To whom it may concern

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Natalie d'Avila

Stability of ultra-high temperature (UHT) processed beverages infused with white tea (*Camellia sinensis*) and grape seed (*Vitis vinifera*) extracts

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Food Technology

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Abstract

Camellia sinensis and grape seed extracts have been associated with health benefits due to their high phenolic content. White tea, derived from young buds and leaves of *Camellia sinensis*, is high in catechins. Grape seeds are also high in catechins, as well as gallic acid and proanthocyanidins. In addition, it has been suggested that grape phenolics may interact synergistically with *Camellia sinensis* extracts, increasing their biological activities.

Although white tea and grape seed extracts can be easily incorporated into beverages, the impact of processing conditions on the stability of bioactive compounds ought to be considered when developing functional beverages. The stability of white tea and grape seed phenolics can be affected by processing and storage conditions, such as temperature, pH and beverage ingredients. Catechins may undergo changes through epimerisation, oxidation and polymerisation, altering the chemical profile of the finished product. The impact of heat treatment on the stability of catechins may be reduced by applying heat for a shorter period of time. Hence, ultra-high temperature (UHT) processing might be a suitable technique to commercially sterilise functional beverages without significantly affecting grape seed and white tea phenolic compounds. There is, however, a lack of information available on the impact of UHT treatment on white tea and grape seed polyphenols. The aim of this project was to evaluate the stability of phenolics, methylxanthines and antioxidant activity in UHT-treated beverages infused with white tea and grape seed (*Vitis vinifera* var. Sauvignon Blanc) extracts during storage.

This study comprised two integrated phases. The first phase aimed at determining the optimum UHT processing temperature based on chemical, microbiological and sensory characteristics of the beverages infused with white tea and grape seed extracts. In this experiment, one commercial formulation (mango-flavoured beverage) was UHT-treated at four temperature levels (110, 120, 130 and 140 °C) for 5 s. The UHT-treated beverages in this study contained catechins, gallic acid, caffeine, and theobromine. The beverages were also expected to contain other phenolics and methylxanthines, which were not investigated. The levels of theobromine and caffeine in the beverages were stable following UHT processing. Even though the impact of UHT-treatment (at 110, 120, 130 and 140 °C for 5 s) on the stability of phenolics in the RTD mango-flavoured beverages was not significant ($P > 0.05$), the levels of GCG increased slightly following UHT processing, and EGCG decreased following treatment at 140 °C, suggesting that epimerisation of catechins may have occurred. Therefore, the optimum UHT treatment conditions chosen for further work were 130 °C for 5 s.

In the second phase of the study, the previously determined UHT processing temperature (130 °C) was used to process pomegranate, mango and nectarine beverages. Microbiological, chemical, and sensory analyses were conducted to investigate the stability of the three UHT-treated beverages stored for 90 days at 20 °C and 40 °C.

Yeasts and moulds were not present in the three UHT-treated beverages stored for up to 42 days. Moulds initially present in the packaging may have contributed to the mould growth in the beverages observed after 63 days of storage, since moulds that have been injured during processing are able to recover during storage. The beverages stored for 90 days at both 20 °C and 40 °C showed acceptable flavour. However, the beverages stored at 40 °C were less well liked ($P < 0.05$), possibly due to the presence of sediment in the beverages stored at 40 °C. The darkening of the beverages stored at 40 °C, possibly caused by oxidation of phenolics, may also have contributed to the lower appearance acceptance scores of the beverages.

Antioxidant capacity of the beverages decreased ($P < 0.05$) after 90 days of storage, while total phenolics ($P < 0.05$) increased slightly. The compositional differences in the formulation of the beverages affected ($P < 0.05$) the phenolic compounds and antioxidant capacity of the products during the 90-day period of storage, suggesting that the different levels of citric acid and ascorbic acid in the beverages could have affected the stability of the phenolics. Epimerisation of catechins may have occurred in the beverages during storage, increasing (+)-catechin levels. Gallic acid also increased ($P < 0.05$) in the beverages during storage, possibly as a product of hydrolysis. Decreases ($P < 0.05$) in the levels of ECG, EGCG, GCG, and EC in the beverages were also observed during storage. Oxidation appeared to be the main reaction responsible for degradation of the catechins in the beverages. In addition, the higher storage temperature (40 °C) increased degradation of catechins and formation of gallic acid. Since catechins are effective radical scavengers, degradation of catechins may partially explain the decrease in antioxidant capacity of the UHT-treated beverages during storage.

In conclusion, the UHT-treated beverages used in this study contained a unique mixture of chemical compounds that may prove beneficial to human health. However, the storage-induced changes in the phenolic profile of the beverages could exert an impact on the efficacy of any potential biological effects of the beverages. These findings contribute to understanding the composition and stability of phenolics and methylxanthines in UHT-treated beverage systems, and this information can be used to improve both the formulation and storage strategies for high-quality beverages infused with white tea and grape seed extracts.

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List of Abbreviations

AAPH	2,2′-Azobis (2-amidino-propane) dihydrochloride
ANOVA	Analysis of variance
cfu	Colony forming units
CG	(-)-Catechin gallate
CIP	Cleaning-in-place
CV	Coefficient of variance
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DAD	Diode array
EC	(-)-Epicatechin
ECG	(-)-Epicatechin gallate
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin gallate
FDA	Food and Drug Administration
FL	Fluorescein
GC	(-)-Gallocatechin
GCG	(-)-Gallocatechin gallate
GLM	General Linear Model
h	Hour
HAT	Radical quenching via hydrogen atom transfer
HPLC	High-performance liquid chromatography
HTST	High-temperature short-time
LDL	Low-density lipoproteins
MF	Microfiltration
ORAC	Oxygen radical absorbance capacity
PDA	Potato dextrose agar
Re	Reynolds number
RTD	Ready-to-drink
s	Seconds

SD	Standard deviation
SET	Single electron transfer
TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluoroacetic acid
UF	Ultrafiltration
UHT	Ultra-high temperature
YGC	Yeast glucose agar with chloramphenicol

Chapter 1 Introduction

The term “functional food”, which originated in Japan in the 1980’s (Gibson & Williams, 2000), is defined as a food product, but not isolated individual components, likely to exert a health or physiological effect when consumed as part of an ordinary diet (Prado et al., 2008).

The demand for functional food products is high as emerging knowledge on nutrient function and bioactive components increases (Day et al., 2009). The functional beverages market is one of the fastest growing segments within the functional food category (Gruenwald, 2009). Plant ingredients with high phenolic content have been incorporated into beverages, such as tea or juices, and marketed as functional products by claiming energy, anti-aging or relaxing effects (Gruenwald, 2009).

Phenolic compounds are secondary metabolites of plants that can reduce the oxidative damage caused by exogenous chemicals and endogenous metabolic processes in the human body (Bravo, 1998; Carvalho et al., 2003; Balasundram et al., 2006). Minimally processed plant foods and beverages, such as juices, are the main sources of phenolic compounds with high antioxidant activity in the human diet (Balasundram et al., 2006; Haleem et al., 2008). Internationally, there are no clear guidelines on daily intake of phenolic compounds. The World Health Organization (WHO), however, suggests that a high intake of plant ingredients is associated with the prevention of chronic diseases, such as coronary heart diseases and diabetes, and has recommended the intake of a minimum of 400 g of fruits and vegetables per day, excluding potatoes and other starchy tubers (WHO, 1990).

Low dietary antioxidant intake within different populations has been associated with low intake of tea, cereals, fruits and vegetables (Phillips et al., 2000; Haleem et al., 2008; Joudalová & Réblová, 2012; Payne et al., 2012). Despite the health benefits associated with the consumption of plant ingredients, the low antioxidant intake of some populations has been reported (Kristjansdottir et al., 2006; Haleem et al., 2008; Wootton-

Beard & Ryan, 2011). It has been estimated that over 80% of the population in the United Kingdom consumes less than 400 g of fruit and vegetables per day, with a mean antioxidant intake of 560 μmol day^{-1} (Haleem et al., 2008). Therefore, the food industry has an important role in increasing the population's natural antioxidants intake and must be committed to delivering convenient, healthier food products that are high in natural antioxidants (Balasundram et al., 2006; Wootton-Beard & Ryan, 2011).

Camellia sinensis and grape seed extracts, which can be successfully incorporated into foods and beverages, have been associated with health benefits due to their high phenolic content (Inoue et al., 2001; Rietveld & Wiseman, 2003; Morré & Morré, 2006; Sakurai et al., 2010). White tea, derived from young buds and leaves of *Camellia sinensis*, is the least processed form of tea and is higher in phenolic compounds than most types of tea derived from *Camellia sinensis*, such as black tea (fully fermented) and oolong tea (semi-fermented) (Higdon, 2007; Sharangi, 2009). Flavanol monomers, also known as catechins, are the most abundant group of phenolic compounds in fresh leaves of *Camellia sinensis* (Hilal & Engelhardt, 2007; Zhao et al., 2011). The biological activity of white tea extract has not been studied as thoroughly as other types of common tea, particularly green tea. However, its antimicrobial, anti-inflammatory and anti-cancer properties have been reported (Santana-Rios et al., 2001; Islam, 2011; Thring et al., 2011). While white tea, derived from *Camellia sinensis,* may not be readily available, grape seed and skins are abundant in various countries, including New Zealand, as by-products of the grape and wine industry, and commercially represent a potential source of natural antioxidants (Balasundram et al., 2006; Bekhit et al., 2011). Grape seeds are high in catechins and proanthocyanidins (Montealegre et al., 2006), and the antioxidant (Shao et al., 2003), antiviral (Bekhit et al., 2011), anti-inflammatory (Sakurai et al., 2010) and anticancer (Morré & Morré, 2006) properties of their phenolics have been reported. In addition, grape phenolics may interact synergistically with *Camellia sinensis* extracts, increasing their biological activities (Morré & Morré, 2006; Bekhit et al., 2011).

Consumers of functional beverages expect the quality of the product to be maintained at a high standard from production to consumption (Kilcast & Subramaniam, 2000). In addition, processed beverages face challenges associated with chemical stability and microbiological deterioration, mainly due to yeasts and moulds (Pitt & Hocking, 2009; Tanaka et al., 2011). Although the market is saturated with a range of effective

preservatives that can potentially be used in beverages, their use is becoming undesirable, as consumers are now demanding products without preservatives. In fact, the use of preservatives in foods and beverages has been discouraged in some countries, such as Australia and Korea (Evans et al., 2010; Shim et al., 2011).

Several preservation techniques have been used to process functional foods and obtain commercially sterile products, such as membrane technology, high pressure processing and heat treatment (Sánchez-Moreno et al., 2005; Bazinet et al., 2010; Cassano et al., 2010). Despite the main goal of preservation techniques, that is, to achieve a shelf stable product, the impact of processing conditions on the stability of bioactive compounds ought to be considered when developing functional foods.

The stability of phenolic compounds in grape and tea extracts is affected by processing and storage conditions, such as temperature, oxygen level, pH, and presence of metal ions (Bazinet et al., 2010; Ananingsih et al., 2011). Catechins in foods may undergo changes through epimerisation, oxidation and polymerisation during processing and storage, changing the chemical profile of the finished product (Bazinet et al., 2010; Ananingsih et al., 2011). Heat pasteurisation is known to cause degradation of phenolic compounds in foods (Rawson et al., 2011); therefore, the impact of heat treatment on the stability of catechins may be reduced by applying heat for a shorter period of time (Bazinet et al., 2010). It is possible that high temperature with short time, particularly ultra-high temperature (UHT) processing, might be able to commercially sterilise functional beverages without significantly affecting grape seed and white tea phenolic compounds. There is, however, lack of information available on the impact of UHT treatment on white tea and grape seed polyphenols.

Thus, there are two main challenges facing the functional beverage industry: firstly, to produce beverages with high and stable phenolic content, and, secondly, to achieve a shelf stable product by using effective processing technologies without the use of chemical preservatives. The present study aims to investigate the impact of UHT-treatment on beverages infused with white tea (*Camellia sinensis*) and grape seed (*Vitis vinifera* var. Sauvignon Blanc) extracts, and the stability of UHT-treated beverages during storage.

Chapter 2 Literature Review

2.1 Plant phenolics

Phenolic compounds constitute one of the most widely distributed groups of substances in plants (Bravo, 1998; Carvalho et al., 2003). Plant phenolics appear to have an important function in a plant's life cycle, including their role as attractants for pollinators and seed-dispersing animals, as well as ultraviolet (UV) protectants (Jaganath & Crozier, 2010). These secondary metabolites of plants are characterised by an aromatic ring possessing one or more hydroxyl substituents (Carvalho et al., 2003).

Free radicals are generated by exogenous chemicals or endogenous metabolic processes in food systems or the human body and may cause oxidative damage by oxidising biomolecules (Carvalho et al., 2003). Phenolic compounds derived from plant metabolism may reduce the oxidative damage on the human body and have been extensively studied due to their beneficial effects to human health (Bagchi et al., 2000; Liu et al., 2002; Cho et al., 2009). The biological actions of phenolics as antioxidants originate from their free radical scavenging, metal chelating, reducing properties and their effects on cell-signaling pathways and gene-expression (Bravo, 1998; Yoneda & Nakatsubo, 1998; Balasundram et al., 2006; Fraga et al., 2010).

2.1.1 Classification of plant phenolics

Phenolic compounds may be classified according to their chemical structure, ranging from simple to complex structures (Carvalho et al., 2003). The main classes of dietary phenolics are flavonoids, phenolic acids, and tannins (King & Young, 1999).

2.1.1.1 Flavonoids

Flavonoids, which represent the largest group of plant phenolics, display a basic structure of 15 carbon atoms with two aromatic rings bound through a three-carbon chain as shown in Figure 2.1 (Bravo, 1998).

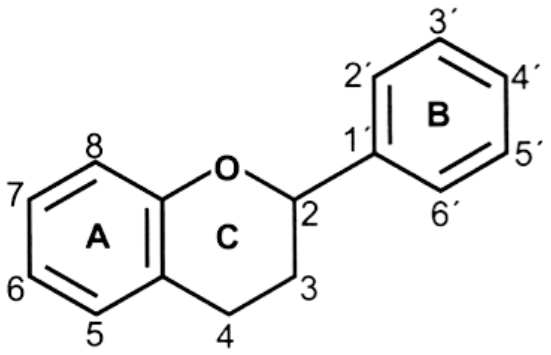


Figure 2.1 General chemical structure and carbon numbering system of flavonoids (A and C = aromatic rings, B = oxygenated heterocyclic ring) (Bravo, 1998).

Flavonoids are present either in a free or conjugated form, in which case they are often esterified to sugar molecules through at least one hydroxyl group, referred to as flavonoid glycosides (Bravo, 1998; Zuanazzi & Montanha, 2003). Flavonoids are of economic interest due to their pharmacological and functional properties (King & Young, 1999; Zuanazzi & Montanha, 2003). Some subclasses of flavonoids are used as leather dyes (Zuanazzi & Montanha, 2003), while others are reported to have anti-inflammatory (Zhang et al., 1997; Thring et al., 2011), antioxidant (Thring et al., 2009), anti-cancer (Sen et al., 2009; Trudel et al., 2012) and antiviral properties (Bekhit et al., 2011).

Flavonoids are grouped into six subclasses based on their chemical structures and biological properties: anthocyanins, flavanols, flavones, isoflavones, flavanones and flavonols (Heim et al., 2002). Anthocyanins are molecules of red, blue and purple pigment, while flavanols, flavonols, flavones and isoflavones are colourless or white to yellow molecules (King & Young, 1999; Zuanazzi & Montanha, 2003). Flavanols (flavan-3-ols) represent the most common flavonoid group consumed by humans and can

be found in tea shoots, chocolate and a wide variety of fruits, including grapes, berries, and apples (Yao et al., 2004; Aron & Kennedy, 2008; Fraga & Oteiza, 2011). The basic chemical structure of flavanols is illustrated in Figure 2.2.

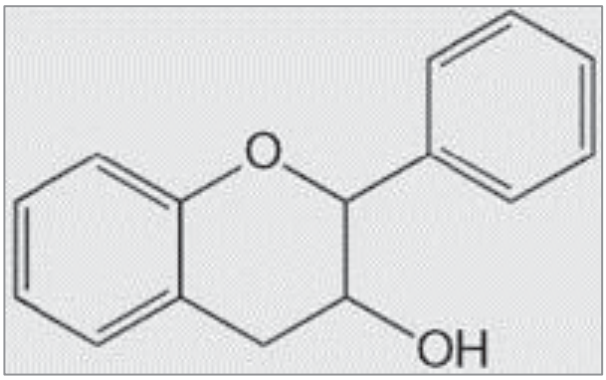


Figure 2.2 Basic chemical structure of flavanols and tannins (Valls et al., 2009).

The most abundant flavanols in plants are (+)-catechin and (-)-epicatechin (Tsanova-Savova et al., 2005; Ghassempour et al., 2011). Catechins are available in nature as two geometrical isomers: catechin in *trans*-form and epicatechin in *cis*-form (Tsuchiya, 2001). These structures exist as four isomers: (+)-catechin, (-)-catechin, (+)-epicatechin, and (-)-epicatechin (Tsuchiya, 2001). Flavanols can undergo esterification with gallic acid to form catechin gallates, and hydroxylation reactions to form gallocatechins, such as (-)-epigallocatechin and (-)-epicatechin gallate (Jaganath & Crozier, 2010). Tannin acyl hydrolase (tannase) catalyses the hydrolysis of the gallic acid esters, releasing gallic acid (Lu et al., 2009). In the presence of tannase, (-)-epigallocatechin 3-gallate (EGCG) and (-)-epicatechin 3-gallate (ECG) are hydrolysed into (-)-epigallocatechin (EGC) and (-)-epicatechin (EC), respectively (Lu et al., 2009).

The solubility of flavonoids is determined by the position occupied by the sugar moiety, the degree of unsaturation, and the nature of the substituent on the aromatic ring (Zuanazzi & Montanha, 2003). Flavonoid glycosides are usually water-soluble and insoluble in most organic solvents, while aglycones (flavonoids without the sugar group) are usually soluble in non-polar organic solvents (Zuanazzi & Montanha, 2003). Catechins are soluble in water and can be extracted with diethyl ether; flavones and flavonols, however, both have low solubility in water (Zuanazzi & Montanha, 2003).

2.1.1.2 Phenolic acids

Phenolic acids, which may occur either in free or in conjugated form, can be divided into two main groups: benzoic acids, containing seven carbon atoms (C6–C1), and cinnamic acids, with nine carbon atoms (C6–C3) (King & Young, 1999; Carvalho et al., 2003). The two major dietary hydroxybenzoic acids are ellagic and gallic acids (King & Young, 1999; Carvalho et al., 2003), which can be found either free or as part of hydrolysable tannins (Santos & Mello, 2003).

2.1.1.3 Tannins

Tannins are water-soluble phenolic compounds of high molecular weight that can bind and precipitate proteins as well as other macromolecules in aqueous solutions (Salminen & Karonen, 2011). The molecular mass of tannins ranges from 500 to 3000 g mol^{-1} (Salminen & Karonen, 2011). The reactivity of tannins is influenced by chain length, hydroxylation pattern, stereochemistry, substitutions, position and extent of inter-monomer linkages (Kraus et al., 2003).

Tannins, also responsible for the astringency perception of foods, can be divided into two main groups: hydrolysable and condensed tannins (Bravo, 1998). Hydrolysable tannins are polymers of gallic or ellagic acids, which can be degraded through pH changes and enzymatic or non-enzymatic hydrolysis into smaller fragments, mainly sugars and phenolic acids (King & Young, 1999).

Condensed tannins consist of chains of two or more flavan-3-ol units linked by C-C and C-O-C bonds (Souquet et al., 1996; Santos-Buelga & Scalbert, 2000). The three rings are distinguished by the letters A, B and C (Figure 2.3) (Santos-Buelga & Scalbert, 2000). Condensed tannins are also called proanthocyanidins as they release anthocyanins when heated in acidic conditions (Souquet et al., 1996). Proanthocyanidins are subdivided into several classes based on their chemical structures. The most common classes are procyanidins, exclusively composed of (+)-catechin or (-)-epicatechin units, and

prodelphinidins, which are derived from (-)-epigallocatechin (Cheynier et al., 1999; King & Young, 1999). Catechin is often described as the most common starter unit in proanthocyanidins (Souquet et al., 1996).

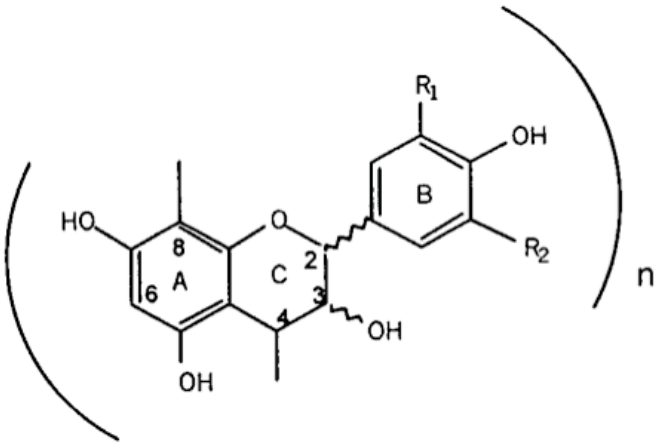


Figure 2.3. Basic structure of proanthocyanidins (Santos-Buelga & Scalbert, 2000).

Proanthocyanidins are called A-, B- or C-type according to the interflavanol linkages between the flavanol units (Santos-Buelga & Scalbert, 2000). Proanthocyanidins of the B-type (dimers) and C-type (trimers) are oligomers or polymers of flavan-3-ols characterised by a single interflavan bond linkage, usually between C4 of the chain-extension unit and C8 or C6 of the starter unit (Santos & Mello, 2003; Ferreira et al., 2010). Proanthocyanidins are classified according to the hydroxylation pattern of the chain-extension units (Santos & Mello, 2003; Ferreira et al., 2010). Proanthocyanidins of the A-type are less common and differ from the B-type by possessing an additional ether linkage between C2 of the chain-extension unit and the starter unit (Santos & Mello, 2003; Ferreira et al., 2010). This feature of two doubly linked flavan-3-ol monomer units introduces a high degree of conformational stability (Santos & Mello, 2003; Ferreira et al., 2010). The flavan-3-ol subunits may carry acyl or glycosyl substituents, whereas gallic acid is the most common acyl substituent (Santos & Mello, 2003).

Proanthocyanidins are mainly found in fruits, grains, wine, beer, and fruit juices (Gu et al., 2004; Prior & Gu, 2005; Pérez-Jiménez et al., 2009). The estimation of proanthocyanidins in foods may be difficult due to their polymeric nature (Prior & Gu, 2005; Pérez-Jiménez et al., 2009). While most condensed tannins are soluble in a variety

of solvents, high molecular weight proanthocyanidins or proanthocyanidins complexed with protein or other molecules may not be extractable, and consequently not accounted for (Pérez-Jiménez et al., 2009). For this reason, available reports may underestimate the content of proanthocyanidin in foods (Prior & Gu, 2005).

The proportion of the constituent units and the average degree of polymerisation (DP) of proanthocyanidin oligomers and polymers in foods can be determined through acid-catalysed degradation in the presence of toluene-α-thiol followed by chromatographic analysis (Souquet et al., 1996; Gu et al., 2003). This reaction releases the terminal units of proanthocyanidins as flavan-3-ols, and the extension units as toluene-α-thiol derivatives (Souquet et al., 1996). The individual oligomeric and polymeric procyanidins in foods can be quantified by normal phase high-performance liquid chromatography (HPLC) (Gu et al., 2002).

2.1.2 Bioavailability and metabolism of flavan-3-ols and proanthocyanidins

Reports on absorption and bioavailability of phenolic compounds in humans revealed that these compounds undergo modifications before passing across the gut barrier (Baba et al., 2002; Gonthier et al., 2003; Serra et al., 2011; Sánchez-Patán et al., 2012; Serra et al., 2012). Proanthocyanidins and flavan-3-ols are thought to be metabolised by endogenous microorganisms in the gastrointestinal tract through numerous catabolic reactions that are still being elucidated, thus generating secondary metabolites that may be responsible for biological actions (Sánchez-Patán et al., 2012). Despite this area of study still being largely undefined, metabolic pathways for the metabolism of catechins, epicatechins and procyanidins have been proposed (Serra et al., 2011; Serra et al., 2012).

The biological actions of flavan-3-ols, proanthocyanidins and their metabolites are dependent on their bioavailability at the postulated target tissue, absorption coefficient and tissue metabolism in the gastrointestinal tract (Fraga & Oteiza, 2011; Ottaviani et al., 2011). The degree of polymerisation (DP) of proanthocyanidins has a significant impact on their absorption through the gut barrier (Deprez et al., 2001; Rasmussen et al., 2005). An *in vitro* study by Deprez et al. (2001) demonstrated that (+)-catechin and

proanthocyanidins dimers and trimers were well absorbed through the Caco-2 human intestinal cell lines, while the permeability of proanthocyanidins polymers (DP < 6) was about ten times lower than monomers, dimers and trimers (Deprez et al., 2001). Similarly, Gonthier et al. (2003) showed that procyanidins were poorly absorbed through the gut barrier of rats compared to flavan-3-ols monomers. Although high molecular weight proanthocyanidins have limited metabolism and bioavailability due to poor absorption through the gut barrier (Gonthier et al., 2003), these compounds can be metabolised into low molecular weight aromatic compounds by colonic microorganisms, producing a range of fermentation products, which may either exert local biological effects in the gut or may be absorbed through the gut barrier (Gonthier et al., 2003; Rasmussen et al., 2005). It is thought that microbial metabolites of procyanidins may exert some biological effects on inner tissues by direct contact with the gut mucosa, protecting it against oxidative stress (Gonthier et al., 2003). Fraga and Oteiza (2011) suggested that the biological effects of non-absorbable procyanidins are related to its interaction with cell membrane proteins and lipids, inducing changes in membrane biophysics that can regulate cell signaling, and by modulating oxidant production. A clinical study by Holt et al. (2002) suggested that procyanidins were possibly degraded into (-)-epicatechin monomers in the human gut after consumption of a flavanol-rich cocoa beverage, but this suggestion was not confirmed by further investigation.

The stereochemical configuration of the phenolic compounds has a significant impact on their absorption, metabolism and biological activities in humans (Fraga et al., 2010; Fraga & Oteiza, 2011; Ottaviani et al., 2011). Ottaviani et al. (2011) suggested that the oral absorption of flavan-3-ols may follow the pattern: (−)-epicatechin > (+)-epicatechin, (+)-catechin > (−)-catechin. In addition, Ottaviani et al. (2011) reported that (-)-epicatechin was the only stereoisomer that caused a significant arterial dilation response *in vivo*. The stereochemical dependence for biological activity (Ottaviani et al., 2011) indicates that isomeric changes, which occur during food processing and storage, may ultimately have an impact on the biological activities of phenolic compounds in functional foods or beverages.

2.1.3 Intercellular mechanisms of action of phenolic compounds

Although intercellular mechanisms of phenolic compounds may be unclear, several mechanisms have been proposed to explain their biological actions (Dashwood et al., 2002; Fraga et al., 2010; Fraga & Oteiza, 2011). Non-specific mechanisms based on their free radical scavenging and metal sequestration properties have been reported (Yoneda & Nakatsubo, 1998; Shao et al., 2003). Available evidence, however, supports the participation of flavan-3-ols and procyanidins in the regulation of cell signaling through specific interactions that modulate the activity and biological reactions of cell signaling proteins (Cilla et al., 2009; Fraga & Oteiza, 2011). Flavan-3-ols and procyanidins modulate pro-inflammatory and oncogenic signals by interaction with cell membrane proteins and lipids (Fraga & Oteiza, 2011). While high molecular weight proanthocyanidins can interact with the cell membrane in the gastrointestinal tract and regulate cell signaling in the gut mucosa, monomers and dimeric procyanidins are transported into the cells, where they modulate pro-inflammatory and oncogenic signals (Fraga et al., 2010; Fraga & Oteiza, 2011). Such mechanisms could partially explain the protective effect of phenolic-rich foods against cardiovascular disease and cancer (Fraga et al., 2010; Fraga & Oteiza, 2011).

An *in vitro* study by Morré and Morré (2006) identified the tumour-specific growth protein (tNOX) as the molecular target in cancer cells for anti-cancer polyphenols. Cilla et al. (2009) reported that fruit beverages containing phenolic compounds had an effect on cell-cycle and cyclins expression, thereby inhibiting Caco-2 cell proliferation. One of the effects of phenolic compounds of interest in nutrition is their influence on factors related to cardiovascular disease and lipid metabolism, in particular cholesterol levels (Zhang et al., 1997; Martin-Carron et al., 1999; Miura et al., 2000). An *in vivo* study conducted by Martin-Carron et al. (1999) showed that a product containing white (*Vitis Vinifera* var. Airen) and red grape (*Vitis Vinifera* var. Cencibel) phenolics was effective in reducing serum total cholesterol and low density lipoprotein (LDL) cholesterol in hypercholesterolemic rats (Martin-Carron et al., 1999). Similarly, Miura et al. (2000) reported that daily consumption of green tea, rich in phenolic compounds, increased resistance of LDL to *in vivo* oxidation, suggesting a protective effect against atherosclerosis and cardiovascular disease (Miura et al., 2000).

2.2 Tea (*Camellia sinensis*)

Camellia sinensis is native to China, and later spread to India and Japan, then to Europe and Russia, entering the New World in the late 17th century (Sharangi, 2009). Different types of tea derived from the leaves of *Camellia sinensis* differ in their appearance, sensory characteristics and chemical contents. *Camellia sinensis* teas may be divided into three groups based on the different processing methods used: non-fermented, semi-fermented and fermented tea (Higdon, 2007; Hilal & Engelhardt, 2007). White and green teas are unfermented products; green tea is, however, made from more mature leaves. Oolong teas are semi-fermented and black teas are fully fermented (Higdon, 2007; Hilal & Engelhardt, 2007). A summary of the common manufacturing processes used to produce *Camellia sinensis* teas is shown in Figure 2.4.

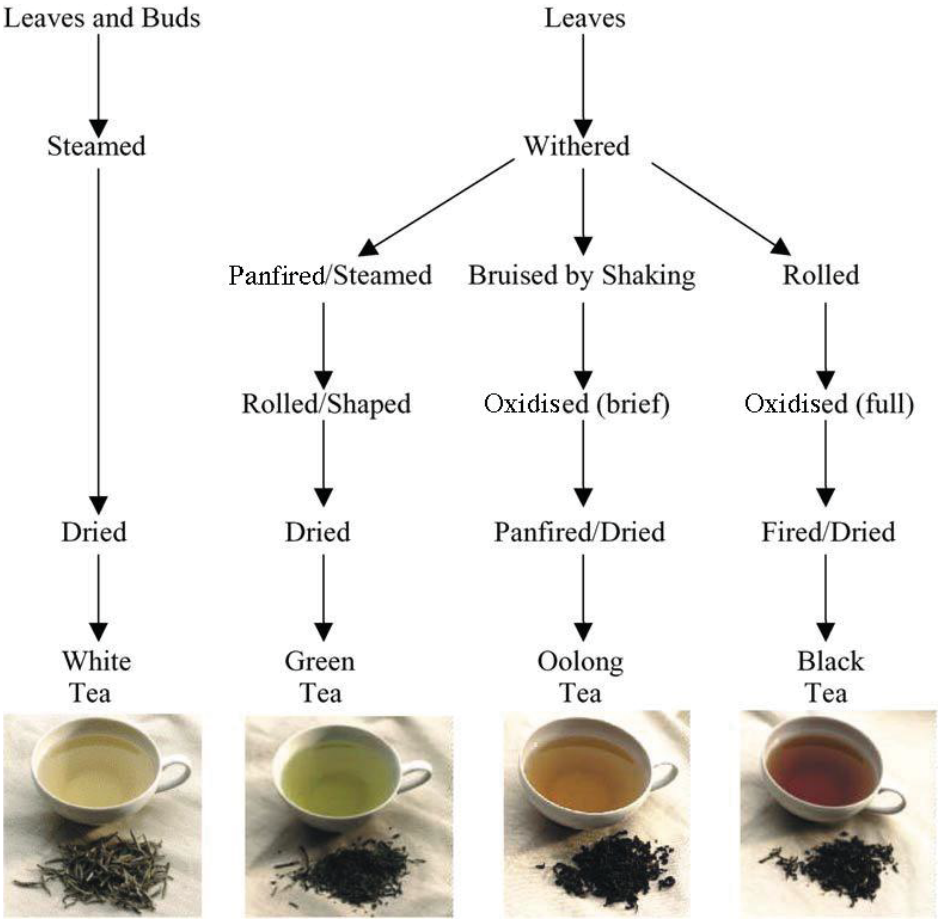


Figure 2.4. Tea (*Camellia sinensis*) manufacturing processes (Santana-Rios et al., 2001).

2.3 Tea (*Camellia sinensis*) processing

White tea is made from buds and young leaves of *Camellia sinensis*, and is the least processed form of tea (Sharangi, 2009). Thus, white tea may retain the highest concentrations of polyphenols (Higdon, 2007). To produce white tea, the fresh leaves of *Camellia sinensis* are heated by either steaming or pan-firing, and then dried (Kuroda & Hara, 2004; Hara, 2011). The heating step inactivates polyphenol oxidases, which is responsible for the oxidation of monomeric catechins (Kuroda & Hara, 2004; Hara, 2011). Hilal and Engelhardt (2007), however, described a processing method, omitting the heating step from the white tea manufacturing process. To produce green tea, the tea is plucked and may be withered in some tea producing countries (Hilal & Engelhardt, 2007). In this process, fresh leaves are exposed to a temperature of 28 °C for about 18 h, which dries the tea, causing chemical changes (Shahidi & Naczk, 1995; Kuroda & Hara, 2004). The green tea leaves are then steamed or pan-fired, rolled and dried (Kuroda & Hara, 2004; Hara, 2011).

Black tea production begins by plucking the tea, mechanically or by hand (Shahidi & Naczk, 1995; Kuroda & Hara, 2004). The tea is then withered, and the dried leaves are rolled. This step causes cellular disruption, which provides adequate conditions for further oxidation of phenolic compounds (Kuroda & Hara, 2004). The broken leaves are then fermented at room temperature usually for 2 to 3 h, which causes non-enzymatic and enzymatic oxidation of phenolic compounds with consequent formation of characteristic colour and flavour of fermented tea (Shahidi & Naczk, 1995; Kuroda & Hara, 2004). Therefore, black tea retains most flavonoids, while catechins are oxidised during this process (Sharangi, 2009). The fermentation process is interrupted by applying heat treatment with controlled time and temperature, followed by grading of the leaves (Shahidi & Naczk, 1995; Kuroda & Hara, 2004). Oolong tea is called “partially fermented tea” because its leaves are oxidised for half the period compared with black tea (Shahidi & Naczk, 1995; Kuroda & Hara, 2004).

Phenolic compounds in tea may change during processing (Chen et al., 1996; Higdon, 2007; Hilal & Engelhardt, 2007). Flavonols, commonly present in tea as glycosides, are less affected by processing (Higdon, 2007; Hilal & Engelhardt, 2007). During

fermentation, flavan-3-ols are oxidised to their corresponding *o*-quinones by catechol oxidase, which are readily condensed to theaflavins, and then to polymeric compounds called thearubugins (Shahidi & Naczk, 1995). On average, tea thearubugins found in hot water extracts are composed of five flavonoid subunits (Shahidi & Naczk, 1995). It seems that low temperature during fermentation increases production of theaflavins and reduces conversion of these compounds to thearubigins during the latter stages of fermentation (Shahidi & Naczk, 1995). Therefore, the degree and rate of oxidation in tea are affected by the content, distribution and composition of flavan-3-ol in fresh tea shoots, the activity of relevant enzymes, the degree of cellular disruption, and fermentation conditions, such as temperature and level of exposure to oxygen (Shahidi & Naczk, 1995).

2.3.1 White tea (*Camellia sinensis*)

White tea (Figure 2.5) is a rare and high-priced product. For this reason, fewer investigations of its biological activities and health benefits are available compared to other types of tea, such as green tea (Rusak et al., 2008; Islam, 2011).

Figure 2.5 Green tea and white tea (*Camellia sinensis*), from left to right (Retrieved October 19, 2012, from http://whiteteacentral.com/whiteteavsgreen.html).

There is no internationally accepted definition for white tea. While in some countries white tea may be defined according to the plucking standards (e.g. only the bud or first leaves are plucked), in China it is defined according to the sub-species it is manufactured from (Hilal & Engelhardt, 2007).

2.3.2 Chemical composition of white tea

The composition of tea leaves depends on variety, geographical origin, environmental conditions and soil conditions (Shahidi & Naczk, 1995). White tea contains flavanols (catechins), phenolic acids, tannins, flavonols and flavone (quercetin, myricetin and kaempferol), and methylxanthine alkaloids (theobromine, theophylline and caffeine) (Santana-Rios et al., 2001; Islam, 2011; Zhao et al., 2011).

Flavanol monomers (catechins) are the most abundant group of phenolic compounds in fresh leaves (Hilal & Engelhardt, 2007; Zhao et al., 2011). The major tea catechins found in fresh leaves are (+)-catechin, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG), and (-)-epicatechin-3-gallate (ECG) (Figure 2.6) (Higdon, 2007; Hilal & Engelhardt, 2007). Islam (2011) reported higher phenolic content for white tea compared to green tea and black tea. Zhao et al. (2011) used an ultra-performance liquid chromatography (UPLC) method equipped with a diode array detector (DAD) and mass spectroscopic (MS) to analyse phenolics and caffeine in 15 tea samples (green pu-erh, green tea, white tea). Zhao et al. (2011) showed that total flavonoid, phenolic acids derivatives and caffeine were higher in white teas than in green pu-erh and green teas, whereas total catechins in white teas and green teas were similar or greater than those in green pu-erh teas. These findings support the argument that unfermented tea usually retains higher concentrations of polyphenols than fermented tea (Higdon, 2007; Sharangi, 2009).

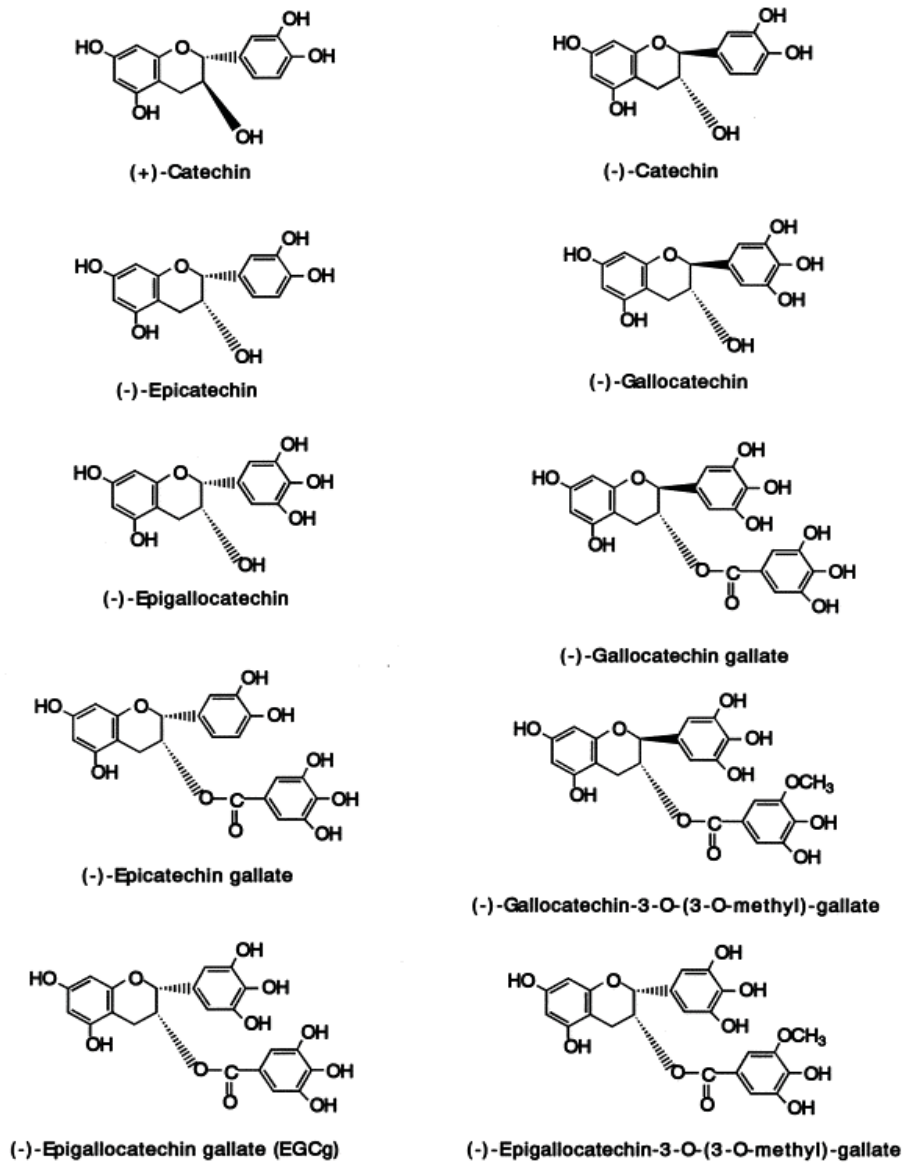


Figure 2.6 Chemical structures of major tea catechins (Saeki et al., 2000).

2.3.3 Tea catechins

Tea catechins (flavan-3-ols) are synthesised by plants and stored in cell vacuoles (Hara, 2011). Catechins constitute up to 30% of the dry weight of tea shoots (Rusak et al., 2008). When the fresh leaves are crushed during manufacturing of black tea, the stable catechins stored in the vacuoles are released and oxidised by polyphenol oxidase, leading to the formation of dimers and polymers (theaflavins and thearubigins) (Hara, 2011).

Processing and storage conditions may cause epimerisation or oxidation of catechins in either plant extracts or processed foods and beverages (Ananingsih et al., 2011). Heat treatment of tea-based beverages causes conversion of EGCG, ECG, EGC, and EC to their corresponding epimers: (-)-gallocatechin gallate (GCG), (-)-catechin gallate (CG), (-)-gallocatechin (GC), and (+)-catechin (Chen et al., 2001). As catechins have been associated with health benefits related to the consumption of teas (Zhang et al., 1997; Cho et al., 2008; Sen et al., 2009; Fraga & Oteiza, 2011), the stability of such compounds must be investigated when developing foods or beverages containing tea extracts.

2.3.4 Biological activities of white and green tea (*Camellia sinensis*)

Camellia sinensis, particularly green tea, has been widely studied for its potential health benefits towards cancer (Inoue et al., 2001; Kuzuhara et al., 2008; Johnson et al., 2010; Trudel et al., 2012) and cardiovascular diseases (Zhang et al., 1997; Miura et al., 2000). The biological effects of *Camellia sinensis* have been attributed to catechins, and EGCG has been identified as a potential inhibitor of expression and activity of pro-matrix-metalloproteinases in human breast cancer cell lines (Sen et al., 2009). In addition, EGCG-enriched green tea beverage has exhibited anti-cancer activity in A549 (lung), HCT15 (colon), BT549 (breast) and PC3 (prostate) human cell lines (Bazinet et al., 2010).

Non-fermented tea, such as white tea, may have higher biological activity than fermented teas due to its higher content of catechins (Almajano et al., 2008). White tea extracts have been reported to have antioxidant (Koutelidakis et al., 2009), antimicrobial (Almajano et al., 2008), anti-inflammatory (Thring et al., 2011) and anti-mutagenic properties (Santana-Rios et al., 2001). Islam (2011) reported that white tea reduced diabetes-associated parameters in rats, such as blood glucose, glucose tolerance, serum lipids and liver glycogen levels. An *in vitro* study by Thring et al. (2011) showed that white tea extract exhibited a protective effect on fibroblast cells against hydrogen peroxide induced damage. In addition, the antimicrobial effects of white tea extracts against *Bacillus cereus*, *Micrococcus Luteus* and *Pseudomonas aeruginosa* strains have

been reported (Almajano et al., 2008). However, there is still limited information on the biological effects of white tea compared to other types of tea.

Tea catechins have been extensively studied due to their potential health benefits (Zhang et al., 1997; Cho et al., 2008; Sen et al., 2009; Fraga & Oteiza, 2011). Reports on green tea catechins support the argument of their chemo-preventive properties (Dashwood et al., 2002; Ramos et al., 2011), which appears to be mediated through common properties with chemical protein chaperones, such as trehalose and glycerol (Kuzuhara et al., 2008). Conformational analysis showed that the hydroxyl groups in EGCG molecule rotate on the four rings; therefore, allowing interaction with various molecular targets, such as proteins, lipids, DNA and RNA oligomers (Kuzuhara et al., 2008). The pharmaceutical industry's interest in the potential health benefits of tea catechins has led to the development of supplements using green tea (Hara, 2011). Furthermore, Hara (2011) predicted a substantial growth in the segment of catechin capsules and tablets in the near future if clinical trials are successful.

2.4 Grape seed extract (*Vitis vinifera*)

2.4.1 Chemical composition of grape seed

White grape varieties and their extracts are good sources of natural antioxidant compounds, which are of growing industrial importance (Montealegre et al., 2006). The phenolic composition of grapes is influenced by grapevine variety, growing season, degree of ripeness, berry size, soil, and climatic conditions such as temperature and light (Montealegre et al., 2006; Xu et al., 2011).

The skins and seeds of white and red grape varieties have similar qualitative and quantitative non-anthocyanin phenolic composition (Rockenbach et al., 2011). Grape seed extracts, however, contain higher total phenolic and flavanol contents compared with those of the skin (Rockenbach et al., 2011) and is rich in oligomeric and polymeric compounds with high antioxidant activity (Montealegre et al., 2006).

Grape seeds contain large quantities of flavanols, such as (+)-catechin, (-)-epicatechin, EGC, ECG, and EGCG, as well as dimeric, trimeric and tetrameric procyanidins (Guendez et al., 2005; Montealegre et al., 2006; Rockenbach et al., 2011). Gallic acid may be present in grape seed in the free form or in the form of esters, such as ECG, and may be released after alkaline hydrolysis of the phenolic extract (Romeyer et al., 1986). Flavanols have been identified as the main phenolic group in grape seeds of Sauvignon Blanc (*Vitis vinifera*) (Montealegre et al., 2006), whereas (+)-catechin has been identified as the most abundant monomeric flavanol compound in grape seeds of different varieties of *Vitis vinifera* (Montealegre et al., 2006; Rockenbach et al., 2011). Similarly, other white grape seed varieties (Ugni blanc and Maccabeo) contain high levels of flavanols and condensed tannins, including low amount of dimeric procyanidins (mostly procyanidin B3), and low amount of free gallic acid (Romeyer et al., 1986). In addition, heat treatment of grape seed extracts may facilitate the release of phenolic compounds by tissue breakdown and inhibition of polyphenol oxidase, therefore increasing the amount of phenolics and caffeine available in the extract (Kim et al., 2006).

2.4.2 Biological activity of grape seed

The antioxidant (Shao et al., 2003), anticoagulant (Bijak et al., 2011) and antimicrobial (Bekhit et al., 2011; Adámez et al., 2012) properties of grape seed extracts have been reported. Grape skins from *Vitis vinifera* var. pinot noir and pinot gris grapes have shown protective effects against influenza virus without any cytotoxic effects on cells (Bekhit et al., 2011). Bijak et al. (2011) conducted *in vitro* experiments to determine the anti-thrombin effects of *Vitis vinifera* extract by measuring performance indicators (thromboplastin, prothrombin and thrombin). The incubation of human plasma with grape seed extract decreased the velocity of fibrin polymerisation in human plasma and prolonged clotting time, suggesting that the extract may have anticoagulant properties (Bijak et al., 2011).

A combination of grape and tea phenolic compounds may increase certain biological activities related to human health (Bekhit et al., 2011). An *in vivo* study by Morré and Morré (2006) reported a synergistic effect between grape extract and green tea phenolics,

which was associated with anti-cancer properties. Morré and Morré (2006) showed that the growth of mammary tumour in mice was inhibited by intra-tumoural injections of green tea infusion combined with grape skin extract.

2.5 Grape seed and tea extracts in foods

Grape seed extract has been used to improve the quality of foods and beverages, such as meats (Mielnik et al., 2006; Carpenter et al., 2007) and breads (Peng et al., 2010), due to its antioxidants and antimicrobial properties, therefore extending their shelf life (Perumalla & Hettiarachchy, 2011). Green tea catechins have also been used as antioxidants to prevent oxidation of canola oil (Chen & Chan, 1996), as well as beef and chicken meat patties (Mitsumoto et al., 2005).

Grape seed extract incorporated with a tea infusion results in a new phenolic profile, where EGC, (-)-epicatechin, ECG and (-)-gallocatechin (GC) are the most abundant phenolic constituents (Bekhit et al., 2011). The new phenolic profile obtained by combining both extracts could have interesting and potentially beneficial health properties, thus necessitating further investigation.

2.6 Ready-to-drink (RTD) tea-based beverage market

Soft drinks include a vast array of non-alcoholic beverage products ranging in composition, from mineral waters, sports drinks, tonics to fruit juices (Stratford & Capell, 2003). Based on the aforementioned definition, ready-to-drink (RTD) grape seed and tea-based beverages may also be classified as soft drinks. While RTD tea drinks are commercially available worldwide (Hara, 2011; Simrany, 2011), grape seed extract is not yet incorporated into commercial products.

The RTD tea-based beverage sector represents a growing market in many countries (Simrany, 2011). Moreover, RTD tea-based beverages have become popular due to an

increasing demand for convenience and consumers' awareness of the health benefits associated with tea consumption (Hara, 2011; Simrany, 2011). Hence, the popularity of RTD tea beverages in the United States was responsible for growth of nearly USD $6 billion in sales over 20 years (1990 to 2010) (Simrany, 2011), as shown in Table 2.1.

Table 2.1 Estimated wholesale value (USD $billion) of the tea industry in the United States (Simrany, 2011).

Year	1990	2010
Traditional Market (supermarket, drug stores & mass merchandisers)	0.87	2.15
RTD Market	0.20	3.30
Food-service Segment	0.50	1.02
Specialty Segment	0.27	1.30
Total Sales	1.84	7.77

In Japan, the amount of bottled tea drinks manufactured in the past two decades has also risen significantly (Figure 2.7). It has been suggested that the high number of scientific reports published on tea catechins and their potential health benefits (Zhang et al., 1997; Miura et al., 2000; Kuzuhara et al., 2008; Sen et al., 2009) may have positively impacted on the growth of the tea drinks market (Hara, 2011). Such health reports are also valuable marketing tools used by the industry to promote tea-based soft drinks.

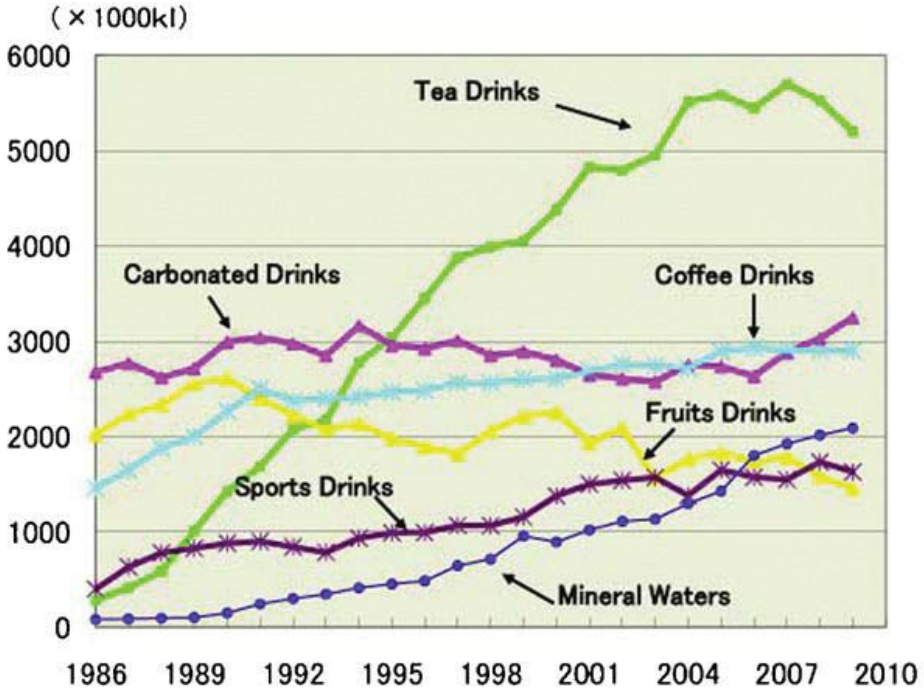


Figure 2.7 Trends of soft drinks production in Japan (1986–2009), expressed as 1000kl (kl = 1000 litres) (Hara, 2011).

2.7 Stability of phenolics and methylxanthines in processed beverages

Consumers expect safety, nutrient composition and overall quality of any food product to be maintained at a high standard from production to consumption (Kilcast & Subramaniam, 2000). In the RTD industry, processing technologies must assure product safety against pathogenic microorganisms, as well as retain bioactive compounds at optimal levels (Day et al., 2009).

High temperature treatments, pH, beverage ingredients, and storage conditions can affect the stability of phenolic compounds in products (Wang & Helliwell, 2000; Bazinet et al., 2010; Rawson et al., 2011), causing changes to the catechins through epimerisation and oxidation (Ananingsih et al., 2011). These factors will be discussed in detail as they may affect the stability of grape seed and white tea phenolic compounds in beverages investigated in this study.

2.7.1 Effect of brewing conditions on phenolics and methylxanthines

The infusion rate of phenolic compounds in water is affected by brewing time and temperature (Labbe et al., 2006). In a study by Yang et al. (2007), green tea, oolong tea and black tea bags were infused in water at 70, 85 and 100 °C eight times for 30 s each time. In this study, the highest content of catechins, gallic acid and caffeine were obtained in the first infusion using water at 85 and 100 °C and in the second infusion with water at 70 °C (Yang et al., 2007). Another study by Sharma et al. (2005a) compared green tea infused with distilled water at 80 °C and 100 °C for 3 min and demonstrated that infusions prepared at 100 °C contained higher levels of catechins, gallic acid and caffeine. However, infusions prepared at a lower temperature (80 °C) were sweeter and preferred by sensory panellists (Sharma et al., 2005a).

Labbe et al. (2006) used a mathematical model to demonstrate the potential to fractionate the compounds of tea by varying the brewing factors, such as time and temperature. Labbe et al. (2006) investigated the solubilisation kinetics of catechins and caffeine during brewing, identifying time-dependent compounds (EGC and EC) and time-temperature dependent compounds (catechin, EGCG, GCG and ECG). This study showed that the brewing duration (5, 10, 20, 40 and 80 minutes) and temperature (50 to 90 °C) affected catechin and caffeine concentrations in green tea extract (Labbe et al., 2006). It was reported that EGCG reached higher concentrations in solutions brewed at 70, 80 and 90 °C, while higher concentrations of caffeine were obtained by heating the solution at 70-80 °C for 20 to 40 minutes (Labbe et al., 2006).

2.7.2 Effect of pH on phenolics in RTD beverages

The stability of catechins in tea-based beverages can be affected by pH (Zhu et al., 1997; Chen et al., 2001). Catechins are usually less stable at higher pH values (Zhu et al., 1997; Chen et al., 2001), degrading almost completely in alkaline solutions above pH 8 (Zhu et al., 1997).

2.7.3 Effect of ingredients on phenolics in RTD beverages

The stability of catechins can be affected by the presence of ingredients commonly added to RTD beverages, such as ascorbic acid, citric acid, sucrose and water (Zhu et al., 1997; Chen et al., 2001; Ananingsih et al., 2011). Citric acid can significantly accelerate degradation of catechins, with sucrose having less effect on catechins (Chen et al., 2001). Ascorbic acid acts as an antioxidant and a pro-oxidant, exhibiting a protective effect on catechins in beverages for the first month of storage, and promoting degradation of catechins after this period (Chen et al., 2001). In addition, water that has not been treated or distilled can cause epimerisation of catechins more easily than purified water, possibly due to pH variability or due to the complexity of ions present in the water, which react with catechins (Wang & Helliwell, 2000). Metal ions can bind to catechins forming metal complexes, which may affect the antioxidant capacity of catechins (Ananingsih et al., 2011).

2.7.4 Effect of heat treatment on phenolics and methylxanthines in RTD beverages

While some phenolics, such as flavone glycosides, flavonol derivatives and phenolic acids, have demonstrated considerable thermal stability in food matrices (Pacheco-Palencia et al., 2009), reports indicate that catechins are affected by heat treatment (Chen et al., 2001; Chamorro et al., 2012).

Kim et al. (2007) reported that epimers of catechins, such as EGCG, EGC, EC, and ECG, decreased after heating tea beverages at 95 or 120 °C for 4 min, while their isomers (GCG, GC, catechin, and catechin gallate (CG)) increased. Conversion of EGCG to GCG has been shown to occur after heat-treating beverages containing green tea at 80, 95 and 120 °C (Chen et al., 2001). Therefore, the GCG content found in canned and bottled tea-based beverages could possibly be an epimerisation product of EGCG formed during heat treatment (Chen et al., 2001). Chen et al. (2001) reported that green tea catechins were degraded by 23% after autoclaving at 120 °C for 20 min. Similarly, Chamorro et al. (2012) reported that autoclaving grape seed extract induced degradation of procyanidins,

resulting in darkening of the samples, which was attributed to oxidation of some catechins (Chamorro et al., 2012).

Bazinet et al. (2010) suggested that using high temperature treatment for a short time (90 °C for 30 s) reduces the impact of heat on the stability of catechins in beverages. Therefore, heat treatment for a short time may be successfully used to process commercial RTD tea-based beverages (Bazinet et al., 2010). Xu and Chang (2010) reported that indirect and direct UHT processing at 143 °C for 60 s did not affect the content of (+)-catechin in soymilk. UHT treatment at 120 °C for 2 s has been successfully used by Sopelana et al. (2011) to extend the shelf-life of coffee brews without affecting caffeine content. Despite the known epimerisation and oxidation effects of heat-treatment on catechins (Chen et al., 2001), there are a limited number of reports on the effect of high temperature short-time (HTST) processing on the stability of white tea or grape seed phenolic compounds. As the impact of thermal treatment on stability of antioxidant compounds is dependent on heating conditions (time-temperature) as well as the physical form of the extracts used (Kim et al., 2006), further investigation is necessary to evaluate the effects of UHT treatment on beverages containing powdered extracts.

Despite the impact of heat treatment on the stability of phenolic compounds, some thermal processing techniques may retard phenolic degradation by inactivation of enzymes (Doğan et al., 2006). Pasteurisation inhibits the activity of polyphenol oxidase enzymes and causes plant tissue to breakdown, facilitating the extraction of phenolic compounds, such as anthocyanins, during processing and, consequently, leading to higher phenolic content in the heat-treated product (Fang et al., 2006). In addition, the effect of heat on antioxidant compounds may vary depending on the physical form of the extracts (e.g. powdered form) (Kim et al., 2006).

2.7.5 Storage conditions and stability of phenolics and methylxanthines

Catechins may undergo oxidative degradation during storage (Kim et al., 2007; Yang et al., 2007). Storage at 25 °C has been shown to decrease EGCG content in aqueous tea

extract (Yang et al., 2007), whereas ECG and GCG decreased rapidly in pasteurised tea-based beverages stored at 25 °C (Bazinet et al., 2010). In addition, pH may also affect the rate of degradation of catechins in tea-based beverages (Bazinet et al., 2010). In fact, EGCG and EGC are more stable in beverages with pH 4, but decrease slowly in beverages with pH 6 (Bazinet et al., 2010). Caffeine content, however, seems to be less affected by storage conditions than catechins (Bazinet et al., 2010).

2.8 Antioxidant capacity of food systems

2.8.1 Autoxidation mechanisms

The autoxidation process of antioxidants is based on chain reactions, including initiation, propagation, branching, and termination of free radicals (Ou et al., 2001; Huang et al., 2005). Chain-breaking antioxidants interrupt the radical chain reaction (propagation and branching) and preventive antioxidants are those that retard or inhibit the formation of free radicals from unstable precursors in the initiation step of the chain reaction, such as metal chelators (Ou et al., 2001; Huang et al., 2005).

A range of methods using different technologies has been proposed to quantify antioxidant capacity of foods and biological samples (Sánchez-Moreno et al., 1998; Ou et al., 2001; Davalos et al., 2004; Huang et al., 2005). Considering the chemical reactions involved, antioxidant capacity assays may be divided into hydrogen atom transfer (HAT) reaction and single electron transfer (ET) reaction based assays (Huang et al., 2005). Oxygen radical absorbance capacity (ORAC), a method commonly used to measure antioxidant capacity of foods, beverages and physiological fluids (Prior et al., 2003; Davalos et al., 2004; Stockham et al., 2011), is based on the HAT reaction (Ou et al., 2001). The hydrogen atom transfer mechanism is based on a chain-breaking antioxidant (AH) that donates a labile hydrogen atom to the radical ROO• before ROO• reacts with the substrate (LH) (Ou et al., 2001; Huang et al., 2005):

$$ROO\bullet + AH \rightarrow ROOH + A\bullet$$
$$ROO\bullet + LH \rightarrow ROOH + L\bullet$$

Thus, the radical A• is stable and does not continue with the autoxidation reactions. It seems that the HAT mechanism plays an important role in the radical chain reaction, and methods based on such reactions may be more relevant to chain-breaking antioxidant capacity (Ou et al., 2001; Huang et al., 2005). Single electron transfer (SET) assays, such as Trolox equivalent antioxidant capacity (TEAC) assay (Re et al., 1999), ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996) and total phenolics by Folin-Ciocalteu reagent (Singleton et al., 1999), involve a redox reaction. In redox reactions, the oxidant abstracts an electron from the antioxidant, causing colour changes (Huang et al., 2005).

Since natural antioxidants are multifunctional, the action of such substances in foods and biological systems is complex and involves a range of mechanisms, such as oxygen scavenging, metal chelation, singlet oxygen quenching or inhibition of oxidative enzymes (Fauconneau et al., 1997; Frankel & Meyer, 2000; Zulueta et al., 2009). For this reason, when evaluating the antioxidant capacity of foods or biological systems, multiple methods should be used to measure different products of oxidation (Frankel & Meyer, 2000). The most commonly used antioxidant capacity methods are described in this section.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay is measured by inhibition of the peroxyl-radical induced oxidation initiated by the thermal decomposition of a radical source (2,2'-azobis (2-amidinopropane) dihydrochloride or AAPH), by measuring the decrease in fluorescence, until zero fluorescence occurs (Ou et al., 2001; Huang et al., 2002). To reduce assay variability, the AAPH solution must be prepared immediately before starting the reaction (Prior et al., 2003).

The original ORAC method described by Cao et al. (1993) was later adapted to an automated version (Cao et al., 1995). The ORAC method was further modified by Ou et al. (2003) with substitution of the fluorescent probe β-phycoerythrin (PE) for the

fluorescein salt, a more stable and inexpensive substance. As the fluorescence intensity of fluorescein salt is pH sensitive and may decrease significantly if pH is below 7, all solutions are prepared in a phosphate buffer (Battey et al., 2001). Later, Huang et al. (2002) validated a fully automated high-throughput ORAC assay, and Davalos et al. (2004) adapted the ORAC assay to manual handling to evaluate antioxidant capacity of wine samples (Davalos et al., 2004).

Ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assay

The ferric reducing antioxidant power (FRAP) assay is based on the reduction of the ferric (Fe^{3+}) complex to the ferrous (Fe^{2+}) form in the presence of antioxidants, resulting in an intense blue colour (Benzie & Strain, 1996). The Trolox equivalent antioxidant capacity (TEAC) assay measures the inhibition of the absorbance of the radical cation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS by antioxidants present in the sample using a spectrophotometer (Cao & Prior, 1998). Although FRAP and TEAC are relatively inexpensive methods compared to ORAC (Zulueta et al., 2009), the ORAC assay has greater specificity and responds to a wider range of antioxidants than the other two methods (Cao & Prior, 1998).

Radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$)

The radical scavenging activity assay using 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) is based on a reaction where the scavenger molecule (antioxidant) donates a hydrogen atom to a stable radical with purple colour ($DPPH^{\bullet}$), causing the reduction of the $DPPH^{\bullet}$ and, consequently, the colour changes from purple to yellow (Sánchez-Moreno et al., 1998). Mishra et al. (2012) compared steady state and fixed time incubation experiments and showed that fixed time experiments may underestimate the radical scavenging activities for slow reacting molecules. This is explained by the fact that reaction kinetics may vary for different antioxidants due to their different lengths of time to reach steady state (Sánchez-Moreno et al., 1998; Mishra et al., 2012). For this reason, the reduction of $DPPH^{\bullet}$ can be measured by monitoring the decrease in its absorbance during the reaction until the plateau is reached (Brand-Williams et al., 1995).

Radical scavenging activity on DPPH is often reported as the amount of antioxidant necessary to decrease the initial $DPPH^{\bullet}$ concentration by 50% (EC_{50}) (Brand-Williams et al., 1995). Sánchez-Moreno et al. (1998) suggested the parameter antiradical efficiency (AE), which involves the potency ($1/EC_{50}$) and the reaction time needed to reach steady state at the concentration corresponding to EC_{50} (TEC_{50}). TEC_{50} is calculated by plotting the time at steady state against the concentration for each antioxidant compound (Sánchez-Moreno et al., 1998).

Correlation of antioxidant assays

A correlation between different antioxidant activity assays is variable because of the complexity of the mixture of phenolic compounds in food systems and because each antioxidant assay uses different technology and measures specific properties of antioxidants (Cao & Prior, 1998). For the same reason, not all antioxidant activities correlate positively with total phenolic contents (Bekhit et al., 2011). If a sample contains a complex mixture of antioxidants, a low correlation between data obtained from HAT and ET assays can be expected due to the different kinetics and reaction mechanisms of the different antioxidants present (Zulueta et al., 2009). Cao and Prior (1998) analysed blood serum samples using three methods (ORAC, TEAC and FRAP) and found no correlation between ORAC and TEAC, and a weak correlation between ORAC and FRAP. Davalos et al. (2004), however, observed good linear correlation (R^2= 0.989) between ORAC levels and total phenolic content of different types of wines analysed.

2.9 Chemical analysis of phenolics and methylxanthines

2.9.1 Extraction of phenolics and methylxanthines

Phenolic and methylxanthine compounds have been extracted from a variety of food matrices, such as tea shoots, fruits and grape skins (Friedman et al., 2005; Sharma et al., 2005b; Tsanova-Savova et al., 2005; Montealegre et al., 2006). Procedures for extraction of phenolic and methylxanthine compounds are summarised in Table 2.2.

The extraction efficiency of phenolic compounds strongly depends on length of extraction and solvents used (Yao et al., 2004; Sharma et al., 2005b). Sharma et al. (2005b) reported that 70% methanol extracted higher amounts of catechins and xanthine alkaloids from tea than water, acetone, acetonitrile or 15% methanol. Similarly, grape juice extracted with methanol contained higher quantities of phenolic compounds compared to ethanol and water extracts (Pinelo et al., 2005). Yao et al. (2004) reported that methanol was the most selective solvent for extracting phenolic compounds from tea shoots compared to chloroform, ethyl acetate and water. In addition, Yao et al. (2004) observed improved peak resolution when the methanol extract was immediately analysed by HPLC instead of when it was dried and re-dissolved for analysis. Another factor that may have an impact on extraction efficiency is pH (Rusak et al., 2008). Low pH may accelerate the extraction of phenolic compounds from plant extracts (Rusak et al., 2008). Rusak et al. (2008) observed that the extraction of phenolic compounds from white tea with water was accelerated by addition of lemon juice, possibly because it reduces the pH of the solution.

Table 2.2 Procedures for extraction of phenolic and methylxanthine compounds from different food matrices prior to HPLC analysis.

Sample type	Compounds analysed	Extraction procedure	Reference
Tea shoots	Catechins, xanthine alkaloids	Extracted with 70% methanol with shaking for 30 s, centrifuged. The supernatant was taken in a flask and the extraction steps repeated to reach 10 mL. Filtered through a 0.5 μm filter.	Sharma et al. (2005b)
Tea shoots	Catechins, caffeine, theobromine, phenolic acids and flavonol glycosides	Extracted with methanol for 4 min at 20 °C, filtered through cotton wool, washed with methanol (3 x 10 mL), and diluted with methanol	Yao et al. (2004)
Grape skins	Catechins, procyanidins, gallic and caffeic acid, and other phenolics	Extracted with ethanol/water/formic acid (50:48.5:1.5), centrifuged, re-extracted with ethyl acetate and diethyl ether, concentrated, and re-suspended in mobile phase and filtered.	Montealegre et al. (2006)
Green tea	Catechins and caffeine	Extracted with water, filtered through a 0.20 μm filter LC13 PVDF, and diluted with HPLC grade water.	Labbe et al. (2006)
Teas	Catechins, caffeine, theobromine, theaflavines	Extracted with water, boiled, stirred, cooled and centrifuged. The supernatant was filtered through a 0.45 μm nylon filter.	Friedman et al. (2005)
Grape extract (powder)	Anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, flavonols and flavanols	Extracted with formic acid: water: methanol (2:28:70) with shaking for 10 min, centrifuged, and filtered through a 0.2 μm membrane filter.	Liang et al. (2011)
Fruits (freeze-dried)	Catechin and epicatechin	Extracted with 80% methanol for 5 min in ultrasonic bath, diluted with water, ultra-centrifuged for 5 min at 14 000 rpm and filtered through a 0.45 μm nylon filter.	Tsanova-Savova et al. (2005)

2.9.2 Analysis of total phenolic content using Folin-Ciocalteu's reagent

Total phenolic content may be determined by spectroscopy using Folin-Ciocalteu's reagent (Singleton & Rossi, 1965; Singleton et al., 1999). This method is based on the reduction of phosphotungstic acid in alkaline solution to phosphotungstic blue. The absorbance of formed phosphotungstic blue is proportional to the number of aromatic phenolic groups (Singleton & Rossi, 1965; Singleton et al., 1999).

Total phenolic content using Folin-Ciocalteu's reagent has been performed alongside antioxidant capacity and HPLC analyses to investigate the phenolic profile of tea samples (Rusak et al., 2008) and grape seed and skin extracts (Rockenbach et al., 2011). However, the Folin-Ciocalteu method can lead to an over-estimation of polyphenol content due to the interference of water-soluble non-phenolic substances that can also reduce the Folin–Ciocalteu complex, such as vitamin C (Singleton et al., 1999).

2.9.3 Analysis of phenolics and methylxanthines by High-Performance Liquid Chromatography (HPLC)

Several proposed methods for analysis of phenolics and methylxanthines are based on liquid chromatography (Yao et al., 2004; Sharma et al., 2005b; Lee et al., 2008b; Munoz et al., 2008; Rusak et al., 2008). Table 2.3 describes the HPLC parameters used by different authors to identify phenolic and methylxanthine compounds in a range of food matrices.

Table 2.3 Parameters of HPLC-DAD systems used for analysis of phenolic and methylxanthine compounds.

Sample type	Compounds analysed	Mobile phase	Stationary phase	Reference
Grape seed and skin	Catechin, epicatechin, procyanidins and anthocyanins	(A) 0.2% TFA in water (v/v) (B) 0.2% TFA in acetonitrile (v/v)	Kromasil 100 C18 (5 µm, 250 mm x 3 mm) at 20 °C	Munoz et al. (2008)
Tea shoots	Catechins and methyl xanthines	(A) Acetonitrile (B) 0.1% Ortho-phosphoric acid in water (w/v)	C-18 Lichrocart (5 µm, 250 x 4.0 mm) at 35 °C	Sharma et al. (2005)
Tea shoots	Catechins, caffeine, theobromine, phenolic acids and flavonol glycosides	(A) Acetonitrile (B) 2% Acetic acid in water (v/v)	Hypersil ODS S5 (5 µm, 250 x 4.6 mm) at 35 °C	Yao et al. (2004)
Grape seed and skin	Catechins, procyanidins, gallic acid, caffeic acid, and other phenolics	(A) 2.5% Acetic acid in water (v/v) (B) Acetonitrile/solvent A (80:20, v/v)	Nucleosil C-18 (5 µm, 250mm x 4.6 mm) at 35 °C	Montealegre et al. (2006)
Juices	Anthocyanins	(A) Acetonitrile (B) 10% Acetic acid and 1% phosphoric acid in water (v/v)	Prodigy ODS (5 µm, 250 x 4.6 mm, Phenomenex)	Lee et al. (2008b)

Column temperature and flow rate affect the separation of phenolic compounds analysed by reversed-phase HPLC (Sharma et al., 2005b; Klein et al., 2012). Sharma et al. (2005b) observed that the retention times of catechins, gallic acid and tea alkaloids in tea samples decreased as the column temperature increased, whereas temperatures beyond 35 °C caused EGCG and EC peaks to merge. Peak resolution is also affected by the solvents used in the mobile phase (Munoz et al., 2008). Acetonitrile has been shown to improve resolution of catechin peaks compared to methanol (Sharma et al., 2005b; Munoz et al., 2008). In addition, the presence of an acid in the mobile phase eliminates peak tailing and improves resolution of catechin peaks (Dalluge et al., 1998). TFA has been shown to improve the definition of the peaks in comparison to acetic acid and phosphoric acid (Klein et al., 2012).

Despite the common use of HPLC equipped with diode array detection (DAD) for characterisation of phenolic compounds and separation of flavanol monomers to trimers (Prior & Gu, 2005), DAD detection is not sufficient to discriminate between compounds with similar spectroscopic characteristics. Therefore, mass spectrometry is recommended when characterising anthocyanins (Valls et al., 2009).

2.10 Microbiological stability of beverages

Microbiological spoilage of foods or beverages depends on both intrinsic and environmental factors (Kilcast & Subramaniam, 2000; Stratford & Capell, 2003). The initial microbial load of products is important as it influences the rate of spoilage. Physical and chemical properties, such as pH, sugar, acidity, presence of preservatives, level of exposure to oxygen, and storage temperature influence the rate of food spoilage. Thus, efficiency of processing method, packaging and handling of product are vital determinants of food spoilage (Kilcast & Subramaniam, 2000).

Aciduric microorganisms (moulds, yeasts, lactic acid bacteria, and acetic acid bacteria) can grow in low pH beverages (Ray, 2003), degrading sugars and nutrients into secondary metabolites, which may inhibit the growth of other microorganisms (Mosqueda-Melgar et al., 2012). Yeasts and moulds are often associated with spoilage of

low pH beverages (Conner & Beuchat, 1987; Deak & Beuchat, 1995; Battey et al., 2001) as they are more aciduric and tolerant of low pH than most species of bacteria (Splittstoesser, 1987; Pitt & Hocking, 2009). Yeasts can grow at pH values ranging from about 2.0 to 8.5, while moulds grow at pH values ranging from about 1.5 to 9.0 (Ray, 2003). Certain acid tolerant bacteria can, however, compete with yeasts and moulds at approximately pH 3.5 (Splittstoesser, 1987; Pitt & Hocking, 2009).

Packaging with low oxygen permeability may protect foods and beverages against growth of yeasts and moulds (Sant'Ana et al., 2010). These microorganisms can cause spoilage when oxygen enters the product by leakage or diffusion through the packaging (Pitt & Hocking, 2009), generating odours, off-flavours, carbon dioxide production, formation of turbidity and sediment (Loureiro & Querol, 1999). Some strains of yeasts and moulds are, however, still capable of multiplying to significant populations under low oxygen conditions (Stratford & Capell, 2003).

Geotrichum, *Penicillium* and *Fusarium* spp are common moulds found in beverages (Tournas et al., 2006a). Common yeasts found in beverages are *Brettanomyces naardenensis*, *B. bruxellensis* (Deak & Beuchat, 1995), *Candida lambica*, *C. sake*, and *Rhodotorula rubra* (Tournas et al., 2006a). Yeasts from the genus *Zygosaccharomyces* are recognised as representing significant spoilage in beverages (Thomas & Davenport, 1985; James & Stratford, 2000). This genus is osmotolerant, and resistant to weak acid preservatives (James & Stratford, 2000). Carbohydrate and potato infused medium is often used for enumeration of yeasts and moulds (Beever & Bollard, 1970). The pH of this medium should be adjusted to approximately 3.5 or, alternatively, antibiotics may be added to inhibit bacterial growth (Tournas et al., 2006b).

The sporulation of yeasts and moulds may occur by sexual or asexual processes (Ray, 2003). Heat-resistant moulds are characterised by the production of heat-resistant structures, such as chlamydospores and ascospores, which survive during thermal processes (Beuchat & Pitt, 2001). Ascospores, usually produced in groups of eight within a closed sac, are the main characteristic of the ascomycete groups of fungi (Beuchat & Pitt, 2001). *Neosartorya fischeri*, *Paecilomyces variotii, Fusarium oxysporum, Byssochlamys fulva, B. nivea, Talaromyces trachyspermus* are common heat-resistant

moulds found in foods and beverages (Conner & Beuchat, 1987; Houbraken & Samson, 2006). Contamination of foods by *Byssochlamys* strains is of public health concern as they can survive pasteurisation temperatures and can produce the mycotoxin patulin in the product during storage (Sant'Ana et al., 2010; Janotová et al., 2011). Mycotoxin is cytotoxic (Mohan et al., 2012) and can induce DNA damage in the brain, liver and kidneys (Melo et al., 2012).

As ascospores are capable of surviving pasteurisation temperatures (Conner & Beuchat, 1987), mould growth in processed beverages may be attributed to the germination of surviving ascospores during storage (Mosqueda-Melgar et al., 2012). However, the ability of ascospores to survive heat treatment can be affected by the presence of organic acids, such as fumaric, tartaric or citric acid, which reduce the pH (Conner & Beuchat, 1987). On the contrary, the presence of non-heat-resistant moulds in a pasteurised product may indicate poor hygiene (Ray, 2003; Stratford & Capell, 2003).

2.11 Preservation techniques used in the beverage industry

Conventional thermal preservation techniques, including pasteurisation and sterilisation, are generally assumed as safe since most have been widely used by the food and beverage industry (Galanakis, 2012). Such conventional techniques can, however, affect levels of bioactive compounds and nutrients in some products (Rawson et al., 2011; Galanakis, 2012).

Due to limitations associated with the use of conventional techniques, several novel technologies with potential to improve the functional properties and stability of bioactives in foods have emerged, such as high pressure processing (HPP), ultraviolet (UV) technology, and pulsed electric field (Fredericks et al., 2011; Rawson et al., 2011; Varela-Santos et al., 2012). However, most of the novel technologies are not suitable to small and medium scale food companies, mainly due to financial constraints or lack of access to specialised equipment. Common preservation techniques used in the beverage industry will be reviewed in the following sections.

2.11.1 Chemical preservatives

Organic acids are added to food and beverages to reduce pH, prevent oxidation or inhibit microbial growth (Søltoft-Jensen & Hansen, 2005). Benzoic, sorbic, nitrous, sulphurous, and propionic acid are commonly used by the food industry to preserve foods or beverages (Pitt & Hocking, 2009). Potassium sorbate, sodium benzoate and sodium metabisulphate have been shown to inhibit growth of *Zygosaccharomyces bailii*, *Alicyclobacillus acidoterrestris* and *Propionibacterium cyclohexanicum*, which are effective spoilers of beverages (Cole et al., 1987; Walker & Phillips, 2008). Their esters, however, are less used (Pitt & Hocking, 2009).

The preservative mechanism of organic acids is explained by the antimicrobial effect of uncharged acid molecules, which dissociate in aqueous environment after entering the neutral cytoplasm, where they accumulate as charged anions, releasing an excess of protons that causes acidification of microbial cells (James & Stratford, 2000; Søltoft-Jensen & Hansen, 2005). Despite the effectiveness of preservatives in inhibiting microbial growth, the use of such substances is undesirable due to a growing demand for products without chemical preservatives (Shim et al., 2011). In fact, the use of preservatives in foods and beverages is discouraged in many countries, such as Australia and Korea (Evans et al., 2010; Shim et al., 2011).

The use of chemical preservatives in specific foods is regulated by law, and must be used within permissible concentration limits and must be safe for human consumption (Pitt & Hocking, 2009). In New Zealand and Australia, the legal limits for food additives are regulated by Standard 1.3.1 (Food Additives) of the Australia New Zealand Food Standards Code (Brooke-Taylor et al., 2003).

2.11.2 Aseptic processing and packaging (APP)

Aseptic processing is commonly used to process milk, fruit juices, yoghurt, ice cream mix and sauces (Awuah et al., 2007). In aseptic processing, the product is sterilised and

delivered to a filling unit in a sterile zone for packaging using sterile containers (Lewis & Heppell, 2000; Gedam et al., 2007). Ultra-high temperature (UHT) treatment is commonly used to achieve commercial sterilisation of beverages before filling and packaging (Prabhakaran & Perera, 2006; Gedam et al., 2007; Sopelana et al., 2011).

Pre-sterilisation of plant and packaging

Processing equipment, packaging equipment and aseptic zone of filling are cleaned and sterilised prior to processing and maintained sterile during production to ensure good sanitary conditions (CAC, 1993). Equipment design must be optimal to contribute towards the production of safe products (CAC, 1993).

The surface of the container material that comes into contact with food or beverage must be sterilised after production and immediately before filling, either inside the packaging machine or externally. If sterilised externally, the packaging must be introduced aseptically into the aseptic zone of the packaging machine (Lewis & Heppell, 2000; Ansari & Datta, 2003). Aseptic packages may be sterilised using physical methods (saturated steam, UV technology), chemical methods (hydrogen peroxide, ethylene oxide), or a combination of both (Lewis & Heppell, 2000; Ansari & Datta, 2003). For instance, hydrogen peroxide and UV technology are commonly used to sterilise packaging (Ansari & Datta, 2003).

Aseptic filling of beverages using bottles

Aseptic filling of beverages is conducted in a sterile area, where the air, the surface of the packaging that comes into contact with the product, and the packaging lines are sterile (Gedam et al., 2007). The filling begins by moving the bottles into a sterile chamber, where they can be sterilised using hydrogen peroxide (Lewis, 1999; Lewis & Heppell, 2000). The bottles are held vertically and passed through a hot-air tunnel that evaporates the remaining hydrogen peroxide. The bottles are inverted again so that all surfaces are rinsed with sterile water, and then held vertically (Lewis & Heppell, 2000). The bottles are filled with product, heat-sealed, and an outer screw cap is placed before bottles leave the sterile chamber (Lewis & Heppell, 2000). The finished product is then stored in a clean and sanitary manner (CAC, 1993).

Packaging materials

The aseptic systems are designed according to the type of packaging used, which must not permit the passage of microorganisms into the product during storage and distribution (CAC, 1993; Lewis & Heppell, 2000). The choice of packaging materials depends on the chemical properties of the materials, including stability to oxidation, combustion and degradation (Lee et al., 2008a). Commonly used packaging materials are plastics, paperboard, flexible pouches, thermoformed plastic containers, and cans (Gedam et al., 2007). Plastic and paper, both carbon-based organic materials, are lighter and more susceptible to chemical reactions than inorganic materials, such as glass and metals (Lee et al., 2008a).

The atoms of plastic materials are bonded by intermolecular forces (or van der Waals forces) responsible for the low-melting and boiling-point of organic materials (Lee et al., 2008a). When compared to glass, plastics are advantageous in reducing transportation costs due to its low weight (Brydson, 1999). However, an important factor associated with the use of plastics is environmental concern, as the materials creates considerable amounts of litter at landfills (Brydson, 1999).

Polyethylene (PE) and polypropylene (PPE) plastic bottles are often used as beverage packaging in aseptic filling system (Lewis & Heppell, 2000). Polyethylene is a polymer of ethylene obtained through different polymerisation processes (Brydson, 1999). Despite its good chemical resistance, oxidation of polyethylene may occur if exposed to UV light, causing embrittlement of the polymer (Brydson, 1999). Polyethylene consisting of linear chains with few side branches usually yields a higher density and lower gas permeability than materials composed of branched chain molecules (Lee et al., 2008a). Processing methods using lower temperatures and pressures as well as metal oxide catalysts and aluminium alkyl result in higher density and high softening points of high-density polyethylene (HDPE) (Brydson, 1999). Linear low-density polyethylenes (LLDPE) have improved resistance to environmental stress-cracking and higher heat deformation resistance compared to LDPE (Brydson, 1999).

2.11.3 Heat treatment

Thermal processing of foods has been commercially used since the early 20th century to inactivate microorganisms and enzymes in foods, achieving product quality and food safety (Sandeep, 2011). The impact of thermal processes on microbial lethality and quality of foods or beverages is usually assessed when designing processes (Lewis & Heppell, 2000). Heat treatment is commonly applied to beverages by continuous flow (Lewis & Heppell, 2000).

Continuous flow systems

In continuous flow systems, commercial sterilisation of beverages is achieved by heating the product until the processing temperature is reached, holding it at this temperature for a specific time to inactivate pathogenic and spoilage microorganisms, and cooling the product rapidly to minimise the impact of heat on nutrients (Emond, 2001; Awuah et al., 2007). This system has the advantage of higher energy efficiency due to rapid heat transfer rates compared to in-container sterilisation (Awuah et al., 2007).

Beverages can be processed by high temperature short-time (HTST) pasteurisation or ultra-high temperature (UHT) using direct or indirect heat exchangers (Awuah et al., 2007). Both pasteurisation and UHT treatment have been used to process commercial beverages rich in antioxidants (Zulueta et al., 2007). The HTST process usually involves heating the product to 72-75 °C for about 15-20 s before it is cooled (Gedam et al., 2007). UHT systems use higher processing temperatures than pasteurisation, usually ranging from 125 °C to 145 °C, allowing shorter holding times to achieve commercial sterility (Emond, 2001). Pasteurisation is usually sufficient to destroy spoilage microorganisms in acidic beverages (pH below 4.6) (Lewis & Heppell, 2000; Emond, 2001). Since pasteurisation uses lower temperatures, heat-treated products with high pH must be stored chilled to maintain product quality and safety during storage (Gedam et al., 2007).

Heating methods

Methods for heating using continuous flow systems include direct steam by injection or infusion, and indirect methods such as plate heat exchangers, tubular heat exchangers, and scraped-surface heat exchangers (Sandeep, 2011). The surface of plate heat exchangers is usually corrugated to increase thermal efficiency by increasing surface contact area for heat transfer and enhancing turbulence in the system (Emond, 2001). While plate heat exchangers are suitable for low viscosity homogenous products, tubular heat exchangers can handle higher viscosity products with some particulates due to smoother corrugations with more gradual angles. Scraped-surface heat exchangers are suitable for high-viscosity and particulate systems (Emond, 2001).

Ultra-high temperature (UHT) processing

Ultra-high temperature (UHT) processing is commonly used to inactivate spores of thermophilic microorganisms in low viscosity foods by combining continuous flow thermal processing with aseptic packaging (Sopelana et al., 2011). An UHT system comprises four main sections: heater, holding tube, regenerator, and cooler (Lewis, 1999). The holding time in the UHT system is determined based on the length of the holding section and flow characteristics of the product such as density, specific gravity, temperature and apparent viscosity (Lewis, 1999; Lewis & Heppell, 2000). This information is used to characterise flow behaviour by calculating the Reynolds number (Re). If Re is less than 2100, the flow is considered laminar, while turbulence occurs when Re is higher than 10000 (Emond, 2001). The flow rate is controlled during processing to ensure holding time is achieved in the continuous-flow system (Emond, 2001). A temperature sensor is usually located at the end of the holding tube so that, if the minimum temperature is not achieved, a flow diversion valve and an alarm is activated, and the product is diverted back to the feed tank (Lewis & Heppell, 2000; Emond, 2001).

An UHT system can be classified as indirect or direct depending on the type of heat transfer medium used (Lewis, 1999). In direct UHT systems, direct contact of steam with the product may be achieved by steam injection or infusion of the product into steam, resulting in rapid heating and dilution of the product (Emond, 2001). In indirect UHT

processes, the heating medium does not come into contact with the product. The main types of indirect heating systems include plate heat exchangers, tubular heat exchangers, and scraped-surface heat exchangers (Lewis, 1999; Lewis & Heppell, 2000).

In both indirect and direct systems, the UHT steriliser is divided into a sterile section (downstream of the holding tube) and a non-sterile section (upstream of the holding tube) (Lewis & Heppell, 2000). During processing, sterility is controlled by not permitting the UHT temperature to drop, and maintaining a sterile atmosphere between processing and packaging (Lewis & Heppell, 2000). The UHT process begins by preheating the product to about 70 or 80 °C. The product remains in the holding tube for a short time, and is then subjected to cooling (Lewis & Heppell, 2000). Higher processing temperatures are obtained by applying higher operating pressures, which are maintained above the saturated vapour pressure at the desired processing temperature during the entire processing period (Lewis & Heppell, 2000).

Heat treatment of beverages

The stability of antioxidant compounds in pasteurised and UHT-treated beverages, such as juices and dairy-based beverages, has been investigated (Schamberger & Labuza, 2007; Zulueta et al., 2007; Zheng & Lu, 2011; Koch et al., 2012). Heat treatment can affect nutrients, such as carotenoids and vitamin C (Mertz et al., 2010), as well as phenolic compounds (Chen et al., 2001; Chamorro et al., 2012).

While some experiments reported no changes in total phenolic content in fruits after pasteurisation (Oliveira et al., 2012), steam pasteurisation of fermented rooibos leaves (known as red bush herbal tea), reduced total phenolic content in the heat-treated product (Koch et al., 2012). The disagreement may be explained by the fact that phenolic compounds with different numbers of phenolic groups respond differently to the Folin-Ciocalteu reagent used in the total phenolic content assay (Singleton et al., 1999).

Although heat treatment may cause degradation of bioactives in beverages (Rawson et al., 2011), the impact of heat treatment on the stability of phenolic compounds may be reduced by applying heat for a short period (Bazinet et al., 2010). Therefore, further investigation is needed to determine the impact of UHT processing on the stability of

phenolic compounds in heat-treated beverages.

2.11.4 Non-thermal preservation technologies

2.11.4.1 Membrane technology

Cold process membrane technologies represent a sustainable alternative to high-temperature treatments (Field, 2010) and have been used to clarify and stabilise beverages, concentrate particular compounds, and separate low and high molecular weight compounds (Rektor et al., 2004; Díaz-Reinoso et al., 2009; Cissé et al., 2011). Membranes are thin permeable and selective barriers composed of ceramic or polymeric material such as polytetrafluoroethylene (PTFE), polypropylene, nylon, polysulfones, and others (Field, 2010). The performance of membranes is influenced by construction material, pore size, temperature, and trans-membrane pressure (Díaz-Reinoso et al., 2009). The pore diameter of microfiltration (MF) membranes ranges from approximately 0.05 to 5 μm allowing the passage of ions, small molecules, macromolecules, and viruses (Field, 2010). The pore diameter of ultrafiltration (UF) membranes ranges from approximately 4 to 100 nm (Field, 2010). Ultrafiltration is, therefore, used for purifying and concentrating macromolecular (10–103 kDa) solutions and suspensions (Field, 2010).

MF has been used to reduce microbial load in clarified beverages with acceptable sensory characteristics (Vaillant et al., 1999; Cissé et al., 2011) and is a common preservation method used for red and white grape juice (Rektor et al., 2004). Changes to physico-chemical characteristics of products ought to be considered when using membrane technology. Cassano et al. (2010) evaluated the physico-chemical composition of cactus pear juice after MF and UF using polyvinylidenfluoride (PVDF) flat sheet membranes with a pore size of 0.20 μm. While suspended solids of cactus pear juice were successfully removed without affecting the pH of the product, a reduction in betacyanin content in the permeate fractions was observed after UF (Cassano et al., 2010). Furthermore, these authors reported that total phenolic content in the permeate

was slightly reduced by about 6% after UF, while MF did not affect total phenolic content of the juice (Cassano et al., 2010). The anthocyanin content of roselle (*Hibiscus sabdariffa* L.) extract, a crop grown in Africa, filtered through a tubular ceramic membrane with pore size diameter of 0.2 μm remained unchanged, while antioxidant activity decreased slightly from 166 to 151 μmol Trolox g^{-1} (Cissé et al., 2011). Similarly, clarification of vinegars by cross-flow MF did not affect the phenolic profile of the permeate, except for procyanidin B2, which was reduced after filtration of the vinegars (López et al., 2005). Therefore, MF may be successfully used to commercially sterilise beverages without causing a significant impact on total phenolic content of the finished product.

Fouling, a limitation of membrane technology, often occurs during filtration (Hughes & Field, 2006) and usually increases by decreasing shear stress, and increasing feed concentration and membrane pore size (Hughes & Field, 2006). In addition, altering the flow regime from laminar to highly turbulent reduces deposition of particles on membrane surfaces and increases permeate flux (Field, 2010). The deposition and removal of biological and non-biological particles during membrane filtration differ significantly (Li & Fane, 2003). Deposition of bacterial cells occurs as they interact with the cake layer and membrane surface (Li et al., 2003). The removal of formed cakes on membrane surfaces can be difficult depending on the ionic condition of the feed and contact time with the membrane surface, but can be partially controlled by reducing flux (Li et al., 2003). Therefore, strategies to limit membrane fouling should be considered, such as adjusting flux and optimising module design (Le-Clech et al., 2006).

2.11.4.2 High pressure processing (HPP)

High pressure processing (HPP) is a non-thermal method used to inactivate microorganisms by applying high hydrostatic pressure (100-800 MPa) to packaged solid or liquid foods in a closed chamber (Ray, 2003). During HPP, pressurisation compresses the volume of the pressurised material and, consequently, the temperature rises due to adiabatic heating (Ray, 2003). Temperature and pressure are then normalised during depressurisation (Ray, 2003). HPP has been used to commercially sterilise carrot juice

(Patterson et al., 2012), pomegranate juice (Varela-Santos et al., 2012) and orange juice (Sánchez-Moreno et al., 2005). Treatment at 300-500 MPa for 5-20 min was sufficient to inactivate yeasts and moulds in fruit beverages (Voldřich et al., 2004), while treatment at 350 MPa for 150 s was sufficient to keep microbial populations below detection limits in pomegranate juice stored at 4 °C for 35 days (Varela-Santos et al., 2012).

HPP causes molecular changes to microbial cells, damaging structures and functions responsible for reproduction and survival (Klotz et al., 2010; Patel et al., 2012). Even though increasing pressure, temperature and time of pressurisation enhances the aforementioned changes (Ray, 2003), injured pathogenic cells may survive and then start to grow after processing under ideal conditions, thus affecting product safety (Klotz et al., 2010; Patel et al., 2012). Gram-positive spore-formers and *Leuconostoc lactis* were found in high pressure-treated carrot juice during storage (Patterson et al., 2012). Further investigation showed that *Leuconostoc lactis* is pressure-resistant as the reduction in juice was only 0.34 $\log_{10}$ cfu/mL after treatment at 600 MPa for 1 min (Patterson et al., 2012). While vegetative cells of thermo-resistant moulds (*Talaromyces avellaneus*) can be sensitive to high pressure, ascospores have demonstrated resistance to pressures between 200 and 600 MPa at 25 °C (Voldřich et al., 2004). Therefore, higher pressure should be applied for a longer period of time, or combined with gentle heating (60 °C) to achieve sufficient inactivation of ascospores (Voldřich et al., 2004).

The effect of HPP on physico-chemical parameters and bioactives in beverages has been reported (Polydera et al., 2005; Corrales et al., 2008; Keenan et al., 2012; Varela-Santos et al., 2012). Valera-Santos et al. (2012) has reported that colour and antioxidant activity of pomegranate juice can be affected during HPP. According to the authors, experimental samples were less red when processing pressure was increased (Varela-Santos et al., 2012). In contrast, minor colour changes were observed for pressurised strawberry and blackberry purées, and the redness of the samples was retained (Patras et al., 2009).

Total phenolic content of HPP-treated foods may increase due to increased extractability of some antioxidant compounds during processing (Patras et al., 2009; Keenan et al., 2012). Patras et al. (2009) observed a 9.8 % increase in phenolics in strawberry purées treated at 600 MPa compared to unprocessed purée. In addition, applying pressure at an

adequate level may significantly reduce polyphenol oxidase enzymes in HPP-treated beverages, thus increasing the stability of phenolic compounds during storage (Keenan et al., 2012). Sánchez-Moreno et al. (2005) reported that orange juice subjected to high pressure processing contained high amount of carotenoids and unchanged vitamin C content. This may be explained by the fact that HPP does not act on covalent bonds of molecules; thus vitamins in foods are not significantly affected (Sánchez-Moreno et al., 2005).

2.11.4.3 Ultraviolet (UV) technology

Ultraviolet (UV) irradiation inactivates microorganisms due to absorption of the radiation by the deoxyribonucleic acid (DNA) of the microbial cell (Ray, 2003). Microorganisms are particularly susceptible to UV light between 200 and 280 nm (Ray, 2003). UV dosage (D), which is defined as time multiplied by irradiance (I), was characterised by Keyser et al. (2008) as UV dosage per volume of liquid, expressed as joule per litre ($J\ L^{-1}$).

UV irradiation has been used to reduce microbial load on poultry skin (Sumner et al., 1996), wine (Fredericks et al., 2011) and juices (Guerrero-Beltran & Barbosa-Canovas, 2006; Keyser et al., 2008). Due to its low penetration power (Ray, 2003), UV technology is more effective at inactivating microorganisms on smooth surfaces within the direct beam of light (Sumner et al., 1996). To overcome the issue of limited penetration depth of UV, thin films are usually required to process liquid foods (Geveke & Torres, 2012). Geveka and Torres (2012) used a centrifugal UV irradiator, which centrifugally forms a thin film, to pasteurise fruit juices.

Reductions in numbers of *Brettanomyces*, *Saccharomyces*, *Acetobacter*, *Lactobacillus*, *Pediococcus* and *Oenococcus* in grape juice and wine were observed by applying a UV-C dosage of 1377 $J\ L^{-1}$ (Fredericks et al., 2011). Geveka and Torres (2012) reported a 5-$\log_{10}$ reduction of *E. coli* in grapefruit juice was achieved by using a UV intensity of 6.0 mW/cm^2 and the energy per volume processed was determined as 2.6 $J\ mL^{-1}$. Keyser et al. (2008) observed that juices with more suspended matter, such as fibre, required higher UV-C dosage levels to achieve effective microbial load reductions. Similarly, Fredericks

et al. (2011) reported that the increase in colour and turbidity of wine and grape juices also decreased the efficacy of UV-C. It appears that suspended particles in beverages could shield microorganisms from UV irradiation (Keyser et al., 2008). Moreover, UV-C efficacy may be greatly decreased in products with high microbial concentrations, possibly due to formation of cell clumps, where the outer-layered cells act as a protective barrier to the cells on the interior of the clump (Keyser et al., 2008; Fredericks et al., 2011).

To reduce microbial loads effectively, UV irradiation may be used in combination with other techniques, including good processing practices and adequate plant sanitation (Sumner et al., 1996; Fredericks et al., 2011). UV irradiation can increase the effectiveness of hydrogen peroxide as a steriliser (Lewis & Heppell, 2000). Therefore, UV and hydrogen peroxide have been combined to sterilise food packaging during aseptic filling (Ansari & Datta, 2003). Marquis and Baldeck (2007) suggested that the synergistic effect of UV and hydrogen peroxide on inactivation of bacterial spores involved uptake of hydrogen peroxide by dormant spores, formation of hydrogen adducts with spore core components, and absorption of UV irradiation by the adducts, thereby damaging the spore enzymes.

2.11.4.4 Hurdle technology

The application of combined preservative factors, so-called hurdle technology, can improve the microbiological stability of foods by either growth inhibition or inactivation of microorganisms in food systems (Leistner, 2004). Common food preservation methods may involve inactivation or removal of microorganisms by fermentation, use of preservatives or oxygen displacement (Leistner, 1992; Sandeep, 2011). Therefore, temperature, water activity, pH, redox potential (Eh), use of preservatives and competitive microorganisms are hurdles which can act synergistically when optimised, thereby improving food quality and safety (Leistner, 1992; Tapia de Daza et al., 1996).

Most conventional and emerging food preservation technologies involve hurdle technology. Examples of hurdle combinations used in the preservation of beverages

include UV technology and pulsed electric fields (PEF) (Noci et al., 2008), heat and PEF (Walkling-Ribeiro et al., 2010), high pressure processing and heat treatment (Voldřich et al., 2004). In addition, a combination of reduced water activity, pH, preservatives and mild heat treatment has successfully been used to preserve so-called minimally processed foods (Tapia de Daza et al., 1996). Voldřich et al. (2004) reported that heat-resistant ascopospores of *Talaromyces avellaneus* in fruit juices, which were resistant to high pressure, were successfully inactivated by combining high pressure treatment (600-700 MPa) with gentle heating (60 °C). Akpomedaye and Ejechi (1998) reported that a combination of two natural antimicrobial extracts (*Zingiber officinale* and *Xylopia aetiopica*), along with heat treatment (100 °C for 2 min), inhibited microbial growth in orange and pineapple juices.

As shelf life stable food products are commonly subjected to heat treatment or aseptic processing (Sandeep, 2011), other hurdles may be combined with these techniques to achieve optimal food preservation that improves food quality and safety (Leistner, 2000).

2.12 Stability and shelf life of beverages

Chemical and physical changes occurring in the product during storage, either due to intrinsic factors, such as interaction of ingredients, or environmental influence, may affect product quality and safety (Kilcast & Subramaniam, 2000). Such changes may be influenced by the preservation technique used, quality of the ingredients, packaging and storage conditions (Sandeep, 2011). Therefore, there is need to assess physico-chemical changes in beverages during storage.

Various instrumental methods can be used to measure the physico-chemical stability of beverages during storage. The main physico-chemical indicator parameters are pH, acidity, total soluble solids and colour (Battey et al., 2001; Hough et al., 2006a; Manzocco et al., 2008). Since the described parameters are often associated with yeast and mould growth (Mosqueda-Melgar et al., 2012), changes in physico-chemical properties of beverages should be monitored during storage trials to evaluate physico-

chemical and microbial stability of the product (Yusof & Chiong, 1997; Hough et al., 2006a).

Product spoilage caused by microorganisms capable of fermenting sugars is usually associated with decreases in total soluble solids (Yusof & Chiong, 1997). In addition, mould growth in beverages may be reduced with increased sugar content and titratable acidity, accompanied by low pH (Battey et al., 2001). Physico-chemical and microbiological deterioration of beverages is often associated with changes in sensory properties of the product (Hough et al., 2006a). Therefore, physico-chemical and microbiological testing accompanied by consumer sensory data enables the estimation of the shelf life of foods and beverages (Hough et al., 2006a).

2.13 Accelerated shelf life and predictive modelling

Accelerated shelf life testing is a practical method of estimating the stability of new long shelf life products (Hough et al., 2006a) and can be applied to any deterioration process that has a valid kinetic model (Kilcast & Subramaniam, 2000). The product is stored under accelerated environmental conditions, where chemical, physical and microbial changes are evaluated. Such changes may then be related to those taking place under prevailing storage conditions using predictive modelling (Hough et al., 2006a). Mathematical modelling may then be used to evaluate chemical, physical and microbial changes in foods (Xiong et al., 1999; Sluis et al., 2005; Wang et al., 2006). Model fitting requires validation to ensure there is reasonable agreement between predicted and observed data (Betts & Walker, 2004). Verification of the model is then conducted by comparison with other studies or against naturally contaminated foods to demonstrate that the model is capable of prediction (Betts & Walker, 2004).

Microbial modelling is often developed to assess the impact of environmental factors on the response of microorganisms in foods (Xiong et al., 1999). Raccach and Mellatdoust (2007) used an algebraic model to determine the shelf life of orange juice, while Battey et al. (2001) predicted the growth of moulds *Aspergillus niger* and *Penicillium spinulosum* in beverages using five variables (pH, titratable acidity, sugar content, and

preservatives) (Battey et al., 2001).

Sluis et al. (2005) proposed a model to predict the effect of storage temperature on the stability of phenolic compounds in apple juice. In another study by Manzocco et al. (2008), the effects of light and temperature on photosensitive soft drinks during storage were observed by using a saffron aqueous model system to simulate a coloured soft drink. Wang et al. (2006) developed a model for predicting the thermo-stability of tea catechins in aqueous systems using a microwave reactor, where degradation and epimerisation reactions followed first-order kinetics (Wang et al., 2006). The rate of reactions, therefore, suggested that decrease of EGCG occurred exponentially with time (Wang et al., 2006).

2.14 Sensory evaluation

Sensory evaluation is an analytical tool used to measure the attributes stimulated by the integrated involvement of properties of food: appearance, odour or aroma, texture and flavour (Drake & Delahunty, 2011). Sensory evaluation is often used during the initial stages of product development to assess changes during the shelf life of a product (Kilcast, 2000).

The three fundamental sensory evaluation methods are discriminative, descriptive and affective analyses (Poste et al., 1991). While discriminative tests are used to compare or determine differences between samples, descriptive tests are more objective methods used to determine the nature and intensity of the differences between samples (Poste et al., 1991; Drake & Delahunty, 2011).

Discriminative tests have been used to determine differences in bitterness, sweetness and sourness between dairy products and orange juice (Prescott et al., 2004). Triangle and ranking tests are common discriminative methods (Drake & Delahunty, 2011) and have been used to assess the perception of bitterness in tea-based beverages (Streit et al., 2007). Descriptive tests have been used to conduct sensory profiling of beverages and chocolates (Donadini et al., 2012), to estimate the shelf life of apple compote (Palazón et

al., 2009) and to determine the intensity of attributes (colour, flavor and bitterness) in heat-treated peanut beverages (Rustom et al., 1996b).

Affective tests measure the preference or acceptance of samples (Drake & Delahunty, 2011) and are often used to obtain consumers' opinions on sensory characteristics when changes in process or formulation of food products occur (Kilcast, 2000; Næs et al., 2010). Affective tests have been used to measure acceptance of processed and unprocessed fruit juices (Mosqueda-Melgar et al., 2012), and to evaluate the effect of product information on the consumer's intention to purchase tea infusions containing grape skin extract (Cheng et al., 2010). Sensory consumer tests comprise discrimination tests based on preference or rating scales and should be conducted with high numbers of consumers, representative of the target market for the product (Drake & Delahunty, 2011), due to the significant variation in preferences (Noble & Lesschaeve, 2010).

When designing a sensory experiment, practicality and sample size ought to be considered (Hein et al., 2008). In addition, product type and consumption context are important factors that can influence consumer liking (Hein et al., 2012).

2.15 Project overview

Available information suggests that both white tea (*Camellia sinensis*) and grape seed (*Vitis vinifera*) extracts are potential sources of beneficial antioxidants (Inoue et al., 2001; Rietveld & Wiseman, 2003; Morré & Morré, 2006; Sakurai et al., 2010). Therefore, a beverage containing a combination of white tea and grape seed extracts is likely to be high in catechins and gallic acid (Hilal & Engelhardt, 2007; Bekhit et al., 2011; Zhao et al., 2011). In addition, a synergistic effect between white tea and grape seed phenolics may increase the biological activities of the extracts (Morré & Morré, 2006; Bekhit et al., 2011).

Processing and storage conditions, including pH and temperature, can affect the stability of catechins in beverages, which can undergo oxidation and epimerisation (Wang & Helliwell, 2000; Bazinet et al., 2010; Rawson et al., 2011). These changes can modify the phenolic profile of the product and, consequently, affect the bioavailability and biological activity of phenolics. Even though drinks, such as juices and dairy-based beverages, are commonly pasteurised and UHT-treated (Schamberger & Labuza, 2007; Zulueta et al., 2007; Zheng & Lu, 2011; Koch et al., 2012), there is no information available on the impact of UHT-processing on the stability of phenolic compounds in beverages containing white tea (*Camellia sinensis*) and grape seed (*Vitis vinifera*) extracts. Therefore, further investigation is necessary to determine the impact of UHT-treatment and storage conditions on RTD beverages infused with white tea and grape seed extracts.

Aims and Objectives

The aim of the project was to evaluate the stability of phenolics, methylxanthines and antioxidant activity in UHT-treated beverages infused with white tea (*Camellia sinensis*) and grape seed (*Vitis vinifera* var. Sauvignon Blanc) extracts during storage.

The specific objectives of the project were:

1. To determine the optimum UHT processing temperature for the production of mango-flavoured beverages containing white tea and grape seed extracts by conducting chemical, microbiological and sensory analysis;

2. To evaluate the stability of three flavoured beverages (pomegranate, mango and nectarine) during storage at 20 °C and 40 °C by measuring pH and total soluble solids, conducting enumeration of yeasts and moulds, detection of heat-resistant moulds, and sensory consumer acceptance;

3. To determine the stability of phenolics and methylxanthines in three flavoured beverages (pomegranate, mango and nectarine) during storage at 20 °C and 40 °C by analysing the beverages for total phenolic content, antioxidant activity, and identifying and quantifying individual phenolics and methylxanthines by reversed-phase HPLC.

Chapter 3 Materials and Methods

3.1 Experimental design

Phase I: **Optimisation of ultra-high temperature (UHT) processing**

The study comprised two integrated phases. The first phase consisted of a single-factor experimental design aimed at determining the optimum UHT processing temperature based on chemical, microbiological and sensory characteristics of the beverages. To determine the optimum UHT processing temperature for beverages containing grape seed and white tea extracts, one commercial formulation (mango-flavoured beverage) was UHT-treated at four temperature levels (110, 120, 130 and 140 °C) for 5 s. Yeasts and moulds, and heat-resistant moulds were analysed using standard methods as described in section 3.3.6. Total soluble solids and pH measurements were performed in duplicate (sections 3.3.4 and 3.3.5). Pre- and post-UHT treated beverages were analysed for total phenolic content (Singleton & Rossi, 1965; Singleton et al., 1999), free radical scavenging activity on α,α-diphenyl-β-picrylhydrazyl ($DPPH^{\bullet}$) (Sánchez-Moreno et al., 1998) and oxygen radical absorbance capacity (ORAC) (Ou et al., 2001; Stockham et al., 2011). A reversed-phase HPLC-DAD method developed by Yao et al. (2004) was modified and validated to identify and quantify catechins, gallic acid, theobromine and caffeine in pre- and post-UHT treated beverages. Consumer sensory preferences were conducted to evaluate consumer preferences between two beverages processed at 110 and 140 °C. Two independent experiments were conducted. Chemical and microbiological analyses were performed in either duplicate or triplicate.

Phase II: **Stability of ultra-high temperature (UHT) processed beverages during storage**

The optimum processing temperature (130 °C) determined in the first phase of the study was subsequently used to process three flavoured beverages (pomegranate, mango and

nectarine). The processed beverages were stored for 90 days at 20 °C and 40 °C, and the stability of antioxidant compounds was investigated after 0, 14, 28, 42, 63 and 90 days of storage. Samples were analysed for total phenolic content, free radical scavenging activity on α,α-diphenyl-β-picrylhydrazyl ($DPPH^{\bullet}$) and oxygen radical absorbance capacity (ORAC). Individual phenolic and methylxanthine compounds were identified and quantified by HPLC-DAD. Measurement of total soluble solids and pH, enumeration of yeasts and moulds, detection of heat-resistant moulds, and sensory acceptance were conducted. Chemical and microbiological analyses were performed in either duplicate or triplicate.

Statistical data analysis

Analysis of variance (ANOVA) was performed using Minitab 16 Statistical Software (Minitab Inc., State College, PA, USA) to determine the effect of processing temperatures on total phenolics, antioxidant activities and individual phenolic compounds obtained in Phase I; and the effects of storage time, storage temperature and type of formulation on the same response variables in Phase II. Sensory acceptance data was also analysed using ANOVA. Significant ($P < 0.05$) differences were separated using Tukey's multiple comparison test.

3.2 Materials

For the microbiological analysis of beverages, sterile Petri dishes and sterile 50-mL graduated tubes were obtained from Thermo Fisher Scientific (New Zealand). Potato dextrose agar (PDA), yeast glucose agar with chloramphenicol (YGC), universal peptone M 66 and absolute ethanol were supplied by Merck (Darmstadt, Germany). Chloramphenicol was obtained from Sigma Chemical Company (St. Louis, MO, USA).

All reagents and chemicals used were of analytical grade or higher. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), disodium phosphate (Na_2HPO_4), Folin-Ciocalteu reagent (2 N), fluorescein sodium salt ($C_{20}H_{10}Na_2O_5$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and gallic acid were all obtained from

Sigma Chemical Company (St. Louis, MO, USA). (+)-Catechin and α,α-diphenyl-β-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol and sodium carbonate (Na_2CO_3) were obtained from Scharlau (Barcelona, Spain) and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was supplied by Cayman Chemicals (Ann Arbor, MI, USA). HPLC grade acetonitrile, trifluoroacetic acid (TFA) and ethanol were obtained from Fisher Scientific (Leicestershire, UK). The standards (-)-epicatechin (EC), (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC) and (-)-epicatechin-3-gallate (ECG) were obtained from Extrasynthése (Genay, France). Gallocatechin gallate (GCG) solution (2000 µg/mL) in methanol, theobromine and caffeine were obtained from Sigma-Aldrich (Steinheim, Germany). All standards used were of 97.5% purity or higher. The water used for all chemical analyses was produced from a Milli-Q water system (Merck Millipore, Darmstadt, Germany).

3.3 Phase I: Optimisation of ultra-high temperature (UHT) processing

A mango-flavoured beverage was processed using the Hipex Mini UHT plant (Thomastown, Australia). Four UHT processing temperatures (110, 120, 130 and 140 °C for 5 s) were used to heat-treat the beverages. For the non-heat-treated control sample, 300 mL were aseptically withdrawn from the heating tank before UHT treatment.

3.3.1 Description of the ultra-high temperature (UHT) equipment

The indirect Hipex Mini UHT (Thomastown, Australia) equipped with a tubular heat exchanger was used. The UHT system comprised the following major components: balance tank, steam-jacketed heating tank, steam boiler, positive displacement pumps, control panel, regeneration section, heating section, holding tube, and a cooling section. The desired holding temperatures were set before each heat-treatment using the control panel.

Cleaning-in-place (CIP) was conducted before and after production according to manufacturer's instructions. A 1% Avoid alkaline solution (Ecolab®, Hamilton, New Zealand) at 65 ± 5 °C was circulated by pumping through the equipment, followed by rinsing with hot potable water. The CIP was conducted at 140 °C, which represented the highest holding temperature used to process the products.

3.3.2 Packaging, raw materials and formulation of beverages

The packaging materials consisted of 250 and 300 mL clear glass bottles and polyethylene terephthalate (PET) lids. The bottles and lids were cleaned using potable water and sterilised by autoclaving at 121 ± 1 °C for 15 min. All the raw materials used to prepare the beverages were obtained from Ti Tonics Limited (New Zealand) and an industrial formulation was used to prepare the beverages. The industrial formulation is confidential and, therefore, has not been described in this document.

3.3.3 Processing

Mixing ingredients and brewing

Ten litres of potable water (65 ± 5 °C) was transferred to the steam-jacketed heating tank of the mini UHT plant. Granulated white sugar was added to the tank before the brewing temperature (71 ± 1 °C) was achieved, and mixed for 3 min until dissolved. Once the brewing temperature (71 ± 1 °C) had been attained, the remaining ingredients were added sequentially as follows: citric acid, white tea extract, grape seed extract, flavouring and colouring. There were no significant temperature changes after the addition of the ingredients to the water as the volume of raw materials was proportionally low compared to the volume of water. The mixture was then heated for 20 min at 71 ± 1 °C. A non-UHT-treated beverage sample (300 mL) was then aseptically withdrawn from the heating tank and stored at 5 ± 1 °C until required for analysis. The temperature of the product

was monitored throughout the brewing step using a digital thermometer (Fluke model 51 II, USA).

The brewing conditions (time-temperature) were standardised for each beverage throughout this phase to enable evaluation of the UHT processing parameters as the only variable factor. The brewing conditions used in this study were ideal for solubilisation of a considerable amount of phenolic compounds as reported by Labbe et al. (2006). According to Lin (2004), non-fermented tea should be infused at 70-80 °C to optimise the solubility of phenolic compounds (as cited in Yang et al., 2007, p. 315).

Ultra-high temperature (UHT) treatment

Four batches of mango-flavoured beverages were produced using different holding temperatures: 110, 120, 130 and 140 °C. The steam-jacketed heating tank valve was automatically opened using the control panel and the beverage was transferred to the pre-heating section. The beverage was heated to between 79 °C to 87 °C by heat regeneration in the UHT system before entering the high temperature heating section. The product was held in the holding tube for 5 s at the selected temperature prior to cooling. The flow rate of the product was 100 L h^{-1}. Cooling was achieved by regeneration of incoming cold product and chilled water. The product outlet temperature was 22 ± 1 °C. The temperature profile of the beverages was monitored by a system of temperature sensors connected to a data logger and temperature was monitored on a digital display.

Cold-filling and storage of beverages

The products were collected from the fill-head at 22 ± 1 °C into sterile one-litre plastic containers. In addition, the first and last portions (approximately one litre) of product flowing through the fill-head were discarded as this was suspected to be diluted with rinse water. The °Brix was measured for every litre of beverage collected using a hand held refractometer (Eclipse B+S 45-07, UK). Product with °Brix below 7.1 was discarded as it was possibly diluted with rinse water. The collected heat-treated product was then manually bottled into clean and sterile glass bottles within 10 min of collection from the fill-head and stored at 5 ± 1 °C until transported to the Food Product Development Laboratory at Massey University, Albany Campus.

The processed, packaged and chilled bottles were transported to Massey University (Albany Campus) within two hours of production and immediately stored at 5 ± 1 °C until required for microbiological and sensory analysis. Three-millilitre (3 mL) aliquots of each sample were aseptically withdrawn from each package within five hours of production, dispensed into three 1.5 mL micro-centrifuge sterile tubes (Thermofisher, New Zealand), and stored at -80 °C until required for chemical analyses.

3.3.4 Total soluble solids

Total soluble solids of the beverages were estimated by measurement of °Brix in duplicate using a hand held refractometer (Eclipse B+S 45-07, UK) at 20 ± 1 °C calibrated with distilled water.

3.3.5 pH measurement

The pH of the beverages was determined in duplicate according to AOAC method 981.12 (2005) using a glass electrode. The pH of the sample was measured directly at 20 ± 1 °C using a Sartorius PB-20 (Japan) pH meter which had been previously calibrated with standard buffer solutions at pH 2.0, 4.0 and 7.0 (AOAC, 2005).

3.3.6 Microbiological analysis

Preparation of media

Media and solutions were prepared according to manufacturer's instructions. Potato dextrose agar (PDA) (39 g/L) was prepared in deionised water. Chloramphenicol (0.01%, w/v) was added to the medium to inhibit bacterial growth (FDA, 1998; Loureiro &

Querol, 1999). The mixture was autoclaved at 121 ± 1 °C for 15 min. The autoclaved agar was kept in a water bath at 45 ± 1 °C until required for use.

Preparation of 0.1% peptone water

Peptone water (0.1%) was prepared in deionised water. The solution was autoclaved at 121 ± 1 °C for 15 min.

Enumeration of yeasts and moulds

Yeasts and moulds in pre- and post-UHT treated beverages were enumerated as described by Tournas, Katsoudas and Miracco (2006b). Samples of beverages were aseptically withdrawn after 4 and 18 days of storage at 5 ± 1 °C. Suitable serial dilutions (10^{-1} to 10^{-2}) of samples were prepared in 0.1% peptone water. Serially diluted and undiluted samples (0.1 mL/plate) were spread-plated in duplicate onto pre-poured solidified PDA plates. The inoculated plates were incubated upright for 5 days at 25 °C (Tournas et al., 2006b). After 5 days, plates with 10 to 150 colonies were counted and the results expressed as colony forming units (cfu) per mL of sample. Plates without visible growth were re-incubated for another 48 h (FDA, 1998).

Detection of heat-resistant moulds

Heat-resistant moulds were detected as described by Beuchat and Pitt (2001) and Houbraken and Samson (2006) with minor modification. From each sample, duplicate 50-mL aliquots were aseptically transferred into sterile 50-mL graduated tubes. The tubes were placed in a water bath at 75-80 °C for 30 min to activate any potential heat-resistant moulds before plating (Beuchat & Pitt, 2001). The surface of the samples in the tubes was well below the surface of the water in the bath to ensure uniform heating. The temperature was monitored by inserting a thermometer into another identical tube containing the same volume of liquid and placed in the water bath.

Following heating, samples were removed from the water bath and cooled rapidly (Beuchat & Pitt, 2001). Each duplicate sample (50 mL) was equally distributed (10 mL) into five Petri dishes, mixed with 10 mL of PDA (58.5 g/L), and the plates allowed to set

(Beuchat & Pitt, 2001). Petri dishes were loosely sealed in a plastic bag to prevent drying and incubated upright at 30 °C for 14 days (Houbraken & Samson, 2006). Incubated plates were examined for growth of heat-resistant fungi at 7 and 14 days (Houbraken & Samson, 2006).

3.3.7 Chemical analyses

Pre- and post-UHT treated mango-flavoured beverages were analysed for total phenolic content, α,α-diphenyl-β-picrylhydrazyl ($DPPH^{\bullet}$) radical-scavenging activity, and oxygen radical absorbance capacity (ORAC). Phenolics and methylxanthines were analysed using reversed-phase HPLC. All chemical analyses were conducted under dim light to avoid degradation of phenolic compounds, which are light sensitive (Cheng et al., 2012).

3.3.7.1 Estimation of total phenolic content

Total phenolic content was estimated by spectroscopy using the Folin-Ciocalteu method described by Singleton et al. (1999). From each sample, 100 µL were transferred into a 15-mL plastic test tube and 900 µL of Milli-Q water was added. A reagent blank was prepared in the same way using Milli-Q water instead of the test sample. Five millilitres of ten-fold concentrated Folin-Ciocalteu reagent were added to each sample, standard and reagent blank, followed by mixing using a vortex mixer for 3 s. After 1 min and before 8 min, 4 mL of sodium carbonate solution (7.5%, w/v) was added. The mixture was mixed by vortexing for 5 s and samples were placed in the dark for 2 h at 20 ± 1 °C. After 2 h, each sample was transferred to a disposable cuvette (1 x 1 x 4.5 cm) and absorbance was measured at 760 nm at 20 ± 1 °C using a UV-1700 spectrophotometer (Shimadzu, Japan) against the reagent blank.

Gallic acid (1 mg/mL) and (+)-catechin (1 mg/mL) standard stock solutions were individually prepared using Milli-Q water, followed by sonication for 10 min. Aliquots of the stock solutions were stored in the dark at -80 °C for 5 months. Gallic acid (0.2

mg/mL) and (+)-catechin (0.2 mg/mL) working solutions were prepared before each experiment by diluting the stock solutions with Milli-Q water. Standard curves were prepared for gallic acid and (+)-catechin, ranging from 0.005 to 0.2 mg/mL. The standard curves ($R^2 > 0.99$) were constructed by plotting the absorbance (760 nm) against the standard concentration. The results were reported as milligrams of gallic acid equivalents per 100 millilitre of sample (mg GAE/100 mL) and milligrams of catechin equivalents per 100 millilitre of sample (mg CAE/100 mL).

3.3.7.2 Determination of radical scavenging activity using α,α-diphenyl-β-picrylhydrazyl (DPPH•)

The free radical scavenging activity using α,α-diphenyl-β-picrylhydrazyl (DPPH•) was measured using the method of Brand-Williams et al. (1995) and modified by Sánchez-Moreno et al. (1998).

Before each assay, samples were diluted with Milli-Q water to 50, 25, 12.5 and 6.25% (v/v). From each diluted sample, 75 µL were added to 2925 µl of freshly prepared 0.025 mg mL^{-1} DPPH solution in absolute methanol, followed by mixing. The control sample consisted of 75 µL of water in 2925 µL of DPPH solution. The reduction of the purple colour of DPPH• was measured at 515 nm against the blank (methanol) at time zero, and after 10, 20, 30, 40 and 60 min using UV-1700 and UV-1800 spectrophotometers (Shimadzu, Japan).

All determinations of free radical scavenging activity on DPPH• were performed in triplicate. Gallic acid and (+)-catechin were used as positive controls. Stock solutions (1 mg/mL) previously prepared in Milli-Q water were individually used to prepare double dilutions using Milli-Q water at 50, 25, 12.5, 6.25, 3.125 and 1.563% (v/v). A calibration curve was prepared using a DPPH stock solution (0.5 mg of DPPH in 10 mL of absolute methanol) to calculate the remaining concentration of DPPH• in the reaction medium at different reaction times (Brand-Williams et al., 1995). Double dilutions of the DPPH stock solution were prepared at 50, 25, 12.5, 6.25, 3.125 and 1.563% (v/v) and absorbance values obtained at 515 nm were plotted against the DPPH• concentration.

The percentage of the remaining DPPH$^{\bullet}$ was calculated by dividing the concentration of DPPH$^{\bullet}$ at the time of steady state by the initial concentration of DPPH$^{\bullet}$ (at time zero), multiplied by 100 (Sánchez-Moreno et al., 1998). The percentage of remaining DPPH$^{\bullet}$ against mL of sample was plotted and the antioxidant capacity of each sample was expressed as the volume (mL) of sample required to decrease 1 g of initial DPPH$^{\bullet}$ concentration by 50% (EC_{50}) (Brand-Williams et al., 1995; Sánchez-Moreno et al., 1998). The lower the value of EC_{50}, the higher the antioxidant capacity of the sample (Sánchez-Moreno et al., 1998).

3.3.7.3 Determination of oxygen radical absorbance capacity (ORAC)

Solutions

Phosphate buffer (75 mM, pH 7.4) was prepared using Milli-Q water and stored for four weeks at 5 ± 1 °C. 2, 2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) solution (153 mM) was prepared in 75 mM phosphate buffer (pH 7.4). AAPH solution was kept on ice during use and it was stable for 8 h (Huang et al., 2002). Sodium fluorescein (FL) stock solution (4 x 10^{-3} mM) was prepared in 75 mM phosphate buffer (pH 7.4). The solution was stable for four weeks at 5 ± 1 °C (Huang et al., 2002). The stock solution was further diluted (1:1000) with 75 mM phosphate buffer (pH 7.4) before each analysis.

Standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) stock solution (0.02M) was prepared in 75 mM phosphate buffer (pH 7.4) (Huang et al., 2002). Aliquots of the Trolox stock solution were stable for 4 months at -80 °C and further standard dilutions (6.25, 12.5, 25, 50, 100 and 200 µM) were prepared before each assay with phosphate buffer (75 mM, pH 7.4).

Procedure

The oxygen radical absorbance capacity (ORAC) method validated by Ou et al. (2001) and adapted by Stockham et al. (2011) was used with minor modifications. The ORAC assay was performed at 37.3 ± 0.3 °C on a FLUOstar OPTIMA microplate reader (BMG

Labtech, Germany) with 96-well black plates (Greiner Bio-one, Germany) equipped with fluorescence filters. The pump was primed with 4 mL of AAPH solution from a beaker reservoir prior to the assay and the instrument sensitivity (gain) was adjusted for each plate. Briefly, 25 µL of diluted samples, 75 mM phosphate buffer (pH 7.4) for the blank, and diluted Trolox were manually pipetted into individual microplate wells (Figure 3.1). To each well, 150 µL of working sodium fluorescein solution was added. The control was prepared by adding 200 µL of 75 mM phosphate buffer (pH 7.4) into the wells. The wells on the outside of the plate were filled with 270 µL of phosphate buffer (pH 7.4) to eliminate the potential effect of heat. The microplate was allowed to equilibrate to 37.3 ± 0.3 °C by pre-heating for 30 min.

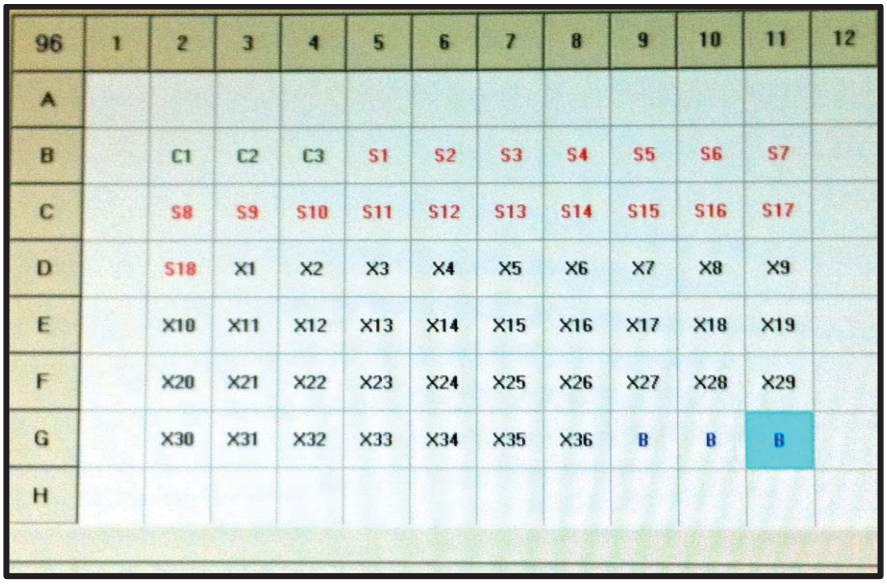


Figure 3.1 Layout of microplates used in the FLUOstar OPTIMA (BMG Labtech, Germany) for ORAC assay, where C, S, X and B represent the positions of wells with control, standards, samples and blank, respectively.

After the incubation period, the intensity of fluorescence was monitored every 60 s (= 1 cycle) for 240 min using an excitation wavelength of 485 nm and emission wavelength of 520 nm. After three cycles (3 min), 25 µL of 153 mM AAPH radical donor was automatically added to all samples except for the control. All samples were then agitated for 5 s.

Fluorescence readings of the blank, control, samples and standards were plotted against time to obtain fluorescence decay curves. Microsoft® Excel version 14.0.0 (Santa Rosa, CA, USA) was used to perform calculations. The area under the curve (AUC) was calculated as described by Stockham et al. (2011) to represent antioxidant capacity using equation 1:

$$AUC = \left(0.5 + \left[\frac{f_1}{f_0} + \frac{f_2}{f_0} + \cdots + \frac{f_i}{f_0}\right]\right) CT \qquad \text{Equation 1}$$

Where f_0 is the initial fluorescence reading at 0 min, f_1, f_2 and f_i are the fluorescence readings at cycles 1, 2 and *i*-th, respectively, and CT is the cycle time in min. The Net AUC was calculated by subtracting the AUC of the blank from the AUC of the sample or standard (Huang et al., 2002; Davalos et al., 2004). Net AUC of Trolox was plotted at five concentration levels against the concentration of Trolox. ORAC values of samples, expressed as micromoles of Trolox equivalents (TE) per litre of sample (μmol TE L^{-1}), were calculated using equation 2:

$$(Net\ AUC\ of\ sample - Intercept) x\ Dilution\ factor \div Slope \quad \text{Equation 2}$$

3.3.7.4 Analysis of phenolic and methylxanthine compounds by High-Performance Liquid Chromatography (HPLC)

The method described by Yao et al. (2004) was modified to identify and quantify catechins, caffeine, theobromine, and gallic acid in beverages. Phenolics and methylxanthines were separated by reversed phase HPLC performed on a 5 μm Grace Smart RP18 column (250 x 4.6 mm) (Grace Davison Discovery Sciences, Deerfield, IL, USA) at 18 °C using a Shimadzu HPLC system (Shimadzu UFLC, Shimadzu Prominence, Japan), consisting of two pumps (LC-20 AD) and an auto sampler (SIL-20ACHT) coupled with an SPD-M20A photodiode array detector.

A discontinuous gradient (Table 3.1) was run from 100% mobile phase A (0.1% TFA in Milli-Q water) to 100% mobile phase B (0.1% TFA in acetonitrile) at a flow rate of 0.75

mL min^{-1}. Phenolics and methylxanthines were detected at 270 nm. The compounds were identified and quantified based on the retention times and peak areas, respectively, of standards (Munoz et al., 2008) Peak areas were integrated using the Shimadzu LC Solutions Software (Shimadzu Prominence, Japan). Standard graphs were generated using Microsoft® Excel version 14.0.0 (Santa Rosa, CA, USA) (Appendix D).

Table 3.1 Gradient program used to separate phenolics and methylxanthines.

Mobile phase (%)		Time (min)
A	**B**	
100	0	0
91.5	8.5	15
90	10	22
89	11	32
85.2	14.8	50
68.2	31.8	60
0	100	68
0	100	78
100	0	95

Note: Mobile phase (A) 0.1% TFA in Milli-Q water, (B) 0.1% TFA in acetonitrile.

Preparation of standard solutions

Gallic acid and (+)-catechin (1 mg/mL) standard stock solutions were prepared in Milli-Q water. Epicatechin (EC), epigallocatechin gallate (EGCG), epigallocatechin (EGC) and epicatechin gallate (ECG) (1 mg/mL) standard stock solutions were prepared in 70% ethanol solution (v/v). Caffeine (126.2 µg/mL) and theobromine (25 µg/mL) standard stock solutions were prepared in methanol. The standard stock solutions were stable for 6 months at -80 °C. A standard mixture using gallic acid, catechin, EC, EGCG, EGC, ECG and caffeine stock solutions was prepared prior to analysis. GCG solution (2000 µg/mL)

and theobromine stock solution were diluted with Milli-Q water prior to analysis. The standards working range is described in Table D1, Appendix D.

Preparation of grape seed and white tea extracts

Grape seed and white tea aqueous extracts were analysed by HPLC-DAD to identify and quantify gallic acid, (+)-catechin, EC, EGCG, EGC, ECG, GCG, caffeine and theobromine. Grape seed and white tea aqueous extracts were individually prepared as previously described for beverages (section 3.3.3). For each extract, one litre of potable water was heated to 71 ± 1 °C in a 2 L stainless steel container using a hot plate. Once the brewing temperature was achieved, the extract was added to the water at 71 ± 1 °C at the same concentration as the formulation of beverages in this study. Ingredients were dissolved by mixing for 1 min and heated for 20 min. The brewing temperature (71 ± 1 °C) was monitored throughout the heating step using a digital thermometer (Fluke model 51 II, USA). After heating, the mixture was rapidly cooled to 20 ± 1 °C by placing the stainless steel container in an ice-bath. From each extract, aliquots (800 µL) were aseptically withdrawn from the stainless steel container, dispensed into 1.5 mL micro-centrifuge sterile tubes (Thermofisher, New Zealand) and stored at -80 °C until required for analysis.

Preparation of samples

Beverage samples, grape seed and white tea aqueous extracts, blank (Milli-Q water), and standards were filtered through 0.20 µm LC13 PVDF filter (Grace Davison Discovery Sciences, Deerfield, IL, USA) prior to analysis. Automatic injections (20 µL) were performed in duplicate.

3.3.8 Sensory evaluation

Paired-preference test was conducted on two separate occasions to determine consumer preferences between two samples. The paired comparison method, also known as two-alternative forced choice, is commonly used in consumer research (Poste et al., 1991; Bi,

2011). Paired comparison method was chosen because paired presentation of samples provides more consistent preference information compared to when a higher number of samples is tested at one time (Windsor et al., 1994; Dessirier & O'Mahony, 1998). Since the beverages were prepared using the same formulation, major sensory differences between samples treated at different temperatures were not expected. Therefore, there was a higher chance of consumers detecting differences between beverages treated differently if fewer samples were evaluated. For this reason, the two treatments (beverages treated at 110 and at 140 °C) were chosen for the paired-preference test.

Consumer recruitment

Consumer sensory panellists, aged 16 years and above, representative of the target population of potential consumers, were invited to participate in the evaluation of beverages during the Open Day at Massey University on 12th May 2012 at Sir Neil Waters Foyer, East Precinct, Albany Campus. The second sensory evaluation was conducted on 10th September 2012, and consumers were recruited by public invitation using the Institute of Food, Nutrition and Human Health (IFNHH) Notice Board at Massey University (Albany Campus).

Sensory methodology

Panellists were served 30 mL each of the two chilled (10 ± 1 °C) beverage samples in identical 50-mL clear plastic sample cups coded with three digit random numbers. Distilled water at ambient temperature (20 ± 1 °C) was used to clean the palate between samples (Noble & Lesschaeve, 2010).

The samples were simultaneously presented to each panellist in a balanced and completely randomised order so that equal number of panellists received the samples in each of the possible orders (Noble & Lesschaeve, 2010). The consumer panellists evaluated the samples from left to right and indicated their preferred sample. The consumer panellists also responded to demographic questions (age, gender and nationality) and tea drinking habits using the paired-preference test form as shown in Appendix B.

The Massey University Human Ethics Committee approved the project to conduct sensory evaluation of the beverages on 9th May 2012 (Appendix A). Participants involved in the study signed a consent form (Appendix B).

3.3.9 Statistical data analysis

Two independent experiments were conducted. Chemical analyses were performed in triplicate (n=6), except for HPLC analysis, which was performed in duplicate (n=4). Microsoft® Excel version 14.0.0 (Santa Rosa, CA, USA) was used to perform linear and non-linear regression analysis of data and to determine the mean, standard deviation (SD) and coefficient of variance (CV%). Data were compared for statistically significant differences using ANOVA on Minitab 16 Statistical Software (Minitab Inc., State College, PA, USA) to determine the effect UHT processing temperatures on response variables. The effect of production trials, as a random factor, on response variables was also determined. Significant ($P < 0.05$) differences between the means were separated using Tukey's multiple comparison test.

Sensory data from each of the two paired-preference tests were analysed using a binomial test model (Bi, 2011) using R statistical software (version 2.15.0 GUI 1.51, Austria, 2012). The probability of selection of a sample is one in two chances (Lawless & Heymann, 2010) and was calculated using equation 3:

$$p = \frac{(1/2)^{n} * n!}{(n-x)!\,x!} \qquad \text{Equation 3}$$

Where n is the total number of consumers, x is the total number of preference judgements for the most preferred sample, and p is the probability of making the number of preference choices for the most preferred sample (Lawless & Heymann, 2010). Minitab 16 Statistical Software (Minitab Inc., State College, PA, USA) was used to estimate the difference between the two proportions obtained from each of the two paired-preference tests.

3.4 Phase II: Stability of ultra-high temperature (UHT) processed beverages during storage

3.4.1 Packaging, raw materials and formulations

Five-hundred millilitres (500 mL) clear polyethylene terephthalate (PET) bottles and appropriate lids (Visy, New Zealand), made from a two-stage stretch blow process without any barriers or additive, were used to package the products.

All the ingredients used in the beverages were obtained from Ti Tonics Limited (New Zealand) with the exception of granulated white sugar (Chelsea Sugar, New Zealand), obtained from a local supermarket in Palmerston North. Three flavoured beverages (pomegranate, nectarine and mango) were prepared using industrial formulations. The industrial formulations are confidential and, therefore, have not been described in this document.

3.4.2 Description of the ultra-high temperature (UHT) equipment

Three flavoured beverages were processed in the Food Pilot Plant at Massey University, Palmerston North, using the Alfa Laval indirect UHT purpose built equipment consisting of shell and tube heat exchanger (Alfa Laval NZ Ltd, Hamilton, New Zealand).

The UHT plant comprised two balance tanks, boiler, positive displacement pump (Hydra-Cell model G13XDSTSSEMA, Hampshire, England), pre-heater, heater, regeneration section/cooling section, holding tube, and a filler located in a laminar flow cabinet (Laftech, Victoria, Australia) equipped with UV lamp (1060 mW/m^2). Thermocouples were connected to the holding tube and the cooling section located before the fill-head. Temperature was monitored on the digital display of the control panel.

Cleaning-in-place (CIP) was performed to clean the equipment before and after production according to manufacturer's instructions. One percent AC-180 caustic solution (Ecolab®, Hamilton, New Zealand) was circulated through the equipment for 15 min at 60 L h^{-1} with processing temperature set at 130 °C. Caustic solution was then rinsed off with hot potable water.

3.4.3 Processing

Mixing ingredients and brewing

Thirteen litres of each of the three flavoured beverages (pomegranate, nectarine and mango) were heat-treated. For each batch, 15 L of reverse osmosis (RO) water were dispensed into a 25 L high-density polyethylene (HDPE) plastic container. The HDPE container was placed in a stainless steel steam-jacketed kettle and immersed in water at 80 ± 2 °C.

Before the brewing temperature (70 ± 2.5 °C) had been attained, granulated white sugar was added to the water in the 25 L plastic container and mixed until dissolved. Once the brewing temperature (70 ± 2.5 °C) had been attained, the remaining ingredients were added sequentially: citric acid, ascorbic acid (in nectarine-flavoured beverages), white tea extract, grape seed extract, flavour and colour. The ingredients were mixed and heated at the brewing temperature (70 ± 2.5 °C) for 20 min, followed by a further 5 min at the same temperature to transfer the product in the HDPE container from the steam-jacketed kettle to the UHT unit. The brewing temperature of the products was kept at 70 ± 2.5 °C and monitored using a digital thermometer (Dick Smith Electronics Q1437, Sydney, Australia).

Ultra-high temperature (UHT) treatment

A schematic flow diagram of the indirect UHT plant used to process the beverages is illustrated in Figure 3.2.

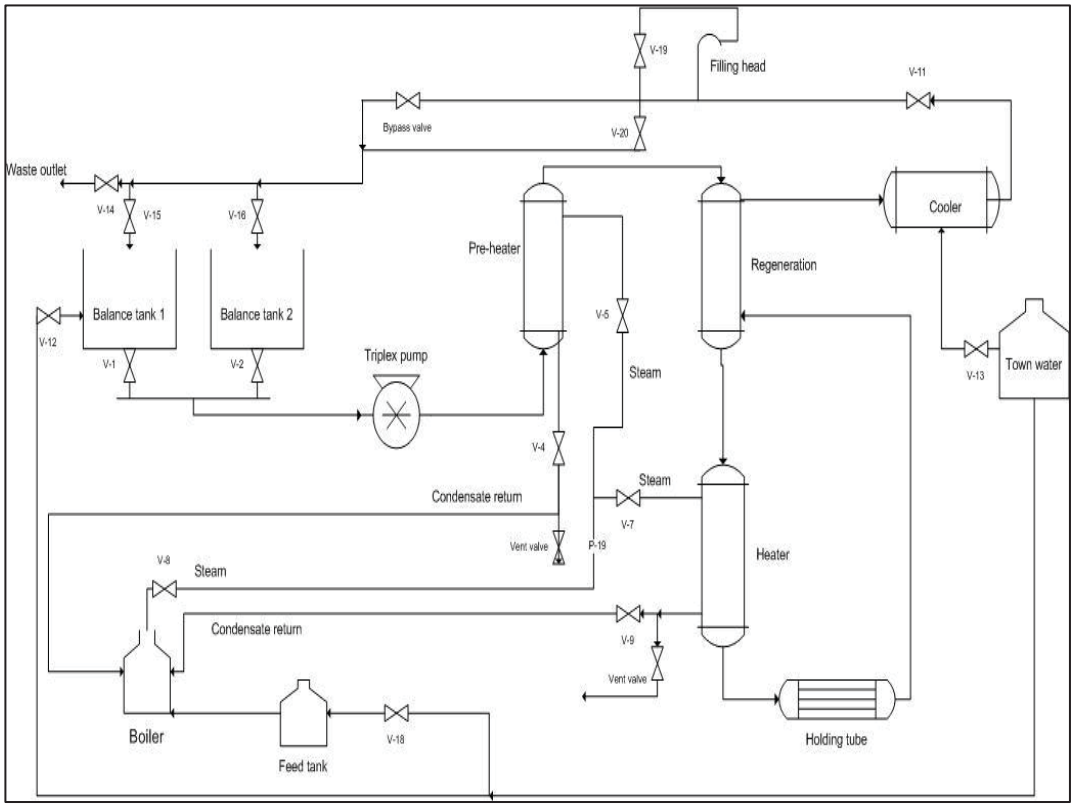


Figure 3.2 Schematic flow diagram of the UHT plant (Alfa Laval NZ Ltd).

Each of the three products was dispensed into the balance tank for UHT processing. The beverages were pre-heated (117 ± 1 °C) and then pumped (60 L h^{-1}) into the holding section. The beverages were held in the holding tube at 130 °C for 5 s, and cooled by regeneration of incoming cold product and chilled water. The product outlet temperature was 17 ± 1 °C. Temperature sensors were located at the start of the holding section and at the end of the cooling section before the fill-head. Temperature profile of the beverages was monitored through the digital display on the control panel. Approximately 20 L of potable water were flushed through the UHT equipment (with holding temperature of 130 °C) between each heat-treated product.

Sterilisation of packaging

Ster-Bac (Ecolab®, Hamilton, New Zealand) solution (200 ppm), a liquid quaternary ammonium compound sanitiser, was prepared using potable water. Bottles and lids were cleaned with hot potable water, soaked in Ster-Bac solution for 30 min, and rinsed with

sterile water. Bottles and lids were then placed in the laminar flow cabinet and exposed to UV-radiation (1060 mW/m^2) for a minimum of 30 min before filling with UHT-treated product.

Cold-filling of beverages

The heat-treated product (17 ± 1 °C) was dispensed from the fill-head and manually bottled in a semi-aseptic environment in the laminar flow cabinet (Figure 3.3). Before filling, the laminar flow cabinet was switched to maximum airflow and UV lights were switched on. Sterile latex surgical gloves were worn during handling and filling.

Figure 3.3 Laminar flow cabinet (Laftech, Victoria, Australia) and filler.

The first and the last portions (approximately one litre each) of the product flowing from the fill-head were discarded as this was presumed to be diluted with water. The °Brix of bottled products was measured using a calibrated pocket digital refractometer (PAL-1, Pocket Series, Atago Ltd., Japan) and products with a °Brix below 7.1 were discarded.

Transportation and storage conditions

Heat-treated packaged beverages were stored at 5 ± 1 °C overnight and transported to Massey University (Albany Campus) within 48 h of production. The beverages were then stored away from direct light in two incubators: at 20 °C (Contherm Digital Series, Contherm Scientific Ltd, New Zealand) and 40 °C (Qualtex, Andrew Thom Ltd, Australia). Samples were analysed for pH, total soluble solids (°Brix), heat-resistant moulds, total phenolics, antioxidant activity, and individual phenolics and methylxanthines by HPLC-DAD after 0, 14, 28, 42, 63 and 90 days of storage at 20 and 40 °C. Samples were also analysed for yeasts and moulds after 0, 7, 14, 21, 28, 42, 63 and 90 days of storage at 20 and 40 °C.

3.4.4 Analyses of UHT-treated beverages during storage

3.4.4.1 Total soluble solids and pH measurement

Total soluble solids (°Brix) and pH were determined according to the procedures previously described in sections 3.3.4 and 3.3.5.

3.4.4.2 Microbiological analysis

Enumeration of yeasts and moulds, and detection of heat-resistant moulds were performed as described in section 3.3.6 using yeast extract glucose chloramphenicol agar (YGC) instead of PDA. The beverage samples stored for 63 and 90 days were plated inside a laminar airflow chamber (Heraus, Germany). The morphology of colonies was observed using a microscope (Axiostar plus, Carl Zeiss). Colonies grown on agar were cut with a sterile sharp scalpel blade, placed onto glass slides, stained with lactophenol-cotton blue dye, and then covered with glass coverslips for microscopic observation (Bencheqroun et al., 2007).

Preparation of media

The YGC medium (40 g/L) was prepared according to manufacturer's instructions (Merck, Darmstadt, Germany) using deionised water. YGC (60g/L) was also used for detection of heat-resistant moulds. All solutions were autoclaved at 121 ± 1 °C for 15 min.

Microbiological analysis of packaging

Bottles and lids (areas that came into contact with product) were analysed for yeasts and moulds (Tournas et al., 2006b), and heat-resistant moulds (Beuchat & Pitt, 2001; Houbraken & Samson, 2006). Sixty-millilitre sterile peptone water (0.1%) was aseptically transferred into each of three bottles, capped and manually shaken for 40 s (R. Liu, personal communication, April 15, 2012, Massey University, Auckland). Peptone water (0.1%) recovered from the bottles was analysed as described in section 3.3.6. Peptone water (0.1%) was aseptically withdrawn from the bottles and suitable serial dilutions (10^{-1} to 10^{-2}) were prepared. Serially diluted and undiluted samples (0.1mL/plate) were spread-plated onto pre-poured solidified YGC agar (Tournas et al., 2006b). The inoculated plates were incubated upright for 5 days at 25 °C (Tournas et al., 2006b). Agar plates with 10 to 150 colonies were counted after 5 days and the results expressed as colony forming units (cfu) per mL of sample. Agar plates without visible growth were re-incubated for another 48 h (FDA, 1998).

For detection of heat-resistant moulds, 50 mL of sterile peptone water (0.1%) were aseptically transferred from each bottle into sterile 50-mL graduated tubes. The tubes were placed in a water bath at 75-80 °C for 30 min (Beuchat & Pitt, 2001). Following heating, samples (50 mL) were removed from the water bath, cooled, then equally distributed (10 mL) into five Petri dishes and mixed with 10 mL of 1.5 strength YGC agar (Beuchat & Pitt, 2001). Petri dishes were loosely sealed in a plastic bag to prevent drying and incubated upright at 30 °C for 14 days (Houbraken & Samson, 2006). Incubated plates were examined for growth of heat-resistant fungi at 7 and 14 days (Houbraken & Samson, 2006).

3.4.4.3 Chemical analyses

Aliquots (800 μL) of mango, pomegranate and nectarine flavoured-beverages stored at 20 and 40 °C were aseptically withdrawn from the original sealed bottles at 0, 14, 28, 42, 63 and 90 days during storage, dispensed into 1.5 mL micro centrifuge sterile tubes (Thermofisher, New Zealand) and stored at -80 °C until required for analysis. Estimation of total phenolic content, determination of free radical scavenging activity on α,α-diphenyl-β-picrylhydrazyl ($DPPH^{\bullet}$) and oxygen radical absorbance capacity (ORAC), as well as identification and quantification of phenolic and methylxanthine compounds by HPLC-DAD were performed as previously described in section 3.3.7.

3.4.4.4 Sensory evaluation

Sensory evaluation of the beverages was conducted in individual sensory booths under white lighting in the purpose-built Sensory Evaluation Laboratory at Massey University, Albany Campus.

Pomegranate-, mango- and nectarine-flavoured beverages stored for 90 days at 20 and 40 °C were evaluated for consumer acceptance. All six beverages had been kept at 5 ± 1 °C for 72 h prior to preparation for consumer sensory testing. The samples were presented monadically to panellists following a completely randomised block design (Wakeling & MacFie, 1995). Twenty millilitres (20 mL) of each of the six beverage samples were served chilled (10 ± 1 °C) in identical clear plastic sample cups (50 mL) coded with three digit random numbers (Noble & Lesschaeve, 2010). Consumer sensory panellists used a nine-point hedonic scale (Kemp et al., 2009) to evaluate flavour and appearance of the beverages (Appendix B). Distilled water at ambient temperature (20 ± 1 °C) was used to clean the palate between samples (Noble & Lesschaeve, 2010).

The data obtained for flavour and appearance acceptance were analysed using ANOVA on Minitab 16 Statistical Software (Minitab Inc., State College, PA, USA). Significant differences ($P < 0.05$) were then separated using Tukey's multiple comparison test.

Chapter 4 Optimisation of ultra-high temperature (UHT) processing conditions of beverages infused with white tea and grape seed extracts (Phase I)

4.1 Introduction

Ultra-high temperature (UHT) processing is commonly used to commercially sterilise low viscosity foods, such as coffee beverages (Sopelana et al., 2011), soymilk (Xu & Chang, 2009) and peanut beverages (Rustom et al., 1996b). This technology has been used to process ready-to-drink (RTD) beverages containing phenolic compounds with high antioxidant capacity (Prabhakaran & Perera, 2006; Schamberger & Labuza, 2007; Zulueta et al., 2007). Although continuous thermal systems are widely used by the beverage industry, there is limited published information on the stability of antioxidant compounds in pasteurised and UHT-treated beverages (Schamberger & Labuza, 2007; Zulueta et al., 2007; Zheng & Lu, 2011; Koch et al., 2012).

The market for plant-based beverages containing antioxidants is rapidly increasing due to their importance in consumer health and wellness, and increased consumers awareness of their biological functions. Products containing grape seeds and white tea are rich in catechins, which may undergo degradation or epimerisation during thermal treatment (Chen et al., 2001; Chamorro et al., 2012). Although there are some reports on the effect of heat on stability of phenolic compounds in white tea and grape seed extracts, there is no published information on the impact of UHT processing on the stability of phenolics in beverages infused with white tea and grape seed extracts together. Therefore, the main objective of this phase of the study was to determine the optimum UHT processing temperature for white tea and grape seed extracts infused beverages. Two independent experiments were conducted as described in section 3.3 to produce two batches of mango-flavoured beverages using the same raw materials. In each experiment, mango

beverages were UHT- treated using four temperatures (110, 120, 130 and 140 °C) for 5 s. Samples were analysed following the procedures previously described in Chapter 3.

4.2 Results and Discussion

4.2.1 Total soluble solids and pH

The total soluble solids (°Brix) and pH of processed mango-flavoured beverages in the first production trial (Phase I) were 7.1 ± 0.05 °Brix and 2.92 ± 0.003, respectively. In the second production trial (Phase I), the °Brix and pH of processed mango-flavoured beverages were 7.1 ± 0.05 °Brix and 2.82 ± 0.004, respectively. The presence of highly reactive hydroxyl groups in phenolics implies that these molecules are susceptible to the effect of pH and, thus, ionisation (Rusak et al., 2008). The low pH of beverages helps to maintain microbial stability of the product during storage as most spoilage bacteria are unlikely to grow in high acid beverages (Stratford & Capell, 2003). However, yeasts and moulds can still grow in low pH (Stratford & Capell, 2003; Tournas et al., 2006a). Hence, the microbial stability of high acid beverages can be achieved by applying UHT treatment combined with aseptic filling using sterile packaging (Stratford & Capell, 2003).

4.2.2 Microbiological analysis of yeasts and moulds

The dehydrated extracts (grape seed and white tea) used to produce the beverages in this study were analysed for presence of yeasts and moulds, and heat-resistant moulds before being added to the beverages. Spread-plating technique was used for enumeration of yeasts and moulds as it provides maximum exposure of cells to atmospheric oxygen (Beuchat & Cousin, 2001). No yeasts, moulds or heat-resistant moulds were detected in any of the extracts. Fungal contamination of up to 10^5 cfu/g can be found in plant extracts (Tournas et al., 2006b), but may be substantially eliminated by thermal

processing and dehydration of the extracts (Tournas et al., 2006b).

In this study, yeasts and moulds were not detected in mango-flavoured beverages, but heat-resistant moulds were detected in non-UHT-treated and UHT-treated mango-flavoured beverages stored for 4 days at 5 ± 1 °C (Table 4.1). Heat-resistant moulds were not detected in beverages processed at 140 °C, while low mould growth was detected in the other beverage samples (Table 4.1). Commercial beverages without added preservatives are susceptible to spoilage by yeasts and moulds (Stratford & Capell, 2003). The chances of contamination by yeasts and moulds are higher in these products due to the presence of sugar and low acidity (Tournas et al., 2006a). In our study, no chemical preservatives were added to the beverages. Weak organic acids, such as citric acid, which was used in the mango-flavoured beverage formulation, can be added to beverages to control yeasts and moulds (Mosqueda-Melgar et al., 2012).

Table 4.1 Heat-resistant moulds (cfu/50 mL) in mango-flavoured beverages.

UHT processing temperature (°C)/ 5 s	7 days	14 days
Non-UHT-treated	nd	1-5
110	1-5	1-5
120	nd	6-10
130	nd	1-5
140	nd	nd

Note: nd = not detected
Plates were incubated for 7 and 14 days at 30 °C.

Although heat treatment has been proven efficient in the inactivation of yeasts and moulds (Sato & Takano, 2000), the predominant forms of heat-resistant moulds (asci or ascospores) in beverages may survive high temperature treatments (Conner & Beuchat, 1987; Sant'Ana et al., 2009). When using heat inactivation, dominant populations of asci structures require additional energy to enable their rupture, which will liberate the ascospores for inactivation (Sant'Ana et al., 2009). While pasteurisation at 94 °C for 30 s

has been shown to inactivate *Byssochlamys fulva* ascospores in juice (Sant'Ana et al., 2009), temperature variations in industrial heat exchangers can result in survival of heat-resistant moulds, even if the raw materials initially contain low levels of ascospores (e.g. less than 10 cfu/100 mL) (Sant'Ana et al., 2009).

While no growth of yeasts, moulds, or heat-resistant moulds were detected in the raw materials (grape seed and white tea extracts) used to produce the beverages, a small count of heat-resistant moulds was observed in the beverages. The presence of moulds was not expected as the UHT temperatures applied to the beverages were likely to inactivate most moulds commonly found in beverages (Rustom et al., 1996a; Sato & Takano, 2000; Pacheco & De Massaguer, 2005). In addition, the growth of moulds in mango-flavoured beverage samples did not follow a clear trend. The results suggested that contamination of samples post UHT processing may have occurred, either during processing in the pilot plant, or during sample plating in the microbiology laboratory. Mould contamination of beverages after UHT treatment has been reported (Sato & Takano, 2000). Sato and Takano (2000) conducted a media fill test, which is the performance of an aseptic manufacturing procedure using a sterile microbiological growth medium, instead of beverage, to test the adequacy of aseptic procedures in preventing contamination during production. Sato and Takano (2000) isolated *Arthrinium sacchari* and *Chaetomium funicola* from UHT-treated (135 °C for 30 s) liquid medium in a tea-based beverages filling production line, which showed low heat resistance. Similar to the present study, Sato and Takano (2000) suggested that the presence of moulds in UHT-treated product could have occurred after UHT processing.

To confirm the presence of heat-resistant moulds in the mango-flavoured beverage samples, the thermal resistance of the strains present in the plated samples should have been determined by isolating the strains, and subjecting them to same heat-treatment conditions used to process the beverages (Sato & Takano, 2000). The number of viable cells in the heat-treated medium is then determined to verify the thermal resistance of the strains (Sato & Takano, 2000). In addition, a media fill test could have been useful to verify if contamination occurred during processing (Sato & Takano, 2000). However, further investigation was not conducted due to the unavailability of equipment and limited time frame of this project. The issue of aerial contamination during plating of heat-resistant moulds has been reported by Beuchat and Pitt (2001) and could have affected our

results as the air in the microbiology laboratory was not filtered. This argument, however, does not explain why mould growth was not detected in beverages processed at 140 °C. In addition, mould growth was not detected in the beverage samples plated using a inoculating laminar airflow chamber during Phase II of this study, which, therefore, suggests that Phase I samples may have suffered aerial contamination in the microbiology laboratory in spite of strict adherence to aseptic techniques.

While heat treatment may be sufficient to provide microbial (yeasts and moulds) stability of beverages and extend their shelf life (Mosqueda-Melgar et al., 2012), fungal contamination of up to 5 $\log_{10}$ cfu/mL has been found in various commercial processed beverages, including fruit juices and soy milk (Tournas et al., 2006a). In this study, the low pH of mango-flavoured beverages (2.82 ± 0.004 and 2.92 ± 0.003, trials 1 and 2, respectively) may have contributed towards the microbial stability of the beverages, and, therefore, treatment of beverages using at least 110 °C for 5 s was sufficient to obtain a stable product. However, the shelf-life stability of the products was not determined since mango-flavoured beverages were only stored for a short period (18 days). Therefore, the optimum UHT temperature must be the highest possible option that does not affect chemical or sensory characteristics of the product as a higher processing temperature may contribute to a longer shelf life of the beverages.

4.2.3 Total phenolic content and antioxidant capacity

Total phenolic and antioxidant capacity levels in non-UHT-treated and UHT-treated mango-flavoured beverages are summarised in Table 4.2. UHT treatment of infused beverages at different temperatures (110, 120, 130 and 140 °C) for 5 s did not affect ($P > 0.05$) total phenolic content or antioxidant capacity (Appendix F).

Table 4.2 Total phenolic content and antioxidant capacity[1] in non-UHT-treated and UHT-treated mango-flavoured beverages.

Processing temperature (°C)/5 s	Total phenolics[2] (mg GAE/100 mL)	Total phenolics[3] (mg CAE/100 mL)	DPPH$^{\bullet}$ EC_{50}[4]	ORAC (µmol TE L^{-1})[5]
Non-UHT-treated	50.89 ± 0.68	46.19 ± 0.76	561.11 ± 24.69	6324.30 ± 476.67
110 °C	51.83 ± 1.06	47.14 ± 1.46	656.45 ± 44.80	5452.56 ± 818.89
120 °C	52.26 ± 1.85	47.58 ± 2.42	615.40 ± 67.25	5902.37 ± 462.40
130 °C	52.57 ± 1.55	47.89 ± 1.98	647.18 ± 33.57	6157.38 ± 443.79
140 °C	52.20 ± 1.36	47.50 ± 1.09	627.45 ± 33.60	6678.82 ± 512.37

[1]Mean values ± standard deviation of triplicate measurements of two independent experiments (n = 6).
[2]GAE= gallic acid equivalent
[3]CAE= catechin equivalent
[4]DPPH$^{\bullet}$ EC_{50} = mL of sample required to decrease 1 g of the initial DPPH$^{\bullet}$ concentration by 50%
[5]TE= Trolox equivalent

Total phenolic content

The total phenolic assay is based on the reduction of phosphotungstic acid in alkaline solution to phosphotungstic blue, which is proportional to the number of aromatic phenolic groups (Singleton & Rossi, 1965; Singleton et al., 1999). Total phenolics in the beverages ranged from 50.89 ± 0.68 to 51.83 ± 1.06 mg GAE/100 mL, and from 46.19 ± 0.76 mg to 47.89 ± 1.98 CAE/100 mL. Grape seed and white tea are good sources of phenolic compounds with high antioxidant activity, such as catechins (Montealegre et al., 2006; Rusak et al., 2008; Travaglia et al., 2011). The total phenolic levels in the UHT-treated beverages (Table 4.2) were similar to those in commercial beverages as reported by Zulueta et al. (2007) and Seeram et al. (2008). Zulueta et al. (2007) reported that UHT-treated fruit beverages contained between 40.2 to 74.1 mg GAE/100 mL, while the total phenolic content in RTD tea-based beverages reported by Seeram et al. (2008) ranged from 40 to 90 mg GAE/100 mL.

In comparison to other tea infusions, the phenolic contents in the mango-flavoured beverages were lower than green tea infusions (89 mg/100 mL) (Bekhit et al., 2011), but higher than other beverages infused with grape skin and green tea, which contained between 10 and 64 mg GAE/100 mL (Bekhit et al., 2011). A variety of tea infusions prepared in boiling water contained from 23.4 to nearly 220 mg GAE/100 mL, while white tea contained 218 mg GAE/100 mL (Almajano et al., 2008). The Folin–Ciocalteau method used in this study is commonly used to estimate total phenolic content in grape and tea samples (Kim et al., 2006; Nishiyama et al., 2010; Xu et al., 2011), although it has low specificity as the reagent reacts with other reducing substances, such as ascorbic acid (Escarpa & González, 2001). For this reason, phenolics in the beverages infused with white tea and grape seed extracts were also quantified by HPLC (section 4.2.4).

Although there was no difference ($P > 0.05$) between total phenolics in non-UHT-treated and UHT-treated beverages, it is thought that thermal treatment of beverages can improve solubility of phenolic compounds of powdered extracts (Kim et al., 2006). However, heat can affect the chemical structure of phenolic compounds, particularly catechins (Wang et al., 2000; Chamorro et al., 2012). Polymerised procyanidins of grape extracts may breakdown during heat treatment, changing from highly to relatively lower

polymerised molecules, thereby modifying the phenolic composition of the product (Chamorro et al., 2012). Since phenolic composition can contribute to antioxidant and pro-oxidant effects of beverages (Maatta-Riihinen et al., 2005; Gollücke et al., 2009), the impact of heat treatment on phenolic composition can affect antioxidant capacity of the beverages. The results of this study showed, however, that the temperatures used to process the beverages (110, 120, 130 and 140 °C) for short time (5 s) did not affect ($P > 0.05$) antioxidant capacity levels.

Antioxidant capacity

Due to the complex phenolic profiles of grape seed and white tea extracts, the beverages produced in the study were expected to contain a heterogeneous chemical composition. Since natural antioxidants are multifunctional, more than one analytical method is recommended to measure the antioxidant capacity of these compounds (Frankel & Meyer, 2000). In this study, the antioxidant capacity of the beverages was estimated by determining free radical scavenging activity using α,α-diphenyl-β-picrylhydrazyl ($DPPH^{\bullet}$) and oxygen radical absorbance capacity (ORAC), in addition to the total phenolic assay (Sánchez-Moreno et al., 1998; Stockham et al., 2011).

The ORAC levels in the non-UHT-treated and UHT-treated mango-flavoured beverages, expressed as μmol Trolox equivalent per litre of sample (μmol TE L^{-1}), ranged from 5452.56 ± 818.89 to 6678.82 ± 512.37 μmol TE L^{-1} (Table 4.2). Stockham et al. (2011) reported similar ORAC levels for tea-based RTD drinks commercially available in the market (2200 to 16000 μmol TE L^{-1}). Processed beverages containing fermented tea (e.g. black tea) extracts usually have lower antioxidant capacity than those prepared with non-fermented tea extracts, such as green and white teas (Stockham et al., 2011). Kodama et al. (2010) reported that commercial brands of RTD green tea-based beverages contained between 6000 and 22000 μmol TE L^{-1}. Beverages containing soy showed higher ORAC levels (Kodama et al., 2010) than those in mango-flavoured beverages. According to Kodama et al. (2010), the antioxidant capacity of commercially available tea-based beverages may be partially attributed to ascorbic acid, which is commonly used as an additive.

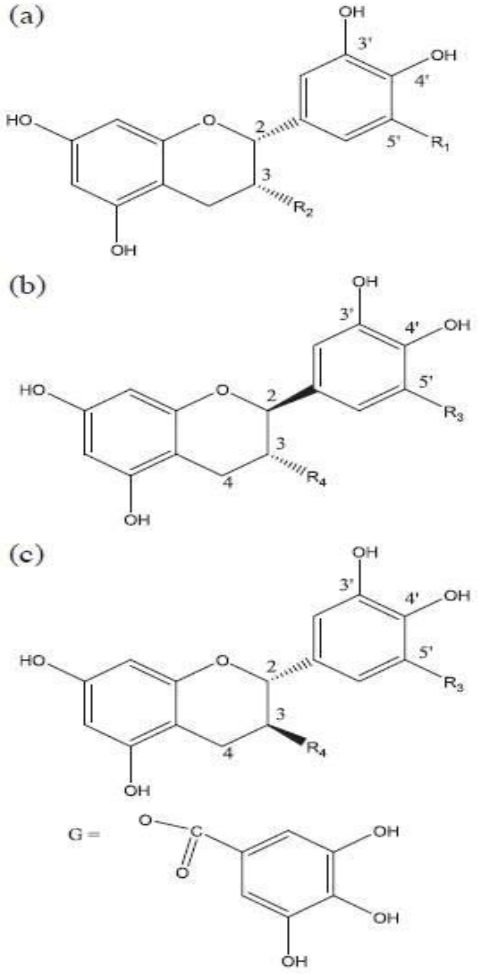
The DPPH free radical scavenging activity results were reported as volume (mL) of sample required to decrease 1 g of the initial DPPH$^{\bullet}$ concentration by 50% (EC_{50}) (Sánchez-Moreno et al., 1998). As shown in Table 4.2, the EC_{50} levels in the beverages ranged from 561.11 ± 24.69 to 656.45 ± 44.80 mL/g of DPPH$^{\bullet}$. Since molecules may demonstrate different DPPH$^{\bullet}$ scavenging activity, Sanchez-Moreno et al. (1998) has classified the kinetic behaviour of antioxidants based on the time needed to reach the steady state at a concentration corresponding to EC_{50} (T_{EC50}) in the DPPH$^{\bullet}$ assay. Gallic acid, which is present in grape seed extracts, has been classified as intermediate, while ascorbic acid, commonly used as an ingredient in RTD beverages, is rapid reacting antioxidant. Quercetin, which is also present in grape seed extracts, has been categorised as slow (Sánchez-Moreno et al., 1998). In our study, the UHT-treated beverages contained grape seed (*Vitis vinifera*) extract, which is high in gallic acid, and white tea. Grape seeds and white tea also contain high amounts of catechins (Guendez et al., 2005; Montealegre et al., 2006; Islam, 2011), while quercetin, which was not quantified in this study, is mainly found in grape skins (Montealegre et al., 2006). Although there is no available information on the free radical scavenging activity of UHT-treated beverages infused with both white tea and grape seed extracts, DPPH$^{\bullet}$ scavenging activity of the beverages was expected since grape seed and white tea contain phenolics with high antioxidant capacity (Guendez et al., 2005; Montealegre et al., 2006).

In this study, there was no correlation between total phenolics and antioxidant capacity data (Table 4.2). When evaluating antioxidant capacity of samples, data obtained from different assays may not always be directly compared because each assay uses a different mechanism to measure antioxidant capacity (Cao & Prior, 1998). While the ORAC assay uses a hydrogen atom transfer (HAT) mechanism with Trolox as the standard (Ou et al., 2001), the DPPH$^{\bullet}$ assay is based on direct reduction via single electron transfers (SET) or radical quenching via hydrogen atom transfer (HAT), resulting in loss of the purple colour (Prior et al., 2005). There are, however, some contradictions on the subject. While inconsistencies between ORAC and DPPH$^{\bullet}$ data have been reported (Seeram et al., 2008), other studies have shown good correlation between antioxidant capacity assays (Gollücke et al., 2009; Granato et al., 2010). Granato et al. (2010) has identified a positive correlation between non-anthocyanin flavonoid content and ORAC and DPPH$^{\bullet}$ levels in wines. Gollücke et al. (2009) found a positive correlation between total

phenolics and DPPH$^{\bullet}$ scavenging activity of grape juice during storage. Steric accessibility is key for the reaction of the antioxidant with DPPH$^{\bullet}$, which is why small molecules that have better access to the radical site have higher results with the DPPH$^{\bullet}$ assay (Prior et al., 2005).

Non-anthocyanin flavonoids contribute significantly towards the measured antioxidant activity of beverages (Granato et al., 2010). Gallic acid, monomeric catechin and epicatechin are major contributors to antioxidant capacity (Lachman et al., 2009). The stereochemical structures of phenolic compounds play an important role in scavenging free radicals (Maatta-Riihinen et al., 2005). Scavenging activities of GCG, GC and (+)-catechin on free radicals generated by AAPH and DPPH are higher than those of their corresponding epimers (EGCG, EGC and EC). In addition, scavenging activity of catechins increases in the following order: EC < (+)-catechin < GCG (Guo et al., 1999). The presence of a gallate group in the molecule (Figure 4.1) plays a major role in its ability to scavenge free radicals (Guo et al., 1999; Locatelli et al., 2009). In fact, the gallate group at 3 position of the C-ring is responsible for the scavenging ability and biological activity of EGCG and ECG (Yang et al., 2001). This partially explains why EGCG is the major contributor to the oxy-radical scavenging activity of green tea (Kang et al., 2010). The increased scavenging ability of EGC and GC when compared to EC and (+)-catechin is related to the hydroxyl group at the 5' position of B-ring (Figure 4.1) (Guo et al., 1999).

Catechol structures contribute significantly towards the antioxidant capacity of phenolic compounds (Maatta-Riihinen et al., 2005). Maatta-Riihinen (2005) has suggested that the number of catechol units in the reaction mixture positively correlates with biological activities of catechins and procyanidins, such as their ability to protect against oxidation of low-density lipoproteins (LDL). The high phenolic content and antioxidant capacity of the UHT-treated beverages may be attributed to their high catechin and procyanidin levels. Therefore, UHT-treated beverages infused with grape seed and white tea extracts may have the ability to protect against *in vivo* oxidation. Further investigation is needed to determine if the UHT-treated beverages exhibit biological activities associated with health benefits.



Notes:

Component	Abbrev.	R_1	R_2	R_3	R_4
(-)-Epigallocatechin gallate	(-)-EGCG	OH	G	-	-
(-)-Epicatechin gallate	(-)-ECG	H	G	-	-
(-)-Epigallocatechin	(-)-EGC	OH	OH	-	-
(-)-Epicatechin	(-)-EC	H	OH	-	-
(-)-Gallocatechin gallate	(-)-GCG	-	-	OH	G
(-)-Gallocatechin	(-)-GC	-	-	OH	OH
(-)-Catechin gallate	(-)-CG	-	-	H	G
(+)-Catechin	(+)-C	-	-	H	OH

Figure 4.1 General chemical structures of catechins: (a) epicatechins, (b) non-epicatechins, (c) (+)-catechin, and G=gallate group (Ananingsih et al., 2011).

4.2.4 Analysis of phenolic and methylxanthine compounds by HPLC

Non-UHT-treated and UHT-treated mango-flavoured beverages were analysed for catechins, gallic acid, caffeine and theobromine as described in section 3.3.7.4, Following the validation of the HPLC method by determining reproducibility and repeatability, expressed as coefficient of variance (CV%) (Appendix D).

Individual phenolics and methylxanthines were quantified in aqueous extracts of grape seed and white tea prepared as described in Chapter 3. As shown in Table 4.3, the grape seed extract contained high levels of (+)-catechin and gallic acid, while EGCG and GC were not detected. White tea extract contained higher levels of caffeine, EGCG, ECG, EGC, as well as lower levels of GCG and (+)-catechin. Similar to our study, Kodama et al. (2010), identified EGC, ECG, and EGCG as the major catechins in *Camellia sinensis* extract.

The concentrations of individual phenolics and methylxanthines was analysed in non-UHT-treated and UHT-treated mango-flavoured beverages (Table 4.4). Since (+)-catechin and EGC peaks co-eluted, their corresponding peak areas were combined and quantified as the sum of (+)-catechin and EGC.

UHT treatment at different temperatures (110, 120, 130 and 140 °C) for 5 s did not affect ($P > 0.05$) the content of individual compounds in the beverage samples (Table 4.4). Overall, the UHT-treated samples were high in EGCG, EC, EGC and (+)-catechin, and contained higher amounts of caffeine than theobromine (Table 4.4). This finding was in agreement with results reported by Friedman et al. (2005), who stated that although the content of theobromine in tea may be variable, it is usually about ten times lower than its caffeine content.

Table 4.3 Concentration[1] of phenolics and methylxantines (mg/L) in white tea and grape seed aqueous extracts.

Aqueous extract	Gallic Acid	Catechin and EGC	Caffeine	EC	EGCG	ECG	Theobromine	GCG
Grape seed	1.65 ± 0.01	13.67 ± 0.42	0.25 ± 0.01	13.47 ± 0.23	nd	nd	0.051 ± 0.05	nd
White tea	0.34 ± 0.00	4.75 ± 0.11	6.43 ± 0.09	2.12 ± 0.15	14.68 ± 0.16	3.98 ± 0.18	0.58 ± 0.03	1.08 ± 0.02

[1]Mean values ± standard deviation of duplicate measurements of two independent experiments (n = 4), expressed as mg/L.
Aqueous extracts were prepared by heating at 71 ± 1 °C for 20 min.
nd = peaks not detected

Table 4.4 Concentration[1] of phenolics and methylxantines (mg/L) in non-UHT-treated and UHT-treated mango-flavoured beverages.

Processing temperature (°C)/5 s	Gallic Acid	Catechin and EGC	Caffeine	EC	EGCG	ECG	Theobromine	GCG
Non-UHT-treated	1.74 ± 0.03	15.48 ± 1.70	6.20 ± 0.86	13.25 ± 0.28	9.30 ± 3.27	1.96 ± 1.0	0.41 ± 0.02	0.20 ± 0.17
110 °C	1.75 ± 0.03	15.93 ± 1.51	6.23 ± 0.78	13.25 ± 0.29	12.11 ± 0.11	2.95 ± 0.27	0.40 ± 0.02	0.57 ± 0.02
120 °C	1.76 ± 0.04	16.16 ± 1.92	6.23 ± 0.73	13.49 ± 0.44	11.89 ± 0.12	2.83 ± 0.12	0.41 ± 0.03	0.53 ± 0.04
130 °C	1.77 ± 0.05	15.89 ± 1.74	6.36 ± 0.92	13.64 ± 0.37	11.72 ± 0.10	2.78 ± 0.27	0.39 ± 0.01	0.56 ± 0.02
140 °C	1.84 ± 0.10	15.84 ± 1.49	6.28 ± 0.97	13.75 ± 0.19	11.14 ± 0.16	2.65 ± 0.41	0.40 ± 0.00	0.56 ± 0.02

[1]Mean values ± standard deviation of duplicate measurements of two independent experiments (n = 4), expressed as mg/L.

The effects of heat on the stability of flavanols reported by others (Kim et al., 2007; Ananingsih et al., 2011) were not observed in the present study. The increase in the concentration of GCG in the beverage, however, tended to be significant ($P = 0.069$) following UHT treatment, while EGCG levels decreased with higher UHT temperature (140 °C), suggesting that epimerisation of catechins may have occurred (Kim et al., 2007; Ananingsih et al., 2011). As previously discussed, the stability of catechins in beverages is related to their sterical structures (Guo et al., 1999). Isomerisation and oxidation are the main reactions that catechins undergo during heating (Wang et al., 2000; Chamorro et al., 2012). Therefore, EGCG, EGC, EC and ECG may undergo epimerisation during heating, and consequently, levels of their respective isomers GCG, GC, catechin, and catechin gallate (CG) increase (Kim et al., 2007; Ananingsih et al., 2011). Meanwhile, oxidation of catechins leads to a decrease in the total catechins in the products, the level of which, according to Kim et al. (2007), is directly proportional to processing temperature.

In this study, two production trials were carried out 4 months apart to produce the beverages, using the same processing parameters and raw materials. Significant differences ($P < 0.05$) in the levels of gallic acid, EC, caffeine, and EGC combined with (+)-catechin were found between the two production trials. The reasons for such differences are not clear and cannot be sufficiently investigated within the scope and time frame of this project. However, since the two batches of beverages were produced four months apart, it is possible that some phytochemicals in the grape seed and white tea extracts had been affected during storage of raw materials at 5 ± 1 °C. The $DPPH^{\bullet}$ scavenging activity of the beverages also varied ($P < 0.05$) between the two productions, being higher in the beverages produced in the second trial. The changes in free radical scavenging activity could be related to modifications in the phenolic profile of the beverages, caused by degradation or epimerisation reactions (Guo et al., 1999). Overall, gallic acid was higher ($P < 0.05$) in the beverages produced during the second trial, while EC, and EGC combined with (+)-catechin were lower ($P < 0.05$) compared with the beverages produced in the first trial. The increased gallic acid levels in the beverages from the second trial may have contributed towards the increase in $DPPH^{\bullet}$ scavenging activity (Guo et al., 1999). While catechins are highly susceptible to degradation and epimerisation, polymerised procyanidins of grape extracts can breakdown during heat

treatment, causing changes from highly to less polymerised molecules (Chamorro et al., 2012), which could increase the levels of monomers. Therefore, the modifications in the composition of oligomers and polymers may affect the free radical scavenging ability in the beverages (Chamorro et al., 2012). In addition, monomeric flavanols may be absorbed more easily than highly polymerised compounds, which may positively contribute towards their *in vivo* biological actions (Deprez et al., 2001; Gonthier et al., 2003). Oxidation, however, may cause degradation of catechins, leading to a decrease in the total catechins (Wang et al., 2000; Kim et al., 2007), and possibly reducing the antioxidant capacity of the product.

Despite the variations in the concentrations of phenolics between the two production trials, similar trends were observed in both occasions. While GCG increased slightly ($P > 0.05$) with UHT treatment in both trials, EGCG decreased ($P > 0.05$) in the beverages treated at 140 °C (Table 4.4). The results suggest that epimerisation of EGCG in the mango-flavoured beverages may have occurred with heat treatment, which would decrease EGCG and consequently increase the level of its isomer GCG (Kim et al., 2007; Ananingsih et al., 2011). Similarly, Chamorro et al. (2012) observed an increase in GC levels after autoclave treatment of grape pomace, indicating that epimerisation of catechins occurred during heat treatment. In our study, however, GC was not quantified due to unavailability of standards. Gallic acid also increased slightly ($P > 0.05$) in beverages treated at 140 °C (Table 4.4). Gallic acid may have been released due to the excision of the gallate group attached to the C-ring of flavonoids (Figure 4.1), or from the hydrolysis of gallotannins, which were not quantified but might have been present in the beverages (Chamorro et al., 2012).

Catechins are more stable in solutions with pH below 3 (Wang et al., 2006) and, therefore, the low pH of the beverages could have contributed towards the stability of phenolic compounds, particularly catechins, during heat processing. In addition, the high level of catechins (Table 4.4) present in the beverages may have suppressed fungal growth and contributed towards the stability of the beverages during storage (Sato & Takano, 2000), as the antimicrobial properties of catechins are well known (Almajano et al., 2008; Perumalla & Hettiarachchy, 2011; Adámez et al., 2012).

4.2.5 Sensory evaluation

Paired-preference test was conducted twice to compare two UHT-treated mango-flavoured beverages processed at 110 and 140 °C. The two beverage samples were chosen for the sensory analysis because there were no significant differences ($P > 0.05$) in antioxidant capacity, phenolics or methylxanthines levels between UHT-treated beverages processed at the different temperatures (110, 120, 130 and 140 °C). Therefore, two independent paired-preference tests were conducted as described in section 3.3.8 to determine any significant sensory differences ($P < 0.05$) between the products as shown in Table C1 (Appendix C).

In the first paired-preference test, the two samples were evaluated by 118 consumer panellists (n=118) with median age of 27.6 years, ranging from 16 to 82 years of age. Of the consumer panellists, 75.4% (n=89) were female, 22.0% (n=26) male, 2.5% (n=3) did not complete the demographic questionnaire, and 57% consumed tea beverages regularly. Overall, there was no difference ($P > 0.05$) in preference between mango-flavoured beverages treated at 110 °C and at 140 °C. Regular consumers of tea beverages (35.6%, n=42) also preferred ($P > 0.05$) both products equally. The second paired-preference test was conducted with 48 consumer sensory panellists (n=48): 45.8% (n=22) were female, 52.1% (n=25) male and 2.1% (n=1) did not complete the demographic questionnaire. The median age of the panellists was 26.4 years, who ranged in age from 18 to 53 years. In the second paired-preference test, 77.1% (n=37) preferred the beverage treated at 110 °C, while 22.9% (n=11) preferred ($P < 0.05$) the beverage treated at 140 °C. The participants involved in the study (n=32, 66.7%) that consumed tea beverages regularly (50.0%, n=24) also preferred ($P < 0.05$) the sample processed at 110 °C.

Although the beverages processed at 110 and 140 °C were equally preferred ($P > 0.05$) in the first paired-preference test, the beverage processed at the lowest temperature (110 °C) was significantly preferred ($P < 0.05$) in the second experiment (Appendix C). The different ($P < 0.05$) outcomes may be related to the lower number of consumers in the second trial. Consumer sensory evaluation is associated with large discrepancies as the participants are not usually trained (Hough et al., 2006b). It is, therefore, recommended to use large numbers of consumer sensory panellists to improve the quality of the data

obtained (Hough et al., 2006b; Noble & Lesschaeve, 2010). While the number of consumer sensory participants may be satisfactory, a higher number in the second experiment would most likely have improved the quality of the data and overall outcomes.

Contextual effects may have also contributed to the different outcomes between the two paired-preference tests. The physical environment in which food or beverage is selected and consumed can alter the perception of the products (Rozin & Tuorila, 1993; King et al., 2004). Meiselman et al. (2000) observed that acceptance of foods can be different when the product is served in different environments. Since the physical environments in the two studies were different, contextual factors of the environment in which the beverages were consumed may have contributed to the different outcomes. The first test was performed during the Open Day at Massey University, where sensory panellists were exposed to context effects of the physical environment, such as lighting and noise, while tasting the samples. Social interaction may have also had an effect on results as flowing discussions between the sensory panellists were permitted during tasting. Therefore, environmental and social factors may have affected the choices of the sensory panellists during the first test.

The second test was conducted in individual sensory booths under white lighting in the purpose-built Sensory Evaluation Laboratory at Massey University. In this environment, the aforementioned contextual effects were controlled under laboratory-based conditions, thus differences within the beverages were the main variable. Therefore, the participants in the second paired-preference test were exposed to less context effects during consumption of the beverages and this may explain the different outcome of the two studies. In addition, it is possible that sensory evaluation performed in sensory booths may have contributed to increased concentration and focus of sensory panellists during the second test. It has been suggested that the exclusion of these contextual variables from research may oversimplify the consumer experience, providing misleading results (Bell et al., 1995; Meiselman et al., 2000). This suggests that the addition of contextual effects in consumer preference tests may provide results that are more similar to choices that consumers would make in "real life" situations (King et al., 2004). For this reason, the results obtained in the first phase, where beverages treated at 110 °C and at 140 °C were equally preferred, were considered more relevant in this study; therefore, sensory

was not a key determining factor for the selection of the optimum UHT-processing temperature.

4.3 General Discussion

Although optimisation of the processing temperature is essential to ensure the stability of phenolics in RTD beverages (Wang et al., 2006; Kim et al., 2007), sensory and microbiological aspects of RTD beverages must also be evaluated. The lowest temperature used to process beverages in this study (110 °C for 5 s) was sufficient to obtain a microbiologically stable product under the experimental conditions used, as yeasts and moulds growth were not detected. The beverages in Phase I were, however, only stored for a short period (18 days); therefore, the microbiological stability of the products during shelf life was not evaluated. Since a higher processing temperature may contribute to a longer shelf life of the beverages, the optimum UHT temperature must be the highest possible option that does not affect chemical or sensory characteristics of the product. Sensory was not a major determining factor for optimising the processing conditions because the general public at the Open Day did not detect any differences between beverages processed at 110 and 140 °C in the first trial. Although heat treatment at different temperatures did not affect ($P > 0.05$) the antioxidant activity in beverages, some changes in phenolic compounds were observed in beverages processed at 140 °C. It appears that catechins in the beverages processed at 140 °C may have undergone epimerisation and hydrolysis, containing less EGCG ($P > 0.05$) and higher ($P > 0.05$) gallic acid compared to beverages processed at 110, 120 and 130 °C. Therefore, the results indicate that UHT treatment at 140 °C or above may negatively affect the content of phenolics in beverages containing white tea and grape seed extracts.

Catechins have demonstrated potential health benefits towards cancer and cardiovascular diseases (Zhang et al., 1997; Dashwood et al., 2002; Hara, 2011), with EGCG playing a major role in the biological activity of *Camellia sinensis* extracts (Sen et al., 2009; Bazinet et al., 2010). Although reports have suggested that a high content of EGCG in beverages may lead to increased health benefits (Sen et al., 2009; Bazinet et al., 2010), it is essential to note that the biological activity of beverages is related to the synergistic

effects between phytochemicals in a mixture, and not just the concentration of each individual component (Shahidi et al., 1992; Peyrat-Maillard et al., 2003).

The aim of this study was to determine the optimum processing conditions, which should have minimal impact on the chemical composition of the beverages. As grape seeds contain high levels of polymerised procyanidins (Chamorro et al. 2012), such compounds are expected to be present in beverages in considerable amounts, but were not investigated in this study due to unavailability of standards. Hence, when we refer to chemical stability of the beverages, we are in fact referring to the stability of phenolics and methylxanthines that were quantified using HPLC-DAD. Since the phenolic profile of the beverages was slightly affected by UHT treatment at 140 °C for 5 s (compared to treatments at 110, 120 and 130 °C), decreasing EGCG and increasing gallic acid, 130 °C was considered the optimum processing temperature to produce beverages infused with white tea and grape seed extracts.

4.4 Conclusion

UHT treatment at 130 °C for 5 s was the optimum parameter to obtain a microbiologically stable beverage without affecting total catechins, gallic acid, theobromine and caffeine in the beverages infused with white tea and grape seed extracts. However, further investigation is needed to determine chemical, microbial and sensory stability of the beverages processed at the optimum UHT conditions (130 °C for 5 s) during storage. Therefore, in the next phase of the study, the shelf stability of three flavoured beverages (mango, pomegranate and nectarine) was investigated during storage at ambient temperature (20 °C) and at 40 °C to simulate accelerated storage.

Chapter 5 Stability of UHT-treated beverages infused with white tea and grape seed extracts during storage (Phase II)

5.1 Introduction

Beverages infused with (*Camellia sinensis*) and grape seed (*Vitis vinifera*) extracts contain catechins, which can undergo oxidation and isomerisation (Sluis et al., 2005; Kim et al., 2007; Li et al., 2012). The stability of catechins in beverages during storage can be affected by temperature, pH, ingredients and metal ions (Wang et al., 2000; Bazinet et al., 2010; Ananingsih et al., 2011). Catechins in beverages can undergo degradation during storage, mainly due to oxidation processes (Wang et al., 2000; Yang et al., 2007). These reactions can lead to changes in the phenolic composition of the beverages, thus affecting the biological properties of the product. In addition, the oxidation of catechins, especially EGCG and EGC, may be accompanied by changes in sensory properties of beverages during storage, including unfavourable colour changes (Wang et al., 2000). The aim of this study was to evaluate the shelf stability of UHT-treated beverages infused with white tea and grape seed extracts, and investigate changes in phenolic and methylxanthine levels in beverages during storage.

The optimum UHT processing conditions established in Phase I of the study (130 °C for 5 s) were used to process the beverages in this experiment. The stability of three flavoured beverages (pomegranate, mango and nectarine) infused with white tea (*Camellia sinensis*) and grape seed (*Vitis vinifera*) extracts were UHT-treated at 130 °C for 5 s, cold-filled into 500 mL PET bottles, and stored in the dark for 90 days at ambient temperature (20 °C) and 40 °C to simulate accelerated storage. PET packaging, rather than glass, was chosen because it is commonly used by the food and beverage industry due to its good mechanical properties, transparency and oxygen barrier properties (Ros-Chumillas et al., 2007). The three industrial formulations (pomegranate, mango and

nectarine) contained the same concentrations of white tea and grape seed extracts, with slightly different levels of citric acid. Nectarine was the only beverage containing ascorbic acid. Beverages were prepared using deionised water to reduce the interaction of metal ions with phenolic compounds. The three flavoured-beverages were analysed before UHT treatment, and after UHT-treatment during storage as described in section 3.4.

5.2 Results and Discussion

5.2.1 Total soluble solids and pH

Data on total soluble solids (°Brix) and pH of the flavoured beverages (pomegranate, mango and nectarine) stored for 90 days at 20 and 40 °C are shown in Table 5.1. The total soluble solids of the three beverages was 7.1 - 7.2 °Brix and did not change during storage (Table 5.1).

The three beverages (pomegranate, mango and nectarine) had different ($P < 0.05$) pH levels at the start and throughout the storage period (Table 5.1). The nectarine-flavoured beverage had the highest pH during storage (3.60 ± 0.00 dropping to 3.14 ± 0.00), followed by pomegranate (3.02 ± 0.00 dropping to 2.76 ± 0.00) and mango (2.96 ± 0.00 dropping to 2.79 ± 0.00) (Table 5.1). The difference in pH levels between the flavoured beverages was expected since each flavoured beverage was prepared using slightly different concentrations of citric acid, while nectarine also contained ascorbic acid.

Storage temperature did not affect ($P > 0.05$) the pH of UHT-treated beverages. Overall, the pH levels of the beverages decreased ($P < 0.05$) with length of storage. The decrease in pH of the UHT-treated beverages during storage (Table 5.1) may have been caused by microbiological growth, such as that of lactic acid bacteria. Lactic acid bacteria can grow in low acid beverages (Stratford & Capell, 2003), producing organic acids (Zalán et al., 2010), thus decreasing the pH of the product (Table 5.1). Although yeasts and moulds did not appear to have germinated continuously during storage, lactic acid bacteria (which were not specifically investigated in this study) may have been present in the packaging, thus contaminating the beverages.

Table 5.1 Total soluble solids (°Brix) and pH of UHT-treated pomegranate-, mango-, and nectarine-flavoured beverages stored for 90 days at 20 and 40 °C.

	Flavour	Storage temperature (°C)	UHT-treated beverages during storage (days)					
			0	14	28	42	63	90
pH	Pomegranate	20 °C	3.02 ± 0.00	2.91 ± 0.00	2.85 ± 0.01	2.86 ± 0.01	2.82 ± 0.00	2.78 ± 0.00
		40 °C	3.02 ± 0.00	2.90 ± 0.0	2.79 ± 0.00	2.89 ± 0.00	2.90 ± 0.00	2.76 ± 0.00
	Mango	20 °C	2.96 ± 0.00	2.91 ± 0.01	2.78 ± 0.00	2.78 ± 0.01	2.89 ± 0.01	2.79 ± 0.00
		40 °C	2.96 ± 0.00	2.91 ± 0.0	2.79 ± 0.00	2.75 ± 0.00	2.90 ± 0.00	2.80 ± 0.00
	Nectarine	20 °C	3.60 ± 0.00	3.29 ± 0.00	3.09 ± 0.01	3.19 ± 0.01	3.40 ± 0.00	3.14 ± 0.00
		40 °C	3.60 ± 0.00	3.36 ± 0.00	3.28 ± 0.01	3.17 ± 0.01	3.35 ± 0.01	3.14 ± 0.00
°Brix	Pomegranate	20 °C	7.2 ± 0.0	7.1 ± 0.0	7.2 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0
		40 °C	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0
	Mango	20 °C	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0
		40 °C	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0
	Nectarine	20 °C	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0
		40 °C	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0	7.1 ± 0.0

Values represent mean ± standard deviation of duplicate measurements.

5.2.2 Microbiological analysis

Enumeration of yeasts and moulds, and detection of heat-resistant moulds

Pomegranate-, mango-, and nectarine-flavoured beverages were analysed for yeasts and moulds, and heat-resistant moulds (Table 5.2). YGC media was used for plating because it is commonly used for analysis of yeasts and moulds in New Zealand (R. Liu, personal communication, April 15, 2012, Massey University, Auckland). Following enumeration, moulds were only found in mango beverages stored for 63 days at 40 °C (40 cfu/ mL), and nectarine beverages stored for 63 days (2.4 x 10^2 cfu/mL) and 90 days (0.23 x 10^2 cfu/mL) at 20 °C. To detect heat-resistant moulds, the beverages were heat-shocked to activate any potential heat-resistant moulds (Beuchat & Pitt, 2001) as described in section 3.3.6. Heat-resistant moulds were detected in all three flavoured beverages at 0 (2 to 20 cfu/50 mL) and 14 days of storage (100 to 400 cfu/50 mL), and in most beverages stored for 28 and 42 days (Table 5.2).

The internal surfaces of the packaging (PET bottles and lids) that came into contact with the beverages were also analysed for yeasts and moulds, and heat-resistant moulds before the sanitation process, as described in section 3.4. Heat-resistant moulds were not detected on the surface of packaging; however, yeasts and moulds ranged from 0.9 to 2.1 x 10^2 cfu/mL (Figure 5.1). Despite the apparent presence of yeasts and moulds on the internal surfaces of the packaging, yeasts and moulds were not detected in non-UHT-treated or UHT-treated beverages stored for up to 42 days. Although the reasons for the presence of yeasts and moulds on packaging may not be easily explained, contamination during storage or transportation could have occurred. The source of contamination of the packaging was not further investigated due to the limited time frame of the study.

Table 5.2 Heat-resistant moulds (cfu/50 mL) in non-UHT treated and UHT-treated pomegranate-, mango-, and nectarine-flavoured beverages (cfu/50 mL) stored for 90 days at 20 and 40 °C.

Flavour	Non-UHT treated	Storage temperature (°C)	UHT-treated beverages during storage (days)					
			0	14	28	42	63	90
Pomegranate	nd	20 °C	14	200-300	200-300	300-400	nd	nd
	nd	40 °C	14	200-300	150-200	300-400	nd	nd
Mango	1	20 °C	2	300-400	150-200	300-400	nd	nd
	1	40 °C	2	300-400	nd	nd	nd	nd
Nectarine	7	20 °C	20	150-200	200-300	50-100	nd	nd
	7	40 °C	20	100-150	nd	50-100	nd	nd

Note: nd = not detected
Plates were incubated at 30 °C for 14 days.

The UHT processing and cold-filling techniques used in the study may have been effective in reducing yeasts and moulds in the products, since these microorganisms were not present in UHT-treated beverages stored for up to 42 days. Moulds were only present after 63 days in mango beverages stored at 40 °C (40 cfu/ mL) and nectarine beverages stored at 20 °C (0.23 to 2.4 x 10^2 cfu/mL). These results are from a different test (enumeration of yeasts and moulds) to that reported on Table 5.2 (detection of heat-resistant moulds). It is possible that the presence of yeasts and moulds in the packaging could have contributed to the mould growth observed in mango- and nectarine-flavoured beverages stored for 63 and 90 days. Yeasts and moulds in beverages that have been metabolically or structurally injured as a result of chemical or physical stress may be able to recover during storage of the product, if the environmental conditions are suitable, causing subsequent product spoilage (Graumlich & Stevenson, 1978; O'Reilly et al., 2000). In this study, a quaternary ammonium-based disinfectant and UV treatment were used to sterilise the packaging prior to the production of the beverages. UV treatment has been shown to reduce nearly 75% of yeasts and moulds in beverages (Pala & Toklucu, 2011). Quaternary ammonium-based disinfectants can effectively reduce yeasts and moulds, such as *Saccharomyces uvarum*, *Candida oleophila* and *Aspergillus niger* (Korukluoglu et al., 2006). As some fungi may be resistant to chemical disinfectants, the concentration and the contact time need to be optimised to destroy the microorganisms on equipment or package surfaces (Korukluoglu et al., 2006).

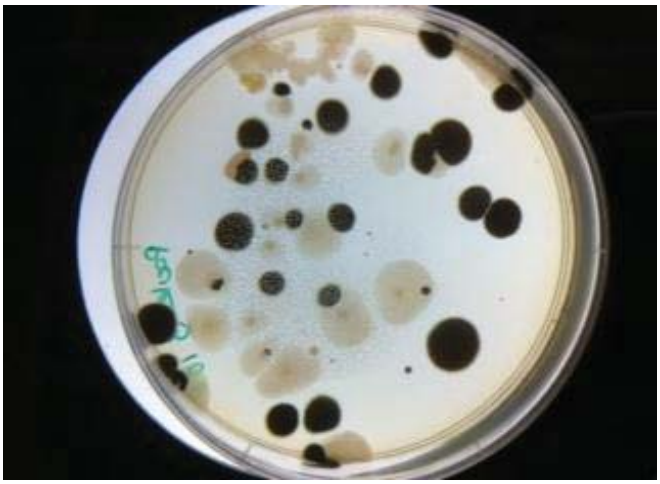


Figure 5.1 Growth of yeasts and moulds from internal surfaces of PET bottles and lids after plating on YGC agar. Plated samples were incubated for 5 days at 25 °C.

While the low pH of the three formulations (pomegranate, nectarine, and mango) may have suppressed the growth of most bacteria in the beverages during storage, fungi are capable of growing at lower pH (< pH 3) (Cole et al., 1987; Stratford & Capell, 2003). Nectarine beverages had slightly higher ($P < 0.05$) pH values (initially at 3.60 ± 0.00) than the other flavours (Table 5.1), which may have provided a more suitable environment for mould growth, hence resulting in the higher counts observed for nectarine beverages stored for 63 days (2.4 x 10^2 cfu/mL) and 90 days (0.23 x 10^2 cfu/mL) at 20 °C.

Morphology

The morphology of the moulds grown on YGC agar after plating mango- and nectarine-flavoured beverage samples were examined after staining the fungi with lacto-cotton blue dye (Bencheqroun et al., 2007). Two moulds were observed in nectarine beverages (Figure 5.2). The circular green velvety moulds grown on YGC agar after plating nectarine beverages (Figure 5.3) had similar morphological characteristics to those of the mango beverages (Figure 5.4). Septate hyphae, branched conidiophore and round conidia (asexual spores) were observed in both moulds (Figure 5.3 and 5.4) (Descals, 1985). The dark brown moulds grown on YGC agar after plating nectarine beverage samples (Figure 5.2) presented elongated conidiophores and ovoid conidia (Figure 5.5) (Descals, 1985). Although the morphological structures observed in Figures 5.3 and 5.4 appeared similar to those of *Penicillium* sp (Kurup et al., 2000; Liu et al., 2011), further investigation (which was outside the scope of this project) would be needed to positively identify the moulds.

Growth of moulds were observed after plating heat shocked beverage samples stored from 0 to 42 days (Table 5.2), suggesting the presence of heat-resistant moulds in the beverages. Microscopic examination of all the moulds grown on YGC agar (Figure 5.6) showed similar hyphal structures and spores characteristics.

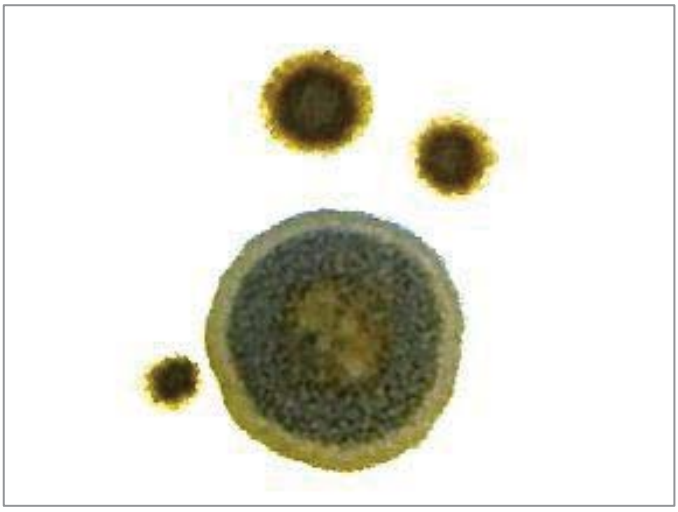


Figure 5.2 Growth of mould on YGC agar after plating nectarine beverage stored for 90 days at 20 °C. Plated samples were incubated for 5 days at 25 °C.

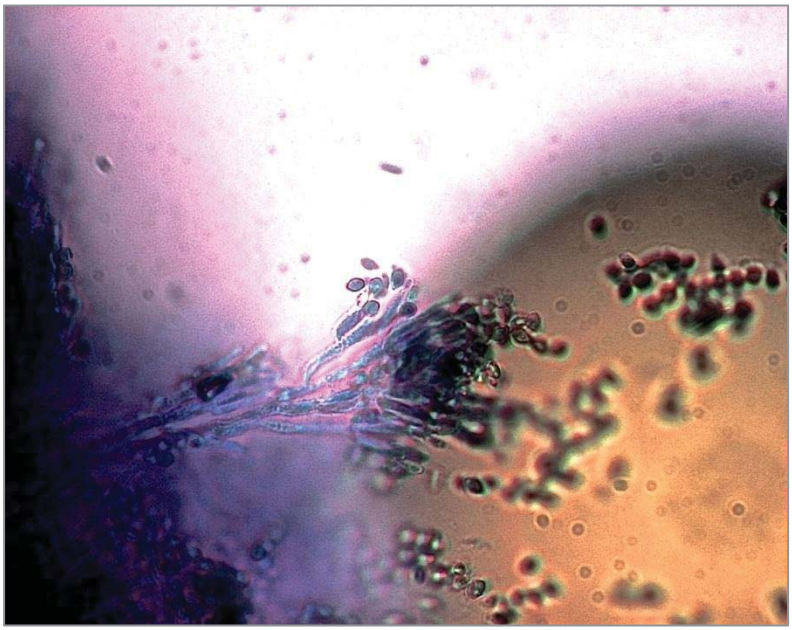


Figure 5.3 Growth of mould on YGC agar after plating nectarine beverage stored at 20 °C for 90 days (Axio Cam MR colour, Carl Zeiss, 100x). Plated samples were incubated for 5 days at 25 °C.

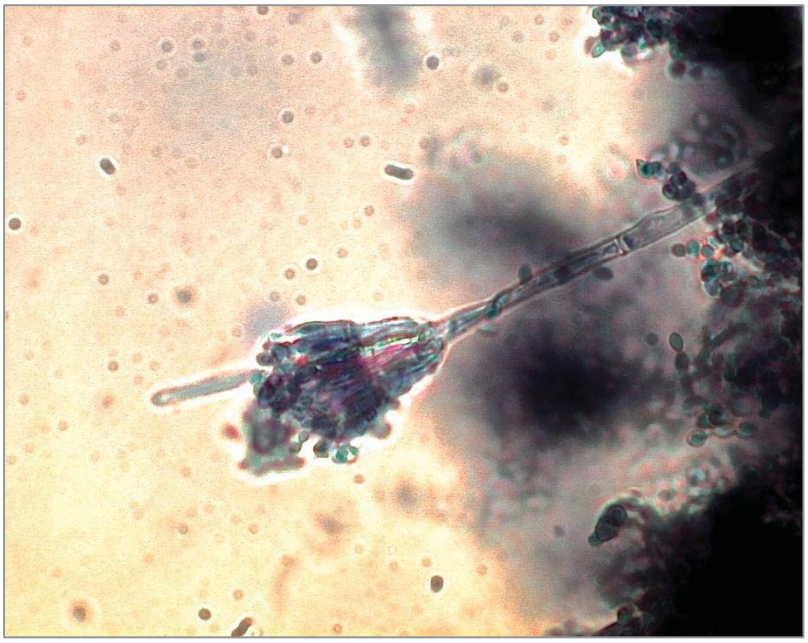


Figure 5.4 Growth of mould on YGC agar after plating mango beverage stored at 20 °C for 63 days (Axio Cam MR colour, Carl Zeiss, 100x). Plated samples were incubated for 5 days at 25 °C.

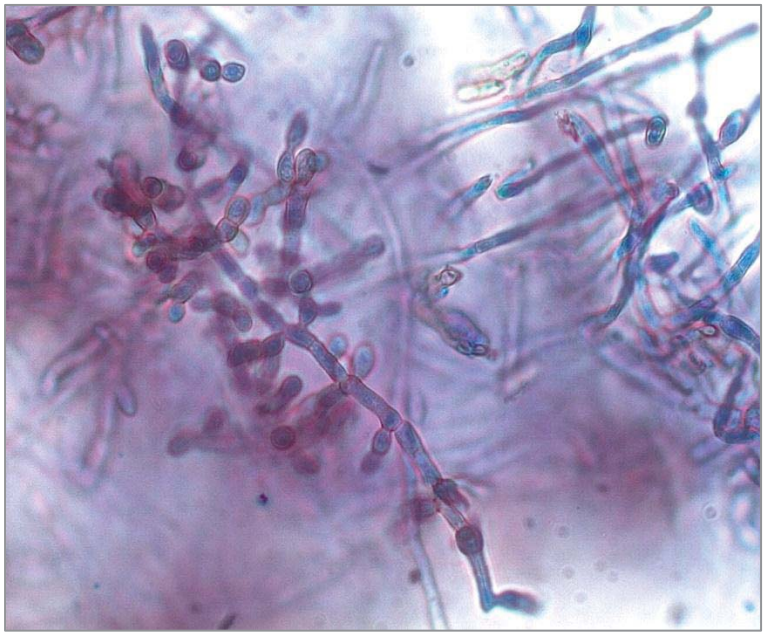


Figure 5.5 Growth of mould on YGC agar after plating nectarine beverage stored for 90 days at 20 °C (Axio Cam MR colour, Carl Zeiss, 100x). Plated samples were incubated for 5 days at 25 °C.

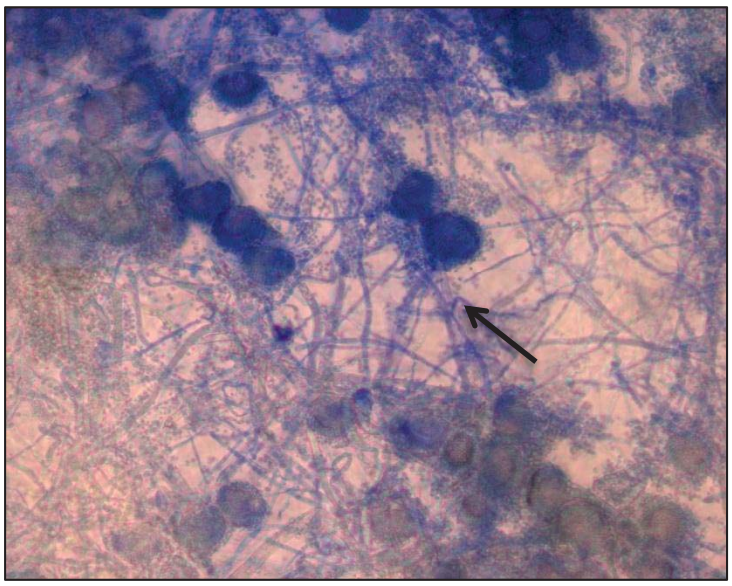


Figure 5.6 Growth of mould on YGC agar after plating heat-shocked mango beverages (Axio Cam MR colour, Carl Zeiss, 40x). Plates were incubated for 14 days at 30 °C.

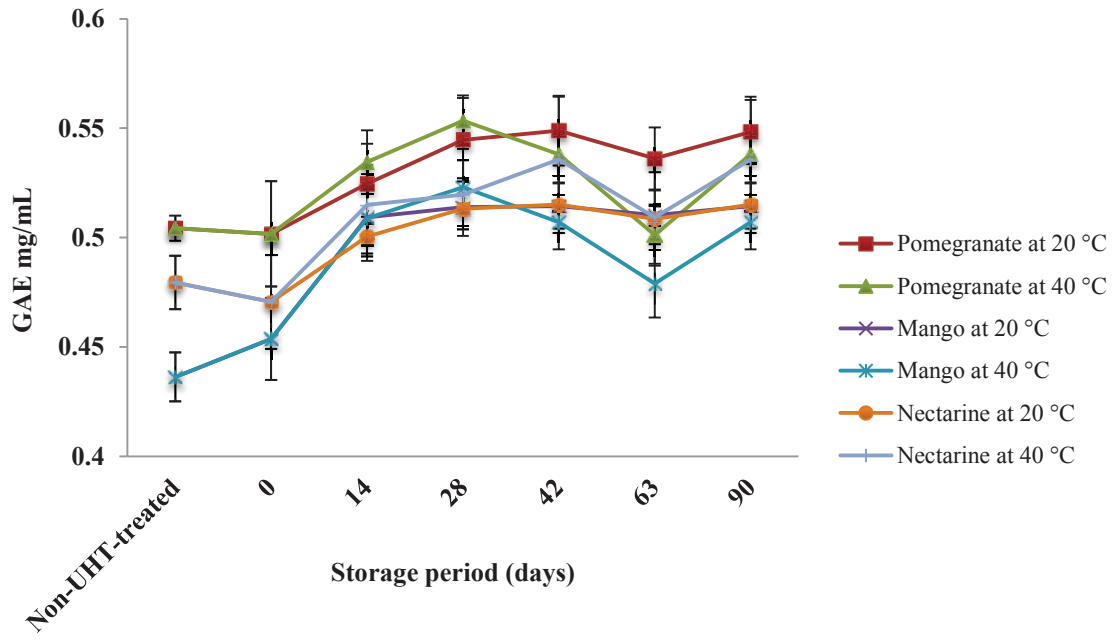
As previously discussed in Chapter 4, aerial contamination of the samples tested for heat-resistant moulds was suspected, as the air in the laboratory was not filtered (Sato & Takano, 2000; Beuchat & Pitt, 2001). Similar to the results obtained in Phase I, the growth of moulds after plating heat shocked beverage samples did not follow a clear trend. No growth of moulds was detected on YGC agar after plating non-UHT-treated pomegranate beverage, while non-UHT-treated mango (1 cfu/50mL) and nectarine (7 cfu/50 mL) beverage samples both showed low mould growth. Mould growth was not detected in mango beverages stored for 28 and 42 days at 40 °C, or nectarine beverages stored for 28 days at 40 °C (Table 5.2). It is noteworthy to mention that beverages stored for 63 and 90 days were plated in a laminar airflow chamber, and heat-resistant moulds were not detected in these samples (Table 5.2). In addition, mould growth was observed on control plates when tests were performed without the use of the laminar airflow chamber, also supporting the assumption that aerial contamination of either non-heat resistant or heat-resistant moulds could have occurred during microbiological analysis in the laboratory, in spite of aseptic techniques being followed. However, the possibility of heat-resistant mould contamination during beverage processing cannot be overlooked and further investigation should be conducted to determine the thermal resistance of the moulds, and potential sources of contamination.

5.2.3 Total phenolic content and antioxidant capacity

Non-UHT-treated and UHT-treated pomegranate, mango-, and nectarine-flavoured beverages were analysed for total phenolics and antioxidant capacity. Mean ± SD are shown in Tables G1 and G2 (Appendix G). Statistical analysis of data is shown in Appendix H. Total phenolics in non-UHT-treated beverages varied from 43.63 ± 1.12 mg GAE/100 mL to 50.43 ± 0.57 mg GAE/100 mL, and 38.81 ± 1.06 mg CAE/100 mL to 45.24 ± 0.54 mg CAE/100 mL (Figure 5.7). Total phenolics in UHT-treated beverages ranged from 45.37 ± 1.87 mg GAE/100 mL to 54.89 ± 1.60 mg GAE/100 mL (Figure 5.7A), and 40.45 ± 1.77 mg CAE/100 mL to 49.48 ± 1.58 mg CAE/100 mL (Figure 5.7B). UHT-treated mango beverage before storage had the lowest level of total phenolics, while UHT-treated pomegranate beverage stored for 42 days at 20 °C had the highest total phenolic content. The ORAC levels in UHT-treated beverages ranged from 4052.55 ± 219.93 μmol TE L^{-1} to 8085.75 ± 584.76 μmol TE L^{-1} (Figure 5.8), while $DPPH^{\bullet}$ scavenging activity, expressed as EC_{50}, ranged from 478.66 ± 10.98 to 685.90 ± 31.49 mL/ g of $DPPH^{\bullet}$ (Figure 5.9).

It is important to note that the reactions involving phytochemicals during processing and storage are strongly dependent on the synergistic effects between phytochemicals in a mixture (Shahidi et al., 1992; Peyrat-Maillard et al., 2003), as well as the matrix in which they are present (Wang et al., 2000; Chen et al., 2001). The beverages produced in this study contained a unique mixture of chemical compounds, which may have different stability to other beverages under the same storage conditions used in this study. To our knowledge, there is no information available on the stability of phenolics and methylxanthines in UHT-treated beverages infused with both white tea (*Camellia sinensis*) and grape seed (*Vitis vinifera*) extracts. For this reason, results obtained in this study were compared to products containing tea (*Camellia sinensis*) and/or grape extracts.

A)



B)

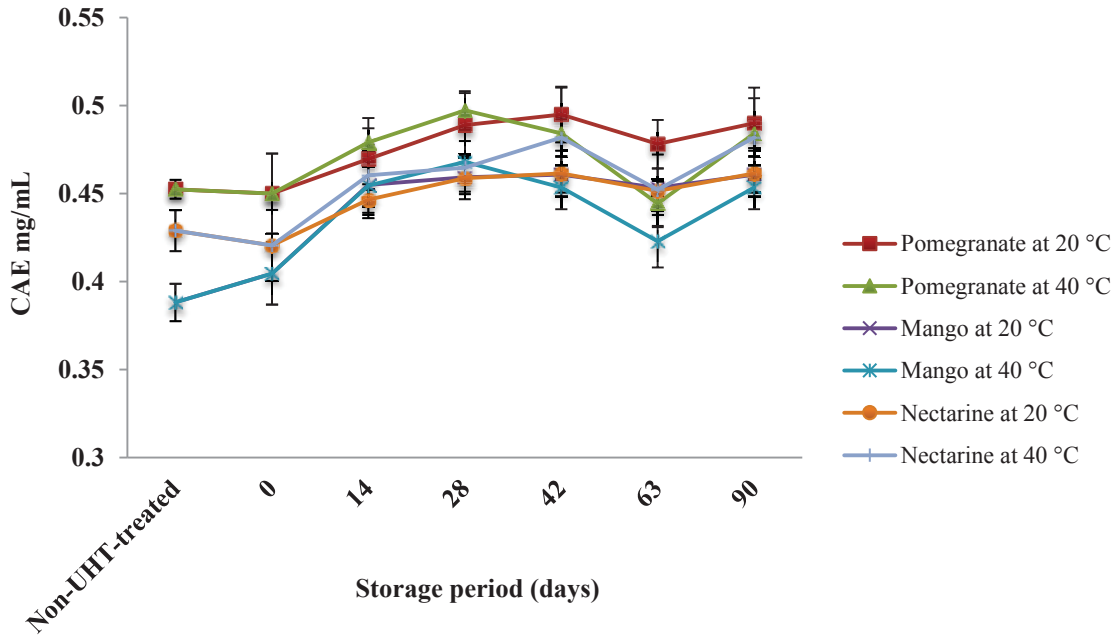


Figure 5.7 Total phenolics in non-UHT-treated pomegranate, mango, and nectarine beverages, and UHT-treated beverages stored for 90 days at 20 and 40 °C, expressed as A) GAE mg/mL and B) CAE mg/mL. Values are the mean ± SD of triplicate measurements.

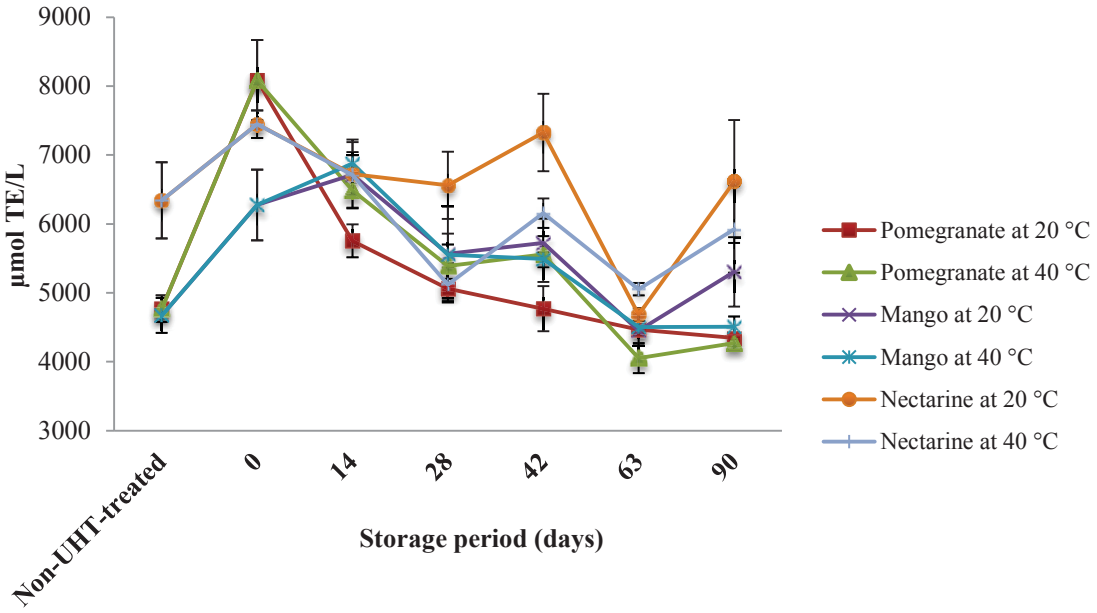


Figure 5.8 Oxygen radical absorbance capacity (ORAC) (µmol TE L^{-1}) in non-UHT-treated pomegranate, mango, and nectarine beverages, and UHT-treated beverages stored for 90 days at 20 and 40 °C. Values are the mean ± SD of triplicate measurements.

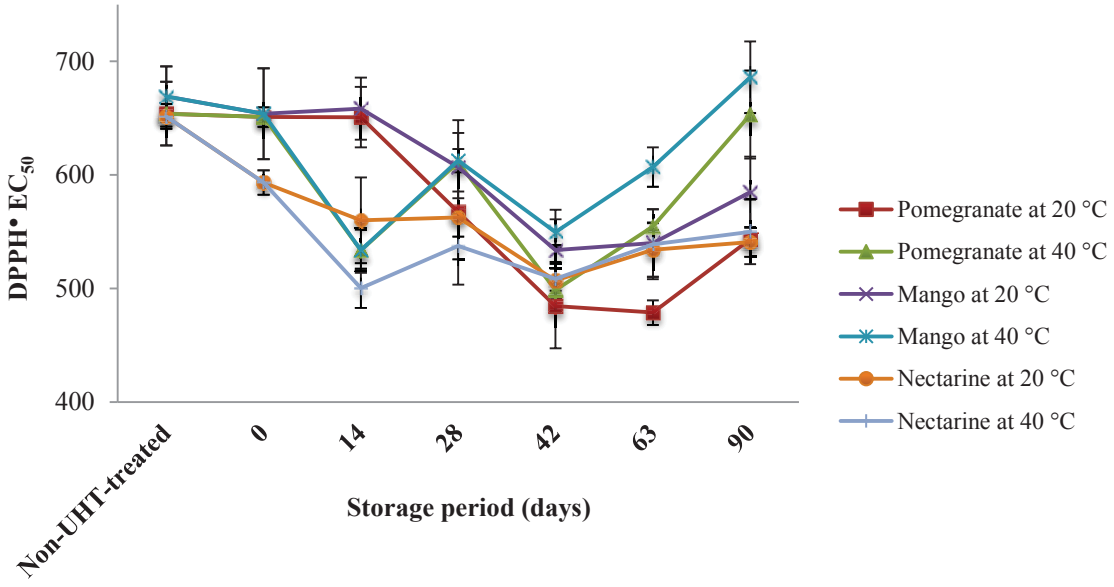


Figure 5.9 Free radical scavenging activity, expressed as DPPH• EC_{50} (mL of sample required to decrease 1 g of the initial DPPH• concentration by 50% in 20 min), in non-UHT-treated pomegranate, mango, and nectarine beverages, and UHT-treated beverages stored for 90 days at 20 and 40 °C. Values are the mean ± SD of triplicate measurements.

Total phenolics and DPPH$^{\bullet}$ scavenging activity were not affected ($P > 0.05$) by UHT processing ($P < 0.05$), while ORAC levels increased ($P < 0.05$) in all three flavoured beverages following UHT processing. Even though total phenolics in beverages were not affected by UHT treatment, heat may have affected the chemical structure of some phenolic compounds, changing the phenolic composition of the beverages (Wang et al., 2000; Chamorro et al., 2012). As discussed in Chapter 4, polymerised procyanidins may breakdown during heat treatment, modifying the phenolic composition (Chamorro et al., 2012), and antioxidant capacity of the product (Maatta-Riihinen et al., 2005; Gollücke et al., 2009). Although procyanidins were not analysed in this study, beverages were expected to contain procyanidins as grape seed extracts usually contain large amounts of these compounds (Prieur et al., 1994; Pérez-Jiménez et al., 2009). Therefore, the impact of heat treatment on the phenolic composition of beverages may have contributed to the detected changes in ORAC levels in the UHT-treated beverages.

Total phenolics, DPPH$^{\bullet}$ scavenging activity and ORAC levels in the UHT-treated beverages were affected ($P < 0.05$) by length of storage and compositional differences in the formulations, but were not affected ($P > 0.05$) by storage temperature. Over the 90 day storage period, the total phenolics increased ($P < 0.05$) by 9.3 - 13.4% in the beverages stored for 90 days at 20 °C, and 7.3 - 13.8% in the beverages stored for 90 days at 40 °C. However, a reduction ($P < 0.05$) in total phenolics was observed after 63 days of storage (Figure 5.7), which may be related to oxidation of phenolic compounds (Wang et al., 2000).

The ORAC levels in the beverages showed an overall decrease ($P < 0.05$) with length of storage (Figure 5.8), while DPPH$^{\bullet}$ scavenging activity decreased ($P < 0.05$) after 63 days of storage (Figure 5.9). The decrease in antioxidant capacity might be due to oxidation of phenolics (Wang et al., 2000). Phenolic compounds other than those measured by HPLC analysis in this study may have also contributed to the antioxidant capacity of the beverages. Grape seeds have been shown to contain oligomeric and polymeric procyanidins (Yilmaz & Toledo, 2003; Montealegre et al., 2006), which were not quantified in this study, but have significant radical scavenging ability (Yilmaz & Toledo, 2003). Therefore, procyanidins may have contributed towards the antioxidant capacity of the beverages. In contrast to our findings, Wang et al. (2000) reported a continuous decrease in total phenolics in steamed and roasted green tea aqueous extracts

stored for 12 days at 50 °C. Gollücke et al. (2008) observed a slight reduction in total phenolics (15%) and DPPH$^{\bullet}$ scavenging activity (16%) in pasteurised red grape juice (*Vitis labrusca*) stored for 10 months at 20-25 °C. The UHT-treated beverages stored for 90 days in this study contained similar total phenolics compared to beverages infused with grape skin and green tea (10-64 mg GAE/100 mL) (Bekhit et al., 2011), and lower ORAC levels than green tea infusions (1253 μmol TE L^{-1}) (Floegel et al., 2011). Since the stability of phenolics are related to the synergistic effects between phytochemicals and ingredients in a mixture (Shahidi et al., 1992; Chen et al., 2001; Peyrat-Maillard et al., 2003), different products, such as green tea extracts or grape juice, are likely to exhibit different phenolic content and antioxidant capacity levels to those of the RTD flavoured beverage formulations in this study.

The overall compositional differences in the formulation of the beverages had an additional effect ($P < 0.05$) on the total phenolics and antioxidant capacity of the beverages during storage. However, the small variation in antioxidant capacity of the beverages may not be biologically significant once consumed. This fact cannot be confirmed, as considerable research needs to be performed to establish the effective concentrations of compounds that can induce biological effects *in vivo*. Total phenolics were higher ($P < 0.05$) in the pomegranate beverage, followed by nectarine and mango. Since the total phenolics assay using Folin-Ciocalteu has low specificity (Escarpa & González, 2001), the variations in total phenolics could be due to the presence of other non-phenolic substances in the beverages, which can react with Folin-Ciocalteu reagent (Singleton et al., 1999; Stevanato et al., 2004). Ascorbic acid and citric acid, which were added to each formulation at different concentrations, may have contributed to differences in the levels of total phenolics (Stevanato et al., 2004). For example, nectarine had higher ($P < 0.05$) DPPH$^{\bullet}$ scavenging activity (Figure 5.9) and ORAC levels (Figure 5.8) than mango and pomegranate during storage, and this could have been due to the ascorbic acid in the formulation, which is an effective radical scavenger (Sánchez-Moreno et al., 1998).

5.2.4 Analysis of phenolic and methylxanthine compounds by HPLC

Catechins, gallic acid, theobromine and caffeine were analysed in non-UHT-treated, and UHT-treated pomegranate, mango and nectarine beverages stored for up to 90 days at 20 and 40 °C. Mean ± SD are shown in Table G3 (Appendix G). UHT treatment did not affect ($P > 0.05$) the levels of ECG, EGCG, GCG, caffeine, theobromine, and (+)-catechin combined with EGC. However, EC and gallic acid increased slightly ($P < 0.05$) in the beverages following UHT treatment. The increase in EC and gallic acid may have been the result of the hydrolysis of phenolics in beverages (Ito et al., 2003).

The length of storage affected ($P < 0.05$) the levels of phenolics and methylxanthines in mango (Figures 5.10 and 5.11), nectarine (Figure 5.12 and 5.13) and pomegranate beverages (Figure 5.14 and 5.15). The levels of compounds varied ($P < 0.05$) according to storage temperature and type of flavoured-beverage (pomegranate, mango and nectarine). The beverages stored at 40 °C had lower ($P < 0.05$) EC, EGCG, ECG, GCG, (+)-catechin and EGC during storage than the beverages stored at 20 °C. Therefore, the higher storage temperature (40 °C) appears to have accelerated the degradation of catechins, decreasing ($P < 0.05$) the total catechins in the beverages after 90 days.

Overall, ECG, EGCG, GCG, and EC decreased ($P < 0.05$) in the UHT-treated beverages during storage. The decrease in antioxidant capacity of the beverages during storage (Figures 5.8 and 5.9) might be due to this decrease in catechins. Gallic acid increased during storage, and was higher in the pomegranate beverage stored at 40 °C (Figure 5.12), mango beverage stored at 40 °C (Figure 5.11), and nectarine beverage stored at 20 °C for 90 days (Figure 5.12).

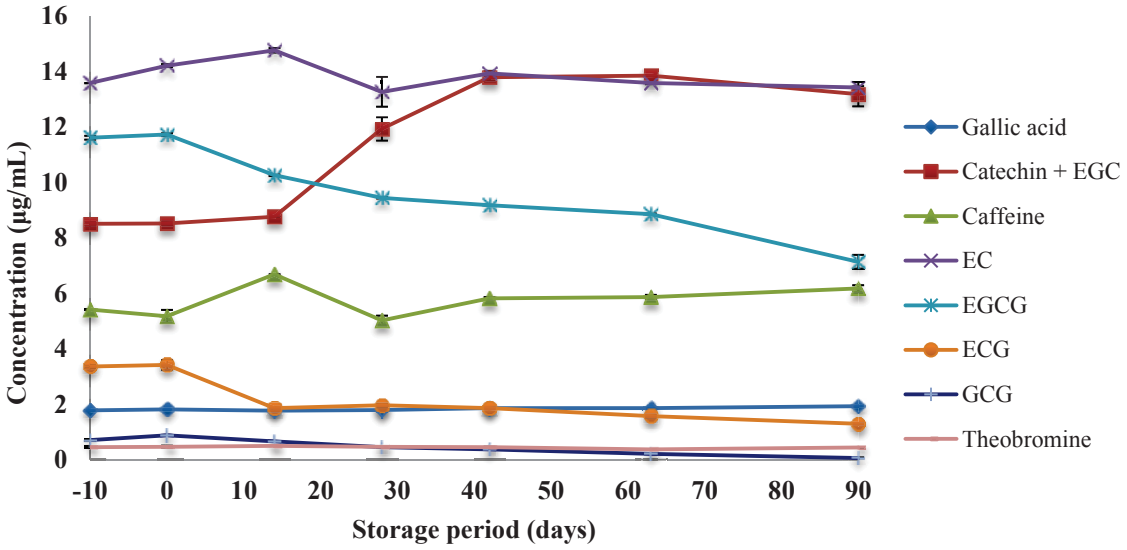


Figure 5.10 Concentrations of phenolics and methylxanthines (µg/mL) in non-UHT-treated (represented as time point -10) and UHT-treated mango beverages stored for 90 days at 20 °C. Each point represents the mean ± SD of triplicate measurements.

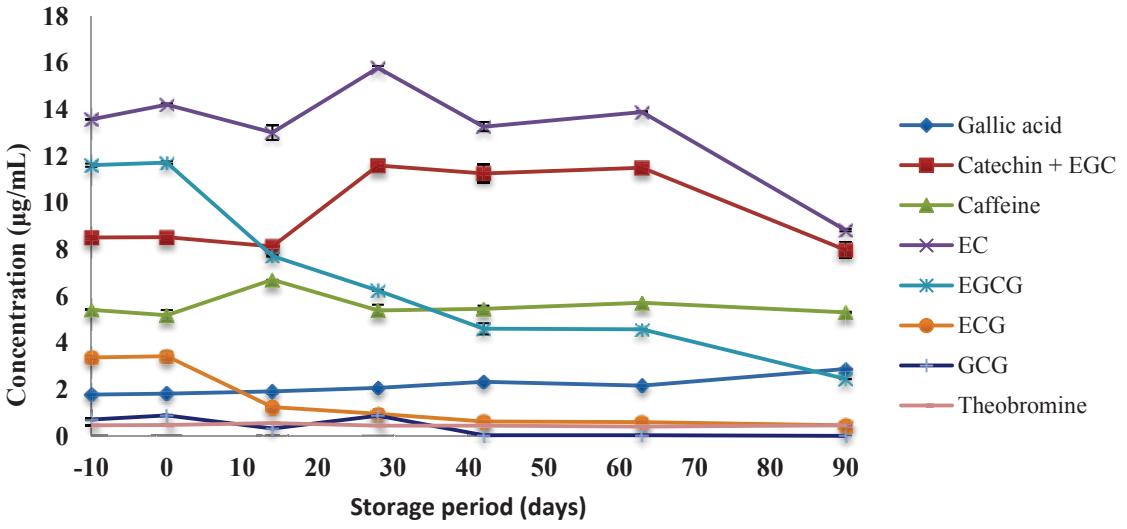


Figure 5.11 Concentrations of phenolics and methylxanthines (µg/mL) in non-UHT-treated (represented as time point -10) and UHT-treated mango beverages stored for 90 days at 40 °C. Each point represents the mean ± SD of triplicate measurements.

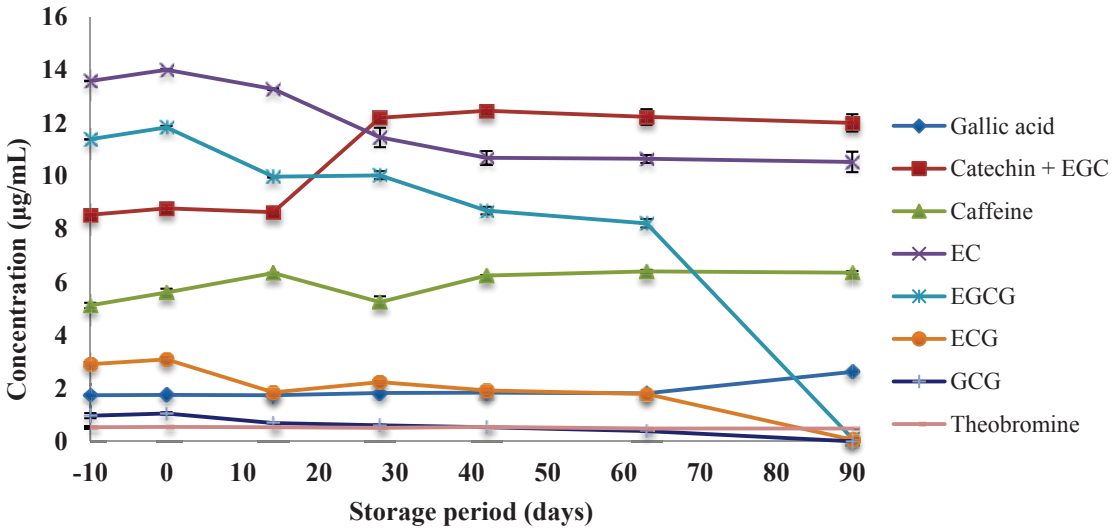


Figure 5.12 Concentrations of phenolics and methylxanthines (µg/mL) in non-UHT-treated (represented as time point -10) and UHT-treated nectarine beverages stored for 90 days at 20 °C. Each point represents the mean ± SD of triplicate measurements.

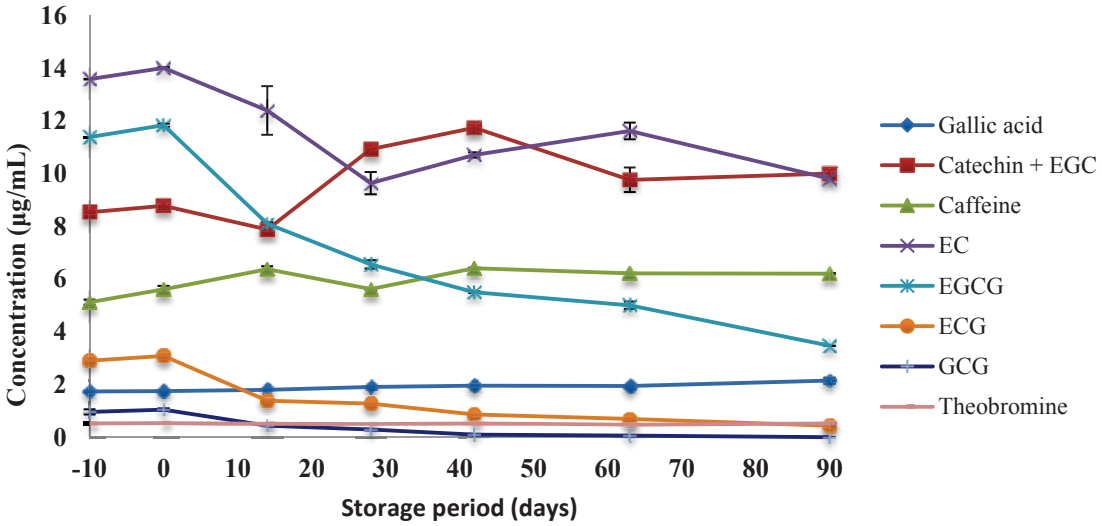


Figure 5.13 Concentrations of phenolics and methylxanthines (µg/mL) in non-UHT-treated (represented as time point -10) and UHT-treated nectarine beverages stored for 90 days at 40 °C. Each point represents the mean ± SD of triplicate measurements.

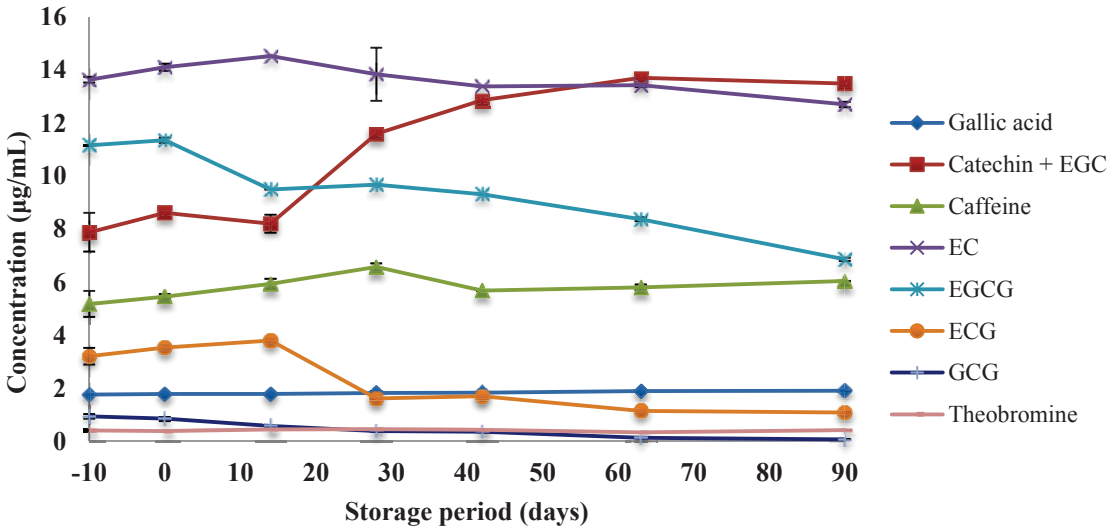


Figure 5.14 Concentrations of phenolics and methylxanthines (µg/mL) in non-UHT-treated (represented as time point -10) and UHT-treated pomegranate beverages stored for 90 days at 20 °C. Each point represents the mean ± SD of triplicate measurements.

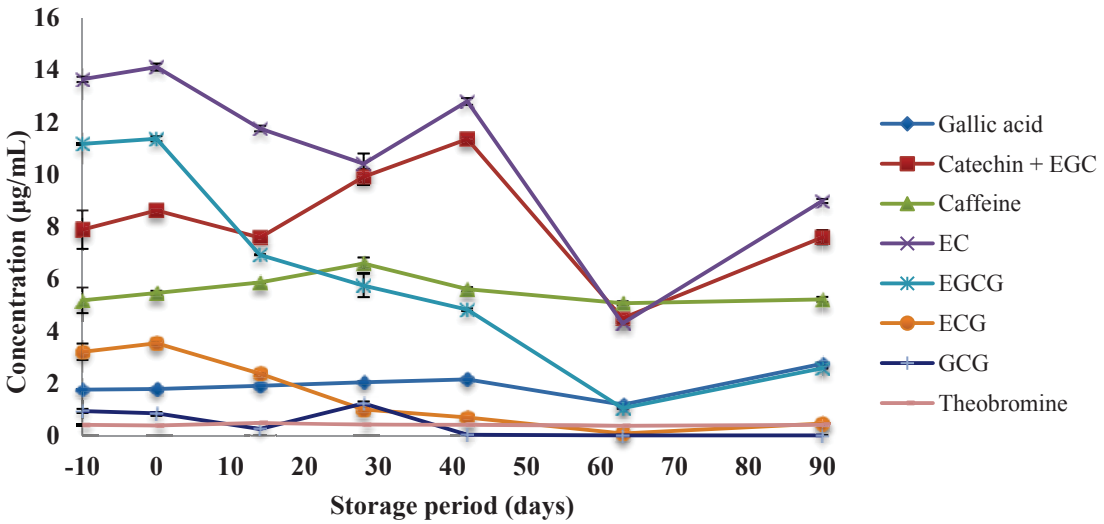


Figure 5.15 Concentrations of phenolics and methylxanthines (µg/mL) in non-UHT-treated (represented as time point -10) and UHT-treated pomegranate beverages stored for 90 days at 40 °C. Each point represents the mean ± SD of triplicate measurements.

The UHT-treated mango and pomegranate beverages stored at 40 °C contained higher ($P < 0.05$) gallic acid than those stored at 20 °C. The higher storage temperature (40 °C) may have facilitated the hydrolysis of flavonoids, causing the excision of the gallate group attached to the C-ring, and releasing gallic acid (Chamorro et al., 2012). The formation of gallic acid as a possible result of hydrolysis has been observed in heat-treated tea leaf extracts stored at room temperature (unspecified temperature) (Ito et al., 2003), and in grape seeds (*Vitis vinifera* var. Xinomavro) stored at 25 °C and 75% relative humidity (Hatzidimitriou et al., 2007). Since gallic acid is an efficient radical scavenger (Hatzidimitriou et al., 2007; Locatelli et al., 2009), the formation of gallic acid may have positively contributed towards the antioxidant capacity of the beverages, which could have been lower otherwise.

The compositional differences in the formulation of the beverages affected ($P < 0.05$) the levels of EC, ECG, (+)-catechin and EGC, but did not impact ($P > 0.05$) on the concentrations of gallic acid, EGCG, and GCG in the beverages during storage. The stability of catechins can be affected by pH and the presence of ingredients in the formulation, such as ascorbic acid and citric acid (Zhu et al., 1997; Chen et al., 2001; Bradshaw et al., 2003). As previously mentioned, each formulation was prepared using a slightly different concentration of citric acid, while ascorbic acid was only added to nectarine beverages. Therefore, the ascorbic acid present in the nectarine formulation may have played a key role in the phenolic changes observed with this product. Ascorbic acid in beverages initially acts as an antioxidant, exhibiting a protective effect on catechins, and later as a pro-oxidant, promoting the degradation of catechins (Chen et al., 2001; Bradshaw et al., 2003). Although the three flavoured beverages contained similar levels of EC and ECG before UHT treatment, nectarine beverages contained less EC throughout the storage period, and less ECG after 90 days of storage (Figure 5.12), suggesting that oxidation of catechins could have occurred. Ascorbic acid may have exhibited pro-oxidant effects in the beverage, which would explain the degradation of catechins in the nectarine beverage. EGCG, which is one of the most unstable catechins (Chen et al., 2001), was 99% degraded in nectarine beverage after 90 days of storage at 20 °C (Figure 5.11). A significant decrease in EGCG content was not observed in the nectarine beverage stored at 40 °C. A possible explanation for this is that the higher temperature (40 °C) might have accelerated the degradation of ascorbic acid in the beverage during storage (Martí et al., 2002). In addition, the presence of citric acid in the

beverages might have also affected the stability of catechins (Su et al., 2003). Chen et al. (2001) evaluated the stability of green tea catechins for 6 months and observed that the combination of sucrose and citric acid, or sucrose and ascorbic acid increased the rate of degradation of catechins. Green tea catechins degraded faster when dissolved in a solution containing sucrose (0.15 g/mL) and citric acid (2 mg/mL) at pH 3.02, compared to when it was dissolved in distilled water or sucrose solution alone (Chen et al., 2001). Therefore, a synergistic effect between phenolics and ingredients in each formulation may have affected the stability of catechins in the beverages.

Similar to our study, Bazinet et al. (2010) observed a decrease in ECG and GCG levels in RTD beverages (pH=4) after 30 days of storage at 25 °C, while Yang et al. (2007) observed a rapid decrease in EGCG, EC, GCG and ECG levels in green tea infusions stored at 25 °C for 36 h. Since the green tea infusions analysed by Yang et al. (2007) were prepared using deionised water only, the higher pH (> 6) of the infusions might have accelerated the degradation of the catechins (Bazinet et al., 2010). In our study, even though the ingredients may have affected the levels of catechins in the UHT-treated beverages, the low pH (Table 5.1) may have contributed towards the stability of catechins during storage, while the higher storage temperature increased ($P < 0.05$) the degradation of catechins. Wang et al. (2000) also reported that EC, EGCG, GCG, ECG, EGC, and (+)-catechin decreased in green tea beverages during 12 days at 50 °C. In our study, (+)-catechin combined with EGC remained at similar levels during the initial 14 days of storage, and then increased in the beverages stored at 20 °C after this period. However, different ($P < 0.05$) trends were observed in the beverages stored at 40 °C, as (+)-catechin and EGC decreased in pomegranate and nectarine beverages stored at 40 °C after 63 days, and in mango beverage stored for 90 days at 40 °C. The conversion of the epi-form catechins to catechins can occur during storage (Wang & Helliwell, 2000). Thus, EGCG, ECG, EGC, and EC may be converted to their corresponding epimers (GCG, CG, GC, and catechin, respectively) (Chen et al., 2001). Wang and Helliwell (2000) observed an increase in non-epicatechins in green tea infusions stored at 40 °C, and a decrease in their corresponding epicatechins, but did not observe the same when infusions were stored at 5 and 25 °C. This finding suggested that epimerisation might be affected by storage temperature (Wang & Helliwell, 2000; Yang et al., 2007). It is possible that an increase in (+)-catechin may have occurred due to epimerisation, resulting in higher (+)-catechin combined with EGC. Ito et al. (2003) observed an

increase in (-)-catechin during storage of green tea infusions, and proposed a mechanism to explain the changes. During storage, ECG can epimerise to CG, and CG is expected to hydrolise to (-)-catechin. Simultaneously, (-)-EC can epimerise to (-)-catechin (Ito et al., 2003). Therefore, both epimerisation and hydrolysis of flavonoids may contribute to an increase in (-)-catechin in beverages. Since (+)-catechin was the only standard available in our study, we are unable to confirm if the HPLC method used for quantification was capable of separating the two catechin forms, (+)-catechin and (-)-catechin. However, it is possible that (+)-catechin in this experiment represents both forms of catechin, which would explain the increase in (+)-catechin levels observed during storage.

Auto-oxidation of catechins has been reported (Yoshioka et al., 1991; Li et al., 2012) and may be the main contributor to the reduction of catechin levels in the beverages observed during storage in this study. Various radicals may be derived from catechins by auto-oxidation (Yoshioka et al., 1991; Li et al., 2012). Flavanols loose one hydrogen radical and form a semiquinone radical with an unpaired electron on the oxygen atom (Wang et al., 2000). Radical formation occurs more easily on the ring displaying three hydroxyl groups, and therefore, radical forming groups are the B-ring of EGC and EGCG (gallyl moiety), and the gallic acid portion of the ECG and EGCG (galloyl moiety) (Yoshioka et al., 1991). The main difference between gallyl and galloyl groups is the proton “Hb” on the C-2 carbon linked to the C-1’ carbon of the gallyl moiety, which interacts with the unpaired electron on the oxygen atom (Figure 5.16). Hb lies in a different plane from the one with the semiquinone radical and the hydrogen atoms “Ha” on C-2’ and C-3’ carbons (Yoshioka et al., 1991; Wang et al., 2000). On the basis of electron spin resonance (ESR) measurement, radicals have been shown to form more easily on the gallyl than on the galloyl moiety (Figure 5.16), which contributes to the tendency of catechins to oxidise in the order EGCG > EGC > ECG > EC (Yoshioka et al., 1991; Wang et al., 2000). An oxidation-polymerisation mechanism may occur, where most of the radicals formed at the initial stage of oxidation may disappear by recombining or changing into other radicals (Yoshioka et al., 1991). Although (+)-catechin and EGC were quantified together in this experiment, their decrease in UHT-treated beverages stored at 40 °C may have been partially caused by oxidation of EGC.

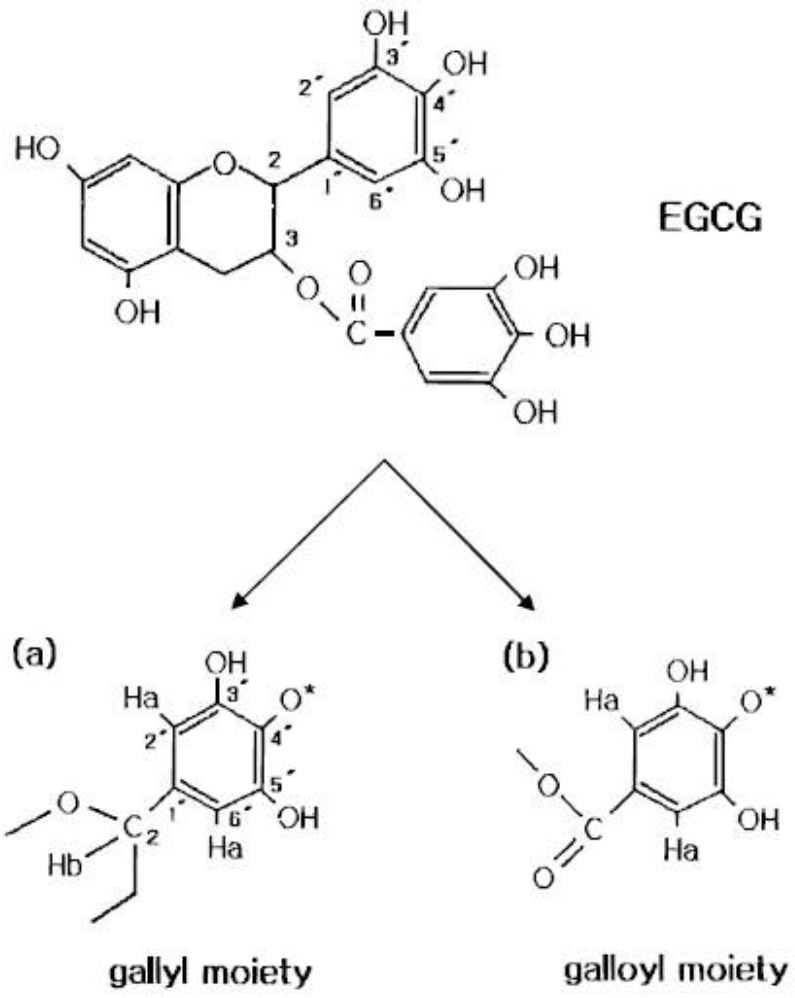


Figure 5.16 Formation of gallyl and galloyl radicals from EGCG (Hb = proton, Ha = hydrogen atom) (Wang et al., 2000).

Theobromine levels were not affected ($P > 0.05$) by storage temperature. Although there were statistical differences ($P < 0.05$) observed in theobromine levels with length of storage and between the different beverages (pomegranate, mango and nectarine) (Figures 5.10 - 5.15), the small changes in theobromine levels are unlikely to be biologically significant. Part of these differences can be explained by measured variation in the assay (CV = 9%). Caffeine levels were also affected ($P < 0.05$) by length of storage, compositional differences in the formulations and storage temperature (Figures 5.10 - 5.15). Caffeine levels in the beverages did not follow a clear trend during storage, and varied ($P < 0.05$) randomly, increasing in all three flavoured beverages after 14 days, and in pomegranate beverage after 28 days of storage. It is possible that the caffeine

variations during storage might have occurred due to possible interference with the caffeine peaks during HPLC analysis. In addition, these differences can be partially attributed to the variation in the assay (CV = 8.5%). Major changes in caffeine levels were not expected since other studies reported that caffeine concentrations in tea-based beverages were generally stable during storage (Bazinet et al., 2010; Sopelana et al., 2011). Similar to our study, Bazinet et al. (2010) reported stable caffeine levels in RTD green tea-based beverages stored for 90 days at 25 °C. In contrast, Li et al. (2012) reported decreases in caffeine levels in green tea aqueous solutions (pH 1 to 3) stored for up to 35 days at or above 40 °C. In this case, the loss of caffeine was attributed to the formation of insoluble caffeine/catechin complexes, resulting in sedimentation (Li et al., 2012).

5.2.5 Sensory evaluation

Sensory consumer acceptance was performed (n=43) to evaluate flavour and appearance of UHT-treated pomegranate-, mango-, and nectarine- flavoured beverages stored for 90 days at 20 and 40 °C (Table 5.3).

Appearance and flavour liking scores differed significantly ($P < 0.05$) between most UHT-treated flavoured beverages (Table 5.3). The flavour of the beverages were rated by sensory panellists (n=43) in decreasing order of acceptance as follows: mango at 20 °C > nectarine at 20 °C > mango at 40 °C > nectarine at 40 °C > pomegranate at 20 °C > pomegranate at 40 °C. There was no difference ($P > 0.05$) in the flavour liking scores between nectarine stored at 20 °C (6.21 ± 1.60), and mango stored at 20 °C (6.61 ± 1.33) and 40 °C (5.56 ± 1.68) (Table 5.3). The flavours of mango (5.56 ± 1.68) and nectarine beverages (5.51 ± 1.99) stored at 40 °C, and pomegranate beverages stored at 20 °C (5.12 ± 1.74) and 40 °C (4.77 ± 2.09) were equally liked ($P > 0.05$). Even though the beverages stored at 40 °C received lower flavour rating scores than those stored at 20 °C, there was no difference ($P > 0.05$) in flavour rating scores between each formulation stored at 20 and 40 °C.

Table 5.3 Hedonic rating scores[1] for flavour and appearance of pomegranate-, mango- and nectarine-flavoured beverages stored for 90 days at 20 and 40 °C.

Flavour	Storage temperature (°C)	Scores for flavour	Scores for appearance
Pomegranate	20 °C	5.12 ± 1.74 ^{c}	6.63 ± 1.50 ^{a}
	40 °C	4.77 ± 2.09 ^{c}	5.40 ± 1.90 ^{bc}
Mango	20 °C	6.61 ± 1.33 ^{a}	6.42 ± 1.68 ^{ab}
	40 °C	5.56 ± 1.68 ^{abc}	4.67 ± 1.99 ^{cd}
Nectarine	20 °C	6.21 ± 1.60 ^{ab}	5.98 ± 1.39 ^{ab}
	40 °C	5.51 ± 1.99 ^{bc}	4.30 ± 1.83 ^{d}

[1]Hedonic rating scores (mean ± standard deviation, n = 43) were measured on a 9-point scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely). Means with different superscripts within each column are significantly different ($P < 0.05$).

The flavour rating scores of the UHT-treated beverages were similar to other products perceived as healthy, such as probiotic and conventional juices, which received flavour rating scores between 5.6 and 5.8, respectively, using 100 mm unstructured line scales (Luckow & Delahunty, 2004), and tropical fruit juices, with scores between 3.51 to 6.90, measured on a nine-point scale (Sabbe et al., 2009).

The appearance of the beverages were rated by sensory panellists (n=43) in decreasing order of acceptance as follows: pomegranate at 20 °C > mango at 20 °C > nectarine at 20 °C > pomegranate at 40 °C > mango at 40 °C > nectarine at 40 °C. The beverages stored at 20 °C received higher ($P < 0.05$) appearance scores than those stored at 40 °C (Table 5.3), but there was no difference ($P < 0.05$) in the appearance scores between pomegranate (6.63), mango (6.42), and nectarine (5.98) beverages stored at 20 °C. The appearance of beverages stored for 90 days at 40 °C was less accepted by sensory panellists than those stored at 20 °C. The development of dark colour and sedimentation in the beverages stored for 90 days at 40 °C (Figure 5.17) may have affected consumers' liking for appearance. This fact was supported by written comments from sensory panellists (25%), such as "particles in the beverages were unappealing" and "I don't like

the appearance of particles in the beverage". Sedimentation was observed in all flavoured beverages stored for 90 days at 40 °C. Sedimentation has been related to complex formation involving phenolics and methylxanthines (Musingo et al., 2001; Li et al., 2012).

When developing beverages with potential health benefits, it is important to evaluate consumer acceptance (Sabbe et al., 2009). Flavour is a key factor when developing novel beverage products, as consumers are not willing to compromise on taste when consuming products perceived as healthy (Luckow & Delahunty, 2004; Verbeke, 2006). High total phenolic content in tea extracts provides a stronger taste of bitterness and astringency to infused beverages, which for teas is considered a desirable quality (Wang et al., 2000). Phenolic compounds, particularly EGCG and EGC, have been considered the key elements that impact on sensory characteristics of processed beverages infused with *Camellia sinensis*, such as colour and flavour (Wang et al., 2000). It has been suggested that oxidation of phenolics is associated with changes in colour (Kim et al., 2007), and a decrease in bitterness and astringency of green tea extracts during storage (Wang et al., 2000). In our study, darkening of the UHT-treated flavoured beverages stored at 40 °C became more evident after 63 days. Similarly, Chamorro et al. (2012) observed darkening of grape seed extract after autoclaving, and Wang et al. (2000) reported the development of brown colour in tea extracts stored at 50 °C for 12 days. The changes in the colour of UHT-treated beverages stored for up to 90 days may be attributed to oxidation of catechins and procyanidins (Kim et al., 2007; Chamorro et al., 2012). The decrease in total phenolic content and catechins observed during storage also suggests that oxidation may have occurred. Therefore, oxidation appeared to be the main reaction affecting the stability of catechins during storage of the UHT-treated beverages under the conditions used in this study.

a.  b.  c. 

Figure 5.17 (a) mango beverages stored for 90 days at 20 and 40 °C; (b) nectarine beverages stored for 90 days at 20 and 40 °C; (c) pomegranate beverages stored for 90 days at 20 and 40 °C.

5.3 Conclusion

UHT treatment can be used to process beverages infused with white tea and grape seed extracts, obtaining stable sensory and microbiological characteristics during storage at room temperature (20 °C) for 90 days. Catechins, however degraded with length of storage, possibly due to oxidation (Kim et al., 2007; Chamorro et al., 2012), while the increase in gallic acid may have been the result of hydrolysis of phenolics (Hatzidimitriou et al., 2007). These reactions may have been accelerated in the beverages stored at 40 °C, where increased degradation of catechins was observed. Phenolics and antioxidant capacity of the UHT-treated beverages were affected ($P < 0.05$) by the compositional differences in the formulations, suggesting that the ingredients used in the RTD beverages, particularly citric acid and ascorbic acid, played a major role in the stability of the phenolics during storage. In conclusion, this study provided a useful approach for understanding catechin stability in UHT-treated beverage systems during storage, which can contribute to improving both the formulation and storage strategies for high-quality beverages infused with white tea and grape seed extracts.

Chapter 6
General Discussion & Recommendations

Camellia sinensis and grape seed extracts are reported to have a variety of potential health benefits, including anti-obesity (Park et al., 2008; Grove & Lambert, 2010) and anti-cancer effects (Dashwood et al., 2002; Kaur et al., 2009), protective effects against cardiovascular diseases (Clifton, 2004; Shao et al., 2003), and improving diabetic markers of glycaemia and inflammation (Kar et al., 2009; Islam, 2011). *In vivo* studies in mice (Koutelidakis et al., 2009) and humans (Serafini et al., 2000) have demonstrated the antioxidant effects of *Camellia sinensis*, while grape seed phenolics have been shown to have both antioxidant (Shao et al., 2003; Sakurai et al., 2010) and anticoagulant properties *in vitro* (Bijak et al., 2011).

It has been suggested that catechins play a major role in the biological activities of *Camellia sinensis* extracts (Sen et al., 2009; Bazinet et al., 2010). Catechins are highly unstable, reacting amongst themselves and with other compounds, thereby modifying the phenolic profile of beverages during processing and storage (Su et al., 2003; Gollücke et al., 2008). Temperature, beverage ingredients and pH also have an effect on the stability of catechins in beverages (Bazinet et al., 2010; Ananingsih et al., 2011; Li et al., 2012), contributing to either oxidative degradation or changes in the stereochemical structures of flavanols. These changes may affect the biological effects of phenolics, including their radical scavenging ability (Maatta-Riihinen et al., 2005). For this reason, ensuring the stability of these phenolics in RTD beverages is of great importance.

In this study, RTD flavoured beverages were prepared using grape seed extract, which contained high levels of (+)-catechin and gallic acid, and white tea extract, which contained mostly caffeine, EGCG, ECG, EGC, GCG and (+)-catechin. Since oxidation and epimerisation of catechins are likely to occur during heat treatment (Wang et al., 2000; Ito et al., 2003; Chamorro et al., 2012), the UHT-processing temperature was optimised to ensure the stability of the phenolics in the beverages. Despite the known effects of heat on the stability of phenolic compounds (Kim et al., 2007; Ananingsih et

al., 2011), the impact of UHT-treatment (at 110, 120, 130 and 140 °C for 5 s) on the stability of grape seed and white tea phenolics in the RTD mango-flavoured beverages was not significant ($P > 0.05$). As expected, the UHT-treated beverages infused with white tea and grape seed extracts were high in EGCG, EC, EGC and (+)-catechin, which are the major contributors to antioxidant capacity (Lachman et al., 2009). Theobromine and caffeine were also stable following UHT-processing. It was observed, however, that the levels of GCG increased slightly following UHT treatment, and EGCG levels decreased slightly with higher UHT processing temperature (140 °C), suggesting that epimerisation of catechins may have occurred (Kim et al., 2007; Ananingsih et al., 2011). When developing UHT-treated beverages with increased shelf stability, the optimum temperature chosen is frequently the highest possible temperature, which does not negatively impact on the chemical and sensory characteristics of the product. Since catechins in the beverages processed at 140 °C for 5 s might have undergone epimerisation, leading to decreased EGCG content, and gallic acid levels increased, the optimum UHT treatment conditions chosen to process the beverages infused with white tea and grape seed extracts were 130 °C for 5 s.

In the second phase of this study, the stability of UHT-treated pomegranate-, mango- and nectarine-flavoured beverages stored for 90 days at 20 and 40 °C was investigated. The UHT treatment (at 130 °C for 5 s) with cold filling used in the study was considered microbiologically effective as yeasts and moulds were only present in low levels (up to 2.4×10^2 cfu/mL) after 63 days of storage. Yeasts and moulds initially present in the packaging may have contributed to the mould growth in the beverages, since moulds that have been injured during processing may be able to recover during storage (Graumlich & Stevenson, 1978; O'Reilly et al., 2000). The data obtained for heat-resistant moulds may have been affected by aerial contamination. Therefore, further investigation should be conducted to confirm the presence of heat-resistant moulds in the beverages or production line, by determining the thermal resistance of moulds and potential sources of contamination.

Total phenolics, $DPPH^{\bullet}$ scavenging activity and ORAC levels in the UHT-treated beverages were not affected ($P > 0.05$) by storage temperature. Antioxidant capacity decreased ($P < 0.05$) after 90 days of storage, while total phenolics ($P < 0.05$) increased. Proanthocyanidins, which are present in grape extracts (Prieur et al., 1994; Pérez-

Jiménez et al., 2009), but were not analysed in this study, may have contributed to the antioxidant capacity and total phenolic content of the beverages. Changes in the phenolic composition of the beverages may have contributed to the variation in antioxidant capacity of beverages during storage (Maatta-Riihinen et al., 2005; Gollücke et al., 2009).

The compositional differences in the formulation of the beverages affected ($P < 0.05$) the phenolic compounds and antioxidant capacity of the products during the 90-day storage period, suggesting that the different levels of citric acid and ascorbic acid in the beverages could have affected the stability of the phenolics. Ascorbic acid, present in the nectarine beverages, may have acted as a pro-oxidant, promoting the degradation of catechins in the nectarine beverages (Li et al., 2012).

Epimerisation of catechins may have occurred in the beverages during storage, increasing (+)-catechin levels combined with EGC. Gallic acid also increased ($P < 0.05$) in the beverages during storage, possibly as a product of hydrolysis. In addition, the higher storage temperature (40 °C) appeared to have increased the formation of gallic acid in beverages. While no major changes were observed in the levels of methylxanthines, ECG, EGCG, GCG, and EC decreased ($P < 0.05$) in the UHT-treated beverages during storage. In addition, the higher storage temperature (40 °C) increased the degradation of catechins. Oxidation seemed to be the main contributor to the decrease in the levels of catechins in the beverages during storage (Yoshioka et al., 1991), and may have also caused the darkening of the beverages stored at 40 °C (Kim et al., 2007; Chamorro et al., 2012). Since catechins are effective radical scavengers (Kang et al., 2010), the decrease in the levels of catechins may partially explain the decrease in antioxidant capacity of the UHT-treated beverages during storage.

Products perceived as healthy are still expected to present acceptable sensory characteristics (Luckow & Delahunty, 2004; Verbeke, 2006). When the sensory acceptability of the three flavoured beverages (pomegranate, mango and nectarine) stored at the two different temperatures was compared, the beverages stored at both 20 °C and 40 °C for 90 days showed acceptable flavour. However, sensory data revealed that the appearance of beverages stored at 20 °C was preferred ($P < 0.05$) compared to those stored at 40 °C, possibly due to the presence of sediment in the beverages stored at 40°C.

Sedimentation might have been caused by complex formation involving phenolics and methylxanthines (Musingo et al., 2001; Li et al., 2012).

Mathematical models have been developed to predict the stability of catechins in different systems (Wang et al., 2006; Harbourne et al., 2008; Ananingsih et al., 2011). However, the prediction of the stability of catechins in these systems was based on fewer ingredients than those present in the RTD beverage systems infused with both white tea and grape seed extracts. Therefore, these models cannot be utilised with RTD beverage systems because the presence of other ingredients in the mixture can affect the stability of phenolics during processing and storage. Furthermore, the pH of the products can also affect the stability of phenolic compounds (Bazinet et al., 2010; Ananingsih et al., 2011; Li et al., 2012). The synergistic or antagonistic effects between phytochemicals and ingredients in the RTD flavoured beverages are also important and should be considered when predicting the stability of phenolics in beverages.

To develop a robust model for prediction of the shelf stability of phenolics in the RTD beverage systems, further investigation should be conducted using optimum beverage formulations. A robust model should consider all occurring reactions (epimerisation, oxidation and polymerisation) as they may occur simultaneously, as well as the impact of the ingredients on the stability of the phenolics. The levels of polymerised proanthocyanidins, which may breakdown in beverages during processing or storage (Chamorro et al., 2012), and other monomeric flavanols, such as catechin gallate (which is a product of epimerisation), should also be investigated as they may contribute to predicting the changes in the phenolic content and antioxidant capacity of the beverages.

As previously discussed, the extent of biological effects of beverages is possibly related to the synergistic effects between phytochemicals and ingredients in a mixture (Shahidi et al., 1992; Peyrat-Maillard et al., 2003). It has been suggested that a synergistic effect between grape and tea phenolic compounds may increase the biological effects related to human health (Morré & Morré, 2006; Bekhit et al., 2011). The UHT-treated beverages stored for 90 days in this study contained total phenolic content similar to those found in herbal infusions (Almajano et al., 2008) and commercial heat-treated fruit juices (Zulueta et al., 2007). The total catechins in the beverages in this study were lower than those in Earl Grey and black tea infusions (Arts et al., 2000), and higher than other commercial

RTD tea-based beverages (Arts et al., 2000). In addition, the beverages stored for 90 days contained less EGCG than green tea infusions, but similar EGCG and EC levels to those found in black tea infusions (Pascual-Teresa et al., 2000). Therefore, the UHT-treated beverages contained considerable amounts of phenolics compared to other beverages. Based on these findings, it is possible that the UHT-treated beverages infused with grape seed and white tea extracts have biological effects that can promote human health, such as the ability to protect against *in vivo* oxidation.

It is recommended that further investigation be carried out to determine if the beverages used in this study exhibit any biological activities with associated health benefits. The absorption and metabolism of the phenolic compounds in the beverages should be investigated to determine the bioavailability of the compounds, as these will impact on the efficacy of any possible biological effects of the product. In addition, the changes in the phenolic compounds in the UHT-treated beverages during storage should be considered as they could impact on the biological effects of the product. Therefore, in addition to the recommended investigations on the efficacy, bioavailability and potential beneficial health effects of the beverages, more work should be done to improve the shelf stability of the beverages, as this is vitally important to ensure the efficacy of the end products.

Chapter 7 Conclusions

The UHT-treated beverages used in this study were infused with both white tea and grape seed extracts and contained a unique mixture of chemical compounds that may prove beneficial to human health. UHT treatment at 130 °C for 5 s was used to obtain stable RTD beverages infused with white tea and grape seed extracts containing caffeine, theobromine, gallic acid and catechins. Although the UHT-treated beverages were acceptable from a sensory perspective, and were microbiologically stable for 90 days, degradation of catechins was observed during storage, increasing when beverages were stored at 40 °C. Oxidation appeared to be the main reaction affecting the stability of catechins in the UHT-treated beverages under the conditions used in this study, and induced changes in the colour of the beverages. Sedimentation observed in the beverages stored at 40 °C also contributed negatively to the appearance acceptance scores of the beverages.

This study provides important information for understanding phenolic stability in UHT-treated beverage systems during storage. The changes in the levels of the phenolics observed in the beverages during storage may affect any potential biological effects. Therefore, understanding the stability of phytochemicals in the beverages can contribute to improving both the formulation and storage strategies for high-quality beverages infused with white tea and grape seed extracts.

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Appendices

Appendix A

Low Risk Notification letter of acknowledgment issued by Massey University Humans Ethics Committee

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

9 May 2012

Natalie d'Avila
7 Paris Place
Birkenhead
AUCKLAND 0626

Dear Natalie

Re: Effect of Ultra High Temperature Processing on Functional Beverages

Thank you for your Low Risk Notification which was received on 9 May 2012.

Your project has been recorded on the Low Risk Database which is reported in the Annual Report of the Massey University Human Ethics Committees.

The low risk notification for this project is valid for a maximum of three years.

Please notify me if situations subsequently occur which cause you to reconsider your initial ethical analysis that it is safe to proceed without approval by one of the University's Human Ethics Committees.

Please note that travel undertaken by students must be approved by the supervisor and the relevant Pro Vice-Chancellor and be in accordance with the Policy and Procedures for Course-Related Student Travel Overseas. In addition, the supervisor must advise the University's Insurance Officer.

A reminder to include the following statement on all public documents:

> *"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher(s) named above are responsible for the ethical conduct of this research.*
>
> *If you have any concerns about the conduct of this research that you wish to raise with someone other than the researcher(s), please contact Professor John O'Neill, Director (Research Ethics), telephone 06 350 5249, e-mail humanethics@massey.ac.nz".*

Please note that if a sponsoring organisation, funding authority or a journal in which you wish to publish requires evidence of committee approval (with an approval number), you will have to provide a full application to one of the University's Human Ethics Committees. You should also note that such an approval can only be provided prior to the commencement of the research.

Yours sincerely

J. O'Neill

John G O'Neill (Professor)
Chair, Human Ethics Chairs' Committee and
Director (Research Ethics)

cc Dr Tony Mutukumira
Institute of Food, Nutrition and Human Health
Albany

Dr Kay Rutherfurd-Markwick
Institute of Food, Nutrition and Human Health
Albany

Prof Richard Archer, HoI
Institute of Food, Nutrition and Human Health
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Research Ethics Office, Massey University, Private Bag 11222, Palmerston North 4442, New Zealand
T +64 6 350 5573 +64 6 350 5575 **F** +64 6 350 5622
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www.massey.ac.nz

Appendix B

Sensory evaluation forms: Information Sheet, participant consent form, preference test form and acceptance test form.

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Effect of ultra-high temperature processing on functional beverages

INFORMATION SHEET

Introduction

My name is Natalie and I am a Masters student in the Institute of Food, Nutrition and Human Health (IFNHH), Massey University. This study is part of my research project and may contribute to the development of a commercial product. You are invited to take part in a study that assesses the impact of heat treatment on sensory characteristics of tea-based beverages. The aim of this consumer study is to evaluate the preference of the products.

Participant involvement

The trial involves tasting and evaluating two beverages. Your participation will take from 5 to 10 minutes.

The beverages you will taste may contain all or some of the following ingredients: sugar, tea, grape extracts, natural colours, flavours, citric acid and ascorbic acid.

You should not take part if you are allergic or may be affected by the consumption of any of the listed ingredients. In the unlikely event of any adverse reaction, medical assistance will be

provided. You may advise one of the researchers of any potentially relevant cultural, religious or ethical beliefs that may prevent you from consuming the foods under consideration.

The information collected in this study will not be linked to any individual's identity and will be used to complete my Masters research project. In case you wish to receive a summary of the findings once data analysis has been completed, please provide your email address.

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- decline to answer any particular question;
- withdraw from the study (at any time);
- ask any questions about the study at any time during participation;
- provide information on the understanding that your name will not be used unless you give permission to the researcher;
- be given access to a summary of the project findings when it is concluded.

Project Contacts

- Natalie d'Avila (Master student)-davila.natalie@gmail.com
- Dr Tony Mutukumira (supervisor) - a.n.mutukumira@massey.ac.nz
- Dr Kay Rutherfurd (co-supervisor)- k.j.rutherfurd@massey.ac.nz

If you have any further questions about this work, please contact one of the researchers above. Your interest is appreciated.

"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher(s) named above are responsible for the ethical conduct of this research.

If you have any concerns about the conduct of this research that you wish to raise with someone other than the researcher(s), please contact Professor John O'Neill, Director, Research Ethics, telephone 06 350 5249, email humanethics@massey.ac.nz".

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Effect of ultra-high temperature processing on functional beverages

PARTICIPANT CONSENT FORM - INDIVIDUAL

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I understand I have the right to withdraw from the study at anytime and to decline to answer any questions.

I agree to voluntarily participate in this study under the conditions set out in the Information Sheet.

Signature: ..... **Date:** .....

Full Name - printed .....

Te Kunenga ki Pūrehuroa
Institute of Food, Nutrition and Human Health
Private Bag 102904, North Shore, 0745, Auckland, New Zealand T +64 9 443 9770 F +64 9 443 9640 www.massey.ac.nz

PREFERENCE TEST

Date: ……

Please taste the two beverage samples from left to right. **Circle the code of the sample that you prefer.** You must choose one sample.

274 **631**

Additional comments:

……

……..

Optional

Age: …… Gender: F / M Nationality: ……...

Do you consume tea products regularly? Yes / No

If you wish to receive a summary of the findings once data analysis has been completed, please provide your email address: .....

ACCEPTANCE TEST

Please evaluate the sample and mark an "X" in the ☐indicating your opinion for each of the following attributes:

Sample code: _____ Date: _____

1. Overall appearance

☐	☐	☐	☐	☐	☐	☐	☐	☐
9=Like extremely	8=Like very much	7=Like moderately	6=Like slightly	5=Neither like nor dislike	4=Dislike slightly	3=Dislike moderately	2=Dislike very much	1=Dislike extremely

2. Overall flavour

☐	☐	☐	☐	☐	☐	☐	☐	☐
9=Like extremely	8=Like very much	7=Like moderately	6=Like slightly	5=Neither like nor dislike	4=Dislike slightly	3=Dislike moderately	2=Dislike very much	1=Dislike extremely

Additional comments:

.....

.....

.....

Appendix C

Table C1. Statistical analysis of paired preference data: proportion test and comparison between two proportions.

Proportion test (R Statistical Software version 2.15.0 GUI 1.51, Austria, 2012)

```
> prop.test(54,118,0.5)
  1-sample proportions test with continuity correction

data:  54 out of 118, null probability 0.5
X-squared = 0.6864, df = 1, p-value = 0.4074
alternative hypothesis: true p is not equal to 0.5
95 percent confidence interval:
 0.3664526 0.5515974
sample estimates:
       p
0.4576271

> prop.test(11,48,0.5)
  1-sample proportions test with continuity correction

data:  11 out of 48, null probability 0.5
X-squared = 13.0208, df = 1, p-value = 0.0003080
alternative hypothesis: true p is not equal to 0.5
95 percent confidence interval:
 0.1251445 0.3766604
sample estimates:
       p
0.2291667
```

Test and CI for Two Proportions

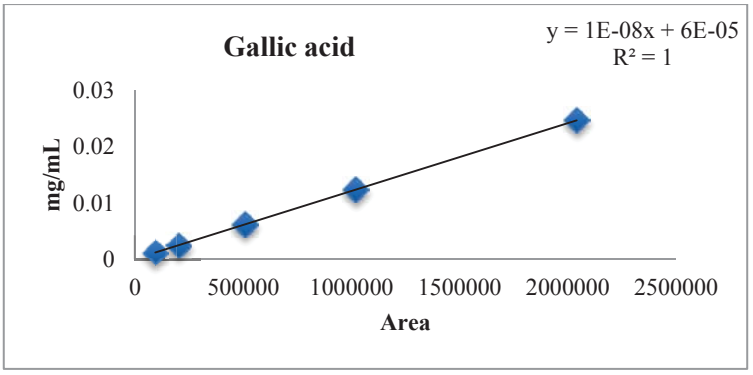
Sample	X	N	Sample p
1	54	118	0.457627
2	11	48	0.229167

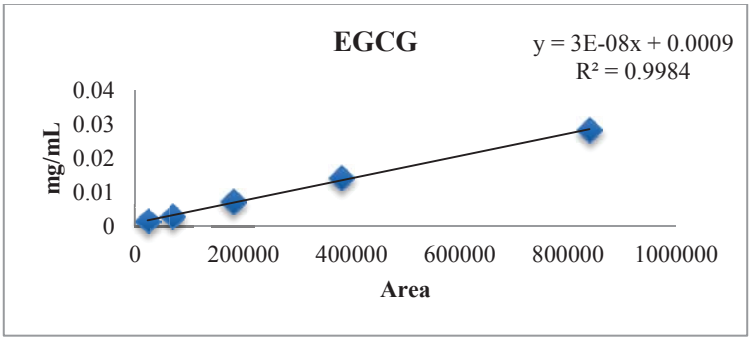
Difference = p (1) - p (2)
Estimate for difference: 0.228460
95% CI for difference: (0.0794049, 0.377516)
Test for difference = 0 (vs not = 0): Z = 2.73 P-Value = 0.006

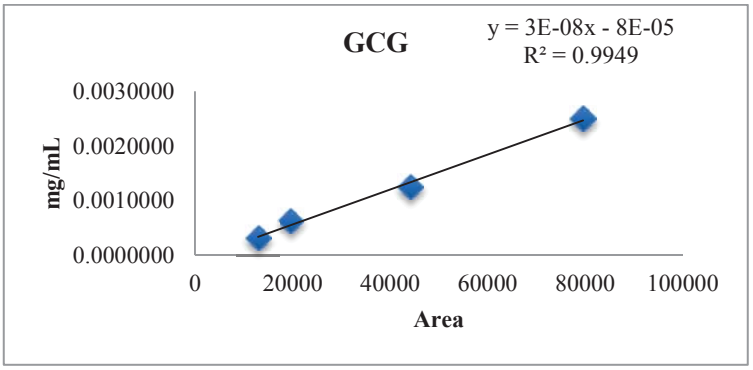
Fisher's exact test: P-Value = 0.008

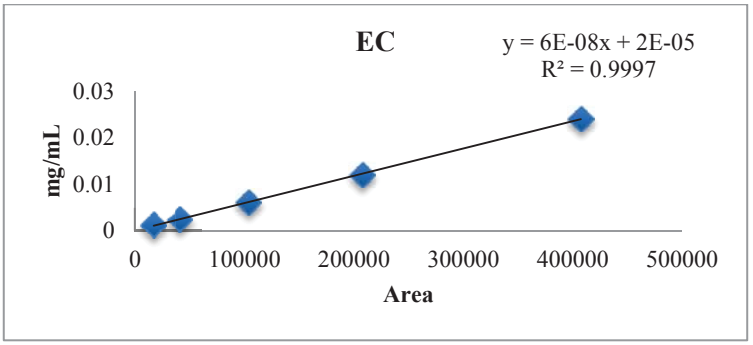
Appendix D
Validation of the HPLC method

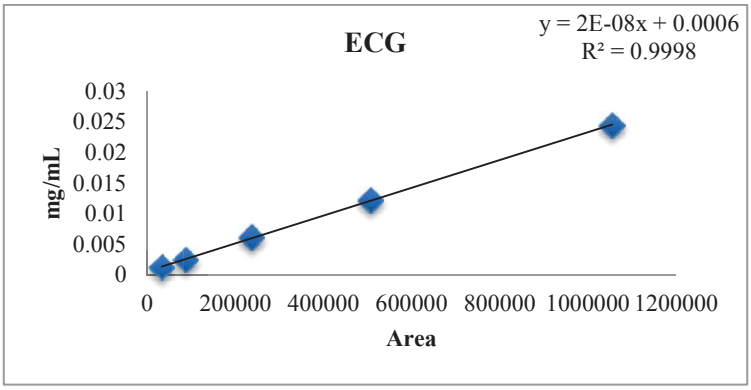
HPLC standard curves

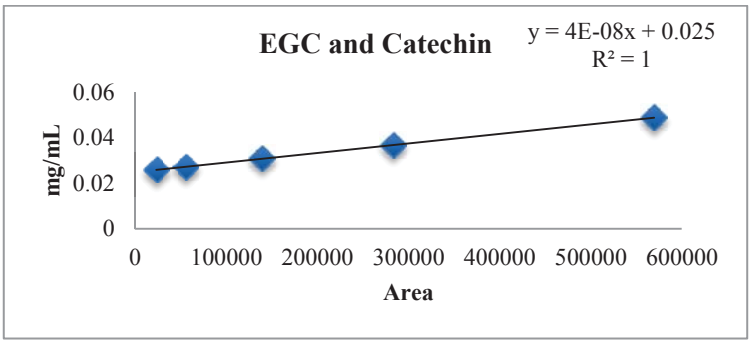


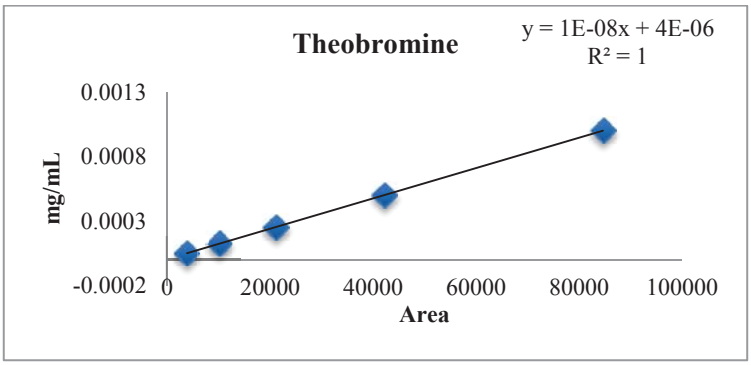







ECG
y = 2E-08x + 0.0006
R² = 0.9998
mg/mL
0.03
0.025
0.02
0.015
0.01
0.005
0
0
200000
400000
600000
800000
1000000
1200000
Area


EGC and Catechin
y = 4E-08x + 0.025
R² = 1
mg/mL
0.06
0.04
0.02
0
0
100000
200000
300000
400000
500000
600000
Area


Theobromine
y = 1E-08x + 4E-06
R² = 1
mg/mL
0.0013
0.0008
0.0003
-0.0002
0
20000
40000
60000
80000
100000
Area

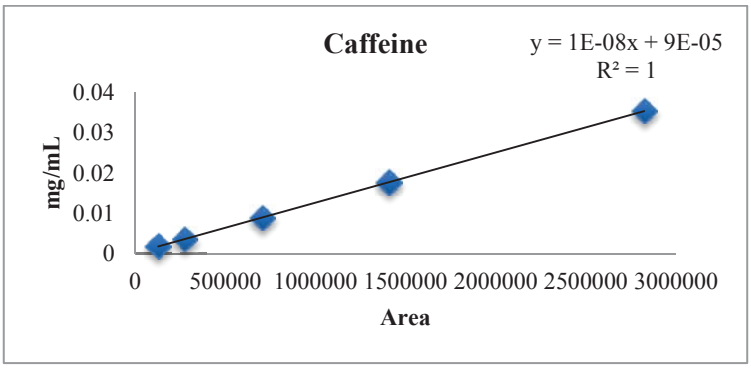

Caffeine
y = 1E-08x + 9E-05
R² = 1
mg/mL
0.04
0.03
0.02
0.01
0
0
500000
1000000
1500000
2000000
2500000
3000000
Area

Table D1. Standards working range: retention time and concentration levels of validated HPLC standards.

Standards	Concentration (µg/mL)					
	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6
Gallic acid	24.72	12.36	6.18	2.47	1.24	
EGC	24.8	12.4	6.2	2.48	1.24	
(+)-Catechin	24	12	6	2.4	1.2	
Caffeine	35.34	17.67	8.83	3.53	1.77	
(-)-Epicatechin	24	12	6	2.4	1.2	
EGCG	28.28	14.14	7.07	2.83	1.41	
ECG	24.48	12.24	6.12	2.45	1.22	
Theobromine	5	1	0.5	0.25	0.13	0.05
GCG		2.5	1.25	0.63	0.31	

Table D2. Validation of gallocatechin gallate (GCG) and theobromine (T) standards working range. The standards were analysed ten times each at five concentration levels. Mean, standard deviation (SD) and coefficient of variance (CV%) of peak areas were calculated.

GCG	mg/mL	Mean	SD	CV %
GCG 1	0.01	234289.1	61160.15	26.1
GCG 2	0.0025	79650.8	2673.06	3.36
GCG 3	0.00125	44254.2	1877.84	4.24
GCG 4	0.000625	19701.4	1840.53	9.34
GCG 5	0.0003125	12961	771.7	5.95
Theobromine				
T 1	0.005	442941.7	7765.2	1.75
T 2	0.001	84718.8	1905.57	2.25
T 3	0.0005	42284.4	467.56	1.11
T 4	0.00025	21223	438.41	2.07
T 5	0.000125	10132	599.53	5.92
T 6	0.00005	3884.6	238.02	6.13

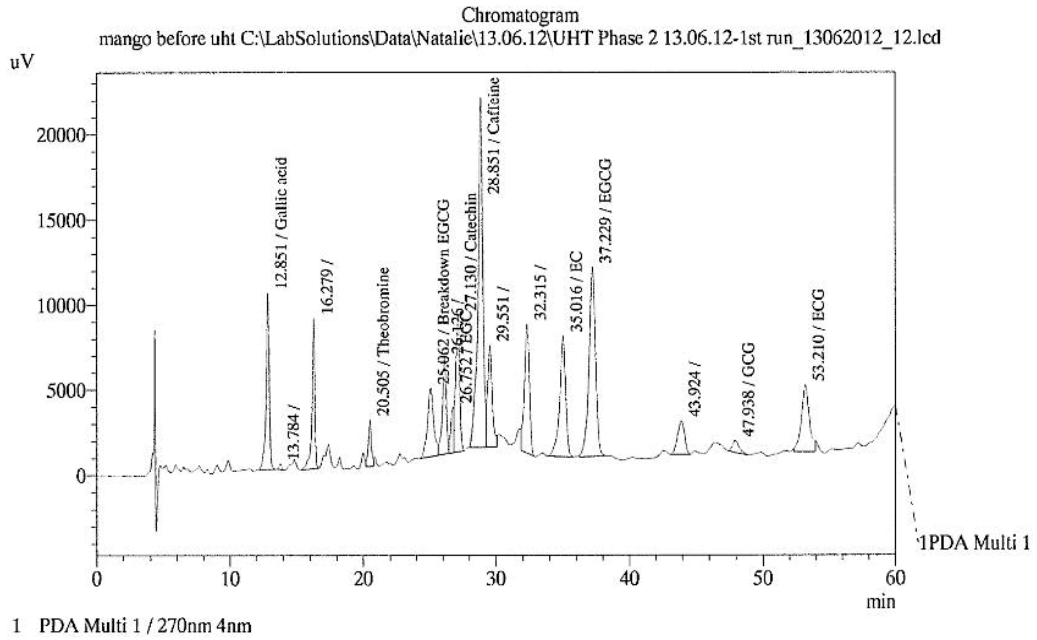
Table D3. Validation of standard mix working range. HPLC standards were analysed ten times each at five concentration levels. Mean, standard deviation (SD) and coefficient of variance (CV%) of peak areas were calculated.

	MIX 1			MIX 2			MIX 3			MIX 4			MIX 5		
Compound	**Average**	**SD**	**CV %**	**Average**	**SD**	**CV%**	**Average**	**SD**	**CV%**	**Average**	**SD**	**CV %**	**Average**	**SD**	**CV %**
Gallic acid	2042635.60	7751.79	0.38	1021089.22	7193.21	0.70	509213.10	4715.06	0.93	200565.50	3480.62	1.74	93956.80	5896.32	6.28
EGC+															
Catechin	569605.20	11989.47	2.10	283492.20	15422.09	5.44	139405.60	7244.89	5.20	55516.60	5024.29	9.05	23432.60	1784.16	7.61
Caffeine	2825721.20	13409.66	0.47	1409502.70	6885.59	0.49	707094.90	3530.83	0.50	276694.20	2601.37	0.94	128805.00	8753.96	6.80
EC	407821.00	9813.26	2.41	207634.00	6046.40	2.91	104045.60	2320.64	2.23	41048.10	1098.11	2.68	17013.20	1385.09	8.14
EGCG	840827.50	29897.16	3.56	381943.20	35262.82	9.23	181671.40	17182.70	9.46	69519.60	6385.07	9.18	23578.70	1826.94	7.75
ECG	1055586.90	22433.05	2.13	507151.60	22637.58	4.46	236952.70	23653.27	9.98	87402.00	2250.89	2.58	33311.50	3252.24	9.76

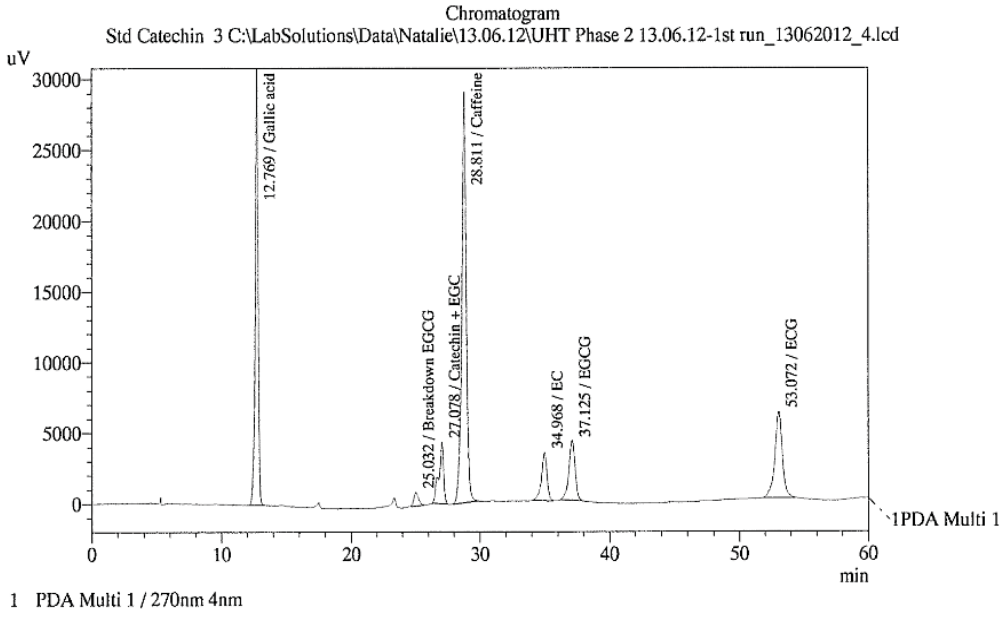
Table D4. Reproducibility assessment of the HPLC method. Two samples (N-40-90 and M-UHT-120) were analysed at five different days, and peak areas were used to calculated mean, standard deviation (SD) and coefficient of variance (CV%). All inter-day CVs % were below 10%.

Sample N-40-90	**Mean (Area)**	**SD**	**CV%**
Gallic Acid	209040.6	8521.64	4.08
Catechin+EGC	175124	5843.49	3.34
Caffeine	540365.6	37460.77	6.93
EC	131126.2	2169.5	1.65
EGCG	70044	4148.05	5.92
ECG	17325.2	1187.18	6.85
Theobromine	50602.8	3434.2	6.79
Sample M-UHT-120	**Mean (Area)**	**SD**	**RSD %**
Gallic Acid	183376.4	4084.96	2.23
Catechin+EGC	254615.8	25402.5	9.98
Caffeine	640271	54294.05	8.48
EC	240242	12511.55	5.21
EGCG	464490	31047.17	6.68
ECG	147626.2	10614.75	7.19
GCG	31766.2	2322.3	7.31
Theobromine	50309	4552.83	9.05

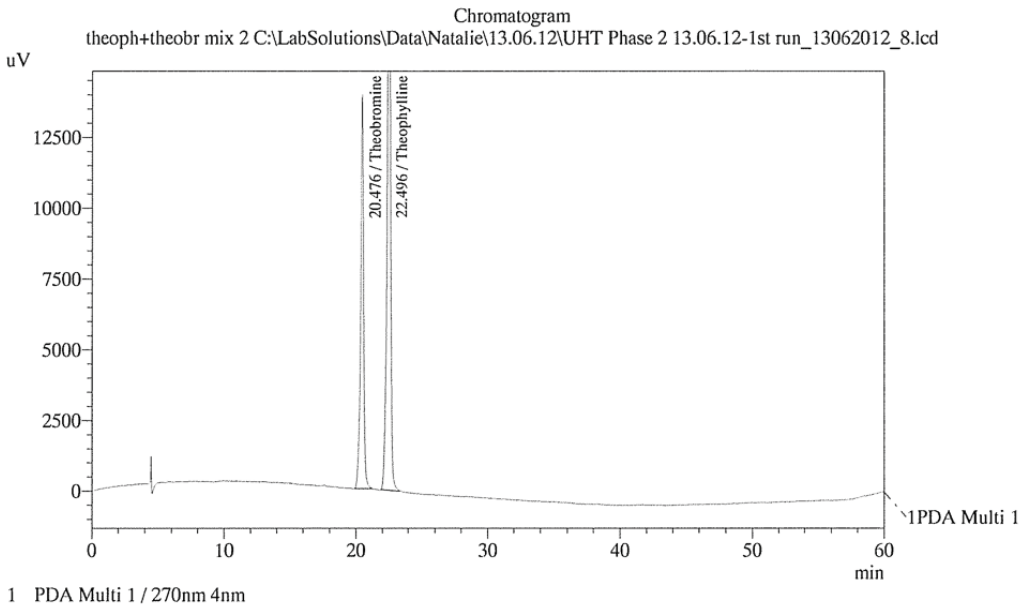
Appendix E
HPLC Chromatograms



UHT-treated beverage sample

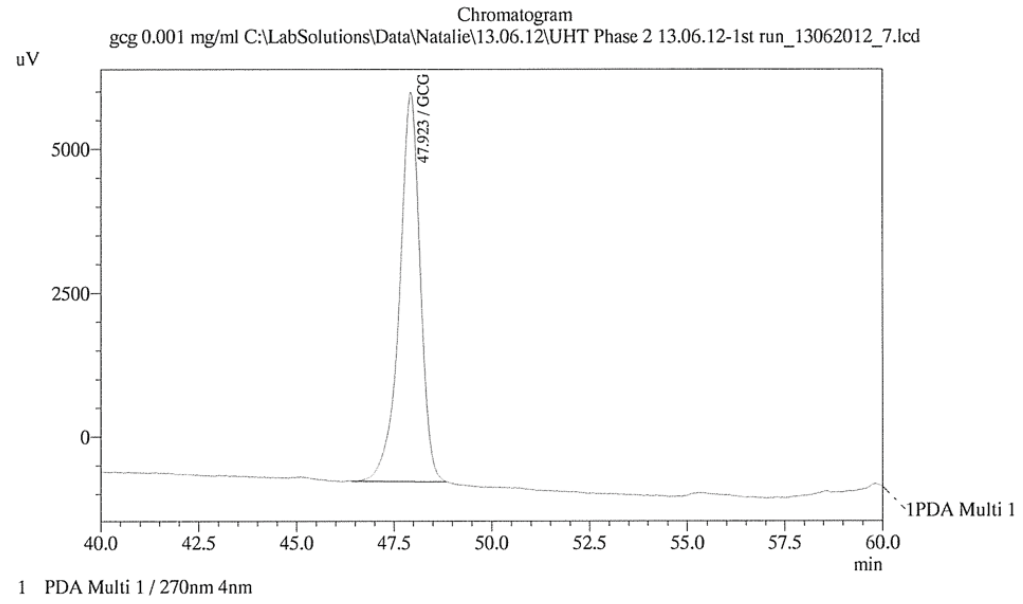


Standards gallic acid, (+)-catechin, EGC, caffeine, EC, EGCG, and ECG



Standards theobromine and theophylline

Note: theophylline was not quantified in this study.



Standard GCG

Appendix F
Statistical analyses of data - Phase I

Statistical analysis of total phenolic content and antioxidant capacity in non-UHT-treated and UHT-treated mango-flavoured beverages (Phase I). ANOVA was used to analyse the effects of UHT temperature (non-UHT-treated, 110, 120, 130 and 140°C) and production trials (1 and 2) as a random factor.

Statistical input for total phenolics (GAE mg/mL) data				
Source	**DF**	**SS**	**F**	**P**
Production	1	0.0001083	0.35	0.586
UHT Temperature	4	0.0010100	0.82	0.575
Production*UHT Temperature	4	0.0012360	1.88	0.154

Statistical input for total phenolics (CAE mg/mL) data				
Source	**DF**	**SS**	**F**	**P**
Production	1	0.0022884	7.33	0.054
UHT Temperature	4	0.0010220	0.82	0.574
Production*UHT Temperature	4	0.0012480	1.88	0.154

Statistical input for DPPH free radical scavenging ability data				
Source	**DF**	**SS**	**F**	**P**
Production	1	38489.5	27.41	0.006
UHT Temperature	4	33602.8	5.98	0.056
Production*UHT Temperature	4	5617.7	9.81	0.000

Statistical input for ORAC data				
Source	**DF**	**SS**	**F**	**P**
Production	1	1650355	1.75	0.256
UHT Temperature	4	5080931	1.35	0.390
Production*UHT Temperature	4	3769344	7.74	0.001

Statistical analysis of HPLC data: phenolics and methylxanthines in non-UHT-treated and UHT-treated mango-flavoured beverages (Phase I). ANOVA was used to analyse the effects of UHT temperature (non-UHT-treated, 110, 120, 130 and 140°C) and production trials (1 and 2) as a random factor.

Statistical input for Gallic acid data				
Source	**DF**	**SS**	**F**	**P**
Production	1	0.0383767	14.41	0.019
UHT Temperature	4	0.0266458	2.50	0.198
Production*UHT Temperature	4	0.0106528	9.85	0.002

Statistical input for EGC and (+)-catechin data				
Source	**DF**	**SS**	**F**	**P**
Production	1	41.4250	438.00	0.000
UHT Temperature	4	0.9445	2.50	0.199
Production*UHT Temperature	4	0.3783	2.14	0.150

Statistical input for caffeine data				
Source	**DF**	**SS**	**F**	**P**
Production	1	10.8278	348.06	0.000
UHT Temperature	4	0.0645	0.52	0.730
Production*UHT Temperature	4	0.1244	6.08	0.010

Statistical input for EC data				
Source	**DF**	**SS**	**F**	**P**
Production	1	1.34891	34.08	0.004
UHT Temperature	4	0.82910	5.24	0.069
Production*UHT Temperature	4	0.15834	4.38	0.026

Statistical input for EGCG data				
Source	**DF**	**SS**	**F**	**P**
Production	1	6.5983	1.03	0.368
UHT Temperature	4	20.6154	0.80	0.581
Production*UHT Temperature	4	25.6423	581.59	0.000

Statistical input for ECG data				
Source	**DF**	**SS**	**F**	**P**
Production	1	0.00138	0.00	0.972
UHT Temperature	4	2.45762	0.62	0.671
Production*UHT Temperature	4	3.94903	329.83	0.000

Statistical input for theobromine data				
Source	**DF**	**SS**	**F**	**P**
Production	1	0.0027839	6.47	0.064
UHT Temperature	4	0.0014403	0.84	0.567
Production*UHT Temperature	4	0.0017222	3.00	0.072

Statistical input for GCG data				
Source	**DF**		**F**	**P**
Production	1	0.014077	0.71	0.446
UHT Temperature	4	0.413972	5.23	0.069
Production*UHT Temperature	4	0.079085	94.66	0.000

Appendix G
Chemical data (Phase II)

Table G1. Total phenolics[1], expressed as GAE mg/mL and CAE mg/mL, in non-UHT-treated and UHT-treated pomegranate, mango, and nectarine beverages stored for 90 days at 20 and 40 °C.

	Flavour	Storage temperature (°C)	Non-UHT-treated	UHT-treated beverages during storage (days)					
				0	14	28	42	63	90
GAE mg/mL	Pomegranate	20 °C	0.50 ± 0.01	0.50 ± 0.02	0.52 ± 0.02	0.54 ± 0.02	0.55 ± 0.02	0.54 ± 0.01	0.55 ± 0.01
		40 °C	0.50 ± 0.01	0.50 ± 0.02	0.53 ± 0.01	0.55 ± 0.01	0.54 ± 0.03	0.50 ± 0.01	0.54 ± 0.03
	Mango	20 °C	0.44 ± 0.01	0.45 ± 0.02	0.51 ± 0.02	0.51 ± 0.01	0.51 ± 0.01	0.51 ± 0.00	0.51 ± 0.01
		40 °C	0.44 ± 0.01	0.45 ± 0.02	0.51 ± 0.02	0.52 ± 0.02	0.51 ± 0.01	0.48 ± 0.02	0.51 ± 0.01
	Nectarine	20 °C	0.48 ± 0.01	0.47 ± 0.02	0.50 ± 0.01	0.51 ± 0.01	0.52 ± 0.01	0.51 ± 0.02	0.52 ± 0.01
		40 °C	0.48 ± 0.01	0.47 ± 0.02	0.51 ± 0.02	0.52 ± 0.02	0.54 ± 0.01	0.51 ± 0.01	0.54 ± 0.01
CAE mg/mL	Pomegranate	20 °C	0.45 ± 0.01	0.45 ± 0.02	0.47 ± 0.02	0.49 ± 0.02	0.49 ± 0.02	0.48 ± 0.01	0.49 ± 0.01
		40 °C	0.45 ± 0.01	0.45 ± 0.02	0.48 ± 0.01	0.50 ± 0.01	0.48 ± 0.03	0.44 ± 0.01	0.48 ± 0.03
	Mango	20 °C	0.39 ± 0.01	0.40 ± 0.02	0.45 ± 0.02	0.46 ± 0.01	0.46 ± 0.01	0.45 ± 0.00	0.46 ± 0.01
		40 °C	0.39 ± 0.01	0.40 ± 0.02	0.45 ± 0.02	0.47 ± 0.02	0.45 ± 0.01	0.42 ± 0.01	0.45 ± 0.01
	Nectarine	20 °C	0.43 ± 0.01	0.42 ± 0.02	0.45 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	0.45 ± 0.02	0.46 ± 0.01
		40 °C	0.43 ± 0.01	0.42 ± 0.02	0.46 ± 0.02	0.46 ± 0.02	0.48 ± 0.01	0.45 ± 0.01	0.48 ± 0.01

[1]Values represent mean ± standard deviation of triplicate measurements.
GAE = gallic acid equivalent, CAE= catechin equivalent.

Table G.2 Antioxidant capacity[1] of non-UHT-treated and UHT-treated beverages stored for 90 days at 20 and 40 °C.

	Flavour	Storage temperature (°C)	Non-UHT-treated	UHT-treated beverages during storage (days)					
				0	14	28	42	63	90
ORAC (μmol TE/L)[2]	Pomegranate	20 °C	4768.75 ± 191.97	8085.75 ± 584.76	5752.81 ± 239.57	5059.83 ± 142.98	4769.54 ± 327.26	4465.37 ± 227.22	4343.56 ± 10.39
		40 °C	4768.75 ± 191.97	8085.75 ± 584.76	6487.22 ± 250.36	5390.79 ± 466.51	5557.31 ± 28.35	4052.55 ± 219.93	4268.35 ± 93.66
	Mango	20 °C	4671.85 ± 251.68	6274.90 ± 511.74	6711.25 ± 477.85	5565.55 ± 136.71	5723.83 ± 351.64	4449.67 ± 72.18	5304.25 ± 504.25
		40 °C	4671.85 ± 251.68	6274.90 ± 511.74	6873.76 ± 170.86	5554.28 ± 694.99	5489.96 ± 332.54	4504.24 ± 271.59	4509.69 ± 146.98
	Nectarine	20 °C	6341.56 ± 554.27	7445.99 ± 199.90	6724.44 ± 499.14	6558.67 ± 486.89	7325.76 ± 562.36	4682.94 ± 92.20	6615.96 ± 891.06
		40 °C	6341.56 ± 554.27	7445.99 ± 199.90	6710.60 ± 286.80	5122.78 ± 245.96	6156.05 ± 215.63	5052.28 ± 91.07	5921.71 ± 629.39
$DPPH^{\bullet}$ EC_{50}[3]	Pomegranate	20 °C	653.91 ± 28.01	650.87 ± 8.56	650.81 ± 26.59	567.30 ± 41.69	484.34 ± 37.21	478.66 ± 10.98	543.20 ± 5.34
		40 °C	653.91 ± 28.01	650.87 ± 8.56	533.47 ± 19.74	611.10 ± 25.76	498.44 ± 19.04	554.50 ± 3.06	653.07 ± 38.73
	Mango	20 °C	668.99 ± 26.53	653.90 ± 40.01	658.34 ± 27.32	606.62 ± 41.43	533.62 ± 35.56	540.07 ± 29.59	584.72 ± 31.23
		40 °C	668.99 ± 26.53	653.90 ± 40.01	533.73 ± 17.95	612.55 ± 10.23	549.54 ± 11.26	606.89 ± 17.31	685.90 ± 31.49
	Nectarine	20 °C	651.37 ± 10.94	593.22 ± 10.82	560.01 ± 37.75	562.53 ± 16.96	506.84 ± 16.49	533.94 ± 24.00	540.93 ± 13.19
		40 °C	651.37 ± 10.94	593.22 ± 10.82	500.09 ± 17.38	537.31 ± 34.00	508.29 ± 28.00	538.90 ± 30.89	549.90 ± 28.46

[1]Values represent mean ± standard deviation of triplicate measurements.
[2]TE = Trolox equivalent. [3]$DPPH^{\bullet}$ EC_{50} = mL of sample required to decrease 1 g of the initial $DPPH^{\bullet}$ concentration by 50% in 20 min.

Table G3. Phenolics and methylxanthines (µg/mL) in non-UHT-treated and UHT-treated beverages stored for 90 days at 20 and 40 °C.

Flavour	Storage time	Gallic acid	(+)Catechin and EGC	Caffeine	EC	EGCG	ECG	GCG	Theobromine
Pomegranate beverages	Non-UHT-treated	1.76	7.37	5.53	13.71	11.18	2.99	0.88	0.43
Stored at 20 °C	Non-UHT-treated	1.76	8.40	4.84	13.56	11.16	3.43	1.00	0.39
	0	1.78	8.61	5.52	14.01	11.43	3.48	0.79	0.37
	0	1.79	8.64	5.39	14.21	11.29	3.60	0.91	0.39
	14	1.78	7.97	5.80	14.55	9.51	3.83	0.57	0.45
	14	1.78	8.45	6.07	14.52	9.50	3.76	0.58	0.45
	28	1.87	11.70	6.67	14.56	9.67	1.62	0.41	0.45
	28	1.78	11.50	6.48	13.14	9.69	1.60	0.36	0.46
	42	1.84	12.74	5.69	13.39	9.33	1.70	0.37	0.45
	42	1.83	12.98	5.68	13.39	9.32	1.69	0.36	0.41
	63	1.90	13.73	5.88	13.39	8.42	1.16	0.13	0.34
	63	1.89	13.68	5.73	13.48	8.32	1.14	0.12	0.34
	90	1.91	13.43	6.04	12.78	6.91	1.10	0.06	0.43
	90	1.91	13.56	6.04	12.63	6.83	1.08	0.08	0.42
Pomegranate beverages	Non-UHT-treated	1.76	7.37	5.53	13.71	11.18	2.99	0.88	0.43
stored at 40 °C	Non-UHT-treated	1.76	8.40	4.84	13.56	11.16	3.43	1.00	0.39
	0	1.78	8.61	5.52	14.01	11.43	3.48	0.79	0.37
	0	1.79	8.64	5.39	14.21	11.29	3.60	0.91	0.39
	14	1.91	7.57	5.87	11.83	6.91	2.36	0.26	0.48
	14	1.91	7.60	5.84	11.68	6.94	2.39	0.25	0.49
	28	2.01	9.70	6.42	10.68	6.06	0.94	1.17	0.42
	28	2.08	10.12	6.75	10.12	5.43	1.05	1.29	0.42
	42	2.13	11.40	5.54	12.88	4.86	0.68	0.03	0.43
	42	2.18	11.31	5.66	12.70	4.78	0.69	0.03	0.40
	63	1.18	4.46	5.01	4.29	1.03	0.09	0.01	0.39
	63	1.19	4.53	5.12	4.30	1.07	0.08	0.01	0.37
	90	2.79	7.79	5.14	8.93	2.57	0.47	0.01	0.44
	90	2.72	7.40	5.29	9.04	2.59	0.46	0.01	0.39
Mango beverages	Non-UHT-treated	1.78	8.56	5.42	13.58	11.56	3.40	0.68	0.46

stored at 20 °C	Non-UHT-treated	1.77	8.45	5.41	13.57	11.66	3.32	0.73	0.45
	0	1.82	8.58	5.01	14.24	11.76	3.30	0.87	0.50
	0	1.82	8.47	5.34	14.17	11.68	3.54	0.90	0.44
	14	1.77	8.77	6.68	14.82	10.27	1.89	0.66	0.49
	14	1.77	8.76	6.68	14.70	10.24	1.83	0.67	0.50
	28	1.80	12.23	4.90	12.88	9.42	2.00	0.44	0.46
	28	1.78	11.63	5.14	13.64	9.47	1.92	0.46	0.46
	42	1.86	13.71	5.79	14.00	9.19	1.84	0.36	0.47
	42	1.86	13.88	5.85	13.87	9.16	1.88	0.39	0.45
	63	1.86	13.84	5.80	13.61	8.87	1.62	0.22	0.37
	63	1.86	13.87	5.92	13.55	8.84	1.53	0.21	0.38
	90	1.94	13.49	6.26	13.46	7.31	1.28	0.05	0.44
	90	1.93	12.87	6.10	13.38	6.96	1.30	0.08	0.44
Mango beverages	Non-UHT-treated	1.78	8.56	5.42	13.58	11.56	3.40	0.68	0.46
stored at 40 °C	Non-UHT-treated	1.77	8.45	5.41	13.57	11.66	3.32	0.73	0.45
	0	1.82	8.58	5.01	14.24	11.76	3.30	0.87	0.50
	0	1.82	8.47	5.34	14.17	11.68	3.54	0.90	0.44
	14	1.91	8.11	6.69	12.79	7.70	1.19	0.32	0.56
	14	1.92	8.15	6.69	13.23	7.75	1.27	0.31	0.54
	28	2.05	11.42	5.20	15.73	6.25	0.97	0.85	0.46
	28	2.07	11.77	5.56	15.84	6.23	0.95	0.89	0.44
	42	2.33	11.52	5.36	13.40	4.76	0.62	0.03	0.45
	42	2.32	10.99	5.55	13.12	4.43	0.63	0.02	0.44
	63	2.14	11.66	5.69	13.92	4.58	0.61	0.03	0.38
	63	2.18	11.34	5.73	13.86	4.56	0.58	0.02	0.41
	90	2.89	7.73	5.28	8.79	2.43	0.42	0.01	0.47
	90	2.85	8.22	5.32	8.87	2.45	0.48	0.00	0.46
Nectarine beverages	Non-UHT-treated	1.74	8.57	5.05	13.59	11.39	2.84	0.90	0.50
stored at 20 °C	Non-UHT-treated	1.74	8.52	5.20	13.61	11.40	2.97	1.03	0.54
	0	1.75	8.69	5.71	13.99	11.80	3.15	1.08	0.55
	0	1.75	8.88	5.52	14.04	11.89	3.03	1.01	0.53
	14	1.72	8.67	6.35	13.30	9.96	1.85	0.69	0.54

	14	1.74	8.62	6.36	13.27	10.01	1.83	0.69	0.52
	28	1.82	12.34	5.10	11.20	9.93	2.20	0.61	0.50
	28	1.81	12.07	5.40	11.73	10.14	2.27	0.60	0.52
	42	1.84	12.37	6.23	10.88	8.80	1.95	0.53	0.55
	42	1.83	12.56	6.27	10.52	8.60	1.88	0.53	0.52
	63	1.81	12.03	6.45	10.55	8.11	1.76	0.39	0.48
	63	1.80	12.44	6.36	10.76	8.34	1.80	0.38	0.48
	90	2.61	11.78	6.40	10.26	0.10	0.05	0.00	0.48
	90	2.64	12.24	6.33	10.81	0.10	0.05	0.00	0.48
Nectarine beverages	Non-UHT-treated	1.74	8.57	5.05	13.59	11.39	2.84	0.90	0.50
stored at 40 °C	Non-UHT-treated	1.74	8.52	5.20	13.61	11.40	2.97	1.03	0.54
	0	1.75	8.69	5.71	13.99	11.80	3.15	1.08	0.55
	0	1.75	8.88	5.52	14.04	11.89	3.03	1.01	0.53
	14	1.80	7.90	6.45	13.06	8.14	1.41	0.45	0.51
	14	1.80	7.86	6.30	11.75	8.07	1.36	0.42	0.51
	28	1.90	10.94	5.61	9.35	6.44	1.30	0.31	0.50
	28	1.92	10.92	5.64	9.94	6.67	1.25	0.27	0.50
	42	1.95	11.89	6.43	10.79	5.49	0.87	0.10	0.54
	42	1.95	11.59	6.40	10.64	5.53	0.85	0.10	0.52
	63	1.94	9.44	6.22	11.40	4.91	0.70	0.07	0.47
	63	1.94	10.10	6.24	11.85	5.11	0.69	0.07	0.47
	90	2.23	9.92	6.23	9.83	3.47	0.43	0.00	0.50
	90	2.06	10.10	6.19	9.82	3.47	0.44	0.00	0.54

Appendix H
Statistical analyses of data - Phase II

Statistical analysis of total phenolic content and antioxidant capacity data (Phase II): ANOVA was used to analyse the effects of storage time (before UHT treatment, and during storage at 0, 14, 28, 42, 63 and 90 days), storage temperature (20°C, 40°C), and flavours (pomegranate, mango, nectarine).

Statistical input for total phenolics (GAE mg/mL) data				
Source	**DF**	**SS**	**F**	**P**
Storage time	6	0.0612725	43.23	0.000
Storage temperature	1	0.0000103	0.04	0.835
Flavour	2	0.0286481	60.64	0.000
Storage temp*Storage time	6	0.0027254	1.92	0.085
Flavour*Storage time	12	0.0066729	2.35	0.011
Flavour*Storage temp	2	0.0014256	3.02	0.054

Statistical input for total phenolics (CAE mg/mL) data				
Source	**DF**	**SS**	**F**	**P**
Storage time	6	0.0517503	39.43	0.000
Storage temperature	1	0.0000041	0.02	0.891
Flavour	2	0.0259359	59.29	0.000
Storage temp*Storage time	6	0.0025633	1.95	0.080
Flavour*Storage time	12	0.0059335	2.26	0.014
Flavour*Storage temp	2	0.0013073	2.99	0.055

Statistical input for DPPH free radical scavenging ability data				
Source	**DF**	**SS**	**F**	**P**
Storage time	6	265999	58.46	0.000
Storage temperature	1	1058	1.40	0.240
Flavour	2	57078	37.63	0.000
Storage temp*Storage time	6	80400	17.67	0.000
Flavour*Storage time	12	27892	3.07	0.001
Flavour*Storage temp	2	4313	2.84	0.063

Statistical input for ORAC data				
Source	**DF**	**SS**	**F**	**P**
Storage time	6	90899339	83.84	0.000
Storage temperature	1	411998	2.28	0.134
Flavour	2	21417554	59.26	0.000
Storage temp*Storage time	6	2013400	1.86	0.096
Flavour*Storage time	12	23234640	10.71	0.000
Flavour*Storage temp	2	1990118	5.51	0.005

Statistical analysis of HPLC data (Phase II): ANOVA was used to analyse the effects of storage time (before UHT treatment, and during storage at 0, 14, 28, 42, 63 and 90 days), storage temperature (20°C, 40°C), and flavours (pomegranate, mango, nectarine).

Statistical input for gallic acid data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	0.15624	2.64	0.081
Storage temperature	1	0.39237	13.24	0.001
Storage time	6	3.32645	18.70	0.000
Flavour*Storage temp	2	0.34186	5.77	0.005
Flavour*Storage time	12	0.44618	1.25	0.272
Storage temp*Storage time	6	0.62495	3.51	0.005

Statistical input for (+)-catechin and EGC data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	11.265	8.76	0.001
Storage temperature	1	65.995	102.60	0.000
Storage time	6	193.946	50.25	0.000
Flavour*Storage temp	2	10.184	7.92	0.001
Flavour*Storage time	12	19.454	2.52	0.010
Storage temp*Storage time	6	69.446	17.99	0.000

Statistical input for caffeine data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	1.18532	21.34	0.000
Storage temperature	1	0.30059	10.82	0.002
Storage time	6	8.82376	52.95	0.000
Flavour*Storage temp	2	0.27753	5.00	0.010
Flavour*Storage time	12	8.88081	26.65	0.000
Storage temp*Storage time	6	1.44664	8.68	0.000

Statistical input for EC data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	42.037	16.02	0.000
Storage temperature	1	32.902	25.08	0.000
Storage time	6	110.283	14.01	0.000
Flavour*Storage temp	2	25.752	9.82	0.000
Flavour*Storage time	12	60.784	3.86	0.000
Storage temp*Storage time	6	27.629	3.51	0.005

Statistical input for EGCG data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	3.840	2.82	0.069
Storage temperature	1	120.567	176.78	0.000
Storage time	6	577.447	141.11	0.000
Flavour*Storage temp	2	15.841	11.61	0.000
Flavour*Storage time	12	33.390	4.08	0.000
Storage temp*Storage time	6	67.258	16.44	0.000

Statistical input for ECG data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	0.6387	10.66	0.000
Storage temperature	1	7.5533	252.09	0.000
Storage time	6	80.5257	447.92	0.000
Flavour*Storage temp	2	0.2228	3.72	0.031
Flavour*Storage time	12	8.5531	23.79	0.000
Storage temp*Storage time	6	4.0369	22.46	0.000

Statistical input for GCG data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	0.06302	2.58	0.085
Storage temperature	1	0.15637	12.80	0.001
Storage time	6	9.09113	124.07	0.000
Flavour*Storage temp	2	0.12745	5.22	0.008
Flavour*Storage time	12	0.58742	4.01	0.000
Storage temp*Storage time	6	0.97332	13.28	0.000

Statistical input for theobromine data				
Source	**DF**	**SS**	**F**	**P**
Flavour	2	0.1412006	227.88	0.000
Storage temperature	1	0.0004233	1.37	0.248
Storage time	6	0.0592192	31.86	0.000
Flavour*Storage temp	2	0.0004210	0.68	0.511
Flavour*Storage time	12	0.0217487	5.85	0.000
Storage temp*Storage time	6	0.0051153	2.75	0.021

Statistical analysis of sensory acceptance data (Phase II): one-way ANOVA was used to analyse appearance and flavour rating scores of beverages (pomegranate, mango, nectarine) stored for 90 days at 20°C and 40°C.

Statistical input for appearance rating scores				
Source	**DF**	**SS**	**F**	**P**
Beverages	5	191.10	12.80	0.000
Statistical input for flavour rating scores				
Source	**DF**	**SS**	**F**	**P**
Beverages	5	99.44	6.45	0.000