bacteria increased the production of lactic acid during storage. The subsequent decrease in viable counts of both streptococci and lactobacilli in the following weeks of storage may be attributed to post-acidification (Kneifel *et al.*, 1993) Although the viability of *S.thermophilus* in all yogurt types throughout the storage period were high, it was observed that the viability of this bacteria in tea yogurts were lower than PY. Since all tea yogurts had greater TA values than PY, the lower viable counts of *S.thermophilus* in tea yogurts could be due to the sensitivity of the bacteria to lactic acid accumulation during fermentation (Chandan & O'Rell, 2006; Vinderola *et al.*, 2002).

The decrease in the viable counts of *Lactobacillus spp.* throughout the storage period is in agreement with several previous studies (Michael *et al.*, 2010; Shori & Baba, 2011; Ranadheera *et al.*, 2012;). It is tempting to speculate that the higher viability of *Lactobacillus spp.* in tea yogurts compared to PY is attributed to the presence of compounds of prebiotic nature in tea extracts. Lee *et al.* (2006) showed that tea polyphenols namely catechin, epicatechin and gallic acid could be metabolized by the gut microflora and was found to be less sensitive towards probiotic strains inclusive of *Lactobacillus spp.* and *Bifidobacteria*, thus suggesting the possibility of tea phenolics as prebiotic substances for yogurt microflora in this study. The idea of tea polyphenol as

a source of prebiotics to stimulate the growth of yogurt bacteria could also be justified by the changes in the phenolic composition between tea extracts and tea yogurts as shown by LCMS analysis (Table 4.16).

CHAPTER 6.0:

CONCLUSION

6.1. Overall conclusion

The addition of tea into yogurt act as buffer which minimises negative effects of lactic acid formed during fermentation on yogurt bacteria. The buffering actions of tea provide additional time for growth and metabolism by yogurt bacteria during milk fermentation and also help to sustain high number of viable bacteria (*Lactobacillus spp*) during the 21 days of storage period. Green tea has the highest content of polyphenols and antioxidant activities followed by white and black teas and this also contribute to sustain viable microbial (*streptococci* and *lactobacilli)* in yogurt during refrigerated storage. Tea polyphenols may be utilised as substrates (prebiotics) by yogurt but this needs to be further confirmed in future studies. Both green and white teas are strongly recommended as a novel ingredient to enhance yogurt's functional properties.

6.2. Future research on tea yogurts

(a) Characterization of organic acids and other by-products produced by altered milk fermentation by yogurt bacteria in the presence of tea extracts.

(b) Rheological studies on tea yogurts. This is important to establish that the presence of tea extracts do not change the textural qualities of yogurt.

(c) Structural analysis of individual phenolic compounds to establish the extent of tea polyphenol metabolism by yogurt bacteria.

(d) Changes in the organoleptic properties of tea-yogurts which are important to ascertain the consumer acceptance of such yogurts.

(e) *In vivo* studies to establish the level of intake of tea-yogurts for optimum health benefits.

CHAPTER 7.0:

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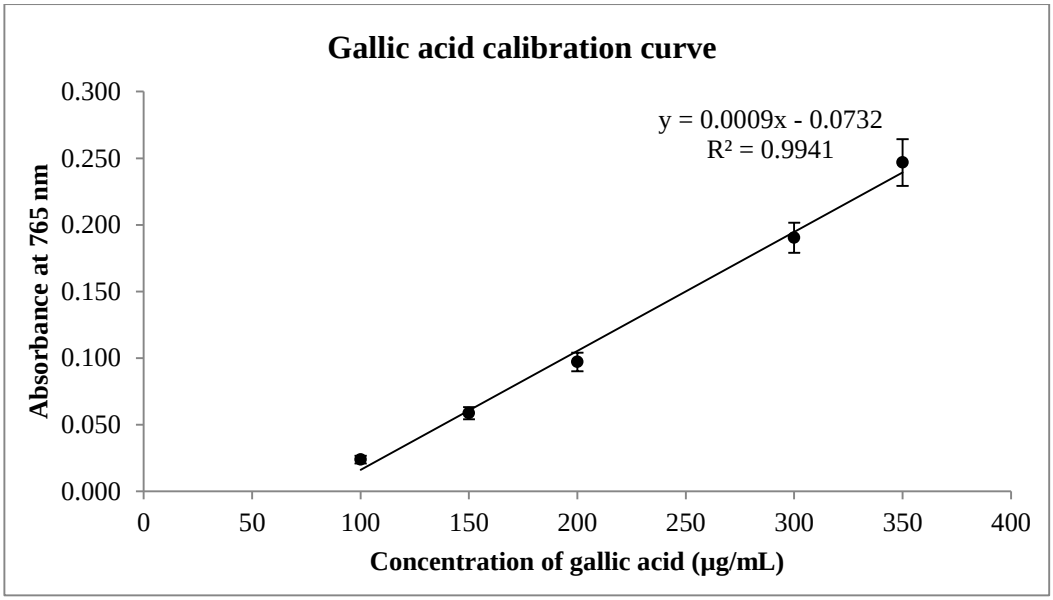
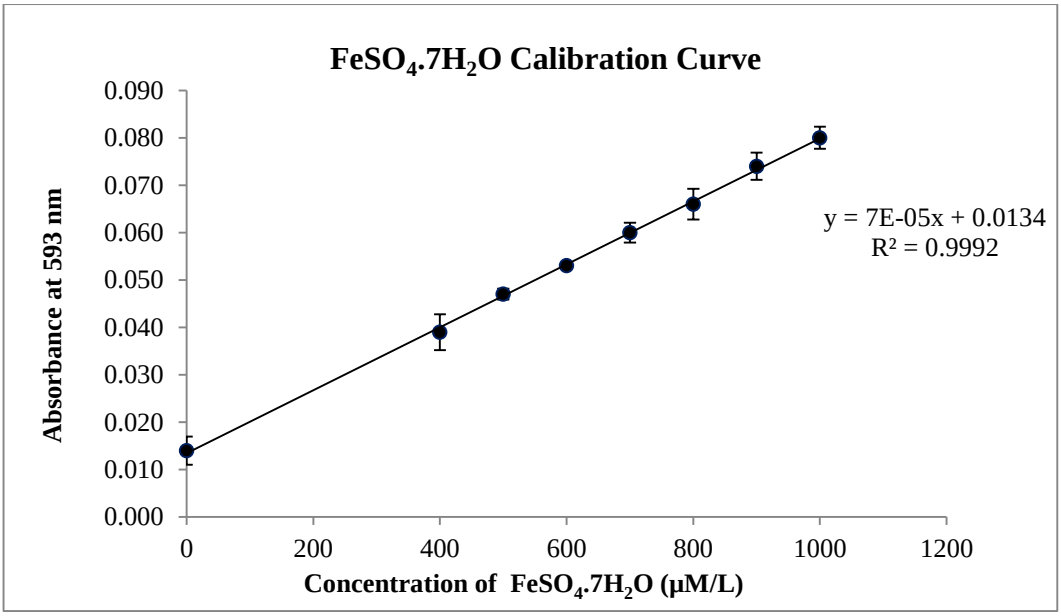
APPENDICES

Appendix 1: Gallic acid calibration curve

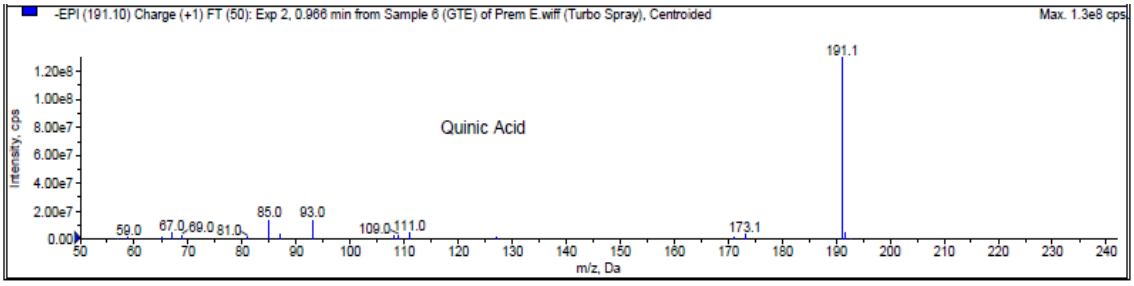


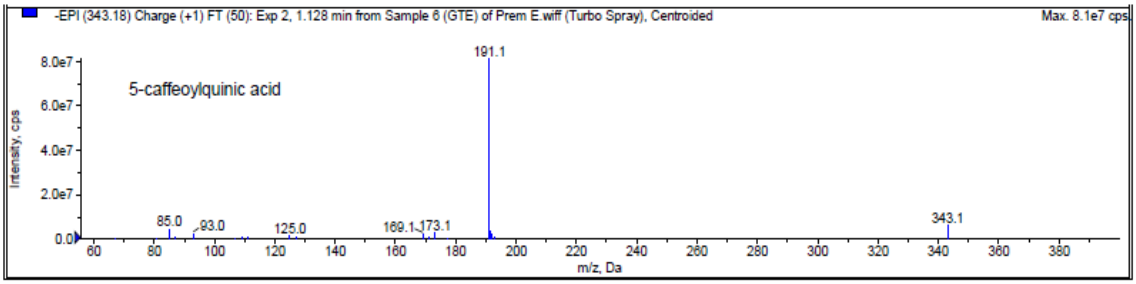
Appendix 2: $FeSO_4.7H_2O$ calibration curve

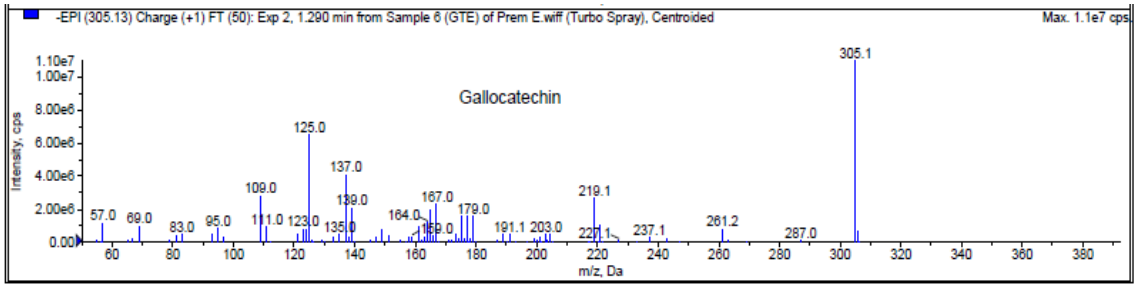


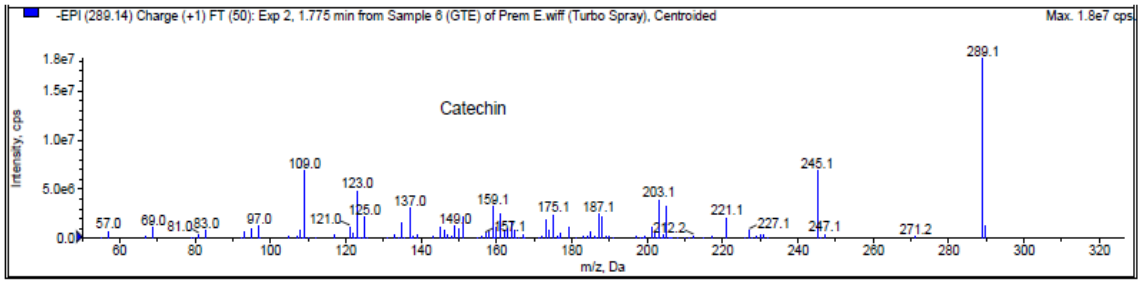
Appendix 3: LCMS analysis

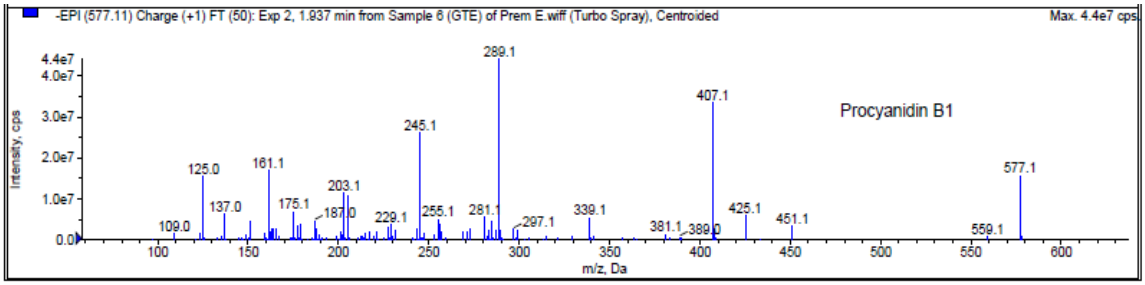
3.1. Mass spectra of each phenolic compound identified in GTE

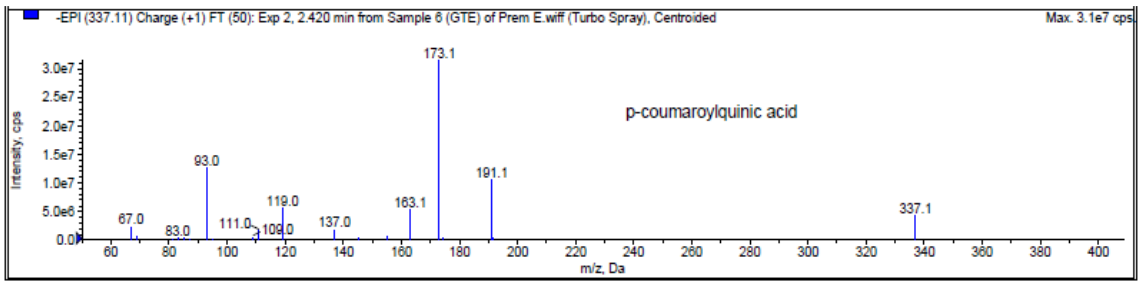


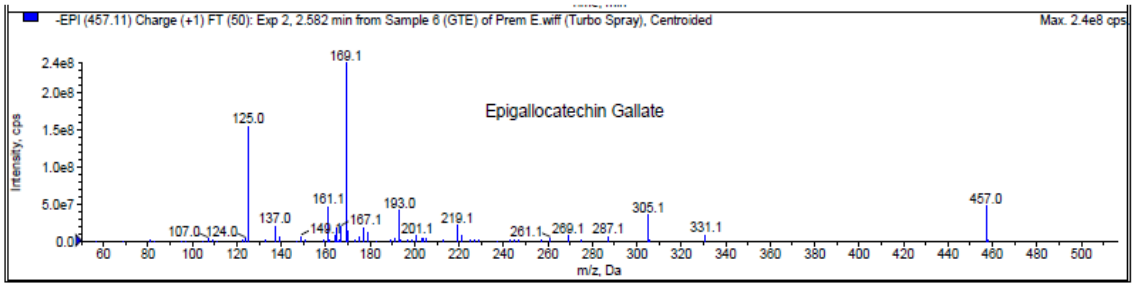


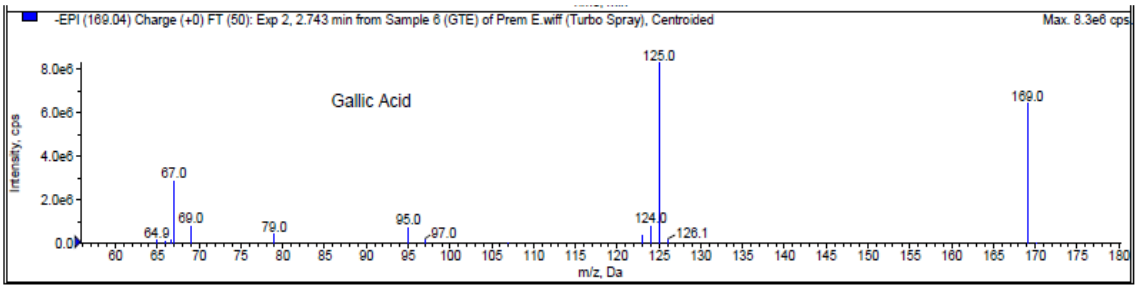


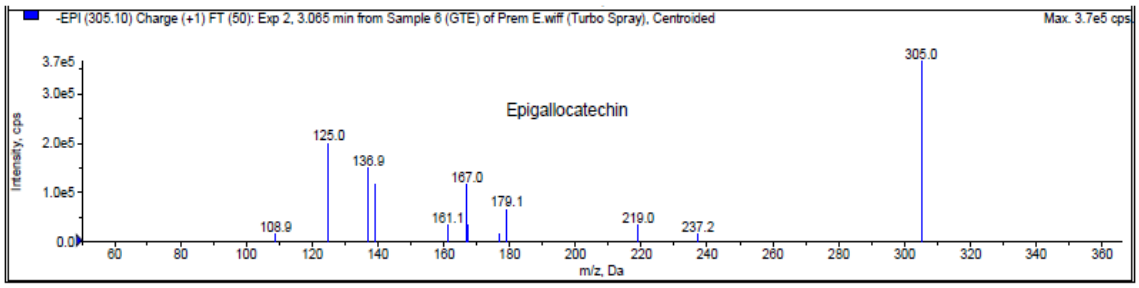


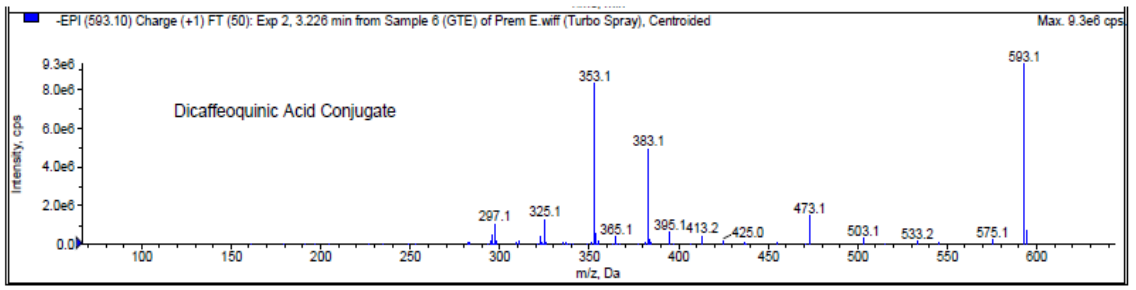


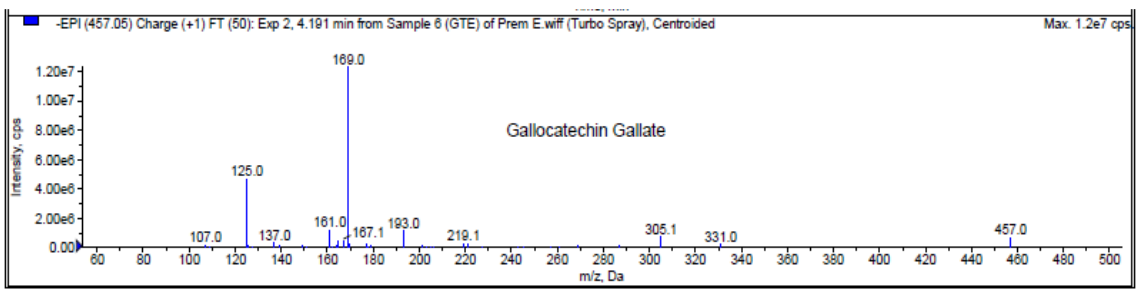

-EPI (337.11) Charge (+1) FT (50): Exp 2, 2.420 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 3.1e7 cps.
p-coumaroylquinic acid
Intensity, cps
m/z, Da

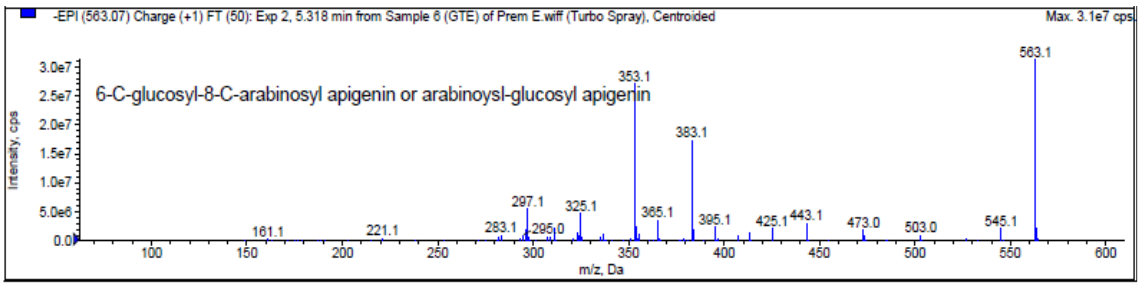

-EPI (457.11) Charge (+1) FT (50): Exp 2, 2.582 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 2.4e8 cps.
Epigallocatechin Gallate
Intensity, cps
m/z, Da

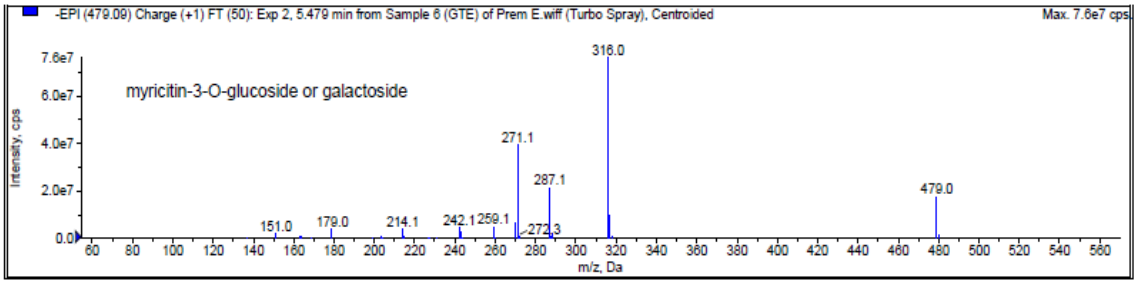

-EPI (169.04) Charge (+0) FT (50): Exp 2, 2.743 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 8.3e6 cps.
Gallic Acid
Intensity, cps
m/z, Da

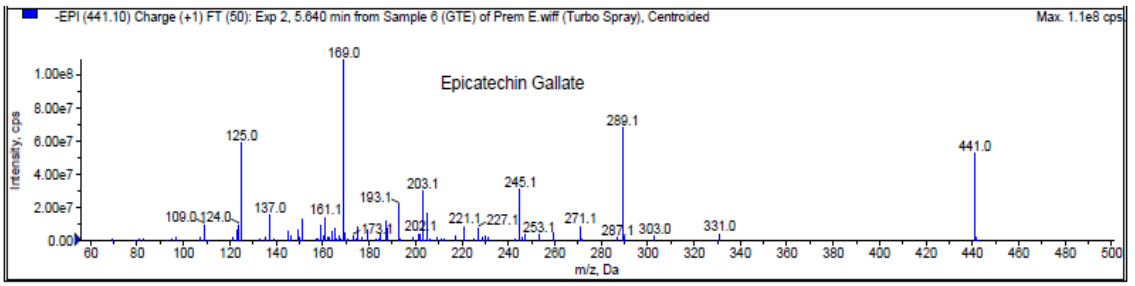

-EPI (305.10) Charge (+1) FT (50): Exp 2, 3.065 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 3.7e5 cps.
Epigallocatechin
Intensity, cps
m/z, Da

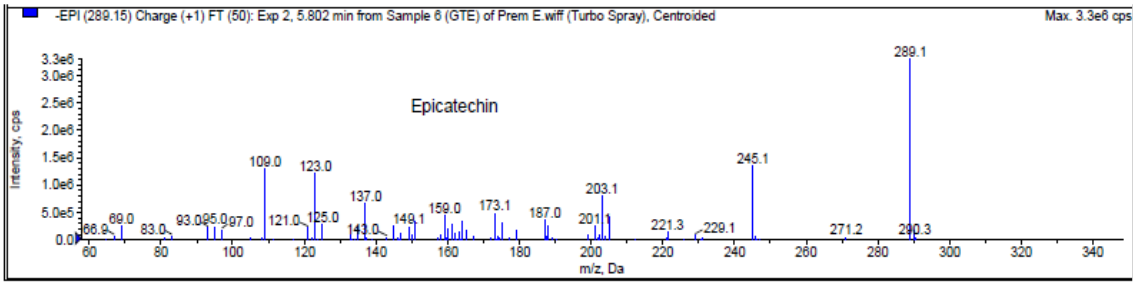

-EPI (593.10) Charge (+1) FT (50): Exp 2, 3.226 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 9.3e6 cps.
Dicaffeoquinic Acid Conjugate
Intensity, cps
m/z, Da

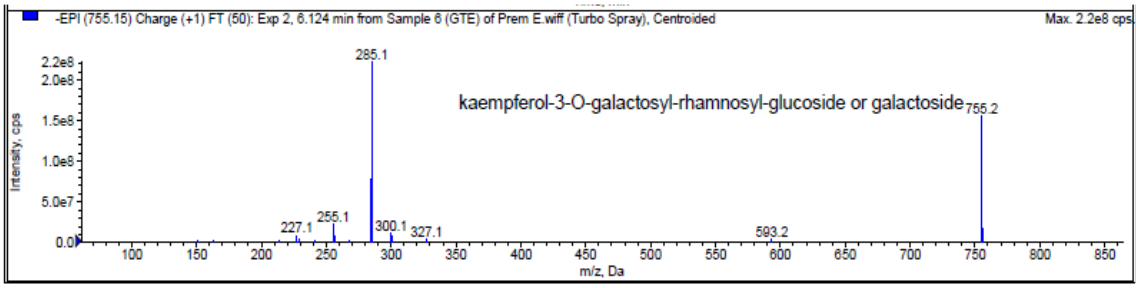

-EPI (457.05) Charge (+1) FT (50): Exp 2, 4.191 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.2e7 cps.
Gallocatechin Gallate
Intensity, cps
m/z, Da

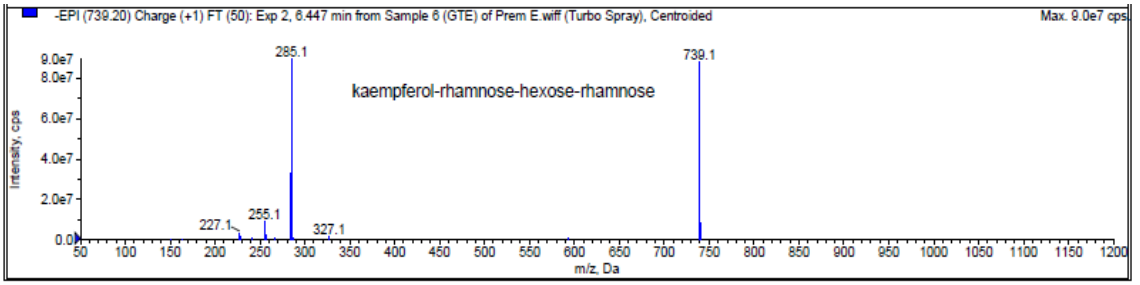

-EPI (563.07) Charge (+1) FT (50): Exp 2, 5.318 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 3.1e7 cps.
6-C-glucosyl-8-C-arabinosyl apigenin or arabinoysl-glucosyl apigenin
Intensity, cps
m/z, Da

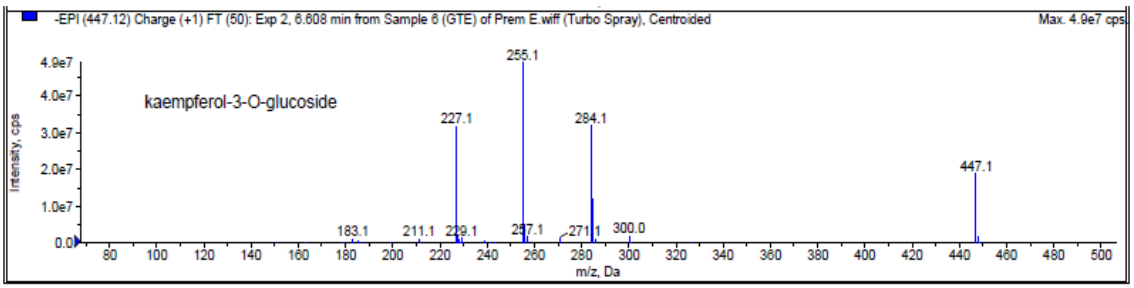

-EPI (479.09) Charge (+1) FT (50): Exp 2, 5.479 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 7.6e7 cps.
myricitin-3-O-glucoside or galactoside
Intensity, cps
m/z, Da


-EPI (441.10) Charge (+1) FT (50): Exp 2, 5.640 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.1e8 cps.
Epicatechin Gallate
Intensity, cps
m/z, Da

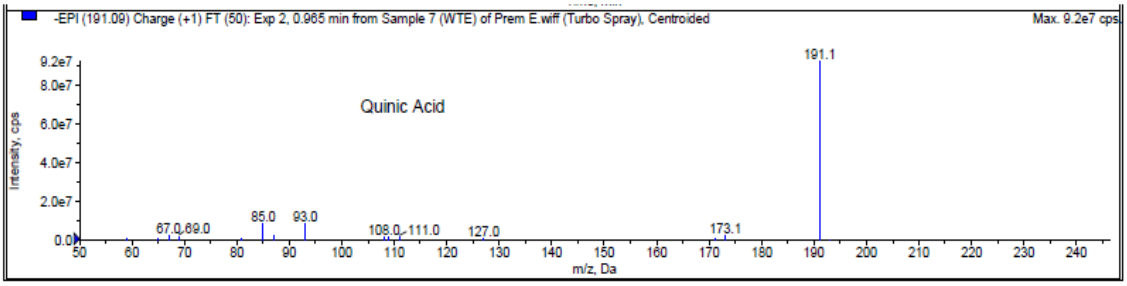

-EPI (289.15) Charge (+1) FT (50): Exp 2, 5.802 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 3.3e6 cps.
Epicatechin
Intensity, cps
m/z, Da

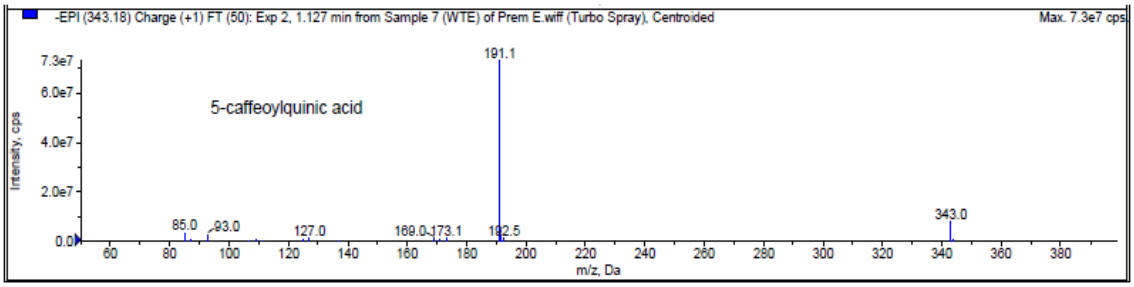

-EPI (755.15) Charge (+1) FT (50): Exp 2, 6.124 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 2.2e8 cps.
kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
Intensity, cps
m/z, Da

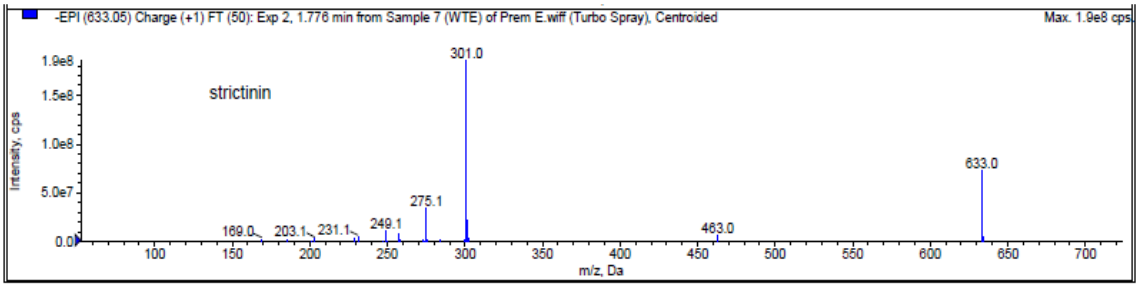

-EPI (739.20) Charge (+1) FT (50): Exp 2, 6.447 min from Sample 6 (GTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 9.0e7 cps.
kaempferol-rhamnose-hexose-rhamnose
Intensity, cps
m/z, Da

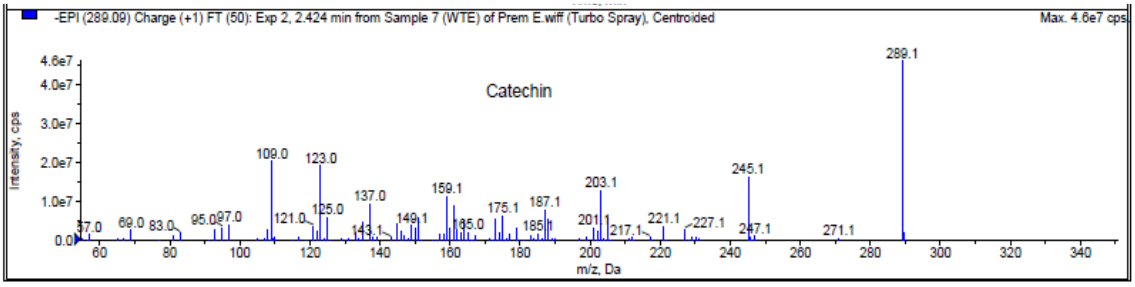


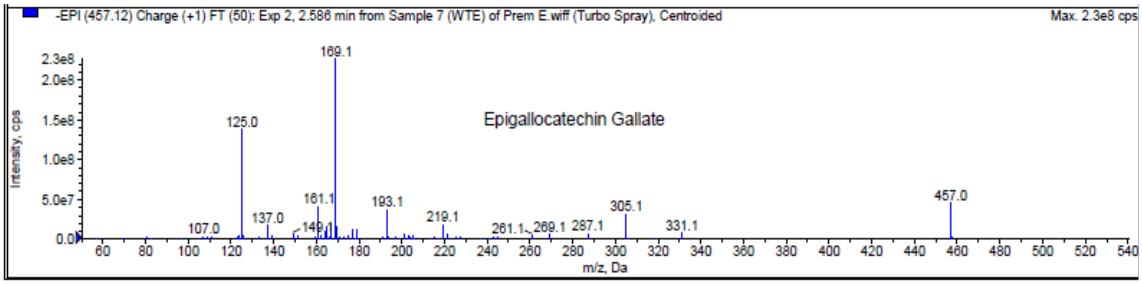
3.2. Mass spectra of each phenolic compound identified in WTE

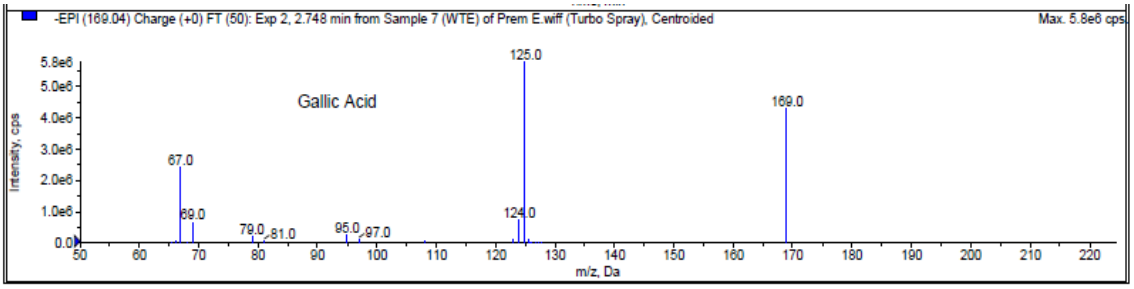


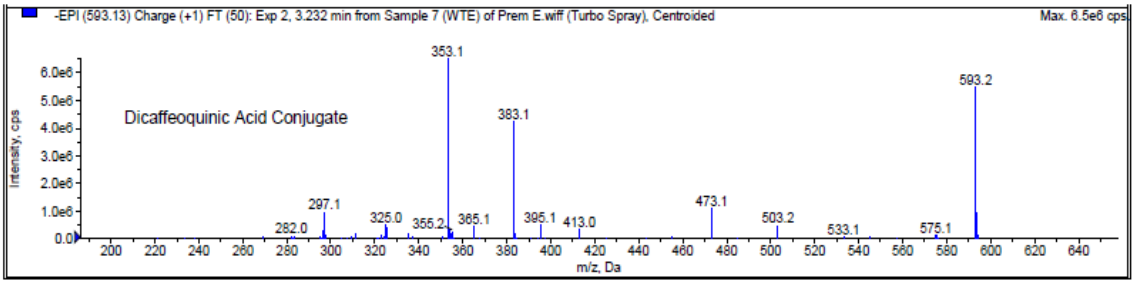


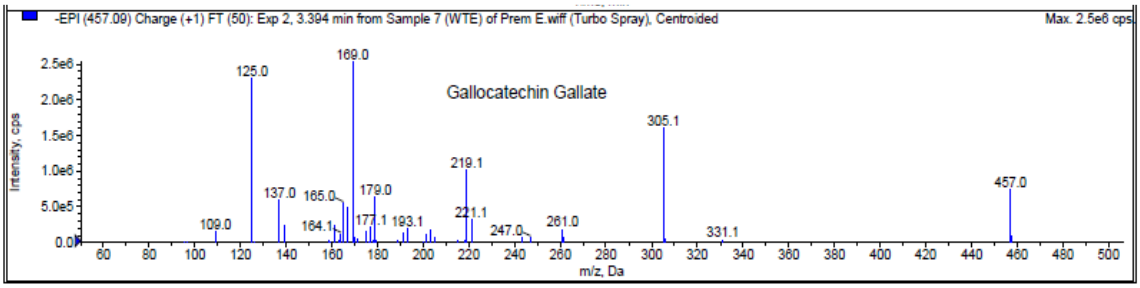


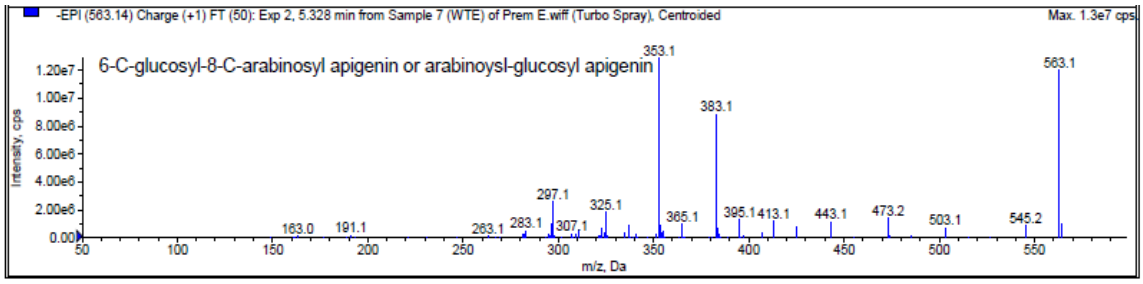


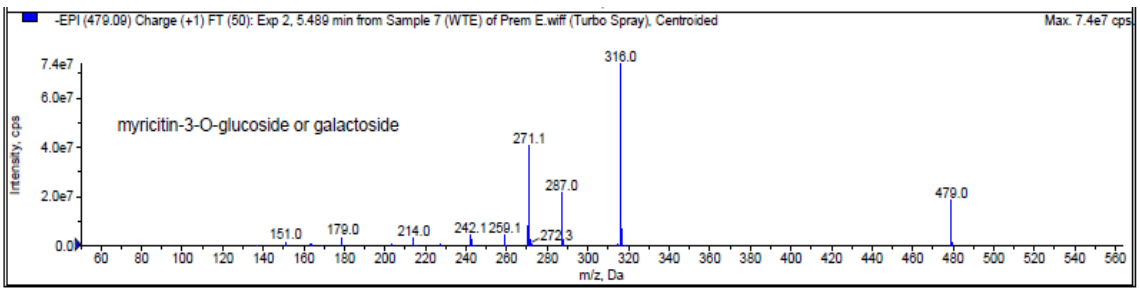

-EPI (457.12) Charge (+1) FT (50): Exp 2, 2.586 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 2.3e8 cps.
Epigallocatechin Gallate
Intensity, cps
m/z, Da

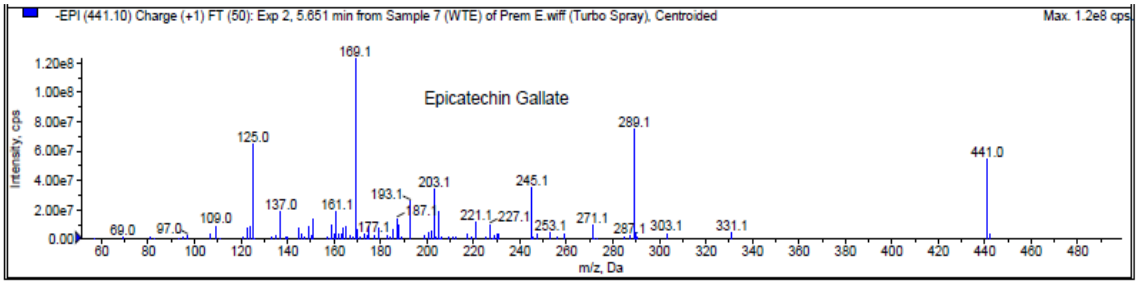

-EPI (169.04) Charge (+0) FT (50): Exp 2, 2.748 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 5.8e6 cps.
Gallic Acid
Intensity, cps
m/z, Da

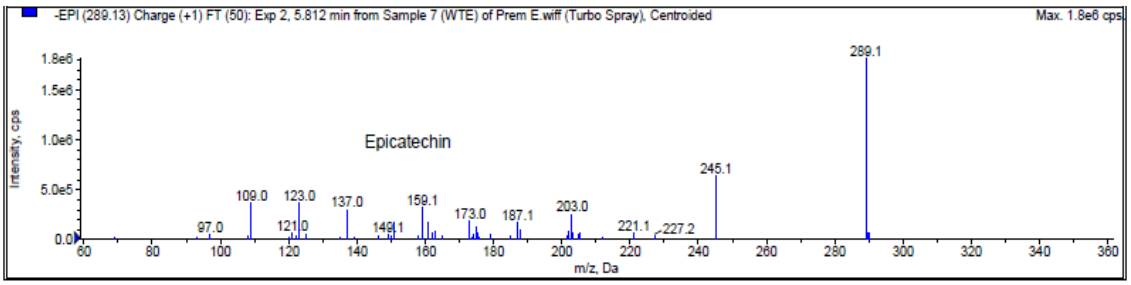

-EPI (593.13) Charge (+1) FT (50): Exp 2, 3.232 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 6.5e6 cps.
Dicaffeoquinic Acid Conjugate
Intensity, cps
m/z, Da

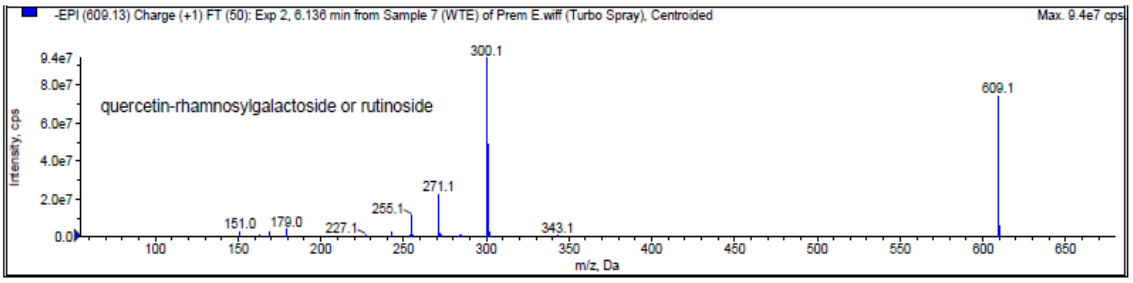

-EPI (457.09) Charge (+1) FT (50): Exp 2, 3.394 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 2.5e6 cps.
Gallocatechin Gallate
Intensity, cps
m/z, Da

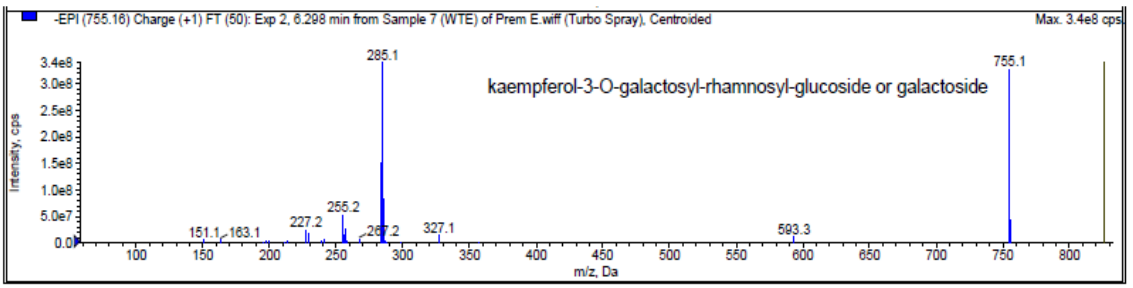

-EPI (563.14) Charge (+1) FT (50): Exp 2, 5.328 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.3e7 cps.
6-C-glucosyl-8-C-arabinosyl apigenin or arabinoysl-glucosyl apigenin
Intensity, cps
m/z, Da

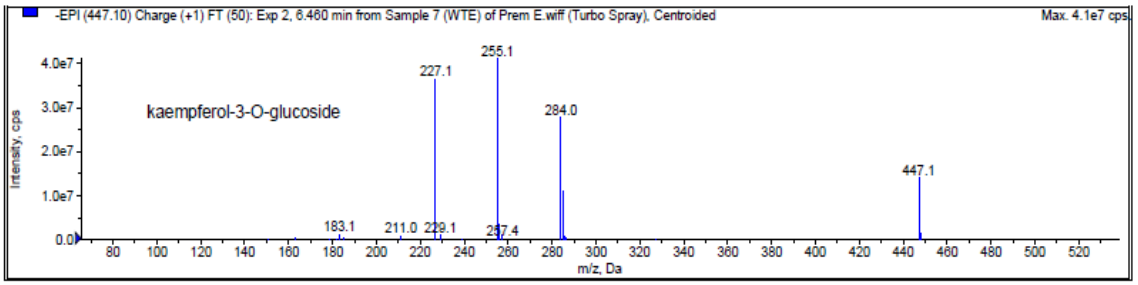

-EPI (479.09) Charge (+1) FT (50): Exp 2, 5.489 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 7.4e7 cps.
myricitin-3-O-glucoside or galactoside
Intensity, cps
m/z, Da

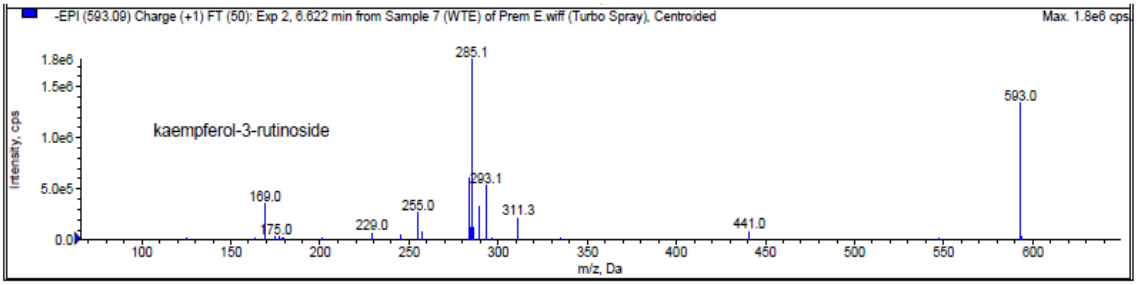

-EPI (441.10) Charge (+1) FT (50): Exp 2, 5.651 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.2e8 cps.
Epicatechin Gallate
Intensity, cps
m/z, Da


-EPI (289.13) Charge (+1) FT (50): Exp 2, 5.812 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.8e6 cps.
Epicatechin
Intensity, cps
m/z, Da

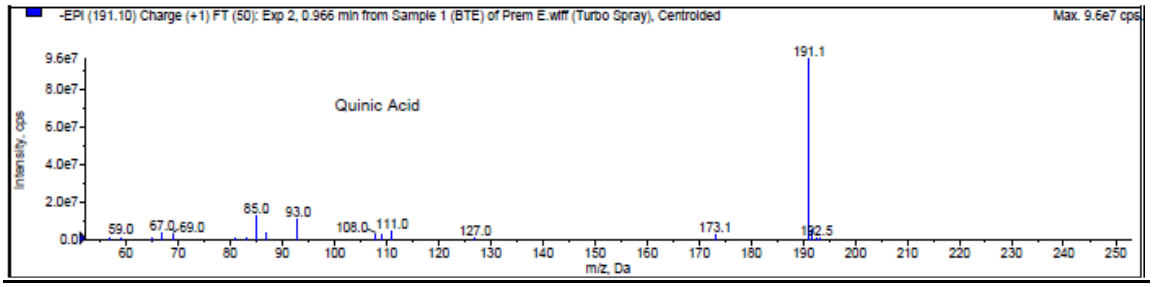

-EPI (609.13) Charge (+1) FT (50): Exp 2, 6.136 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 9.4e7 cps.
quercetin-rhamnosylgalactoside or rutinoside
Intensity, cps
m/z, Da

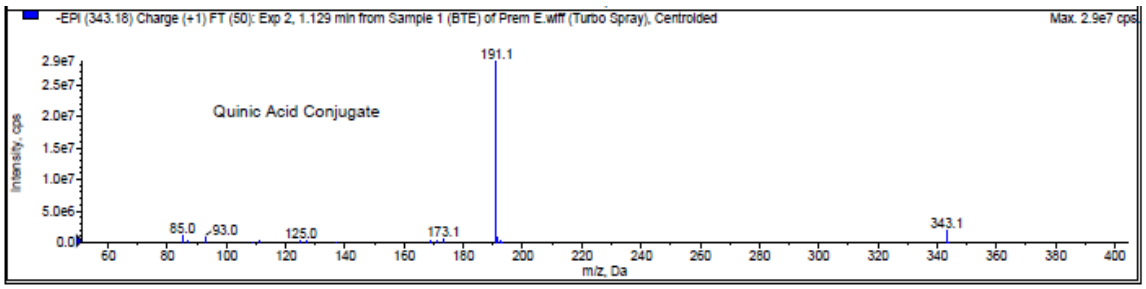

-EPI (755.16) Charge (+1) FT (50): Exp 2, 6.298 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 3.4e8 cps.
kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
Intensity, cps
m/z, Da

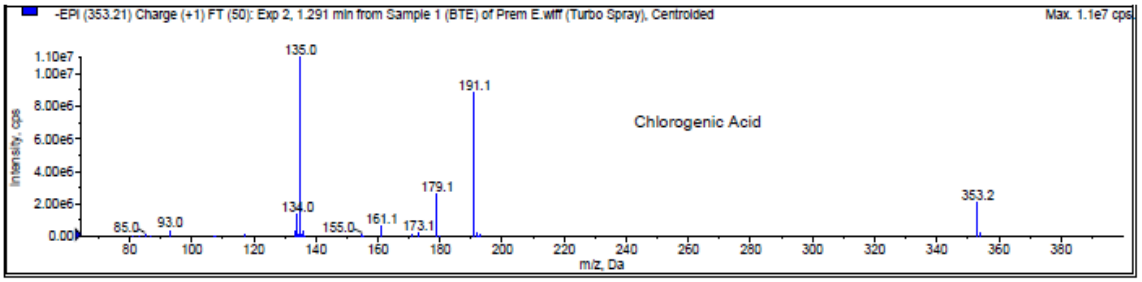

-EPI (447.10) Charge (+1) FT (50): Exp 2, 6.460 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 4.1e7 cps.
kaempferol-3-O-glucoside
Intensity, cps
m/z, Da

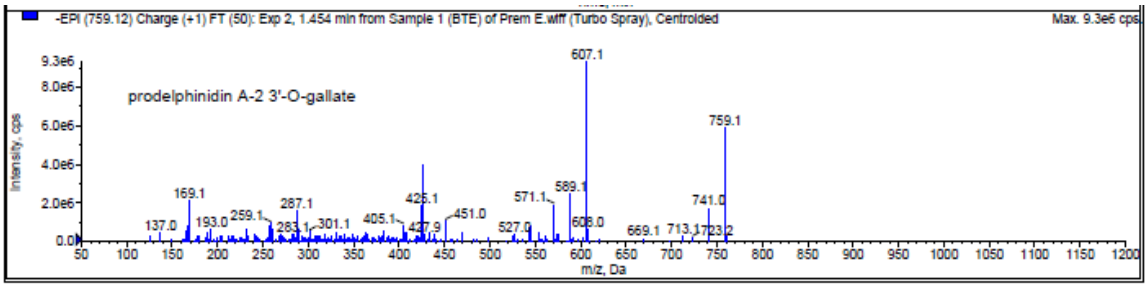

-EPI (593.09) Charge (+1) FT (50): Exp 2, 6.622 min from Sample 7 (WTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.8e6 cps.
kaempferol-3-rutinoside
Intensity, cps
m/z, Da

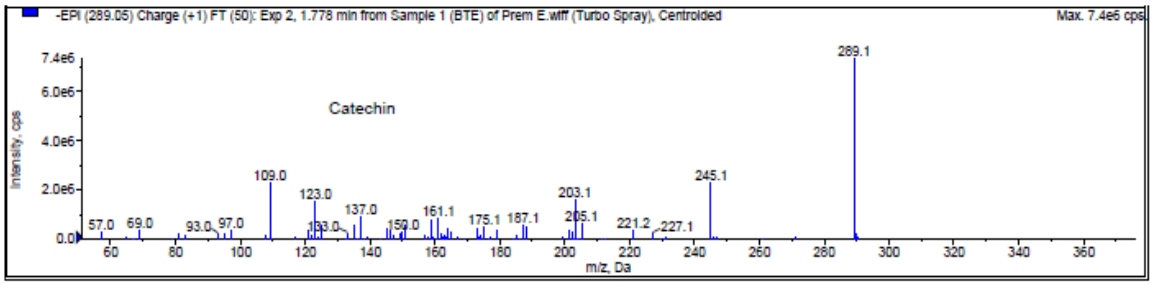
3.3. Mass spectra of each phenolic compound identified in BTE

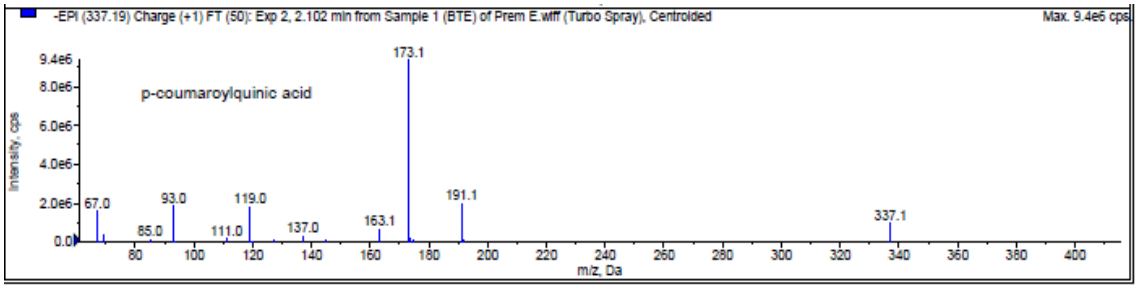


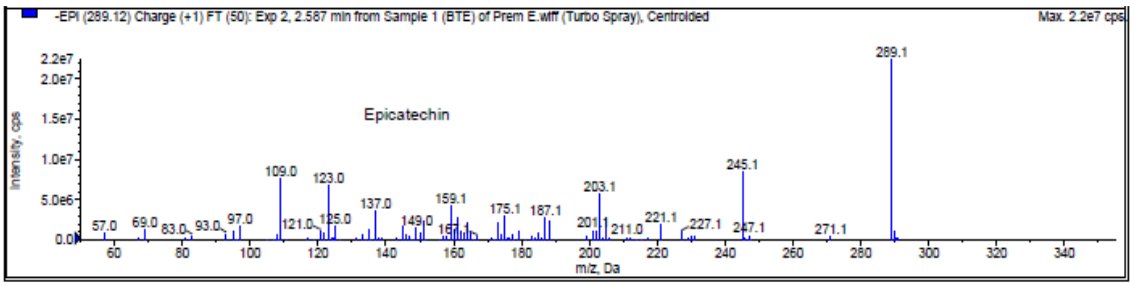


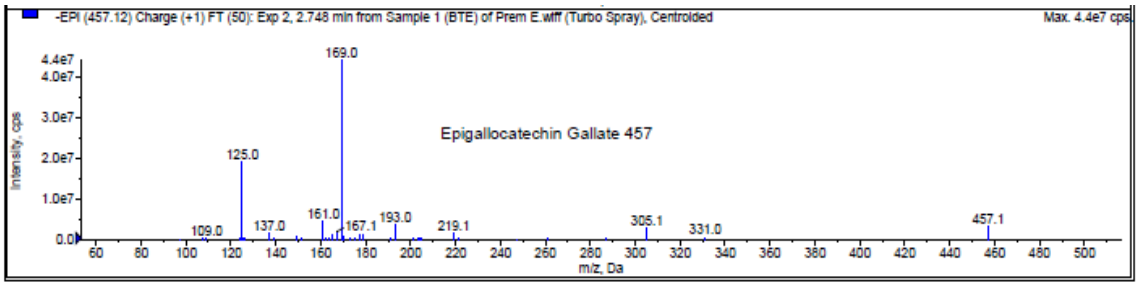


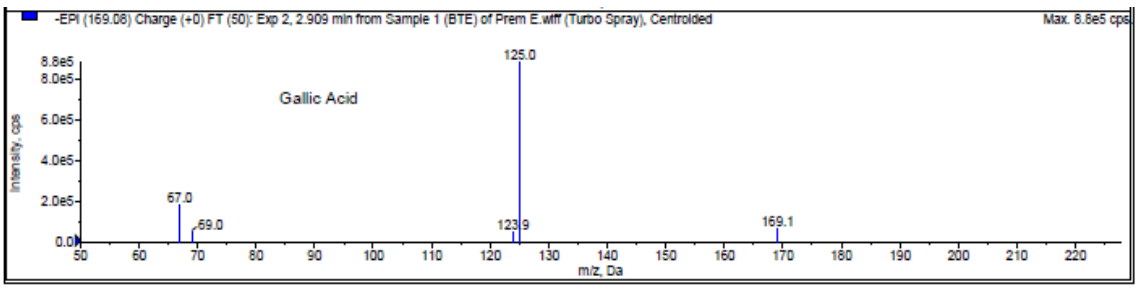


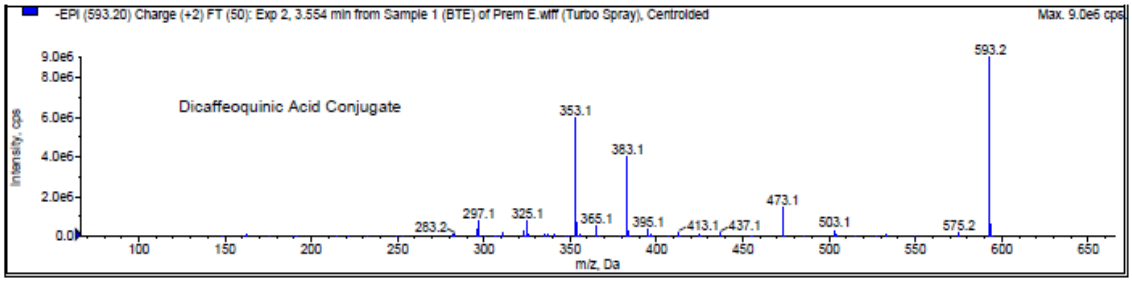


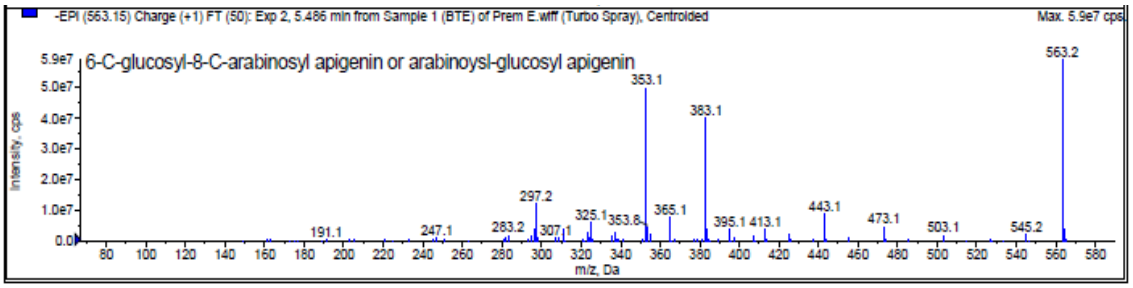


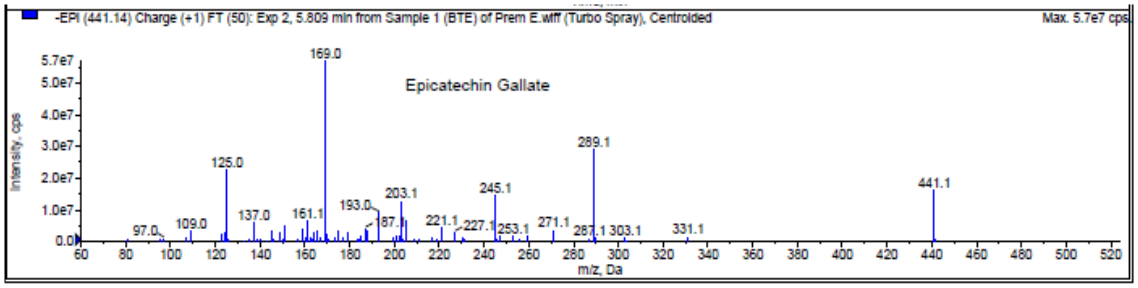

-EPI (289.12) Charge (+1) FT (50): Exp 2, 2.587 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centrolded
Max. 2.2e7 cps.
Epicatechin
Intensity, cps
m/z, Da
289.1
245.1
109.0
123.0
203.1
137.0
159.1
175.1
187.1
57.0
69.0
83.0
93.0
97.0
121.0
125.0
149.0
201.1
211.0
221.1
227.1
247.1
271.1

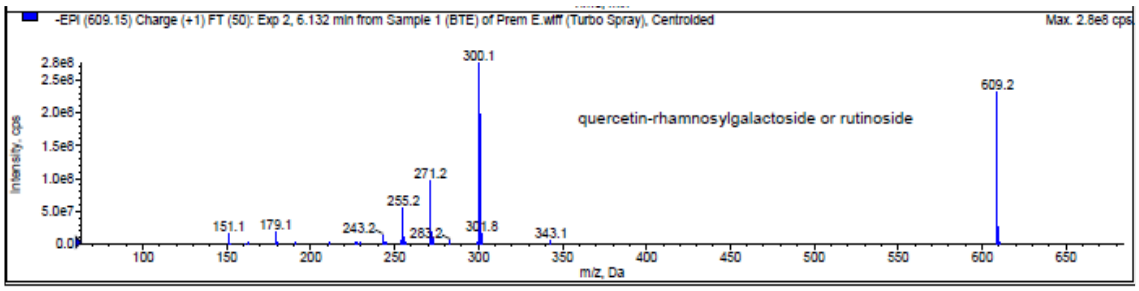

-EPI (457.12) Charge (+1) FT (50): Exp 2, 2.748 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centrolded
Max. 4.4e7 cps.
Epigallocatechin Gallate 457
Intensity, cps
m/z, Da
169.0
125.0
109.0
137.0
161.0
167.1
193.0
219.1
305.1
331.0
457.1

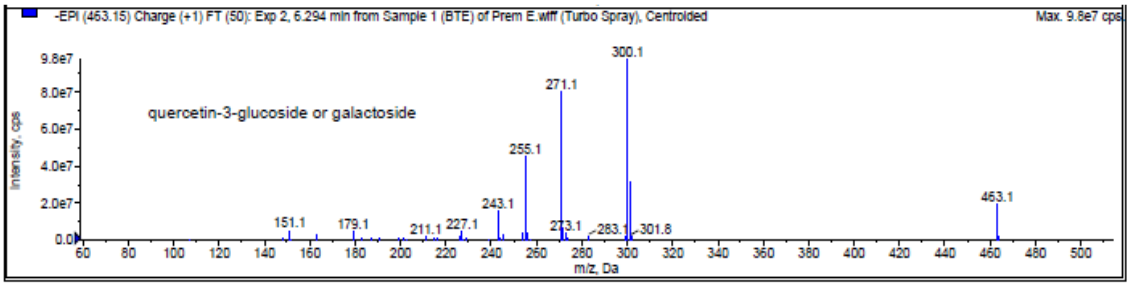

-EPI (169.08) Charge (+0) FT (50): Exp 2, 2.909 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centrolded
Max. 8.8e5 cps.
Gallic Acid
Intensity, cps
m/z, Da
125.0
67.0
69.0
123.9
169.1

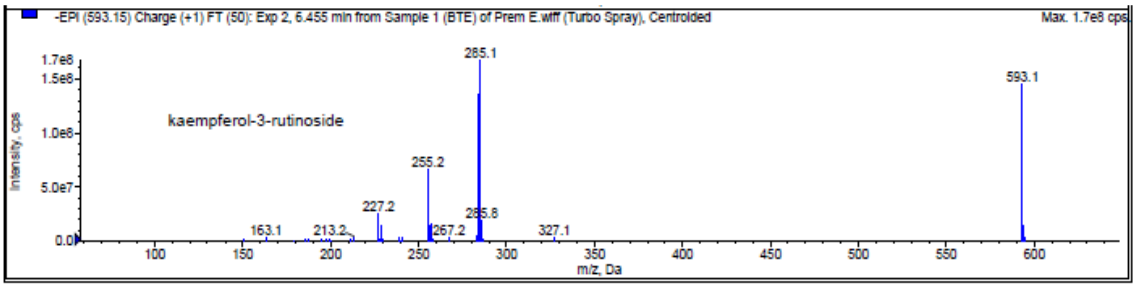

-EPI (593.20) Charge (+2) FT (50): Exp 2, 3.554 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centrolded
Max. 9.0e6 cps.
Dicaffeoquinic Acid Conjugate
Intensity, cps
m/z, Da
593.2
353.1
383.1
473.1
263.2
297.1
325.1
365.1
395.1
413.1
437.1
503.1
575.2

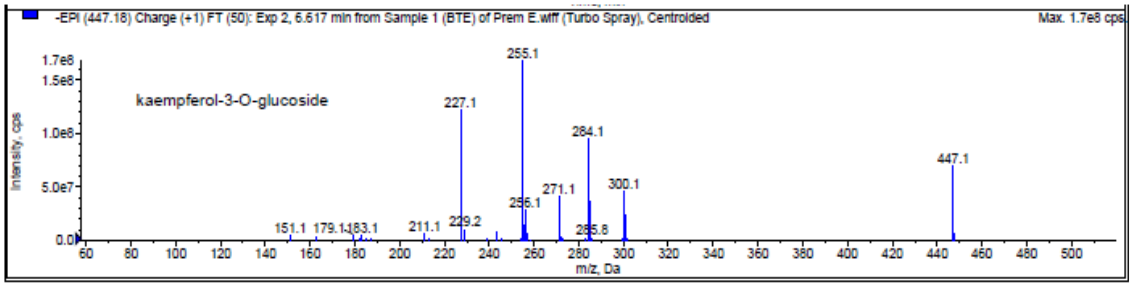

-EPI (563.15) Charge (+1) FT (50): Exp 2, 5.486 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centrolded
Max. 5.9e7 cps.
6-C-glucosyl-8-C-arabinosyl apigenin or arabinoysl-glucosyl apigenin
Intensity, cps
m/z, Da
563.2
353.1
383.1
297.2
443.1
191.1
247.1
263.2
307.1
325.1
353.8
365.1
395.1
413.1
473.1
503.1
545.2

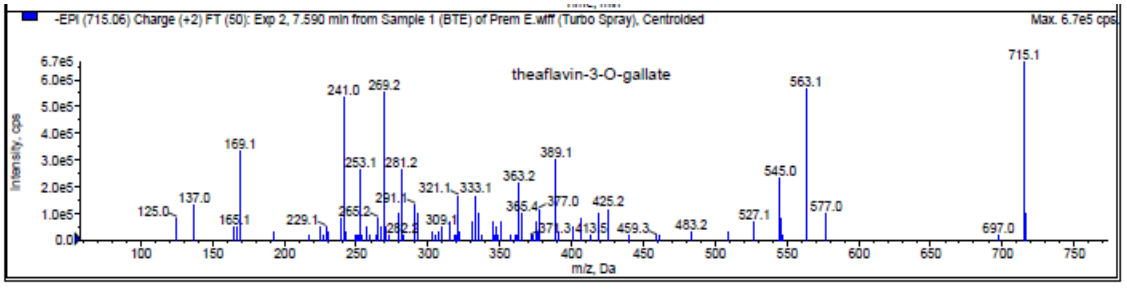

-EPI (441.14) Charge (+1) FT (50): Exp 2, 5.809 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centrolded
Max. 5.7e7 cps.
Epicatechin Gallate
Intensity, cps
m/z, Da
169.0
289.1
125.0
203.1
245.1
441.1
193.0
137.0
161.1
97.0
109.0
221.1
227.1
253.1
271.1
287.1
303.1
331.1


-EPI (609.15) Charge (+1) FT (50): Exp 2, 6.132 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 2.8e8 cps.
quercetin-rhamnosylgalactoside or rutinoside
Intensity, cps
m/z, Da

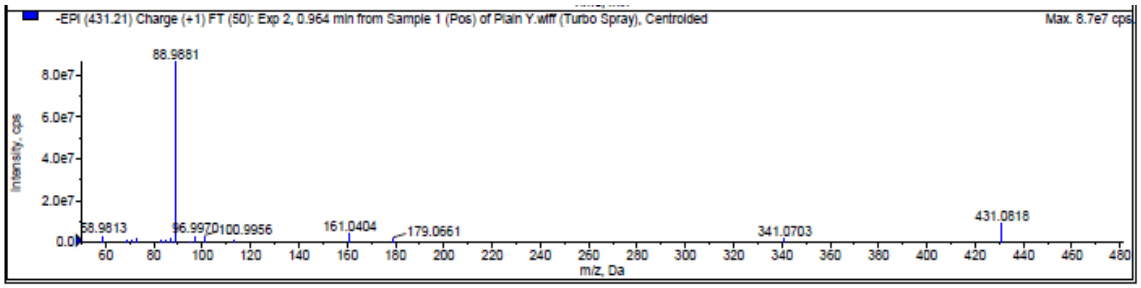

-EPI (463.15) Charge (+1) FT (50): Exp 2, 6.294 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 9.8e7 cps.
quercetin-3-glucoside or galactoside
Intensity, cps
m/z, Da

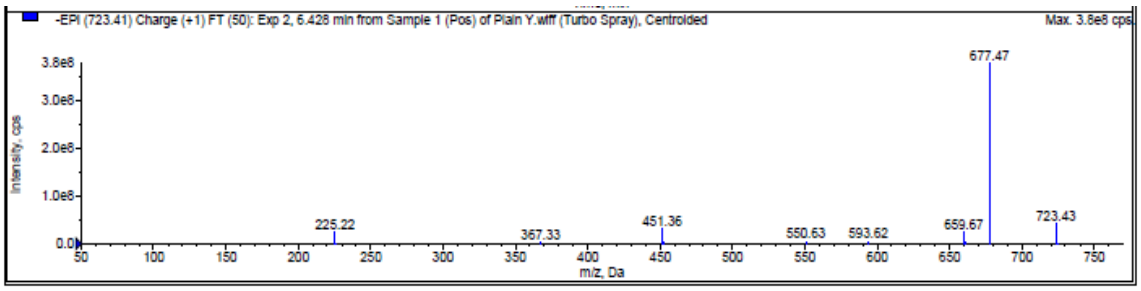

-EPI (593.15) Charge (+1) FT (50): Exp 2, 6.455 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.7e8 cps.
kaempferol-3-rutinoside
Intensity, cps
m/z, Da


-EPI (447.18) Charge (+1) FT (50): Exp 2, 6.617 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 1.7e8 cps.
kaempferol-3-O-glucoside
Intensity, cps
m/z, Da

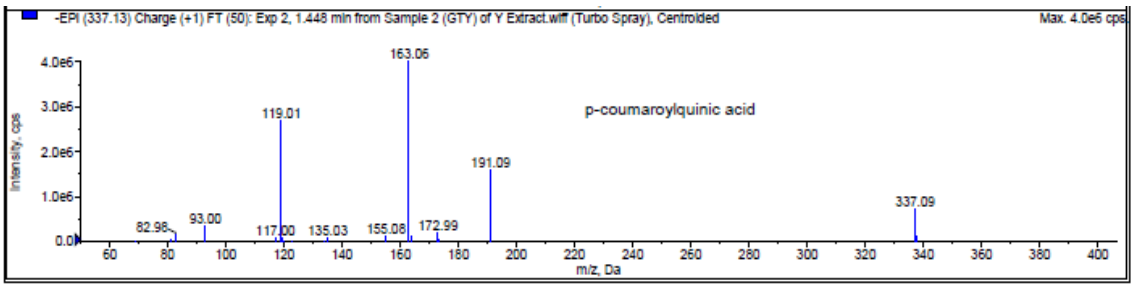

-EPI (715.06) Charge (+2) FT (50): Exp 2, 7.590 min from Sample 1 (BTE) of Prem E.wiff (Turbo Spray), Centroided
Max. 6.7e5 cps.
theaflavin-3-O-gallate
Intensity, cps
m/z, Da

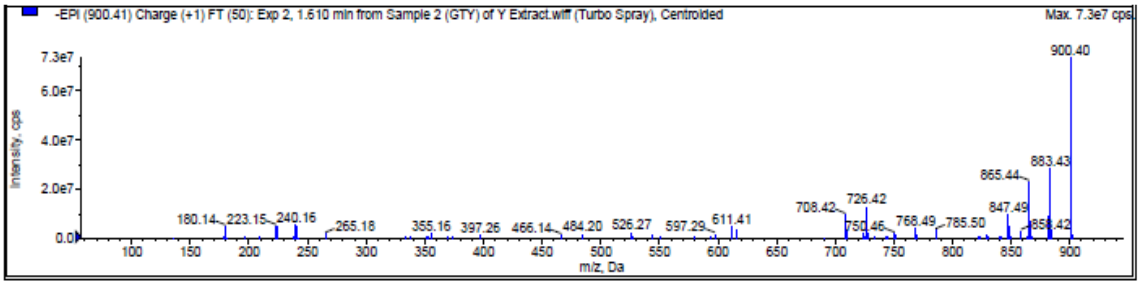
3.4. Mass spectra of each phenolic compound identified in PY

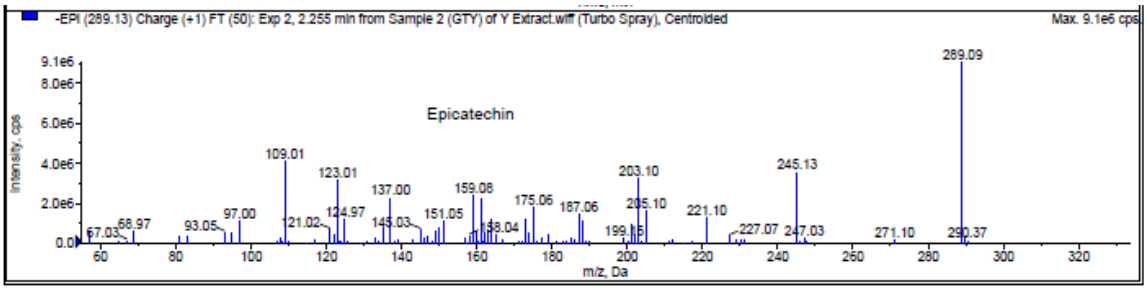


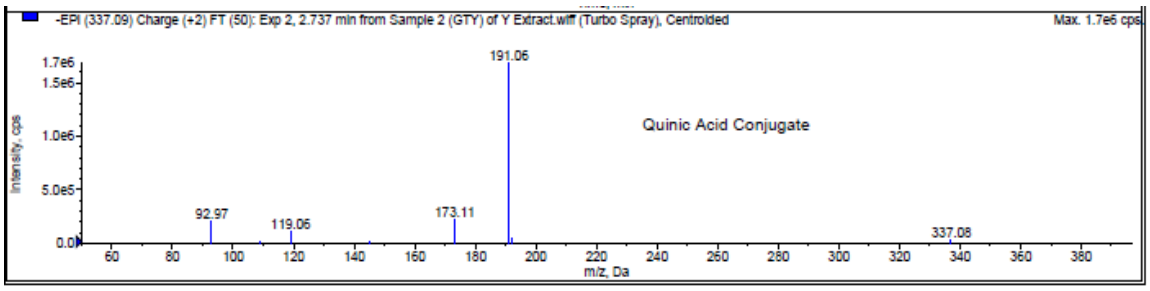


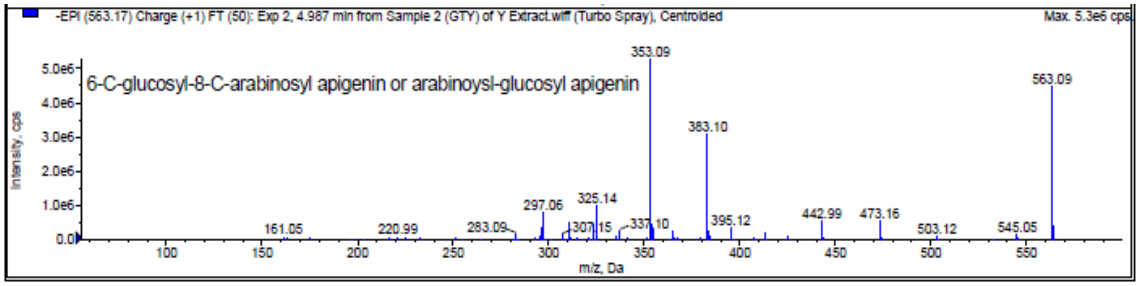
3.5. Mass spectra of each phenolic compound identified in GTY

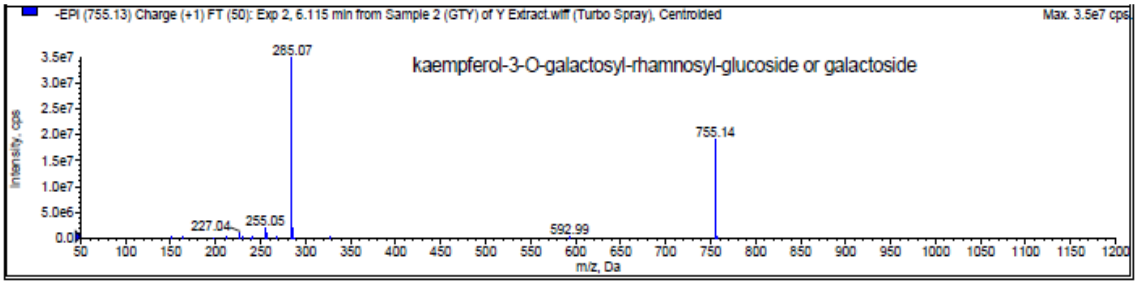


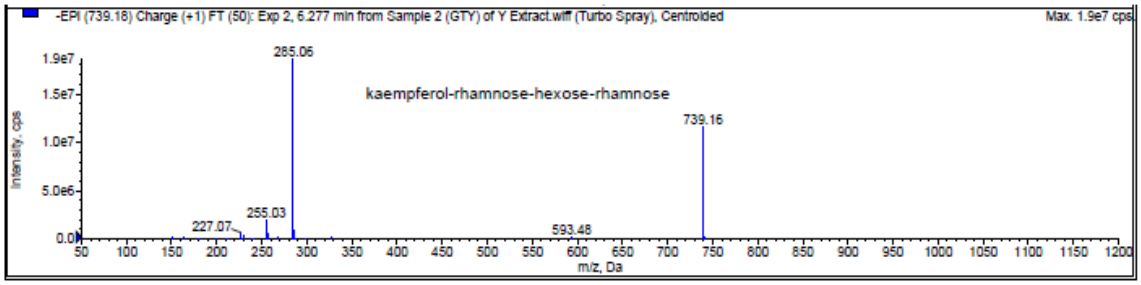


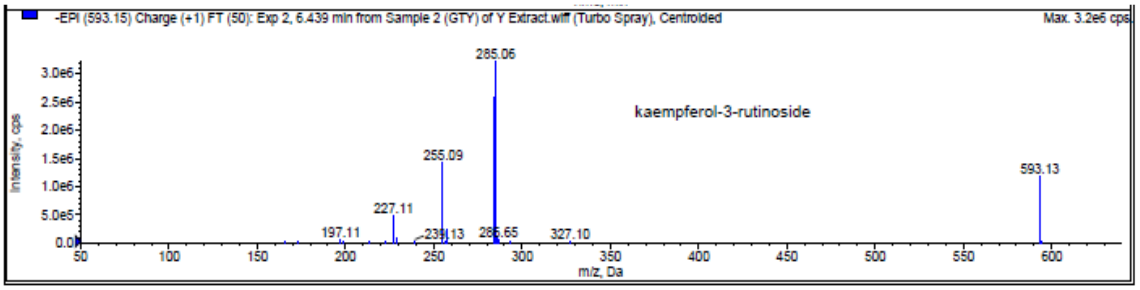


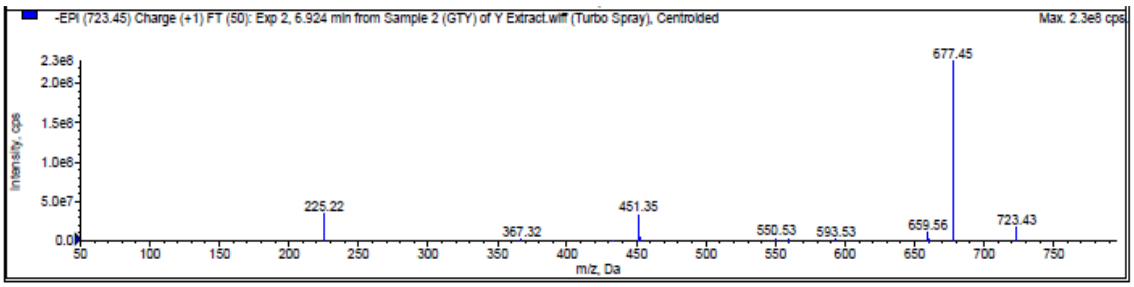

-EPI (337.09) Charge (+2) FT (50): Exp 2, 2.737 min from Sample 2 (GTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.7e6 cps.
Quinic Acid Conjugate
Intensity, cps
m/z, Da
191.06
92.97
119.06
173.11
337.08

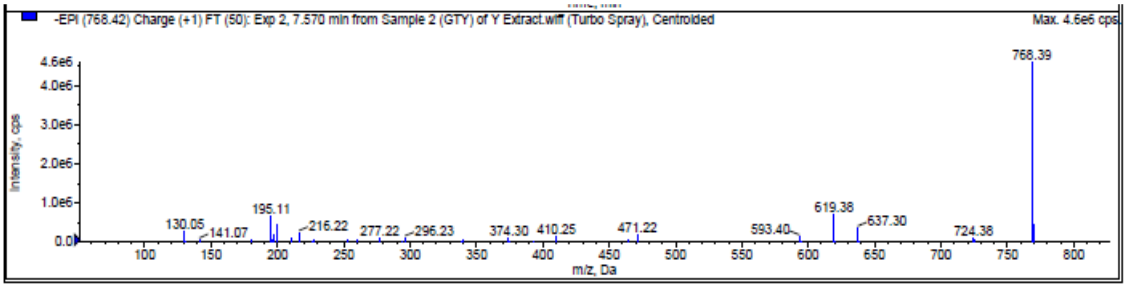

-EPI (563.17) Charge (+1) FT (50): Exp 2, 4.987 min from Sample 2 (GTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 5.3e6 cps.
6-C-glucosyl-8-C-arabinosyl apigenin or arabinoysl-glucosyl apigenin
Intensity, cps
m/z, Da
353.09
563.09
383.10
297.06
325.14
161.05
220.99
283.09
307.15
337.10
395.12
442.99
473.16
503.12
545.05

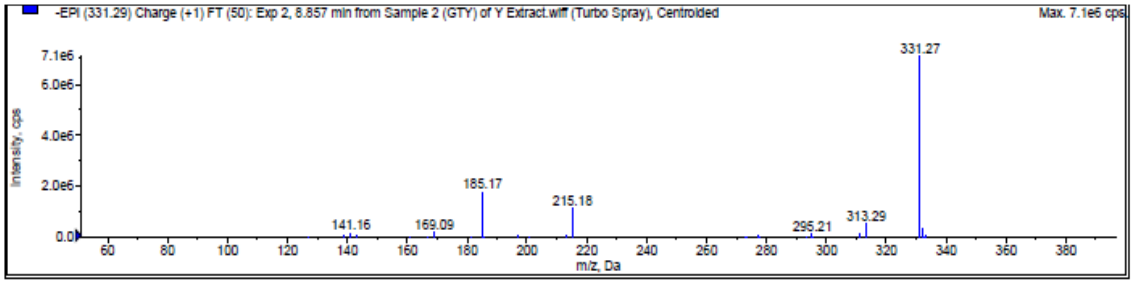

-EPI (755.13) Charge (+1) FT (50): Exp 2, 6.115 min from Sample 2 (GTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 3.5e7 cps.
kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
Intensity, cps
m/z, Da
285.07
755.14
227.04
255.05
592.99


-EPI (739.18) Charge (+1) FT (50): Exp 2, 6.277 min from Sample 2 (GTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.9e7 cps.
kaempferol-rhamnose-hexose-rhamnose
Intensity, cps
m/z, Da
285.06
739.16
227.07
255.03
593.48

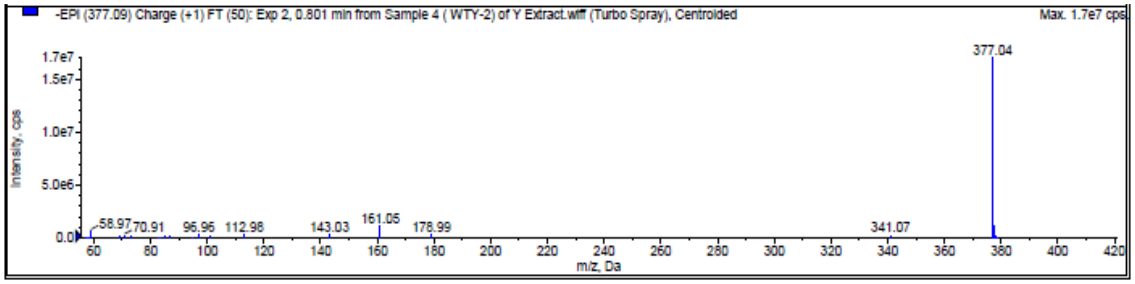

-EPI (593.15) Charge (+1) FT (50): Exp 2, 6.439 min from Sample 2 (GTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 3.2e6 cps.
kaempferol-3-rutinoside
Intensity, cps
m/z, Da
285.06
255.09
593.13
227.11
197.11
239.13
286.65
327.10

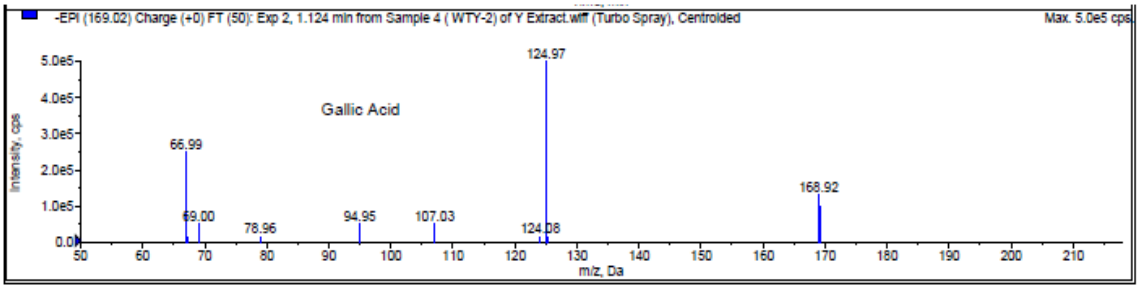


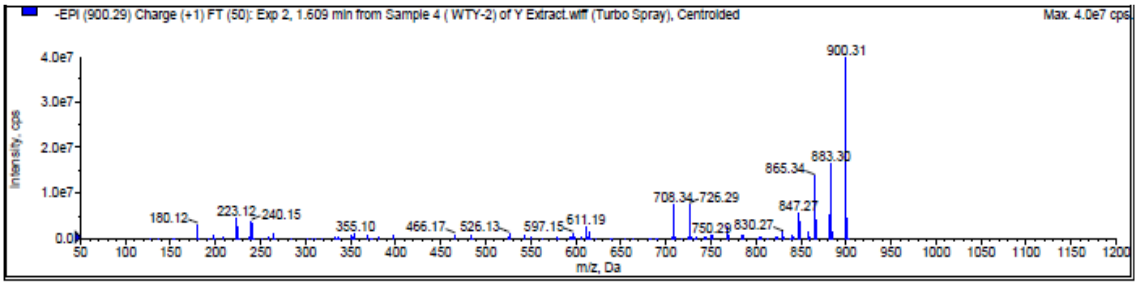


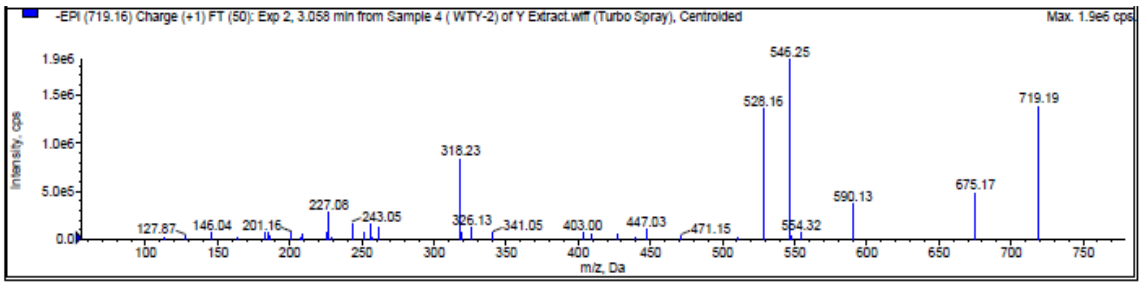


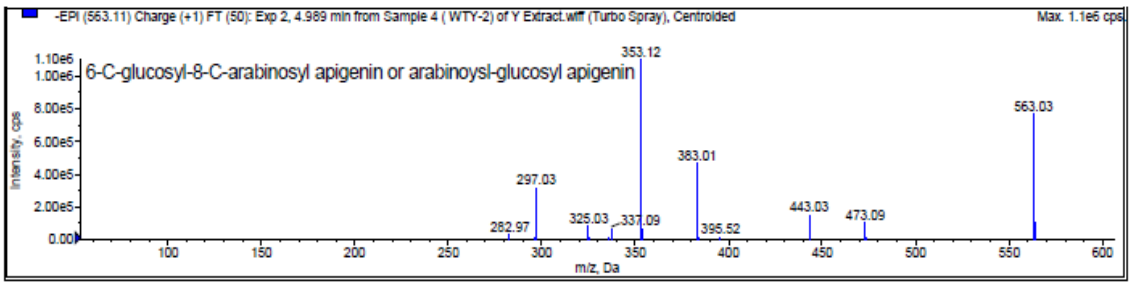
3.6. Mass spectra of each phenolic compound identified in WTY

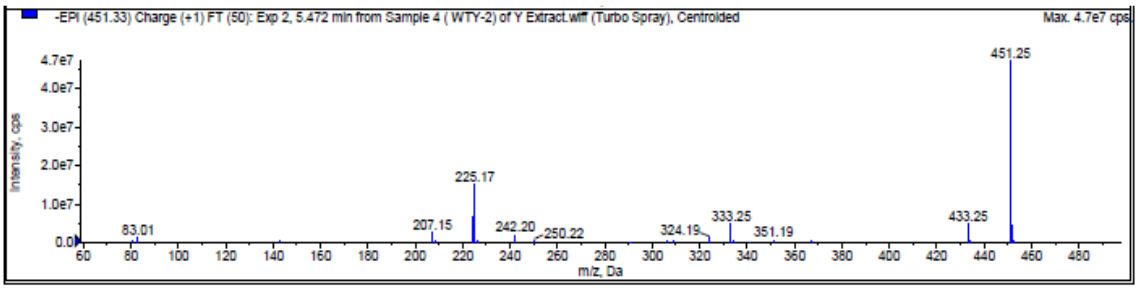


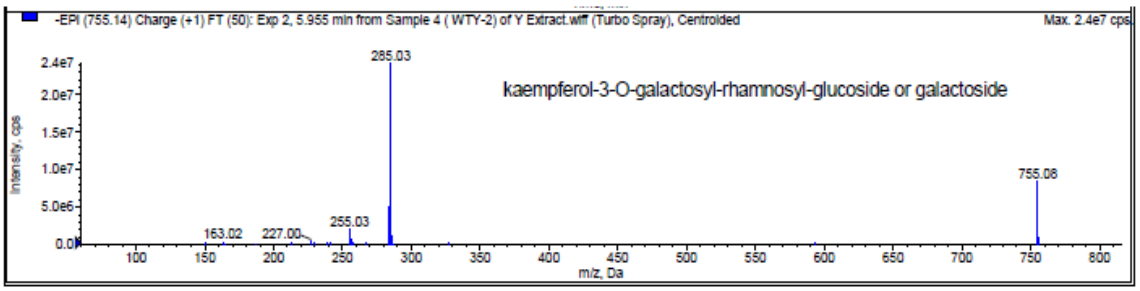


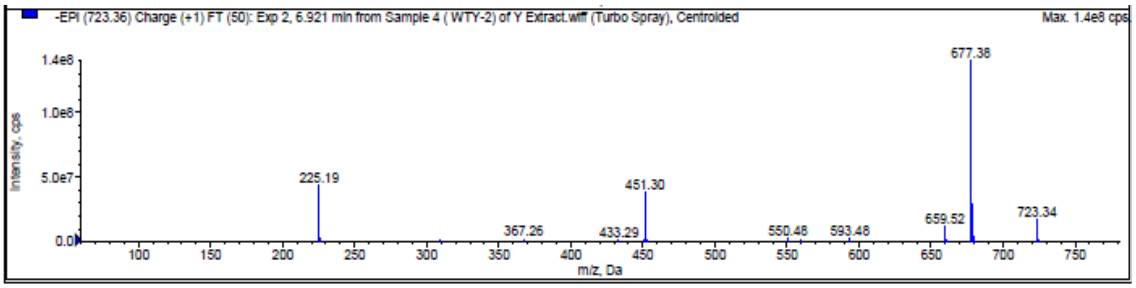


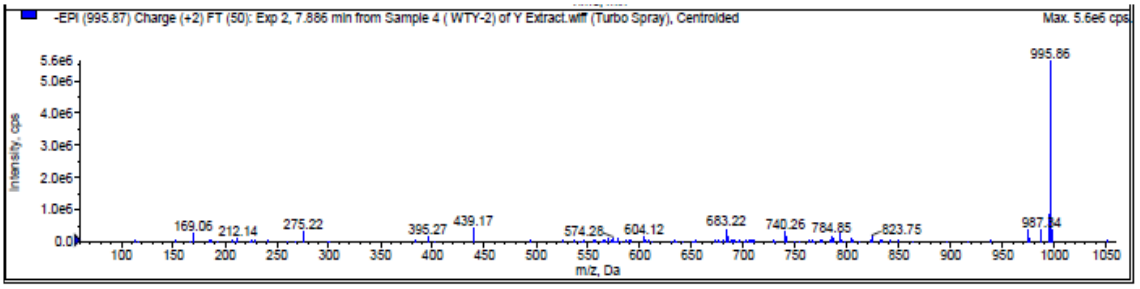

-EPI (719.16) Charge (+1) FT (50): Exp 2, 3.058 min from Sample 4 (WTY-2) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.9e6 cps.
Intensity, cps
m/z, Da
546.25
528.16
719.19
318.23
675.17
590.13
227.06
243.05
326.13
341.05
403.00
447.03
471.15
554.32
127.87
146.04
201.16


-EPI (563.11) Charge (+1) FT (50): Exp 2, 4.989 min from Sample 4 (WTY-2) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.1e6 cps.
6-C-glucosyl-8-C-arabinosyl apigenin or arabinoysl-glucosyl apigenin
Intensity, cps
m/z, Da
353.12
563.03
383.01
297.03
443.03
473.09
325.03
337.09
282.97
395.52

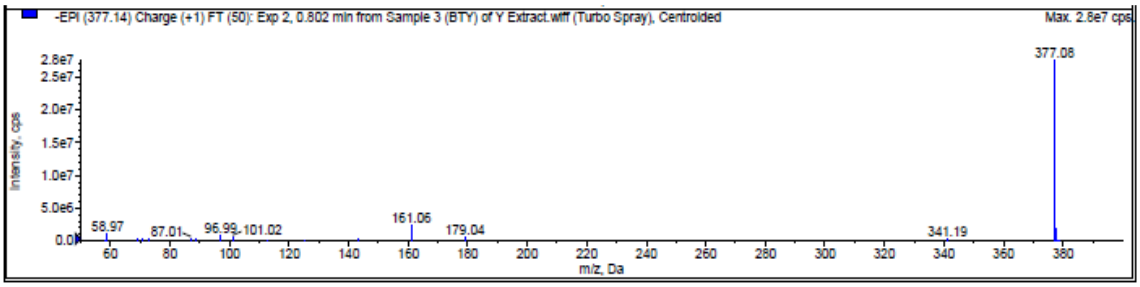

-EPI (451.33) Charge (+1) FT (50): Exp 2, 5.472 min from Sample 4 (WTY-2) of Y Extract.wiff (Turbo Spray), Centroided
Max. 4.7e7 cps.
Intensity, cps
m/z, Da
451.25
225.17
433.25
333.25
83.01
207.15
242.20
250.22
324.19
351.19

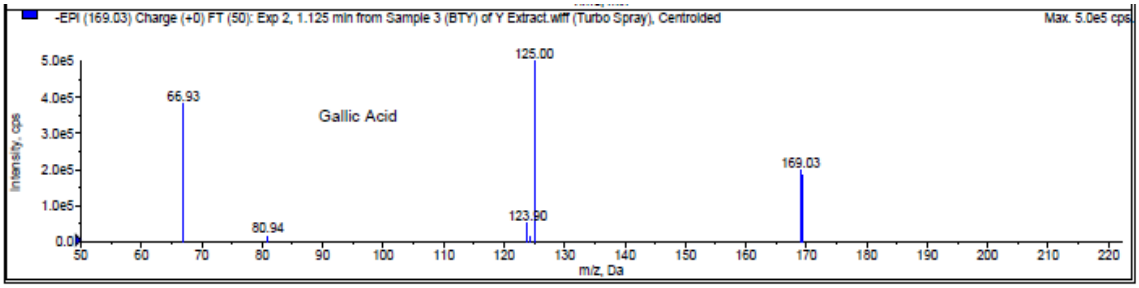

-EPI (755.14) Charge (+1) FT (50): Exp 2, 5.955 min from Sample 4 (WTY-2) of Y Extract.wiff (Turbo Spray), Centroided
Max. 2.4e7 cps.
kaempferol-3-O-galactosyl-rhamnosyl-glucoside or galactoside
Intensity, cps
m/z, Da
285.03
755.08
255.03
163.02
227.00

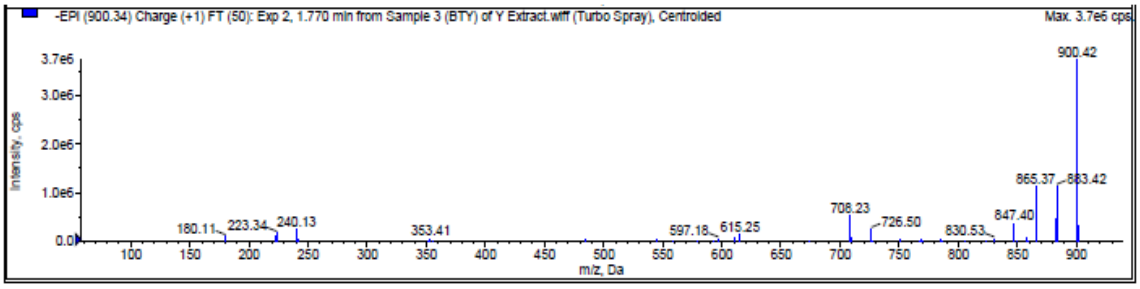

-EPI (723.36) Charge (+1) FT (50): Exp 2, 6.921 min from Sample 4 (WTY-2) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.4e8 cps.
Intensity, cps
m/z, Da
677.38
225.19
451.30
723.34
659.52
367.26
433.29
550.48
593.48

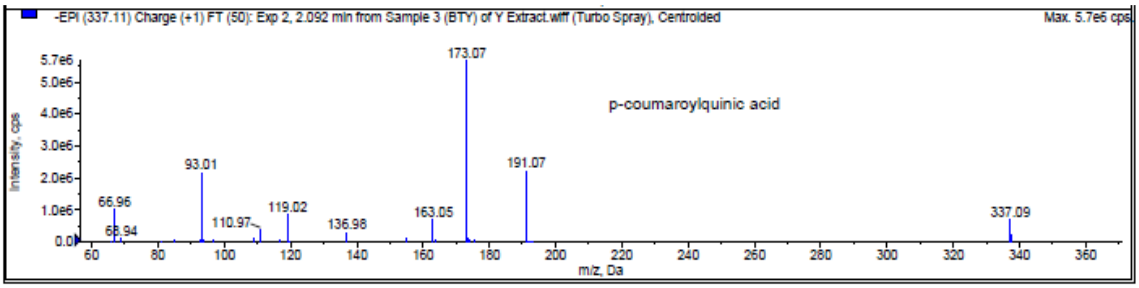

-EPI (995.87) Charge (+2) FT (50): Exp 2, 7.886 min from Sample 4 (WTY-2) of Y Extract.wiff (Turbo Spray), Centroided
Max. 5.6e6 cps.
Intensity, cps
m/z, Da
995.86
987.84
169.06
212.14
275.22
395.27
439.17
574.28
604.12
683.22
740.26
784.85
823.75

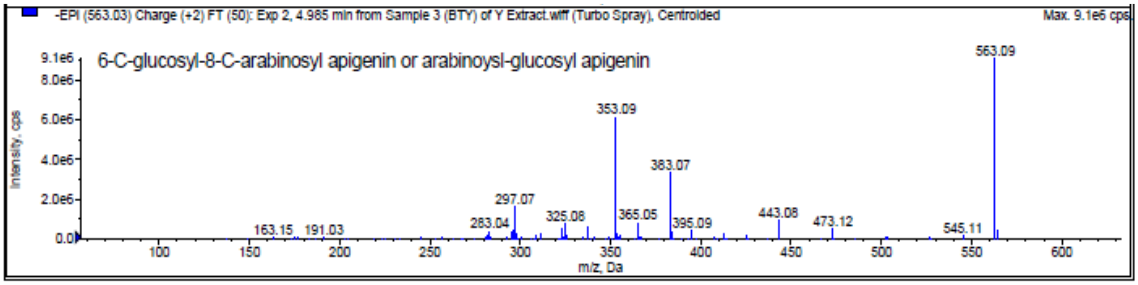
3.7. Mass spectra of each phenolic compound identified in BTY

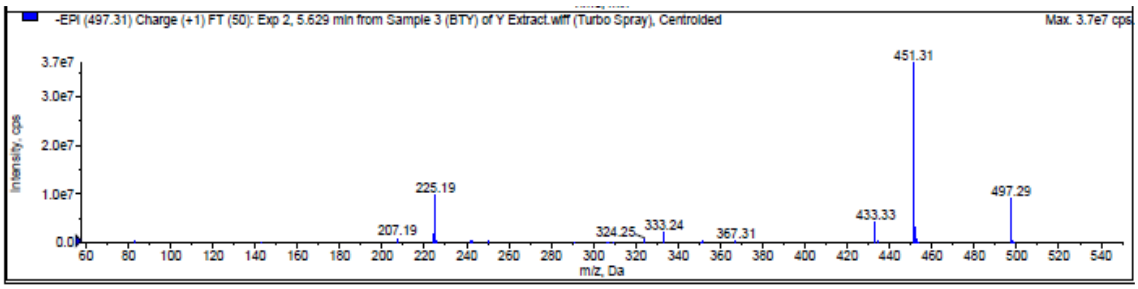


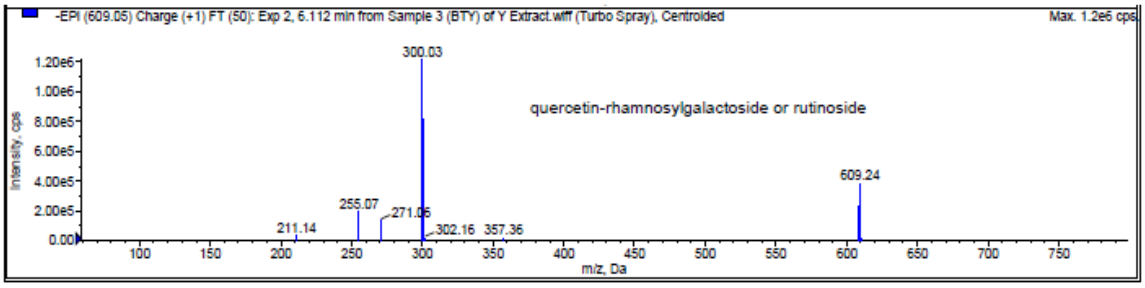


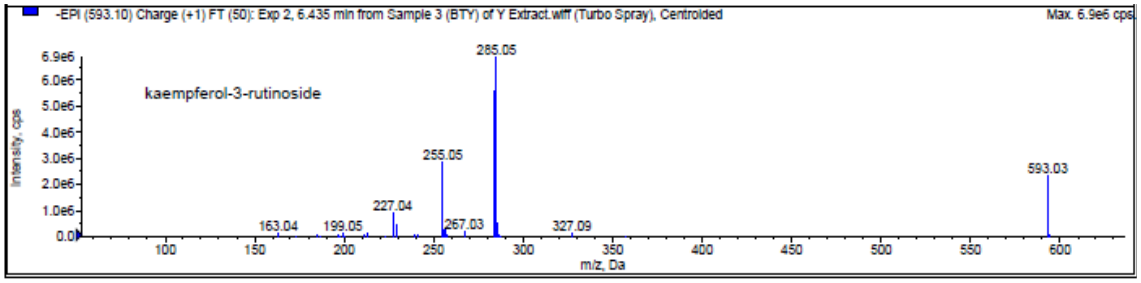


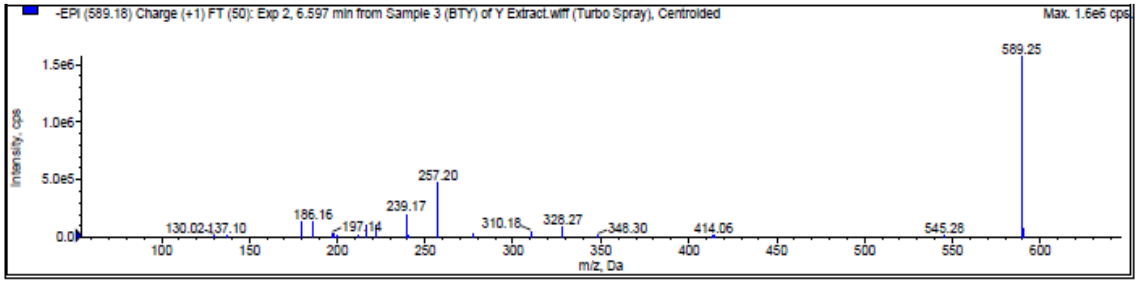


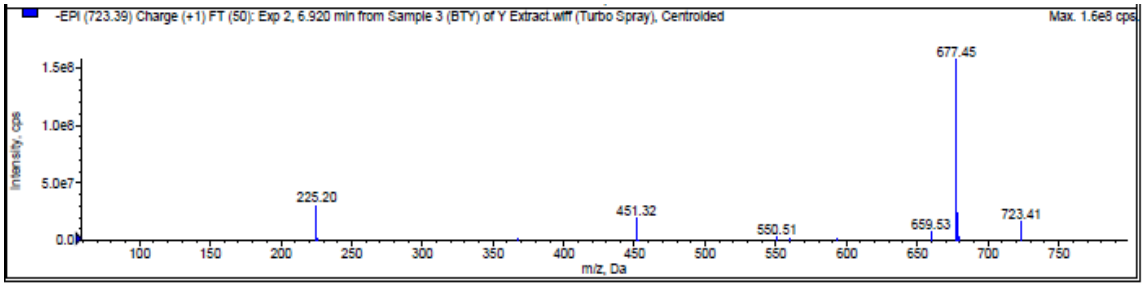


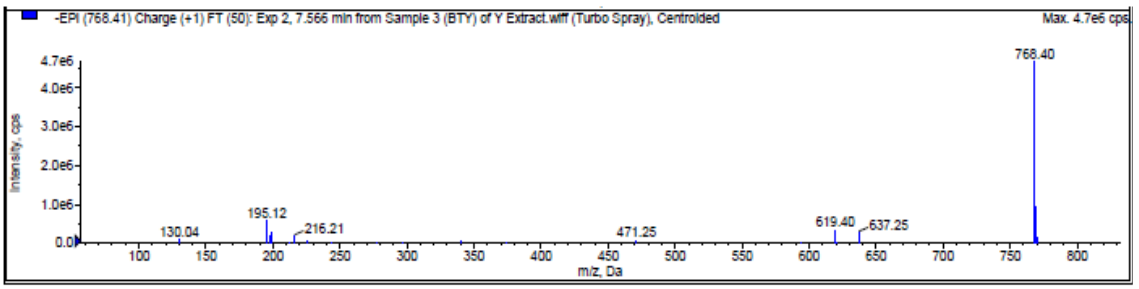

-EPI (497.31) Charge (+1) FT (50): Exp 2, 5.629 min from Sample 3 (BTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 3.7e7 cps
Intensity, cps
m/z, Da
451.31
225.19
497.29
433.33
207.19
324.25
333.24
367.31

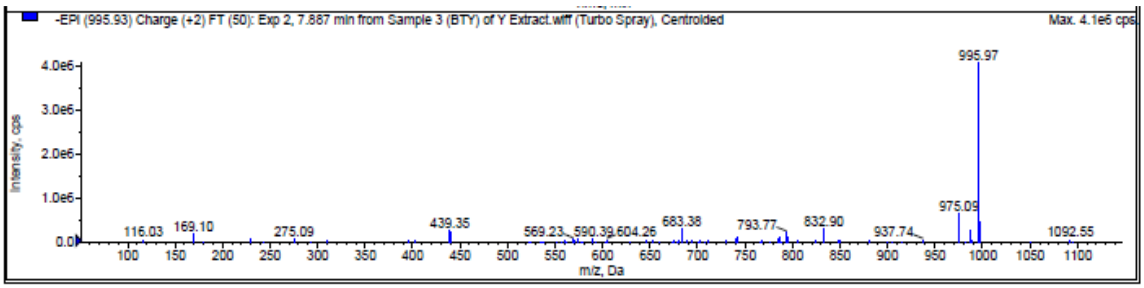

-EPI (609.05) Charge (+1) FT (50): Exp 2, 6.112 min from Sample 3 (BTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.2e6 cps
Intensity, cps
m/z, Da
300.03
quercetin-rhamnosylgalactoside or rutinoside
609.24
255.07
271.06
211.14
302.16
357.36


-EPI (593.10) Charge (+1) FT (50): Exp 2, 6.435 min from Sample 3 (BTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 6.9e6 cps
Intensity, cps
m/z, Da
285.05
kaempferol-3-rutinoside
255.05
593.03
227.04
163.04
199.05
267.03
327.09


-EPI (589.18) Charge (+1) FT (50): Exp 2, 6.597 min from Sample 3 (BTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.6e6 cps
Intensity, cps
m/z, Da
589.25
257.20
239.17
186.16
130.02
137.10
197.14
310.18
328.27
348.30
414.06
545.28


-EPI (723.39) Charge (+1) FT (50): Exp 2, 6.920 min from Sample 3 (BTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 1.6e8 cps
Intensity, cps
m/z, Da
677.45
225.20
451.32
550.51
659.53
723.41


-EPI (768.41) Charge (+1) FT (50): Exp 2, 7.566 min from Sample 3 (BTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 4.7e6 cps
Intensity, cps
m/z, Da
768.40
195.12
130.04
216.21
471.25
619.40
637.25


-EPI (995.93) Charge (+2) FT (50): Exp 2, 7.887 min from Sample 3 (BTY) of Y Extract.wiff (Turbo Spray), Centroided
Max. 4.1e6 cps
Intensity, cps
4.0e6
3.0e6
2.0e6
1.0e6
0.0
995.97
975.09
116.03
169.10
275.09
439.35
569.23
590.39
604.26
683.38
793.77
832.90
937.74
1092.55
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
1050
1100
m/z, Da