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BIOCHEMISTRY OF ANTIOXIDANTS: ANTIOXIDANT CAPACITY MEASUREMENT METHODS AND THEIR APPLICATION TO DEVELOP USEFUL INDICATORS OF STABILITY AND FUNCTIONALITY IN FOOD MATRICES

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**BIOCHEMISTRY OF ANTIOXIDANTS:
ANTIOXIDANT CAPACITY MEASUREMENT METHODS AND THEIR
APPLICATION TO DEVELOP USEFUL INDICATORS OF STABILITY
AND FUNCTIONALITY IN FOOD MATRICES**

by

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Biochemistry of antioxidants: antioxidant capacity measurement and their application to develop useful indicators of stability and functionality in food matrices

Kwestan Muhammad

Abstract

Antioxidant properties of green tea (GT) have been widely reported. The antioxidant capacity (AOC) of green tea was investigated to include the effect of infusion time over 24 hours. The AOC was measured by the FRAP, DPPH, TEAC, and CBA assays. It was proven according that after 2 hours of brewing, tea has higher AOC and Total phenolic content (TPC), these significantly decreases after 4 hours.

GT has a high amount of polyphenols with potent AOC. However, interactions between polyphenols and food matrix may decrease their potential benefit. The objective of this experiment was to test the hypothesis that the addition of milk (full fat, semi-skimmed, and skimmed) may affect the phenolic content and AOC was measured. The results indicated the plain GT had highest activity; then tea with FFM had a significantly higher amount of AO than others.

Plant extracts possess health promoting properties. The objective of this study was to determine the TPC and AOA of different concentrations of spice extracts (fennel, clove, cardamom, cinnamon, ginger, anise, and black pepper) with DPPH, TEAC and Rancimat methods. At low concentration, black pepper had a highest activity but at high concentration, ginger showed the highest activity among the extracts. The TPC for spice extract was greater for anise. Results provided evidence that the studied spices may be used as a natural AO.

In recent decades, saliva has emerged as a new way to diagnose and investigate basic health problems. In this study, salivary TPC and AOC were measured after consumption a single cup of green tea with and without of milk. In a healthy adult crossover design. The salivary AOC and TPC were measured before and after consumption up to 3 hours. Results indicated that milk decreased AOC of GT when compared with the control water. The activity reached peak 1 hour after ingestion and then decreased returning to the baseline. Results confirmed that saliva could be used as an easier and safer alternative to blood to assess AOA in humans.

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Without help from the particular that mentioned above, I would face many difficulties while doing this.

Author's Declaration

At no time during the registration for the degree of Research Master has the author been registered for any other University award without prior agreement of the Graduate Committee.

A programme of advanced study was undertaken, which included a course in molecular and cellular biology, Biochemistry, and instruction on antioxidant capacity methodologies and postgraduate courses on research skills and methods, methods and laboratory methods teaching and practice.

Conferences attended at Plymouth University included a postgraduate society conference and one on translational biomedicine; and a Biochemical Society conference on 'Analysis of free radicals, radical modifications and Redox signalling'

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Signed

Date

Dedication

This thesis is dedicated to my love life Waleed without his love, support, and encouragement I would not had succeed. Also, dedicate it to my family especially my dearest brother Daniel.

List of abbreviations

| Abbreviation | Description |
|--------------|---|
| AAI | Antioxidant activity index |
| ABAP | 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride |
| ABTS | 2,2'-azinobis-(3-ethylbenzotiazoline-6-sulphonic acid) |
| AOC | Antioxidant capacity |
| BHA | Butylated hydroxyanisol |
| BHT | Batylated hydroxytoluene |
| C | (±)-catechin |
| CG | (-)-catechin gallate |
| DPPH | 1,1-Diphenyl-2-picrylhydrazyl |
| EC | (-)-epicatechin |
| ECG | (-)-epicatechin gallate |
| EGC | (-)-epigallocatechin |
| EGCG | (-)-epigallocatechin gallate |
| FCR | Folin-Ciocaltu's reagent |
| FFM | Full fat milk |
| FRAP | Ferric-reducing antioxidant power |
| GAE | Gallic acid equivalent |
| GC | (-)-galocatechin |
| GCG | (-)-galocatechin gallate |
| GT | Green tea |
| LDLs | Low-density lipoproteins |
| ORAC | Oxygen radical absorbing capacity |
| PG | Propyl galate |
| SKM | Skimmed milk |
| SSKM | Semi-skimmed milk |
| TBARS | Thiobarbituric acid-reactive substances |
| TBHQ | Tertiary butyl hydroquinone |
| TEAC | Trolox equivalent antioxidant capacity |
| TOSC | Total oxyradical scavenging capacity |
| TPC | Total phenol content |
| TRAP | Total radical trapping parameter |
| Trolox | ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) |
| FCR | Folin-Ciocaleu's reagent |
| CBA | Crocin Bleaching assay |

Chapter 1

Introduction:

In recent decades, there has been an increasing tendency towards using natural antioxidants instead of synthetic ones, because natural compounds are simpler in comparison to synthetic materials and substances. Synthetic compounds require a long time to complete their natural cycle and return to nature thus causing environmental pollution, but the natural substances are much cheaper and safer than the synthetic ones (Oliveira et al. 2008).

Reactive oxygen species (ROS) such as hydroxyl radical $\cdot\text{OH}$, peroxy radical $\text{ROO}\cdot$ and superoxide anion radical ($\text{O}_2^{\cdot-}$) are generated in vivo by normal aerobic metabolism and exogenous sources such as environmental pollution, UV light, diet, and smoking cigarettes. These ROS may cause oxidative stress, damage to DNA, protein, lipid and other molecules. In addition, they have an important role in various physiological processes such as aging, cardiovascular disease, cancer, and toxicity of many compounds (Gliszczynska-Swiglo 2006).

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl galate (PG) and tertiary butyl hydroquinone (TBHQ) are the synthetic antioxidants which are most widely used in food to prevent oxidation (Pizzale et al. 2002). However, most of them are heat sensitive, volatile and unstable. Consequently there has been growing interest in using natural antioxidants instead of synthetic ones (Ruiz-Navajas et al. 2011). Furthermore, many clinical and epidemiological studies have shown that the consumption of fruit and vegetables reduces the risk of chronic diseases such as cardiovascular disease,

and diabetes, as most fruits, vegetables and plants contain phenolic compounds that have important biological activities such as scavenging free radicals, quenching lipid peroxidation and preventing oxidative damage to DNA. Moreover the public's perception is that natural antioxidants are better and safer than synthetic ones (Viuda-Martos et al. 2010).

During the past two decades, green tea and spices have been the focus of research looking for a new source of potent antioxidants. They have protective effect on many free radicals including DPPH, ABTS, and ABAP radical preventing cell death in keratinocytes and fibroblasts (Ling et al. 2009).

Phenolic and polyphenolic compounds are found in all foods: although at low level in most of them. Studies have proved that high levels of phenolics are present in coffee, chocolate, tea, spice, fruit and some oils. Phenolic compounds are used as natural antioxidants as well as having antimicrobial activity (Oliveira et al. 2008).

Lipid peroxidation is one of the main reasons for quality of food loss during storage. This process is connected with the formation of free radicals that lead to the generation of aldehydes which are responsible for the development of rancidity and colour changes. Thus, it leads to loss of colour, protein solubility and decrease of nutritional value (Fasseas et al. 2008).

Natural antioxidants such as Lycopene has been shown to have a strong antioxidant capacity, and can be found in various plants such as tomatoes and red peppers. In addition, tea catechins can prevent lipid peroxidation (Tsong 2006). Furthermore, spices have been shown to possess medicinal properties and have been used effectively in the indigenous medicine of India and Turkey (Chatterjee et al. 2005).

(Halliwell and Gutteridge 1999) reported that the main reason for food deterioration is lipid peroxidation occurring between unsaturated lipids and oxygen molecules causing lipid deterioration. This is the main reason why the food industry aim to reduce autoxidation by freezing, vacuum packaging, refrigeration, and packaging under inert gas in absence of oxygen. However, none of these ways is either practical or economic. Thus, antioxidants are used to extend the shelf life of food, protect nutritional values and reduce waste (Daker et al. 2008).

The population of the south of France, despite their high-fat diet and smoking habits, have a low frequency of coronary heart disease. One of the reasons suggested for this is the high consumption of red wine as many studies have shown; red wine prevents oxidation of low-density lipoproteins (LDLs) in vivo. In addition, it has been shown high intakes of monounsaturated fats (found in red meat, nut, olive oil and milk products) can significantly reduced plasma cholesterol and have also been demonstrated to decrease oxidation of low-density lipoprotein (Rice-Evans 1995).

Research aims and objectives

This project aimed to assess the antioxidant activity of natural compounds (green tea, and spices) and the effect of matrix (full fat milk, semi-skimmed milk, and skimmed milk) on this activity. The objectives were as follows:

1. to assess the antioxidant capacity of brewed green tea at room temperature over a period of time.
2. to validation the FRAP assay at 25 °C rather than the commonly used 37 °C.
3. to modify the crocin bleaching assay to work at 25 °C and validate the calculation.
4. to measure the effect of addition of three types of milk (full fat, semi skimmed, skimmed milk) on the antioxidant capacity of green tea.
5. to measure the antioxidant capacity of saliva before and after ingestion of green tea, and the effect of the adding milk (full fat and skimmed milk) on salivary antioxidant capacity.
6. to evaluate the efficacy of spice extracts for preventing oxidation of oil (thus extending the storage shelf life).
7. to measure the antioxidant lipid activity of different concentrations of spice extracts (fennel, clove, cardamom, cinnamon, ginger, anise, and black pepper) using the Rancimat method.
8. to determine the phenolic content and radical scavenging capacity of spice extracts at different concentrations.

Literature review

A brief history of free radicals

In 1789, Lavoisier introduced the term “radical” to designate a group of elements that combined with oxygen in acids. The term has continued to be used by chemists to signify a group of atoms that remain unaltered during a series of reactions (Ihde 1966).

Determination of molecular weight by the vapour density method in 1860 led to the determination of chemical structures believed to be radicals. In 1900, the radical Triphenylmethyl was the first organic radical identified by Moses Gomberg (1866-1947) who reported his results on the reaction of Triphenylmethyl halides with metals (Gomberg 1900).

Chemists at the British Rubber producer Research Association in the 1940s reported on the effect of free radicals on the rancidity of oils and fats. An increase of interest in free radicals in biological context began with the discover of superoxide dismutase (SOD) in 1958 (Bateman 1954). Nowadays the term radicals are used in place of free radicals. Today thousands of biological processes and industrial are understood to be dependent on reactions involving free radicals.

Free Radicals

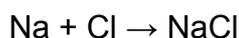
Since Moses Gomberg discovered the first organic free radical, research has showed that free radicals are present in our bodies, in the environment and in chemical reactions. The past two decades have seen an explosion in interest in free radicals as important factors in both biology and chemistry. Molecules, atoms or ions, which have unpaired electrons in their outer shell, are called free radicals. They are unstable and may have negative, positive or zero charges.

First, it is necessary to clarify the concepts of antioxidant, pro-oxidant, oxidant and reductant.

Antioxidant Concepts

Reductant and oxidant

Reduction is a chemical reaction defined as a gain of electrons. Oxidation is a chemical reaction defined as a loss of electrons. A reducing agent or reductant is a substance that donates electrons and an oxidizing agent or oxidant is a substance that accepts electrons. Oxidation and reduction never happen alone. Reduction and oxidation reactions are called redox reactions (Prior and Cao 1999).



So: Na is reductant

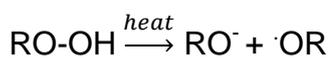
Cl is oxidant

Figure 1: An example a Redox reaction

Formation and destruction of free radicals

Initiation

A covalent bond is a chemical bond, which is made by sharing two electrons between two atoms. Some molecules, which contain a weak covalent bond can break to give two atoms each containing unpaired electrons in their outer shells. This is called homolysis also known as homolytic bond cleavage.

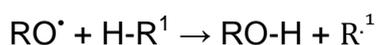


(A peroxide)

The initiation reaction is the formation of radical from non-radical species. The energy source could be heat, light, UV or x-rays.

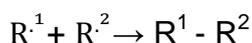
Propagation

To form stable radical or non-radical products, the radical which has been formed in the initiation stage, can undergo addition or abstraction reactions. They make strong bonds in products by breaking down weak bonds in the reactants.



Termination

In propagation reactions, there is one radical reactant and one radical product whereas in termination there are two radical reactants and no new radical product (if the combination of radicals produce non-radical products).



Free radicals in nature

Enzymes in the body can convert oxygen to hydroxyl radicals, which are highly reactive with proteins, DNA and fats, causing cell damage; as well as too much exposure environmental pollution, ultraviolet light, cigarette smoke, excessive intakes of iron and food additive can produce harmful radical in the body (Kaur et al. 2008).

Biological implications

Oxygen is essential to aerobic life but also imposes toxicity. Reduction of O_2 from many sources can produce the superoxide anion free radical ($O_2^{\cdot-}$) which is primary source of radicals and a damaging intermediate. This damage to cells can contribute to numerous degenerative disease including coronary heart, inflammatory disease, cancer, diabetes mellitus and reperfusion injury (Daker et al. 2008).

Table 1: free radicals and their names (Gliszczynska-Swiglo 2006)

| Radical | Name |
|----------------|------------------------------|
| $\cdot OH$ | Hydroxyl radical |
| $RO\cdot$ | Lipid alkoxy radical |
| $ROO\cdot$ | Lipid peroxy radical |
| $R\cdot$ | Lipid carbon central radical |
| $O_2^{\cdot-}$ | Super oxide radical |
| 1O_2 | Singlet oxygen |

Source of free radicals

Free radicals are characterised having one or more unpaired electrons and unstable. They are seeking for electron to complete their outer shell to become stable. Sources of free radicals are divided into two types: endogenous and exogenous sources Endogenous free radicals are produced as by products of the mitochondrial electron transport chain during normal aerobic metabolism.

Exogenous sources are generated through smoking, UV and food additives (Halliwell 1996).

Table 2: Sources of free radicals

| Endogenous | Exogenous |
|----------------------------------|--------------------------------|
| Mitochondrial electron transport | Drug metabolism |
| Transition metal ion | Radiation (X-ray and UV light) |
| Inflammation | Cigarette smoke |
| Enzymes such as xanthine oxidase | Food additives and iron intake |

Antioxidants

An antioxidant is a molecule that can inhibit the oxidation of other molecules by donating or accepting an electron to the free radical. Therefore, antioxidants are reducing agents, but not all reducing agents are antioxidants. The human body has number endogenous sources of antioxidants which have an important roles to play in preventing damage and quenching free radicals. Common dietary antioxidants that are beneficial in biological systems in vitro are vitamin C, vitamin E, polyphenolics and phenolics compounds. In addition, flavonoids, quercetin, myricetin and gallic acid have been shown to be as a common antioxidants due to their hydroxyl groups. In general, the greater the antioxidant activity is, the more the number of hydroxyl groups which are found in the structure of the compound.

Antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays. The single electron based assays involve one redox reaction with oxidant indicator of reaction endpoint however most hydrogen atom transfer based assays monitor competitive reaction kinetics and quantify hydrogen atom donating capacity whereas ET-based assay measure antioxidant's reaction capacity (Prior and Cao 1999). Table 3 shows the methods that either based HAT or ET based reaction assay.

Table 3: Antioxidant assays based on reaction mechanisms

| Assay involving HAT-based reaction | Assay ET-based reaction |
|---|---|
| ORAC (oxygen radical absorbance capacity) | TEAC (Trolox equivalent antioxidant capacity) |
| TRAP (total radical trapping antioxidant potential) | FRAP (ferric reducing antioxidant potential) |
| Crocin bleaching assay | DPPH (diphenyl-1-picryl hydrazyl) |
| IOU (inhibited oxygen uptake) | Total phenol content |
| Inhibition of linoleic acid oxidation | Copper (II) reduction capacity |

There are two types of antioxidant defence mechanisms in having organisms are enzymatic and non-enzymatic systems and compounds defence systems (Prior and Cao 1999).

Table 4: Functions of enzymatic and non-enzymatic antioxidants (Katalinic et al. 2005) and (Prior and Cao 1999).

| Enzymatic | non-enzymatic |
|--|--|
| SOD: removes O_2^- | Vitamin C: free radical scavenger recycles vitamin E |
| GSH: removes H_2O_2 and organic hydroperoxides | Vitamin E major chain-breaking antioxidant in cell membrane |
| CAT: removes H_2O_2 | Glutathione multiple roles in cellular antioxidant defence |
| | uric acid scavenger of OH radicals |
| | α -lipoic acid recycles vitamin C; effective glutathione substitute |
| | Carotenoids scavenger of ROS; singlet oxygen quencher |

In general, antioxidants can be divided into two groups: the first prevent the initiation of the chain reaction and the other slow down or delay propagation (Tubaro et al. 1998). However oxidation is essential to most living organism for producing energy for biological process but ROS are produced during normal metabolism may cause various human disease (Tubaro et al. 1998) and (Halliwell and Aruoma 1991).

Antioxidant and pro-oxidant

A pro-oxidant is a substance that can cause oxidative damage of nucleic acids, proteins and lipids, which may resulting in disease. A pro-oxidant can be

inhibited by presence of an antioxidant. Pro-oxidant is a synonym for reactive species. Antioxidants can reduce pro-oxidants with the formed products having low or no toxicity (Prior and Cao 1999).

Antioxidants are small molecules, while others are proteins or enzymes. Antioxidants are classified from the viewpoint of their mechanisms into preventing antioxidants, repair and de novo antioxidants and scavenging antioxidants (Niki 2010). Antioxidant capacity is defined as the ability of a compound to reduce pro-oxidant reactive species of pathologic significance. Recently, antioxidants have become a topic of increasing interest because dietary antioxidants including vitamin E, polyphenolic compounds, vitamin C, and carotenoids, are believed to be effective nutrients in the prevention of oxidative stress leading to many diseases such as inflammation, cardiovascular disease, cancer, and aging-related disorders (Huang et al. 2005). Deficiency or an absence of antioxidant defences may cause a situation of increased oxidative stress and this might be associated to a variety of disorders, including cancer and coronary heart disease. Because of normal aerobic metabolism, harmful reactive oxygen species (ROS) are produced. These free radicals are removed in vivo by antioxidants (Benzie and Strain 1996).

Antioxidant defences include enzymes that catalyse reactions in which reactive species are removed, (e.g. catalase); proteins that limit the availability of pro-oxidants such as iron and copper ions, (e.g. ferritin); and the expression of stress proteins (often referred to as heat-shock proteins). However, we are concerned with the low-molecular-mass compounds antioxidants that eliminate ROS/RNS (Reactive nitrogen species). These may be synthesised in vivo, e.g.

melanin, or derived from the diet, e.g. vitamin C (ascorbic acid), plant phenols such as the catechins, and carotenoids such as β -carotene.

Antioxidants and food

Fruit and vegetables contain many biological active compounds with physiological and biochemical functions that benefit human health and can significantly delay or prevent many diseases. Most of them characterized by low caloric value and high level of antioxidants (Floegel et al. 2011).

In kiwi fruit, the level of vitamin C is higher than that of lemons, oranges, strawberries, and grapefruit. Phenolic compounds are different in chemical structure and characteristically present in foods such as fruits and vegetables, wine, tea, and spices. Some research papers have reported that phenol compounds have effects in vivo and in vitro including metal-chelating and antioxidant activity (Samman et al. 2001) and (Tavarini et al. 2008).

Oliveira et al. (2008) determined phenolic compounds in green walnut husks and identified thirteen phenolic compounds: gallic acid, juglone, myricetin, chlorogenic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, ferulic acid, sinapic acid, chlorogenic acid and caffeic acid.

Bee products such as honey, royal jelly and propolis might also be used as natural antioxidants. Honey can be considered as a good source of natural phenolic and flavonoids compounds. Polyphenol composition is indicated by the colour of honey: higher phenolic and flavonoid concentrations are found in a dark coloured honey. The type and concentration of phenolic substances in honey depend on the flower source of the nectar, environmental factors (season, climate, soil type) genetic factors and processing methods. More generally, the

antioxidant activity of honey depends on the temperature, type of substrate, concentration, light and physical state of the system (Ruiz-Navajas et al. 2011). The advantages of using natural antioxidants is that they are more acceptable to the public who are concerned about the addition of synthetic antioxidants which are more expensive, heat sensitive and less effective when compared with natural ones (Viuda-Martos et al. 2010).

Polyphenols, or polyphenolic compounds which are synthesized by plants, are polyhydroxylated phytochemicals that can be divided into two classes flavonoids and phenolic acids. Flavonoids may be divided into several types flavanols which are also called catechins, flavanones, flavonols and anthocyanidins. Hence, polyphenols and flavonoids act as antioxidants in blood and tissues in the body that is due to their capacity to donate hydrogen atoms or electrons. Antioxidant flavonoids and polyphenols in human diet (vegetables, tea, fruit, and wine) are much higher than the amount of antioxidants of these foods such as vitamin E, vitamin C, and carotenoids. Recent studies have shown that ascorbate may be less effective than catechins in terms of regenerating α -tocopherol in micellar solution. Despite the strong antioxidant activity of flavonoids in vitro their capacity in vivo is limited. The main reasons being low absorption of flavonoids in humans in contrast to vitamin C and E, and the short half-life flavonoids in human plasma (Lotito and Frei 2006).

Oxidative stress

Oxidative stress is the imbalance between reactive oxygen species ROS and the biological system's ability to detoxify the reactive intermediates. Oxidative stress has been associated with the development of many diseases such as diabetes, heart disease, eye disease, sclerosis and is thought to cause the

pathogenesis of a number of human diseases and neuronal degeneration such as Parkinson's and Alzheimer's diseases. There is a continuous production of ROS by normal metabolism in the normal healthy person. ROS are needed to maintain biological homeostasis but on the other hand, overproduction of ROS can cause damage to cell structure and function (Butterfield et al. 2010) and (Wiernsperger 2003).

Much research has indicated that fruit and vegetables reduce the risk of chronic diseases. Antioxidants present in vegetables and fruit such as vitamin C, and E, polyphenols and carotenoids, prevent harmful ROS in the body during normal cellular function. However, it has been suggested that carotenoids might not act as antioxidant in vivo (Yeum et al. 2010).

There is a continuous production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) even in a healthy person. An important source of ROS is the mitochondrial respiratory chain (electron transfer chain), where a small fraction of respired oxygen is reduced to superoxide instead of water. Superoxide can be converted to hydrogen peroxide (H_2O_2) in a reaction catalysed by the enzyme superoxide dismutase. Other important ROS are the hydroxyl radical (OH^\cdot) and singlet oxygen (1O_2). ROS and RNS are capable of oxidising and hence damaging, just about anything in the body such as DNA, proteins and lipids. The human body has a range of antioxidant defence systems, whose function is to limit this damage by eliminating ROS and RNS. Despite the presence of 'repair' systems, and damage accumulates with age, for example the number of mitochondrial DNA variants in individual cells increases with age, and many of these have substantial deletions (Ozawa 1998).

Interaction between antioxidants and food matrix

The oxidative deterioration of proteins has been considerably studied in biological systems, but there are gaps in the knowledge of antioxidants interaction with meat proteins (Estévez and Cava 2004). The same group also reported contradictory effects when the antioxidants were used in a food product matrix that already contained natural antioxidants, such as alpha-tocopherols (Estévez et al. 2007). This may be due to the interaction between different antioxidant compounds. The activity of plant phenolics could be reduced due to their oxidation, and subsequent prooxidant activity of the oxidation products that could act promoting oxidative reactions as it may be the case in systems with high oxidative instability (Huang and Frankel 1997). Other compounds in the matrix may play a role in oxidation reactions, as do fatty acid profiles. These reaction effects have been reported to be concentration-dependent. Studies on the effect of antioxidant activity of tomato extracts showed no effect of lycopene and β -carotene concentrations, but the effect appeared when these interacted with small amounts of tomato-occurring compounds. Heat processing (e.g. frying) could also reduce the levels of natural and added antioxidants in fats, and this seems to be dependent on the saturation level and heating regimes, as studied by Barrera-Arellano et al. (2002) for tocopherols and by Gomez-Alonso et al. (2003) for phenolic compounds. The matrix may then play a relevant role on the antioxidant-prooxidant activity, as it may influence interactions of compounds and possibly interfere with reaction kinetics.

These sorts of reactions could have negative effects not only in foods, but also in the human body as shown by Samman et al. (2001) where volunteers

consumed phenol rich extracts from rosemary and tea added to the meat component of their meals; they showed that these extracts decreased nonhaeme-iron absorption, and thus impaired the dietary utilization of this nutrient. The measurement of the types of compounds and their concentrations is common to establish the potential functionality and mechanisms of plant extracts, for example total phenolic contents, quantification of products of deterioration or fat oxidation, or even colour as an indicator of oxidation of food components, particularly haem proteins. There are additional methods for determining antioxidant activity which are commonly used, but their principles are not always well understood (Prior and Cao 1999).

Methods to measure antioxidant capacity

Different assays have been introduced to assess antioxidant activity of biological samples and foods. In past two decades many spectrophotometric assays have appeared for measuring food antioxidant such as DPPH, ABTS, ORAC, and FRAP. They all work on almost the same principle: redox active compounds are generated, and the ability of the sample to scavenge radicals, and synthetic colour radicals, can be monitored by spectrophotometry. FRAP, DPPH, and ABTS assays are based on transferring electrons and involve the reduction of oxidant (Floegel et al. 2011).

Numerous methods have been developed to measure antioxidant capacity. They are based on chemical reaction or use of a biological response. The most common methods used to assess antioxidant capacity of food and beverages are Trolox equivalent activity (TEAC), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ABTS, chemiluminescence, crocin bleaching assay, Electron spin

resonance (ESR) and Ferric reducing antioxidant potential (FRAP) which are commonly used for measuring the antioxidant capacity of plasma.

TEAC-Trolox equivalent antioxidant capacity:

TEAC is decolourization assay based on the ability of compound to scavenge blue/green ABTS radical cation, which is formed after adding potassium persulfate (ABTS^{•+} reduced by antioxidant). This means the TEAC assay is an inhibition assay after generation of the free radical sample is then added and the inhibition of free radical action measured, and this inhibition is referred to as the antioxidant activity of the sample (Katalinic et al. 2005). The reaction between ABTS^{•+} and antioxidant samples can be determined spectrophotometrically at 734 nm and compound with Trolox (water soluble analogue vitamin E) (Arts et al. 2003).

DPPH quenching assay

Goldschmidt and Renn discovered DPPH in 1922. It is now used as a colorimetric reagent for redox process and ERS standard because it does not react with oxygen (Ionita 2005). The DPPH measurement is the ability of antioxidant samples to donate hydrogen or electrons to the DPPH (1,1-diphenyl-2-picryl-hydrazyl) resulting in bleaching of DPPH. The higher the antioxidant activity is, the greater the bleaching of the purple DPPH.

This method has been used to determine the antioxidant activity of wines, spices (oregano, thyme, sage, rosemary and cloves) crocin (extracted from saffron) and also human plasma. DPPH has a deep violet colour and becomes pale, yellow or colourless when neutralized in solution. DPPH is insoluble in water but can be dissolved in ethanol and methanol and has an absorption peak at 510-520 nm. Decrease in absorbance indicates a high antioxidant activity of the samples (Viuda-Martos et al. 2010) and (Fasseas et al. 2008).

FRAP (Ferric reducing antioxidant power) assay

In the FRAP assay there are no free radicals involved but it depends on the reduction of ferric iron (Fe^{3+}) to ferrous (Fe^{2+}) at low pH, causing coloured ferrous-tripyridyltriazine which can be monitored at the 595 nm wave length. Then by the presence of antioxidants it shown the blue colour (Floegel et al. 2011). Trolox used as a standard in this assay ferric salt $\text{Fe(III)(TPTZ)}_2\text{Cl}_3$ (TPTZ = 2,4,6-tripyridyls-triazine) is used as an oxidant at low pH (Benzie and Strain 1996). In another words, the FRAP assay is the ability of compounds to reduce Fe^{3+} to Fe^{2+} which develops a blue colour (i.e. antioxidant donating electron) to measure FRAP value FRAP reagent is warmed up to 37°C and a reagent blank is read at 593 nm (Katalinic et al. 2005). One FRAP unit is defined as the reduction of Fe^{+3} to Fe^{+2} used to determine antioxidant capacity of plasma and serum.

Katalinic et al. (2005) used the FRAP assay to compared female and male antioxidant capacity of heart, kidney, liver and brain tissues in rats. The FRAP assay does not involve prooxidant and oxidizable substrate: the FRAP assay is a reductant in chemical terms but in biological terms is an antioxidant (Prior and Cao 1999).

Crocin bleaching assay (CBA)

The crocin bleaching assay is a method used for measuring the total antioxidant capacity of plant extracts, biological fluids and pure natural compounds. Crocin is responsible for the red colour of saffron and gardenia and it is a water-soluble carotenoid and widely used for biological analysis of clinical samples (Fasseas et al. 2008). Gardenia and saffron are known as herbal medicines. The yellow pigment have been used for culinary purposes in order to colour candy rice, jelly, juice and noodles in China, Japan and India because it is a water-soluble.

Phytochemical studies have been show that the chemical responsible for saffron and gardenia is crocin (Chen et al. 2008). Chemical studies have shown crocin to be responsible for numerous pharmacological effects such as reduced risk of cardiovascular diseases and protection against of tumour cell proliferation and hypatocytes. The crocin bleaching assay is a competition kinetic procedure for measuring antioxidant capacity. The crocin assay is based on bleaching due to the compound reacting with the peroxy radical carotenoid of crocin (oxidation of crocin solution) by (ABAP) free radicals and could inhibit of the oxidation by antioxidants. Oxidizable and reducing radicals can bleach crocin but crocin is not bleached by superoxide (O_2^-) and methyl radicals because of the advantage of microplate-based technology make the crocin assay a precise and rapid method for a number of samples at the same time thus it is particularly important for clinical tests (Chatterjee et al. 2005).

This assay is based on the competition between the bleaching crocin by ABAP, which is a peroxy radical generator during thermal decomposition of ABAP and antioxidants inhibition of bleaching (oxidation) by trapping the radicals formed. Crocin absorbs a short wavelength 440-450 nm. Some food pigments such as carotenoids absorb light of the same range as the crocin. Hence the method can also be made for their allowing applicant to both hydrophilic and lipophilic antioxidants (Di Majo et al. 2008).

Total phenol content

Phenols are characterised by having an aromatic ring ($-C_6H_5$) bonded to one hydroxyl ($-OH$) group. Phenolic compound acts as antioxidants. Folin-Ciocalteu's reagent is also called the Gallic Acid Equivalence method (GAE). It is used to indicate the presence or absence of phenolic groups. Initially Folin-Ciocalteu's reagent was intended for the analysis of proteins which contain a

phenol group (tryosin residue). Actually, the Folin-Ciocalteu's reagent measures a sample's reducing capacity. Many researchers have found excellent linear correlations between the antioxidant activity and total phenols. Clearly, the FCR is not special to phenolic compounds it can be reduced by nonphenolics such as vitamin C, Cu(I), etc.

Polyphenols or polyphenolic compounds are polyhydroxylated phytochemicals that can be divided into two classes: flavonoids and phenolic acids. Flavonoids may be divided into several types: flavanols which are also called catechins, flavanones, flavonols and anthocyanidins (Lotito and Frei 2006). Phenolic classes such as flavonoids, phenolic acid, simple phenols, and anthocyanins have radical scavenging properties and are considered as food antioxidants. It is also reported that they have anti-inflammatory, anti-fungal, and antibacterial properties.

Folin-Ciocalteu's reagent reacts with phenolic compounds only in the presence of sodium carbonate (i.e. phenolic is able to reduce Folin-Ciocalteu's reagent that leads to formation of blue compounds between phenolate and Folin-Ciocalteu's reagent). The development of the blue colour is due to transfer of electrons. However, the colour development is very slow but can be accelerated by heating. The light absorption at the blue wavelength is proportional with the concentration of phenol of the compound. Phenolic compounds in the plant kingdom have become an object of interest among natural antioxidants because of the widespread occurrence of phenolic compounds (Zovko Končić et al. 2010).

Folin-Ciocalteu's method has been applied to many foods such as tea, vegetables, spices, fruit and wine (Ferreira et al. 2004) and (Hossain et al. 2011). Tsai et al. (2008) found that the green tea has the highest total phenol

content among twelve dried herbs (Juhua, honeysuckle, lavender, rose, osmanthus, duzhong, lemongrass, mat and rosemary).

Rancimat method

Lipid peroxidation is a main cause of deterioration of food flavour and the development of unpleasant taste. The food industry seeks to inhibit food oxidation by using food additives. Heat process can accelerate oxidation which can evaluate stability index by Rancimat equipment.

All fats and oil can oxidize and the rate of oxidation depends on storage conditions, the presence of antioxidants and the degree of unsaturation. The rancimat method is also known as the oil stability index (OSI) and is a rapid method to determine oxidative stability of fat and oil. Oxidation processes in fats and oils are very slow. The advantage of the rancimat test is that it obtains the result in few hours instead waiting of few weeks or months (Velasco, Andersen & Skibsted, 2004).

Carboxylic acid is generated from oil or fat samples when heated to 100-120 °C under air flow at 20-15 l/h and these, when trapped in water, can monitored by electron-conductivity. The change in conductivity, which indicates the formation of volatile acids, means that the oxidation has occurred. Differential scanning calorimetry (DSC) has also been used to determine oxidation of oil and fats (Velasco et al., 2009).

Lipid oxidation

Oxidation is one of the fundamental reactions in lipid autoxidation. The compounds formed by coincidence cause rancidity, generate unpleasant

flavours and reduce nutrition value and hence may make the food product be rejected by the consumer (Sun et al. 2001).

The process of lipid peroxidation is very slow at room temperature but can be accelerated by several physical and chemical parameters such as increasing temperature, rise in oxygen level partial pressure and metal catalysts (Viuda-Martos et al. 2010).

TBARS

TBARS is the common method for determining plasma lipid peroxidation that occurs with free radical generation MDA is generated when free radicals damage lipids then MDA can react with TBA under high temperature and low pH to form a complex (MDA-TBA complex) and can be measured with fluorometry or spectrophotometry (Sun et al. 2001). In other words if free radicals steal electrons from lipid in cell membranes this process causes cell damage.

Unsaturated fatty acids are more susceptible to lipid peroxidation than the saturated ones. The rate of TBARS inhibition of unsaturated fatty acids is less than saturated fatty acids (Rael et al. 2004).

Green tea

Tea is the most common beverage consumed in the world next to water. Tea can be divided into three types according to the level of fermentation: green tea (unfermented), black tea (fermented) and oolong tea (partially fermented) (Sharma et al. 2008). Among them green tea has the highest catechins (8.0-14.4 g /100 g dry tea leaves) followed by oolong tea (4.14-4.92 g /100 g dry tea leaves) and black tea has the lowest catechins (0.24-0.51 g /100 g dry tea leaves) (Chen et al. 2000) and (Toschi et al. 2000). The chemical content of green tea varies according to climate, position of the leaf on the harvest, and

season. It mainly consists of carbohydrate, proteins, vitamins (C, E, B) and xanthine bases such as theophylline and caffeine. It also, contains gallic acid and phenolic acids such as flavonols, caffeic acid, and chlorogenic acid (Peters et al. 2010).

Many epidemiological studies have indicated that tea consumption could prevent chronic diseases including cardiovascular disorders, cancer, diabetes, and obesity. Catechins are known to be a physiological active agent in tea exhibiting biological activity in humans and animals such as modulation of plasma lipid, antioxidant capacity and insulin sensitivity (Peters et al. 2010). The major polyphenols in green tea are: (-)-epigallocatechin-3-gallate (EGCG), (+)-gallocatechin (GC), (-)-epicatechin 3-gallate, (+)-catechin, (-)-epicatechin (Gupta et al. 2002) and (Sharma et al. 2008). These have shown a strong antioxidant capacity in vitro and in vivo (Zaveri 2006).

Many studies have demonstrated the biological effects of tea in vivo and in vitro and have been approved that tea is a main source of dietary polyphenols and a major source of flavones. Catechin gallate esters and the catechins polyphenols in plant can act as hydrogen donating antioxidants, metal chelators, reducing agents and single oxygen quenchers.

It has been suggested that green tea has superior health effect than the black tea because of higher antioxidant activity and higher content of (-)-epigallocatechin gallate. In addition, green tea leaves after harvest are immediately heated with rolling to prevent the oxidation catalysed by the enzyme, polyphenol oxidase, which is able to oxidize the catechins, thus giving green tea the highest catechins. Black tea, when fermented, gives the enzyme

more time to act so that most of the catechins are converted into thearubigins and theaflavins which give back tea colour.

Generally green tea contains 30% w/w of catechins but black tea contains 3-10% w/w. Recently in Taiwan it has become popular to make the tea using cold water. However, Lin et al. (2008) found that cold water extracts were less effective in antioxidant capacity than hot water extracted green tea.

Health benefit of green tea

Many researchers have demonstrated that drinking green tea reduces the risk of developing many diseases such as diabetes because green tea increases insulin activity and also have proved consumption of green tea and oolong tea significantly reduces risk of developing hypertension. In addition, science shows green tea can control obesity (Zaveri 2006) and (Cheng 2006).

Numerous clinical studies have shown that tea possess different pharmacological properties including anti-aging, anti-bacterial, anti-viral, anti-arteriosclerotic, anti-bacterial, and anti-cancer effects. Furthermore it has been shown that green tea consumption increase the acid resistance of teeth, which can damage cariogenic bacteria (Koo and Cho 2004).

Experimental studies have shown an inverse relationship between consumption of green tea and risk of stomach cancer as well as colorectal cancer. Green tea also provides protection against Parkinson's and Alzheimer's disease and can reduce cholesterol level in serum in vivo and in vitro.

The daily amount of consumption of (0.6-1.5)l/day of green tea will increase plasma antioxidant capacity, protect DNA from damage, and reduce lipid peroxidation in healthy subjects (Ellinger et al. 2011).

Chapter 2

Rational, general materials and methods

Experimental rationale

Over the last decade, dietary polyphenolic substances have been implicated in many nutritional and health effects. Numerous reports have been published describing many effects in animal, clinical, and in vitro studies. Generally, antioxidants include molecules that are naturally present in most herbs, vegetable, and fruits. These compounds have a strong ability to prevent or delay the oxidation process by inhibiting the propagation of lipid peroxidation or formation of free radicals. Individual methods are not satisfactory because no one method can indicate with measurements the complexity of the system. A battery of assays is normally necessary, but comparison between methods can then be difficult. Therefore, there is a need to develop methods and approaches which would contribute to the interpretation of antioxidant capacity of natural extracts. There is also a need to understand the role of interactions between antioxidant compounds and the matrix on the mechanisms of oxidation and peroxidation in biological systems. The approach was then to select spices and apply a battery of tests to compare their antioxidant capacity. Method development included a validation for a crocin assay at a different temperature, and an innovative treatment of the data in some of the colour decay assays. The effect of the matrix was studied in a simple system where differing fat levels of milk in tea were used. An additional aim of this research was to explore an easy and inexpensive alternatives to measure total antioxidant capacity on body fluids using saliva to include matrix and interaction effects using several

antioxidants. Finally potential interactions and with each other in vitro, and their potential benefit in real life were investigated.

Chemicals

ABTS (2,2'-azobis-(3-ethylbenzthioline-6-sulfonic acid)diammonium salt), potassium persulfate, Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ascorbic acid, DPPH (1,1-Diphenyl-2-picrylhydrazyl), acetic acid, sodium hydroxide, ironchloride (III), TPTZ, gallic acid, Folin-Ciocalteu's reagent, sodium carbonate, ethanol, and saffron were purchased from Sigma (Poole, UK). ABAP (2,2'-azobis(2-methylpropionamide) dihydrochloride) from Cayman chemical, spices from Kalsec, potassium dihydrogen orthophosphate, FeSO₄.7H₂O was purchased at BDH (British Drug House, UK). Green tea (Qi green tea) was obtained from local shop (Rickard Lane, Plymouth, UK). The Salivette kits were purchased from Sarstedt, UK. Milk (So organic milk) was purchased from local supermarket (Sainsbury's, Plymouth, UK).

Preparation of chemicals

Preparation of FRAP reagent

The FRAP reagent was prepared by adding 3.4 ml of acetic acid to 100 ml of water then adjusting the pH to 3.6 by adding 2 mM of NaOH. The solution was then made up to 250 ml with water, then 0.081 g of FeCl₃ add to the solution, followed by 0.039 g of TPTZ after dissolving in dilute acid. The FRAP reagent was stored up to two weeks at 4°C.

Preparation of crocin extract

Crocine extract was prepared according to Chatterjee et al. (2005). 40µM saffron was dissolved in 50 ml of 50 mM phosphate buffer, and the concentration was

checked by measuring the absorbance of a two fold dilution of the solution at 440 nm two fold dilution based on extinction coefficient of $89 \text{ mM}^{-1} \text{ cm}^{-1}$ by adding more crocin until obtained absorbance was then samples kept at $-20 \text{ }^{\circ}\text{C}$.

Preparation of ABTS radical cation

ABTS was dissolved in water to give a 7 mM solution. $\text{ABTS}^{+\cdot}$ was produced by adding of potassium persulfate to a final concentration of 2.45 mM to the ABTS solution. The mixture was allowed to stand in the dark at room temperature for (12-16h) before used. Under these conditions the radical is stable for several days. The $\text{ABTS}^{+\cdot}$ radical cation was diluted with water to give an absorbance of 0.6-0.7 at 734 nm.

Preparation of 'dummy' Folin-Ciocalteu's reagent

Five ml of 85% phosphoric acid was added to 10 ml concentrated hydrochloric acid. Then 15 g of lithium sulphate, 5 ml water and a drop of bromine were added. Then Reflux for 15 minutes cool to room temperature and completed to 100 ml with water.

Methods

Determination of total phenol content

The method of Viuda-Martos et al. (2010) was used to determine the total phenol content of green tea by using Folin-Ciocalteu's reagent. Briefly, a volume of 25 μl of sample was added to Eppendorf tubes followed by 625 μl of Folin-Ciocalteu's reagent, which was diluted 10 fold with water, and then 500 μl of 7.5% sodium carbonate was added. Tubes were mixed well, and then incubated at $50 \text{ }^{\circ}\text{C}$ for 5 min. The absorbance at 760 nm was then measured in a plate reader (VERSA max microplate reader) and compared to a calibration

curve prepared using (0-3) mM gallic acid. Each measurement was carried out in triplicate.

DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay

The assay was performed according to the method reported by Viuda-Martos et al. (2010). Briefly, 1 ml of 6×10^{-5} M of ethanolic solution of DPPH was added to 25 μ l sample. The mixture was mixed well, and allowed to stand in the dark at room temperature for 1 hour. The absorbance measurements were taken at 517 nm using a plate reader (VERSA max microplate reader), with ethanol as a blank. A control was measured without added antioxidant. Ascorbic acid (0-10) mM was used as a reference. Each measurement was performed in triplicate.

FRAP (The ferric reducing antioxidant potential) assay

The reducing ability of samples was determined using the FRAP assay which quantifies the ability of samples to reduce Fe^{3+} TPTZ to Fe^{2+} TPTZ, which produces a blue colour that be measured at 595 nm. The method was performed as described by Benzie and Strain (1996) with slight modifications. The sample and FRAP reagent were warmed to 25 °C and then 10 μ l of sample or the standard were added followed by 260 μ l FRAP reagent. A water blank was used. Absorbance at 595 nm was measured for 45 min in a plate reader (VERSA max microplate reader). The measurements were compared to a standard curve prepared using 0-2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The FRAP assay was compared at the two different temperatures 25 °C for 45 min and 37 °C for 15 min. The results show at 45 min at 25 °C essentially equivalent to 15 min at 37 °C as shown in Table 5 and Figure 3. That is the reason for doing the FRAP assay at 25 °C rather than 37 °C was much easier to do.

Table 5: FRAP value \pm SD of green tea at 25 °C and 37 °C

| Green tea (g/l) | FRAP value mM at 25 °C | FRAP value mM at 37 °C |
|-----------------|------------------------|------------------------|
| 0.1 | 0.11 \pm 0.01 | 0.14 \pm 0.03 |
| 0.2 | 0.23 \pm 0.01 | 0.21 \pm 0.01 |
| 0.4 | 0.41 \pm 0.01 | 0.35 \pm 0.01 |
| 0.6 | 0.58 \pm 0.02 | 0.55 \pm 0.02 |
| 0.8 | 0.77 \pm 0.03 | 0.66 \pm 0.01 |
| 1 | 0.92 \pm 0.02 | 0.81 \pm 0.04 |

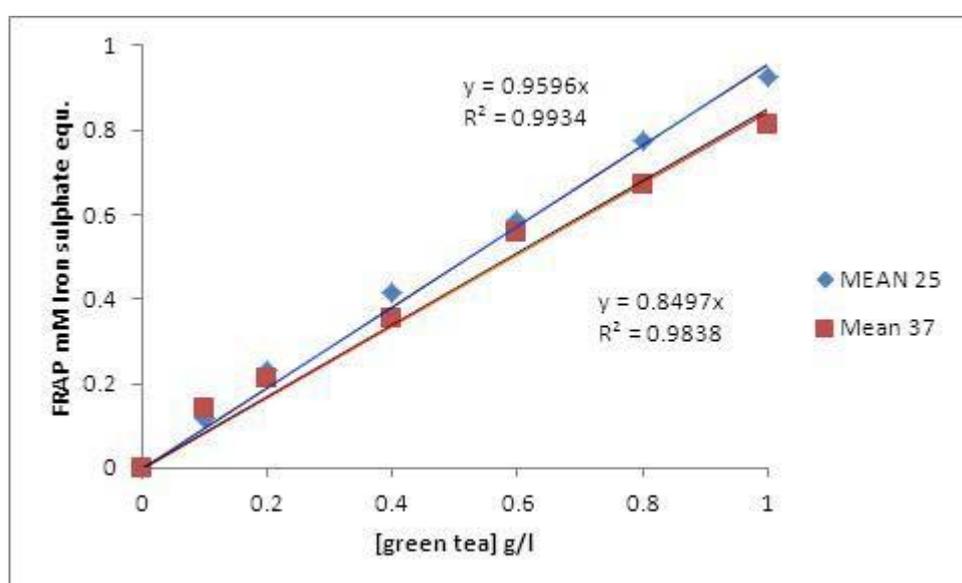


Figure 2: The relationship between antioxidant capacity and the concentration of green tea comparing 25 °C and 37 °C.

TEAC assay (Trolox equivalent antioxidant capacity)

Antioxidant capacity was measured according the method described by Re et al. (1999). The experiments were performed on a plate reader (VERSA max microplate reader) at 25 °C for 10 min. The mixture was allowed to stand in the dark room temperature for (12-16) hours before use. Under these conditions the

radical is stable for a few days. ABTS radical cation was diluted with water to an absorbance of 0.6 to 0.7 at 734 nm. Afterwards, 2000 μ l of ABTS radical cation was added to 20 μ l of the sample or the standard. Trolox (0-10) mM was used as a reference standard.

Crocin bleaching assay (CBA)

This assay was performed based on the method described by Chatterjee et al. (2005) based on the measurement of bleaching the carotenoid crocin with slight modifications. Ten mM of Trolox was dissolved in 50 mM phosphate buffer pH 7.5, Trolox is has poor solubility at this concentration and up to 20 min was required for it to dissolve properly. ABAP solution was freshly prepared using 0.1 g of ABAP which was dissolved in 2 ml of 50 mM phosphate buffer. The following were added to the wells of a 96 well plates, 100 μ l of crocin solution, 100 μ l of sample or standard and 50 μ l of ABAP. The absorbance was measured at 440 nm using a plate reader (VERSA max microplate reader) at 25 °C for 30 min. The blank was crocin, and buffer was used in place of an antioxidant.

Chapter 3

The effect of infusion time on antioxidant capacity of green tea

Introduction

Tea is the most common beverage in the world next to water, and contains high levels of polyphenolic compounds (Sharma et al. 2008). Black tea is most common in western countries, whereas large quantities of green tea are consumed in Asian countries. Recently, the consumption of green tea has increased throughout the world due to its perceived health benefits.

Green tea is an excellent source of methylxanthines, especially caffeine. The age of the leaf is an important factor because old leaves contain more EGCG and total catechins, but less caffeine than young ones (Lin et al. 2003). (Toschi et al. 2000) reported that the antioxidant activity of green tea is higher in those teas which contains a higher amounts of EGC and EGCG.

Some studies have investigated the effect of brewing time on green tea. Sharma et al. (2005) reported that temperature had an effects on catechin extraction especially EGCG, EGC, and EC. Furthermore, Labbé et al. (2006) studied the effect on different temperatures and brewing duration on the catechin solubilisation from green tea. They found that the concentration of catechin and caffeine depends on the temperature and duration of brewing, reporting that 500 µg/ml of catechin was extracted after 5 min of brewing at 50 °C, and 1000 and, 1342 µg/ml at 70, and 90 °C, respectively. Kim et al. (2007) reported 85 °C as the temperature for pasteurization and extraction of

canned ready_to_drink green tea gives lower catechin extraction. Pasteurization at 85 °C reduced EGCG by 2% and EGC by 0.85% if compared to 120 °C, which reduced EGCG by 40.2% and EGC by 16.7% thus by increasing temperature the amount of catechin decreased. Thus, the purpose of this study was to assess the stability of antioxidant capacity of brewed green tea over a period of time.

Experimental design

Green tea (Qi green tea) was kept under nitrogen after opening to prevent auto-oxidation. In this experiment, 0.4 g of green tea was added to 40 ml of hot water (just after boiling) at (75-80 °C), stirred for 3 minutes, sealed and left at room temperature for 24 hours. Before analysis samples were centrifuged (MSE Microcentaur, 13000 rpm) for 5 min, and stored at -20 °C. For treatment, each samples were prepared six times. In order to collect six replicates at each one of the sampling times (0-24) hours, and to allow for sample preparation time consistently, two groups of three samples were taken with a 2 min delay between groups. Total phenol content and antioxidant capacity were measured using five different assays (FRAP, TPC, CBA, TEAC, and DPPH). Each sample was analysed in triplicate.

Statistical analysis

The data were analysed by one-way ANOVA analysis followed by Tukey's test (95% confidence level). A paired Tukey's test was used to find the significant differences ($P < 0.05$) between each samples. The statistical analysis were made using Minitab (version 16).

Chemicals and Methods

These are the same as outlined in Chapter two.

Results and discussion

The TEAC assay described above provides a measure of the antioxidant activity of green tea which was determined by decolourization of ABTS^{•+} after 10 min from starting the reaction. The amount of antioxidant activity of the green tea was calculated using the Beer-Lambert law. Consequently, antioxidant capacity is the level of reduction of the ABTS^{•+} radical cation. To establish if the brewing time has an effect on the antioxidant capacity of green tea, an ANOVA test was performed considering the samples over a period of time. Time had a significant effect ($p < 0.05$) on the antioxidant capacity of the green tea. The mean values were found to increase from an initial value of 96 ± 13 mM to 149 ± 13 mM at 2 hours, and then decreased from 4 hours sample onwards. While samples at the first three times were significantly different by Tukey's test, samples at times 4, 6, 8, 22 and 24 hours had no significant difference between them. The results are presented in Table 6.

Time after brewing has a significant effect on the total phenolic content of green tea as confirmed by one-way ANOVA ($p < 0.05$). The mean value was found to increase from an initial value 5.43 mM gallic acid equivalent at time zero to a peak 6.32 mM gallic acid equivalent at 2 hours, but they were no significant differences after 2 hours. There is a direct correlation between antioxidant capacity and total phenolics because phenolics or polar compounds are associated with higher antioxidant activity. The reason phenolic compounds could be considered as antioxidants is that their presence at low concentrations compared to the oxidizable substrate can significantly delay oxidation or scavenging radicals by producing stable radicals after donating an electron. The antioxidant capacity of phenolic compounds depends on the rate of hydrogen withdrawal linked to the bond-dissociation energy (BDE) of the hydrogen-

phenolic bond (Bortolomeazzi et al. 2007). The time of harvesting has an effect on the polyphenol levels, tea leaves which are harvested in the summer are higher in polyphenols than those harvested in spring (Demeule et al. 2002). In addition, other factors such as tea vintage, soil for tea cultivation or the exposure to the sun are uncontrolled factors which may influence catechin content of green tea (Kumamoto and Sonda 1998).

Reducing ability of brewed green tea during 24 hours measured by FRAP assay. Statistical test one-way ANOVA ($p < 0.05$) followed by Tukey's test showed that time has a significant effect on the antioxidant activity of green tea. The mean value increased from 0.46 ± 0.01 to 0.48 ± 0.01 mM Iron sulphate equivalent after 2 hours, and then significantly decreased from that time onwards.

Scavenging ability of green tea was measured by the DPPH assay. Brewing time had a significant effect on the antioxidant capacity of green tea measured in this way ($p < 0.05$). The brewed green tea after 2 hours showed the highest scavenging ability, with a mean value of 195 ± 23 mM, which decreased thereafter. (Lin et al. 2008) studied the effect of different brewing methods (hot and cold brewing) on antioxidant capacity of green tea at different concentrations. They found the hot water extracts had higher antioxidant capacity and reducing power. Nevertheless, cold brewed extracts had the same effect in scavenging ability on DPPH, hydroxyl radical and chelating ability on ferrous.

The crocin bleaching assay is a competition kinetic procedure that measures the ability of green tea to react with peroxy radicals. In our case, the antioxidants of green tea compete with the carotenoid crocin for peroxy radical produced by a diazo-compound. Hence, the bleaching rate of crocin by peroxy

radicals is measured in the absence of antioxidants. This kinetic approach provides a more precise evaluation of the efficiency of antioxidant defence. It was shown statistically using one-way ANOVA that time had a significant effect ($p < 0.05$) on brewing tea, the highest antioxidant measurements on the brewed tea were obtained after 2 hours when the mean value was 183.2 unit/l; after that, the antioxidants in the green tea significantly decreased over the remaining 24 hours.

Labbé et al. (2006) reported that catechins (green tea) could be classified into two groups according to the changes during brewing. The first group is the time dependent catechins such as EC and EGC, The second group is time/temperature dependent compounds such as EGCG, ECG, and GCG. Stability of green tea infusion (catechin) has reported that epimerization can be observed at 40 °C over prolonged storage. Thus temperature and heating time could influenced the epimerization of catechin in green tea infusions (Wang and Helliwell 2000). In addition, metal ions can effect of catechin antioxidant activity by binding to the catechins. Catechins reacts with metal ions forming metal ion complexes. Kumamoto et al. (2001) reported that the antioxidant activity of EGCG increased in the presence of Mn^{2+} and Cu^{2+} but the activity decreased in the presence of Fe^{2+} because the metal ions bound to EGCG and changed its oxidation potential. Formation of phenoxy radical can occurs easily at EGCG and ECG because of their gallate group. The presence of Mn^{2+} and Cu^{2+} can help this reaction and increase the antioxidant activity of these catechins. Ryan and Hynes (2007) used UV-visible to measure electron transfer of Fe^{3+} to ECG and EGCG and they noticed the powerful antioxidant properties of ligands with one molecule to either ECG or EGCG reduced four Fe^{3+} species (i.e. one molecule able to reduced four Fe^{3+}). Considering that total amount of catechin

reduced by increasing temperature, that is due to oxidation occurred during heating process besides the epimerization. This oxidation can change tea colour to be darker instead of green and deeper yellow (Kim et al. 2007) and (Zhu et al. 1997). Many studies have been reported that antioxidant, antimutagenic, anti-inflammatory, antibacterial, and antitoxin properties of tea are mainly related to phenolic compounds (Yang 1999), (Dufresne and Farnworth 2000), (Balentine et al. 1997), and (Yen and Chen 1994).

Conclusion

Although based on different approaches, all five assays used demonstrated the effect of time on the antioxidant activity of brewed green tea; all assays showed clearly that the antioxidant activity level is highest after two hours of brewing and then significantly decreases after 2 hours at 25 °C of brewing and then significantly decreased up to 24 h. One possible reason for low antioxidant capacity at 0 h compared with after two hours was that the time too short for full antioxidant released.

Table 6: Antioxidant activity and total phenolic content of brewed green tea during a period of time by five different assays (DPPH, TEAC, CBA, and FRAP) (n = 6)

| Methods | Time (h) | | | | | | |
|---------|--------------------------|--------------------------|---------------------------|----------------------------|----------------------------|----------------------------|---------------------------|
| | 0 | 2 | 4 | 6 | 8 | 22 | 24 |
| TEAC | 96 ± 13 ^a | 149 ± 13 ^b | 126 ± 12 ^c | 130 ± 6 ^c | 118 ± 6 ^c | 120 ± 7 ^c | 118 ± 4 ^c |
| TPC | 5.4 ± 0.3 ^a | 6.3 ± 0.1 ^b | 6.1 ± 0.01 ^b | 6.1 ± 0.5 ^b | 6.2 ± 0.1 ^b | 6.3 ± 0.1 ^b | 6.2 ± 0.001 ^b |
| FRAP | 0.46 ± 0.01 ^a | 0.48 ± 0.01 ^b | 0.46 ± 0.01 ^b | 0.46 ± 0.01 ^a | 0.46 ± 0.01 ^a | 0.46 ± 0.0 ^a | 0.45 ± 0.001 ^a |
| DPPH | 136 ± 19 ^a | 195 ± 23 ^b | 106 ± 18 ^a | 99 ± 27 ^a | 108 ± 28 ^a | 108 ± 13 ^a | 95 ± 44 ^a |
| CBA | 177.8 ± 21 ^a | 183.3 ± 14 ^a | 136.5 ± 12.6 ^b | 122.2 ± 17.8 ^{bc} | 119.1 ± 17.8 ^{bc} | 100.4 ± 17.6 ^{bc} | 107.1 ± 14.1 ^c |

Values that do not share the same letter in the same row are significantly different according to pairwise Tukey's test 95% confidence. Results shown as a mean ± SD antioxidant unit/l for CBA, for DPPH is DPPH radical quenched mM, TEAC: ABTS radical quenched mM, TPC: gallic acid mM equivalent, FRAP: Iron sulphate mM equivalent.

Chapter 4

Effects of full fat, semi-skimmed, and skimmed cow milk on the total antioxidant capacity of green tea

Introduction

Experimental evidence has suggested that tea (black and green) is associated with a reduction in the risk of coronary diseases because they possess high level of antioxidants that which protect tissue against lipid peroxidation and oxygen free radicals (Ryan and Petit 2010). The food matrix may also be relevant to the antioxidant-prooxidant activity, as it may influence interactions between compounds and possibly interfere with reaction kinetics.

These sort of interactions could have negative effects not only in foods, but also in the human body, as shown by a study by Samman et al. (2001) where volunteers consumed phenol rich extracts from rosemary and tea, added to the meat component of their meals; they showed that extracts decreased nonhaem-iron absorption, and thus impaired the dietary utilization of this nutrient.

The measurement of the types of compounds and their concentrations is fundamental to the establishment of the potential functionality and mechanisms of plant extracts, for example total phenolic contents, quantification of products of deterioration or fat oxidation, or even colour as an indicator of oxidation of food components, particularly haem proteins. Additionally, methods for determination of antioxidant activities are commonly applied, but their principles are not always well understood (Prior and Cao 1999).

There are various methods for tea preparation in different countries. In the United Kingdom (UK), tea is consumed with the addition of milk but in China, Turkey, Japan, and most of Arab countries, tea is mostly consumed without adding milk (Sharma et al. 2008).

There are many studies indicating that the antioxidant capacity of tea is decreased by the addition of milk for example, Zulueta et al. (2009) reported that milk has an antioxidant activity and that this is due to the casein. Ryan and Petit (2010) studied the effect of adding bovine milk (whole, semi-skimmed, and skimmed) to five brands of black tea. Each tea analysed was a significant source of antioxidants. However, adding milk to tea infusions decreased the antioxidant activity. Skimmed milk decreased the antioxidant activity more than whole and semi-skimmed milk. Variation in temperature did not affect their activity. In addition, Serafini et al. (2009) measured plasma antioxidant capacity and levels of caffeic and ferulic acids after consumption of 200 g blueberry with 200 ml of whole milk and without milk, using water as a control. They found ingestion of blueberry significantly increased the plasma antioxidant levels and enhanced plasma level of caffeic and ferulic acids, whereas ingestion with whole milk reduced plasma antioxidant capacity. Furthermore, Ryan and Sutherland (2011) reported the addition of soya milk to five different black teas caused a significant increase in antioxidant capacity, and also demonstrated that addition of sugar and sweetener did not significantly change total antioxidant capacity.

Sharma et al. (2008) studied the interaction of milk and sugar with black tea, and reported that black tea possessed a higher total phenol content than black tea with milk. They also demonstrated that the scavenging activity of black tea against the DPPH radical was highest followed by black tea with sugar. Addition

of milk to the black tea decreased the DPPH radical scavenging activity. It was reported that the concentration of catechins increases in plasma rapidly after consumption of green tea and black tea (van het Hof et al. 1998). Lorenz et al. (2007) studied the effect of adding milk to black tea. Flow-mediated dilation (FMD) was measured in 16 healthy females before and after consumption of 500 ml of black tea, black tea with 10% of skimmed milk and boiling water. They found that black tea significantly improved FMD compared with water control but addition of skimmed milk blunted the effect of black tea.

Given the evidence in the literature, this hypothesis tested here is that milk of different fat content (whole milk 3-4% fat, semi-skimmed milk 1.5-2% fat, and skimmed milk < 0.5% fat) would affect the antioxidant activity of green tea. Hence, the objective of the work was to determine the effect of milk with various fat contents on the antioxidant capacity of green tea using the five different assays previously described. Four types of brewed tea were prepared: plain green tea, green tea with whole milk, green tea with semi skimmed milk, and green tea with skimmed milk.

Materials and methods

These are outlined in the chapter 2.

Experimental design

Tea was prepared following a consistent method as described below, after which one of three different types of milk with different fat content were added to it. Total phenol content and antioxidant capacity were measured by different assays (FRAP, TEAC, CBA, and DPPH). For the preparation of the green tea, 40 ml of hot water (75-80) °C were added to 0.4 ± 0.001 g of green tea (Qi green tea), and stirred for 3 min. Then 4 ml of milk (So organic) that purchased at Sainsbury's, Plymouth, UK (full fat, semi-skimmed, or skimmed) were adde,

stirred for 2 min and centrifuged for 5 min (Denley Bs 400, 4000 rpm). This was repeated six times. Water was used as a control. Dummy reagents were prepared for all four of methods to allow compensation for the turbidity of milk in the samples. For the FRAP and ABTS assays, the dummy was water, for the DPPH assay it was ethanol, while for total phenol content a dummy reagent was prepared as described in Chapter two. Each experiment was repeated six times with the same milk and same conditions; each sample tested in triplicate.

Statistical analysis

Analysis of variance (ANOVA) two-way followed by Tukey's test 95% confidence was applied to the data to determine differences ($p < 0.05$). Pairwise tests were used to find significant differences between samples. The statistical analysis was carried using Minitab (version 16).

Results and discussion

Many researchers have described the potential antioxidant properties of polyphenols. These compounds act as antioxidants by donating a hydrogen atom to free radicals. The results from the experiment carried out have shown type of matrix have a significant effect on the total phenolic content of green tea. This is obtained ($p < 0.05$) by two way ANOVA, General Linear Model followed with Tukey's test 95% confidence. There was a linear relationship between the concentration of green tea and its phenolic content. It was also demonstrated that plain green tea contains a high total phenolic content and green tea with skimmed milk have a lower level of phenolics. There was no significant difference between plain green tea and full fat milk ($p > 0.05$). The data of green tea with skimmed milk are the most interesting, because these are consistent with the phenolics in the green tea being titrated against something in the milk. The Total phenolic content of green tea and green tea mixed with skimmed milk

are presented in Table 7 and Figure 4 the data for green tea with full fat and semi-skimmed milk are not shown. Both linear and quadratic regressions were applied to all matrices. The R- sq for all was the same except for green tea with skimmed milk, with linear regression R-sq was 92.6% which was improved 96.8% when the quadratic regression was applied. Similar results found by Sharma et al. (2008) who studied the effect of milk and sugar on black tea. It has been reported that interactions between polyphenols from the tea and polar milk proteins which is non-covalent or covalent interaction that either increase or decrease antioxidant potential of polyphenols. Both of the covalent and non-covalent interactions can precipitate protein via multisite or multidentate interaction this could produce a mask of the flavonoids on the milk and polyphenols (i.e. catechins). This effect depends on the molar ratio of phenolics to protein (Sharma et al. 2008).

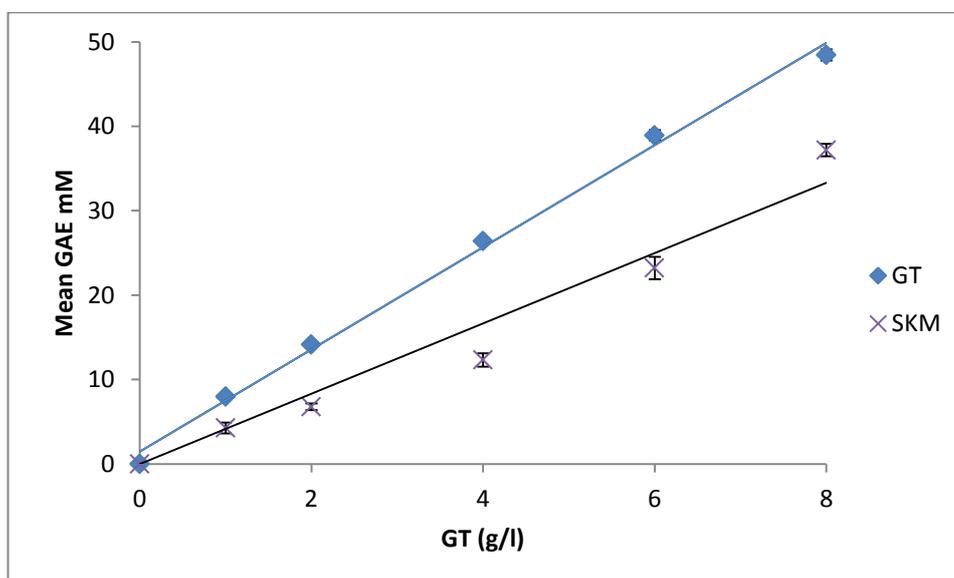


Figure 3: Total phenolic content of plain green tea and green tea mix with skimmed milk. Gt is plain green tea, SKM is green tea mixed with skimmed milk.

The TEAC value was determined at 10 min by using the Beer-Lambert law. However, earlier authors have used the percentage inhibition of ABTS radical

cation (the concentration of the antioxidant required to scavenge 50% of ABTS radical cation) according to this formula:

$$\% \text{ Inhibition} = [(A_{c(0)} - A_{A(t)}) / A_{c(0)}] \times 100$$

where $A_{c(0)}$ is the absorbance of the control at 0 min; $A_{A(t)}$ is the absorbance of the sample or Trolox standard at 6 min.

The results shown that concentration of green tea, and the matrix have significant effects on the TEAC value ($p < 0.05$) by two way ANOVA, General Linear Model followed with Tukey's test 95% confidence and found that plain green tea highest antioxidant activity and green tea with skimmed milk showed lower activity. No significant differences were found between adding semi-skimmed milk and skimmed milk. Nevertheless, the antioxidant of plain green tea was significant higher than in full fat milk but at lower concentration green tea with adding full fat milk had a higher scavenging activity than the plain green tea. Table 8 and Figure 5 showed the results of ABTS radical quenched concentrations (mM) of green tea and skimmed milk. In addition, the ABTS quenching was significantly increased by increasing concentration of green tea. Linear regression was applied for plain green tea 97% skimmed milk was 95% for the semi-skimmed milk was 94% and for full fat milk $R^2 = 93\%$. Similar result was found by Dubeau et al. (2010) who studied the effect of adding milk to green tea, Darjeeling, and English breakfast teas by TEAC test. Almajano et al. (2007) measured the antioxidant activity of milk proteins (β -casein and α -casein, bovine serum albumin (BSA), α -lactalbumin and β -lactoglobulin) themselves by the TEAC assay. They reported that they had antioxidant activity and their radical scavenging ability improved by adding epigallocatechin gallate during storage at 30 °C. However, von Staszewski et al. (2011) reported that no

antioxidant capacity of whey protein alone when they used the DPPH assay. They explained that was due to the low protein concentration was tested or different radical scavenging was used.

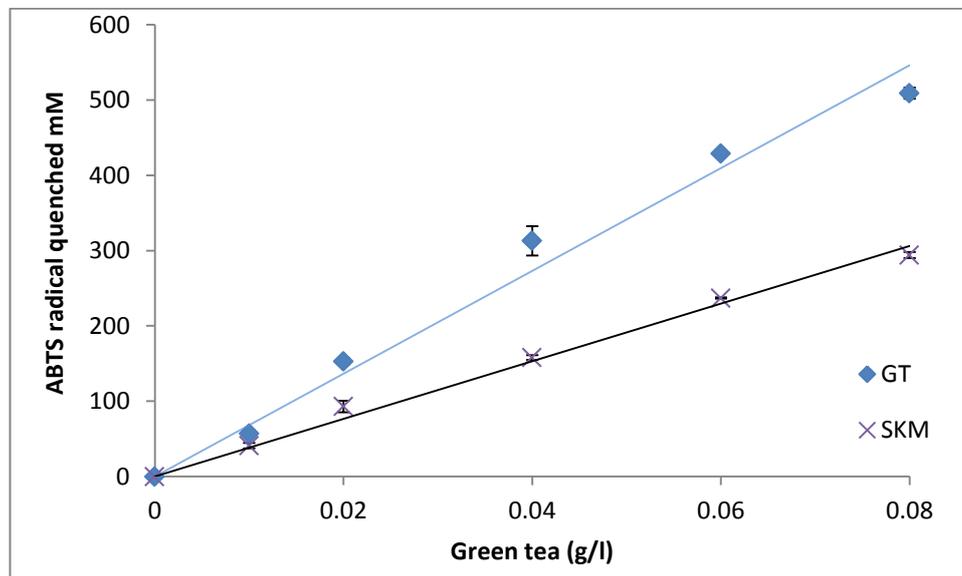


Figure 4: antioxidant capacity of plain green tea, and green tea with skimmed milk measured by TEAC assay. GT is plain green tea, SKM is green tea mixed with skimmed milk

The free radical scavenging potential of green tea and green tea with (full fat, semi-skimmed, and skimmed) at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 g/l) was evaluated using the DPPH assay. The value of DPPH radical quenched was calculated by using the Beer-Lambert law. However, earlier authors have used the percentage inhibition of DPPH radical according to this formula:

$$\% \text{ Inhibition} = [(A_{C(0)} - A_{A(t)}) / A_{C(0)}] \times 100$$

Where $A_{C(0)}$ is the absorbance of the control at 0 min; $A_{A(t)}$ is the absorbance of the sample or ascorbic acid standard. The data are represented in Figure 10

was calculated according to percentage inhibition, results were not shown for green tea with full fat milk and semi-skimmed milk.

The data were analysed by two-way ANOVA with Tukey's method 95% confidence. The results shown that concentration, matrices and reaction between matrices and concentration are significantly different ($p < 0.05$). There were significant difference activities at different concentration. The radical scavenging activity increased with increase concentration of extracts. From the experiments performed it was observed that the antioxidant capacity for all samples increase with increasing concentration of extracts (GT with FFM, GT with SSKM, plain GT, and GT with SKM). Statistically, plain green tea had a highest scavenging activity, followed by green tea with full fat milk, green tea with semi-skimmed milk and green tea with skimmed milk. At low concentration full fat milk and semi-skimmed milk had the same activity. The results are presented in a Table 9 and Figure 6. Linear regression was applied for the plain green tea R- sq 98.6% for green tea with full fat milk was 98%, green tea with semi skimmed milk was 98% and 99% for green tea with skimmed milk. In DPPH assay adding milk to green tea may mask scavenging activity because of the present of protein in milk, thus the radical scavenging do not reach their highest scavenging activity because of presence of protein. And also observed that degree of masking of antioxidant activity depend on the polyphenol composition and the type of milk (Arts et al. 2002). In addition, the interaction between protein and polyphenolic compounds can created by hydrophobic association that might be stabilized by hydrogen bonding (Murray et al. 1994). Furthermore, antioxidant activity of green tea may influenced by many factors such as leaf size, time, stirring duration, and teabag porosity.

Similar results found by von Staszewski et al. (2011) when they were combined green tea infusion with whey protein. They noticed that decreased in DPPH radical scavenging. They conclude that the degree of inhibition of green tea antioxidant capacity by whey protein depend on the polyphenol composition and not polyphenol content. Also similar observation obtained by Sharma et al. (2008) who studied effect of milk and sugar on black tea. They found that addition of milk reduced the ability of black tea to scavenge DPPH radical.

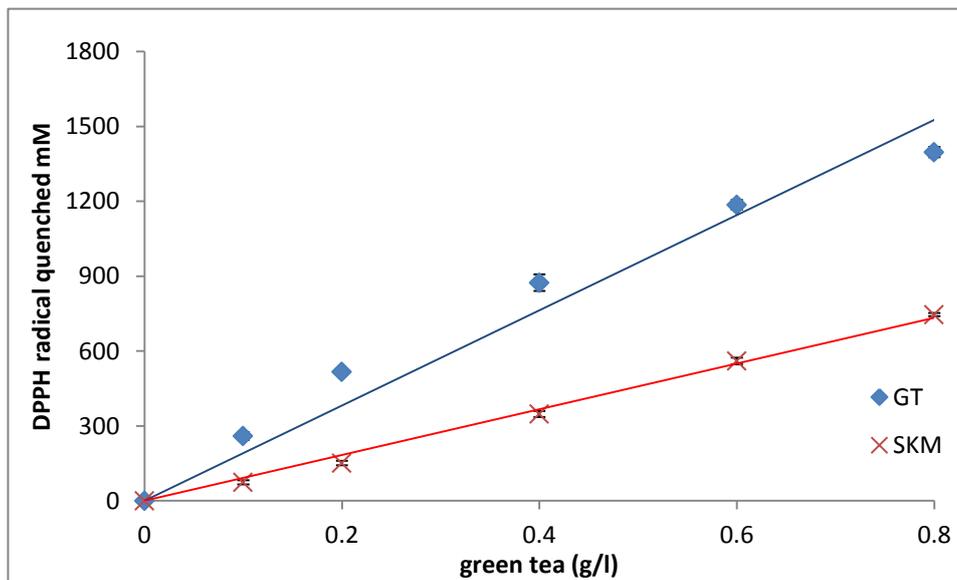


Figure 5: Scavenging capacity of plain green tea and green tea with skimmed milk measured by DPPH assay. GT is plain green tea, SKM is green tea with skimmed milk.

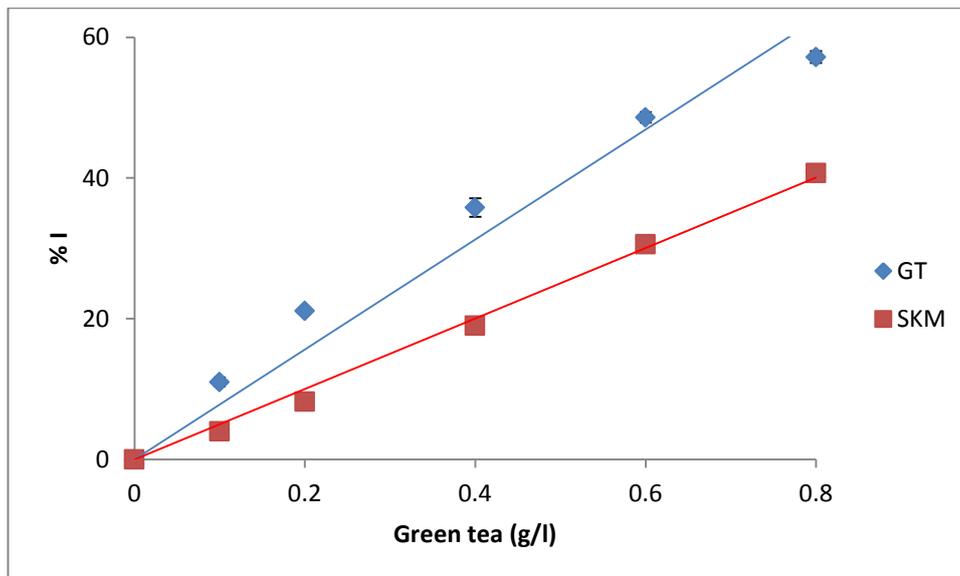


Figure 10: Inhibition percentage of green tea against DPPH radical measured by DPPH assay. GT is plain green tea, SKM is green tea with skimmed milk.

The ability of green tea to reduce Fe(III)-2,4,6- Tri(2-Pyr- idyl)-s-triazine (iron chloride-TPTZ) complex to Fe(II)-TPTZ (iron-sulphate TPTZ) were determined in three different matrices with six different concentrations and analysed using Two-way ANOVA, General Linear Model followed by Tukey's test. The results show that the matrices and concentration have significant effect on the antioxidant activity ($P < 0.05$). The reducing capacity of plain green tea was significantly higher (antioxidant capacity = $7.704 + 81.13$ concentration $R\text{-Sq} = 98.4\%$) than green tea with milk, and green tea with skimmed milk showed lowest reducing activities (antioxidant capacity = $1.044 + 72.75$ concentration $R\text{-Sq} = 97.7\%$) is presented in Figure 7 and Table 10. The same result was found by Ryan and Petit (2010) . When they studied the effect of adding milk (full fat milk, semi-skimmed, and skimmed milk) to 5 brand of black tea by FRAP assay.

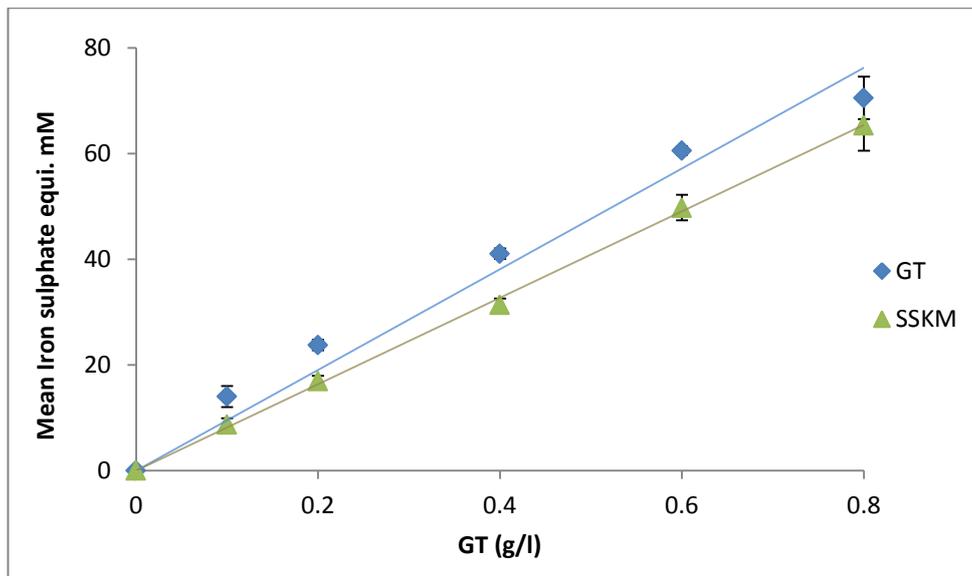


Figure 6: antioxidant activity of plain green tea and green tea with semi-kimmed milk measured by FRAP assay. GT is plain green tea, SSKM is green tea mixed with semi-skimmed milk.

Thermal decomposition of ABAP produce peroxy radical that can cause damage of crocin structure resulting in bleaching crocin it could be detected spectrophotometrically at 440 nm. Green tea could significantly inhibit bleaching by scavenging peroxy radical. The ability of green tea to react with peroxy radical in different matrix were significantly different ($p < 0.05$) in term of matrices and concentrations were tested by Two-way ANOVA. In the ranking of antioxidant activity obtained by crocin bleaching assay plain green tea showed a highest antioxidant capacity but green tea with skimmed milk showed lowest antioxidant capacity. The results are shown in Figure 8 and Table 11. This decreased (effect) was not noticed by Kyle et al. (2007) who reported that the addition of milk did not effect on the FRAP values of tea. In contrast, Langley-Evans (2000) found that the antioxidant activity was decreased after adding of skimmed milk. It has been reported that polyphenols having a high affinity for prolin-rich proteins such as caseins (Luck et al. 1994). Jöbstl et al. (2006)

reported that noncovalent cross-linking of EGCG by caseins, highlighting the interaction of tea catechin with casein in milk. Casein have a higher antioxidative amino acid such as tryptophan, lysine, methionine, histidine and tyrosine but are low in cysteine (Rival et al. 2000) and (Uchida and Kawakishi 1992).

In the present study, the highest antioxidant potential was noted in plain green tea then green tea with full fat milk had higher activity that green tea with semi-skimmed and skimmed milk. Milk is contain a number fat-soluble antioxidants (tocopherols, carotenoids, and retinols). Therefore, removing fat content in milk may take away a number of fat-soluble antioxidant and that is decrease its antioxidant potential.

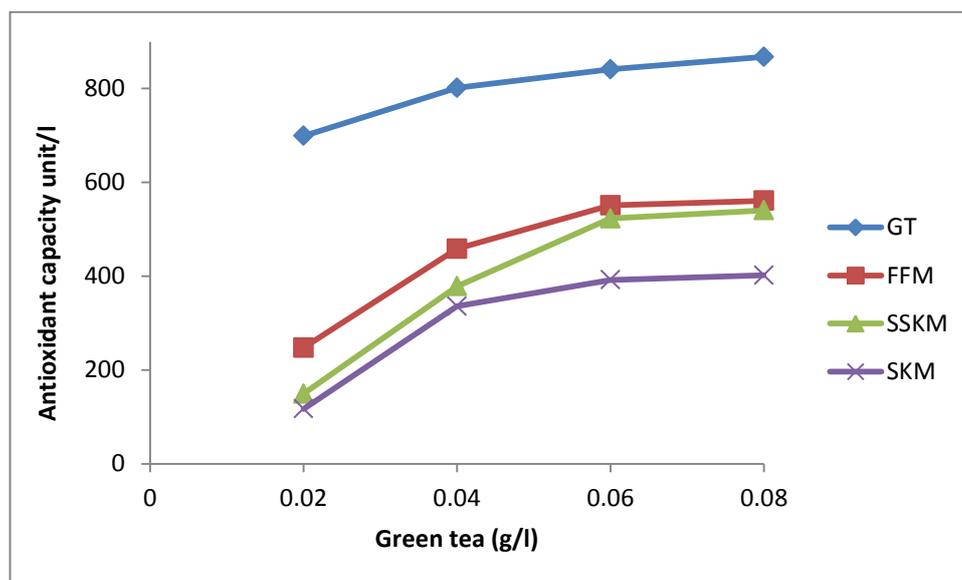


Figure 7: antioxidant capacity of green tea and green tea with fullfat , semi-skimmed, and skimmed milk determined by crocin bleaching assay. GT is plain green tea, FFM is green tea with full fat milk, SSK is green tea with semi-skimmed milk, and SKM is green tea mixed with skimmed milk.

Conclusion

Green tea is rich in antioxidant and the addition of milk can effect the antioxidant capacity and reduce antioxidant activity. The effects were much higher with skimmed milk compared to full fat milk. The degree of which the addition of milk decreased the antioxidant capacity of green tea depends on the amount of added and the level of fat content of the milk. Thus, it seems the polyphenols have a strong affinity to interact with the some component (protein) in milk. It has been reported that milk has antioxidants, with the fat removing process may remove some antioxidants. The results showed that the food matrix influence the antioxidant capacity of green tea polyphenols because they cannot able to reach their optimum scavenging capacity. Thus, it could be healthier to drink green tea without milk.

Chapter 5

Measuring antioxidant capacity in human saliva

Introduction

Human saliva is a mouth fluid, which has many functions. It is involved in homeostasis, and has a vital role in maintaining oral health. In addition, saliva protects oral mucous against damage, and has an minor role in preliminary food digestion by the presence of α -amylase and other enzymes. It also defends against pathogenic microorganisms. In the presence of defence proteins that react either in a specific way (immunoglobulins) or in a non-specific way (lysozyme, lactoferrin, hystatins, cystatins, peroxydase, and others) at inhibits the growth of microorganisms (Van Nieuw Amerongen et al. 2004) and (Lawrence 2002). Saliva has an important role in maintaining teeth enamel mineralization: several proteins (mucins, proline_rich proteins (PRPs) and statherin) help to maintain Ca^{+2} sovrasaturation in saliva (Amerongen and Veerman 2002). In humans, salivary glands can divided in two main types, the major and minor salivary glands. The major salivary glands are parotid, sublingual, and submandibular. Minor salivary glands are the von Ebner glands and Blandin-Nuhn mucous glands (Carranza et al. 2005).

The composition of saliva varies according to the mucous or serous component of the glands (Hu et al. 2004). The relative contribution from each type of gland to the total unstimulated saliva secretion varies as follows, 65% for submandibular, 23% parotid, 8% von Ebner, and 4% sublingual (Schipper et al. 2007). Healthy adult subjects produce approximately 0.5 ml/min, which is 500-1500 ml of saliva per day. Several physiological and pathological factors can

affect saliva secretion in both qualitative and quantitative term such as taste stimulation, smell, chewing, age, drugs, oral hygiene, physical exercise, psychological state and hormone status. The rate of salivary secretion can also increased by stimulation with acidic food, which increases the concentration of chloride, sodium, and bicarbonate, and decreases the concentration of salivary phosphate and potassium. The type of saliva is different according to the type of salivary gland, with differences in the concentration of ions, salts, and total protein (Aps and Martens 2005).

Saliva is the first biological medium to interact with external materials that are taken by animals and human as part of drink food and inhaled volatile substances. Salivary antioxidants can be divided into two types, water-soluble antioxidants and lipid-soluble antioxidants. Water-soluble antioxidants include many different molecules and enzymes, such as uric acid (the most important molecule in saliva), ascorbic acid (the second) and protease. Lipid-soluble antioxidants are present at very low concentrations (Nagler et al. 2002) and (Zappacosta et al. 1999). Saliva contains many biochemical systems reported to be involved in soft-tissue repair, and numerous antibacterial components such as lactoferrin, lysozyme and salivary peroxidase (Zappacosta et al. 1999). A limit of research have been done about measuring antioxidant capacity of saliva for example Astaneie et al. (2005) reported no significant differences in the TBARS level of plasma and saliva between diabetic and healthy control subjects. However, while other studies also found no significant difference between Type 1 diabetes mellitus and healthy controls, the plasma antioxidant capacity was 16% lower in a Type 1 diabetes (Vessby et al. 2002). Zappacosta et al. (1999) found no significant difference concentration of uric acid in saliva and total radical-trapping antioxidant activity between smokers and non-

smokers but glutathione concentration was significantly higher in smokers. They also demonstrated that the concentration of glutathione in saliva reduces after a single cigarette. Furthermore, Reznick et al. (2006) analysed levels of superoxide dismutase activity (SOD) and total antioxidant status (TAS) in both saliva for Type 1 diabetes mellitus and found TAS, and SOD was significantly increased. Astaneie et al. (2005) examined levels of lipid peroxidation and antioxidants in saliva and plasma for two groups: the first group had Type 1 diabetes mellitus, and the second group were a control; they had found no significant differences of TBARS levels between the two groups but the total antioxidant activity (ferric reducing ability) of saliva and plasma of the diabetic group was significantly higher than the healthy group. Recently, there has been an increasing interest in using saliva to diagnose disease. This is due to the fact that saliva can be a simply and non-invasive collected. It has been demonstrated that c-erbB-2 soluble fragments and 15-3 cancer antigen in breast cancer can be measured in saliva (Tabak 2001). Blood is a traditional source of genomic DNA but also saliva has investigated as an alternative source of DNA, deriving from the oral cells (Ng et al. 2006) and it has been proven to be a useful source for biomarker profiling (Tabak 2001) and (Khalil Boutaga 2007).

Several epidemiologic studies have reported that ethanol consumption can reduce the risk of coronary heart disease (CHD) because ethanol increases high density lipoprotein (HDL)-cholesterol plasma levels to enhance blood fibrinolysis and decrease platelet aggregation. The effects of drinking beer on plasma antioxidant capacity were investigated by (Ghiselli et al. 2000), who showed that after 1h of beer consumption, plasma antioxidant significantly increased, but after 2 hours it returned to the baseline. Beer and wine contain

phenolic compounds that once absorbed, can contribute to the plasma antioxidant capacity. A few studies have been done to assess antioxidant capacity in coffee, which has a high consumption in many countries. Coffee contains a large number of phenolic acids such as chlorogenic, ferulic, caffeic, and *p*-coumaric acids. Research by Nardini et al. (2002). Has shown an increase of total plasma caffeic acid concentration in 10 healthy males one hour after consumption of coffee. Furthermore, Natella et al. (2007) informed that after drinking 200 ml of coffee, plasma antioxidants measured as TRAP significantly increased.

The aim of this study was to measure total phenolic content in saliva and saliva antioxidant capacity after consumption of a single cup of green tea, and establish the effect of the addition of full fat milk or skimmed milk, using different assays (FRAP, TEAC, DPPH, and crocin bleaching assay) over a period of time.

Experimental design

Salivary antioxidant capacity and total phenol content were measured by different methods (FRAP, TEAC, CBA and DPPH) after taking a single cup of green tea with or without milk (full fat milk and skimmed milk). On six different days, in the morning after overnight fasting before brushing teeth, the subject (healthy non-smoker adult) was given a single cup of green tea. To prepare the tea (200 ml of hot water (75-80 °C) was added to 2 ± 0.02 g of green tea or green tea plus 10 ml of milk (full fat milk, or skimmed milk) and was then stirred for 3 min. Milk (So organic) was purchased at Sainsbury's. The control was 200 ml of hot water. Saliva samples were obtained by using a Salivette kit (Sarstedt, UK) in which the cotton pad was held under the tongue for 5 min before and after (0, 60, 120, and 180 min) drinking the green tea. Then samples stored at

2-8 °C until all collected then centrifuged (Denley BS 400, 4000 rpm) for 5 min and the samples of saliva were stored at -20 °C.

Material and Methods

These are the same as outlined in Chapter 2.

Statistical analysis

The data is presented as mean of six duplicates \pm SD and Analysis of Variance (General Linear Model). Statistical analysis was conducted using Minitab version 16. A p value of less than 0.05 using Tukey's test was considered statistically significant.

Results and Discussion

Salivary antioxidant capacity after consumption of 200 ml of green tea, the effects of addition 10 ml of full fat milk or skimmed milk, and water was control measured by TEAC, DPPH, FRAP, crocin bleaching assay and measuring of the phenolic content every hour up to 3 hours. According to the TEAC assay, the addition of milk and time had statistically significant effects ($p < 0.05$). Salivary antioxidant reached a peak after one hour and then started decreasing onwards. Plain green tea showed a high antioxidant capacity, and there was no significant difference in the salivary antioxidant after taking plain green tea compared to green tea with full fat milk ($p > 0.05$). However, salivary antioxidant capacity significantly decreased after drinking plain green tea with skimmed milk ($p < 0.05$). The results are shown in Table 12. Total phenolic content and the salivary antioxidant baseline and value after consumption of green tea or green tea with adding 10 ml of milk (full fat, and skimmed milk) were measured by different assays (FRAP, TEAC, DPPH and CBA). Water was used as a control. The data were analysed by a paired t-test (Minitab 16). However, we have the suspicion that the saliva was contaminated by the green tea in the mouth

because it was taken immediately after drinking tea. The results are shown in Tables 15, 16, 17, and 18.

Salivary scavenging activity measured by the DPPH assay showed that plain green tea gave the highest activity but no significant differences were noted in salivary scavenging on adding full fat milk and skimmed milk ($p > 0.05$) but adding skimmed milk to the green tea was significantly decreased antioxidant activity. Salivary scavenging activity reached to the peak one hour after taking the green tea and then returned to the baseline. The results shown in Table 12. Researchers have found that the reaction time of the DPPH radical with antioxidant can be divided into three types fast, slow, and intermediate types (Brand-Williams et al. 1995). Many studies measured the scavenging ability on DPPH after 30 min (incubation of the sample with the reagent) (Deng et al. 2011), (Floegel et al. 2011) and (Sharma and Bhat 2009). In this study, the salivary scavenging activity was measured after 60 min. Study by Cao et al. (1998) investigated the effects of fruit and vegetables on plasma antioxidant capacity in 36 healthy non-smokers consumed a control diet 10 servings of fruit and vegetables for two weeks and on the same diet but with the addition of two servings of broccoli in days 6-10. They found that for both diets the plasma antioxidants were significantly increased when measured by ORAC assay. The same researchers, in another study Cao et al. (1998) investigated the serum antioxidant capacity of 8 elderly after feeding them with 294 g of spinach, 240 g strawberries or 300 ml of red wine compared with a control diet or 1250 mg of vitamin C. Then they found that the serum antioxidant capacity significantly increased during 4 hours after the consumption of wine or of strawberries, and spinach by ORAC, TEAC, and FRAP assay.

The ability of saliva to reduce Fe^{3+} to Fe^{2+} was measured using the FRAP assay. Time and milk addition has significantly effects ($p < 0.05$). Highest antioxidant activity was found after one hour after which it decreased. Statistically, plain green tea led to a higher reducing ability of the saliva, and this was significantly decreased by adding milk. No significant difference was found between green tea with full fat milk and green tea with skimmed milk ($p > 0.05$). The results shown in Table 13. Research by Benzie et al. (1999) showed a 4% increased in the antioxidant capacity of plasma and urine 40 min after drinking green tea assessed by FRAP assay. It also reported that the maximum total phenolic content at 60-90 min. Plasma antioxidant capacity was significantly higher after the consumption of tomatoes with olive oil for one week consumption, than consumption of tomato's with sunflower oil measured with a FRAP assay (Lee et al. 2000). The authors conclude that the plasma antioxidants can be affected by the polyphenols contained in olive oil. Another research by Serafini et al. (2003) reported plasma FRAP significantly increased by 20% in 12 healthy subjects after 1 hour from consumption of 100 g of dark chocolate but did not increase after the consumption of 100 g of dark chocolate with 200 ml of whole milk or 200g of milk chocolate. Other authors Rein et al. (2000) reported plasma antioxidant which was measured as TRAP, to increase 31% after 2 hours in 17 healthy subjects after they had consumed 80 g of procyanidin-rich, semisweet chocolate, returning to the baseline value after 4 hours. Also Benzie et al. (1999) showed a 4% increase in the antioxidant capacity of plasma and urine after 40 min of drinking green tea assessed by the FRAP assay, and that the maximum total phenol antioxidant capacity is reached at 60-90 min.

The crocin bleaching assay was also used to measured the salivary antioxidant capacity, and it was found that time, milk addition, interaction between time and

matrices were significantly different ($p < 0.05$). The antioxidant capacity peaked after one hour and then significantly decreased as shown in Table 13. Statistically it was found that plain green tea led to a higher antioxidant capacity followed by green tea with full fat milk and green tea with skimmed milk ($p < 0.05$). It is difficult to interpret and compare results due to the lack of a similar study in which antioxidant capacity in saliva was measured in this way.

Salivary total phenolic content was measured after consumption of plain green tea, green tea with full fat and skimmed milk. Salivary phenolic content reached peak after one hour of consumption of tea and then significantly decreased return to the baseline. Plain green tea had a highest phenolic content. Statistically, there are no significant difference between salivary phenolic content of plain green tea and green tea with full fat milk ($p > 0.05$). A significant difference was found between salivary phenolic content of plain green tea and green tea with adding skimmed milk. The data are presented in Table 14. Day and Stansbie (1995) studied serum antioxidant capacity in 6 healthy men, and showed a 24% antioxidant increase in serum after the consumption of 250 ml of white wine, in this study ethanol was used as a control and had no effects. In contrast, Serafini et al. (1998) found polyphenol concentration and plasma TRAP, in 10 healthy subjects after 50 min of consumption of alcohol free red wine significantly increased, but did not find any increase after the ingestion of alcohol-free white wine. In the same research, it was determined that the serum antioxidant capacity, measured by chemiluminescence in 9 subjects increased by 18% after 1 hour and by 11% after 2 hours of drinking 300 ml red wine, and 4% and 7% respectively 1 and 2 hours after drinking of white wine. Whitehead et al. (1995) found a significant increase of plasma FRAP and total phenol in 9 volunteers after 30 min ingestion of 100 ml red wine and malt whisky.

Leenen (2000) reported the effects of single consumption green and black tea with and without milk on 21 healthy subjects, using water as control on the human plasma, found after 60 min of consumption of black tea plasma antioxidant significantly increased and green tea but in higher observed (amount) but milk did not effect on plasma antioxidant. In contrast, study by Serafini et al. (1996) on the consumption of 300 ml green tea or black tea and the addition of 100 of whole milk to the tea by two group of 5 healthy adults, experiments were repeated on a separated day the antioxidant capacity in plasma (TRAP) measured before and after 30, 50 and 80 min found plasma antioxidant capacity significantly increased after consumption tea peak at 30-50 min. The addition of milk to tea had totally inhibited the increase in plasma. Some studies demonstrated that polyphenols possess a high binding affinity for caseins (proline-rich proteins) (Luck et al. 1994). In addition, study by Jöbstl et al. (2006) showed the noncovalent cross-linking of epigallocatechin gallate (EGCG) by caseins, highlighting the interaction between catechins from tea and milk caseins.

Hollman et al. (1996) noticed that the concentration of quercetin in plasma increased after consumption of black tea, but also was not affected by the addition of milk to the tea. However, Serafini et al. (1996) reported that plasma antioxidant activity increased after consumption of black tea and was reduced by the addition of milk. It is possible that the supressing effect of milk on plasma antioxidant capacity is related to the reduction of the absorption of total antioxidant and is not due to catechins and quercetin in particular. Although addition of milk might not inhibit the quercetin or catechine concentration. Probably it has effects on the other antioxidant components such as the thearubigins and theaflavins in affect the total antioxidant capacity of tea (Ryan

and Petit 2010). A few researches have reported about measuring antioxidant capacity in saliva. Thus, it is hard to compare this result to others Therefore, further experiments are needed in order to clarify and understand the mechanism of activity over time.

Conclusion

Saliva is important for tasting, chewing, and first step in digestion. The present study suggests that saliva could be used as a biomarker to diagnose antioxidant capacity which is advantage because blood tests required needles injection and can possibly cause contamination. This biological fluid has appeared easier to manipulate, collect and it is a good alternative source to blood to diagnose diseases and also measuring antioxidant activity. In addition, analysis of saliva can provide important information of function of different organs within the body.

Table 7: Salivary scavenging capacity measured by DPPH and TEAC assays after 1, 2, and 3 hours of ingestion of 200 ml of green tea and green tea with adding 10 ml of full fat milk and skimmed milk, water was control (n = 6 independent experiments)

| Methods | Time (h) | | |
|----------------------------|----------|----------|----------|
| | 1 | 2 | 3 |
| TEAC | | | |
| GT ^A | 150 ± 17 | 123 ± 13 | 101 ± 15 |
| FFM ^A | 123 ± 14 | 112 ± 14 | 105 ± 16 |
| SKM ^B | 100 ± 24 | 86 ± 21 | 56 ± 29 |
| Water Control ^C | 55 ± 2.5 | 52 ± 1.4 | 53 ± 1.8 |
| DPPH | | | |
| GT ^A | 325 ± 39 | 156 ± 54 | 54 ± 25 |
| FFM ^A | 288 ± 57 | 150 ± 59 | 41 ± 41 |
| SKM ^B | 165 ± 18 | 116 ± 18 | 33 ± 15 |
| Water Control ^C | 12 ± 0.6 | 11 ± 0.3 | 10 ± 1 |

Values followed by the same letter in the same column show statistically significant difference ($p > 0.05$) by Tukey's test 95% confidence. Results (mean ± SD) have shown as ABTS radical quenched mM for TEAC assay and DPPH radical quenched mM for DPPH assay. GT is saliva after taking plain green tea. FFT is saliva after ingestion green tea with full fat milk, SKM is saliva after drinking green tea mixed with skimmed milk.

Table 8: Salivary antioxidant capacity measured by FRAP and crocin bleaching assays after 1, 2, and 3 hours of ingestion of 200 ml of green tea and green tea with adding 10 ml of full fat milk and skimmed milk, water was control (n = 6 independent experiments)

| ASSAY | Time (h) | | |
|-------------------------------|----------|----------|----------|
| | 1 | 2 | 3 |
| FRAP | | | |
| GT ^A | 289 ± 27 | 271 ± 26 | 263 ± 37 |
| FFM ^B | 263 ± 32 | 246 ± 22 | 236 ± 33 |
| SKM ^B | 254 ± 14 | 245 ± 20 | 225 ± 12 |
| Water Control ^B | 253 ± 21 | 226 ± 6 | 214 ± 14 |
| Crocin bleaching assay | | | |
| GT ^A | 111 ± 3 | 104 ± 3 | 96 ± 5 |
| FFM ^B | 110 ± 8 | 92 ± 12 | 73 ± 9 |
| SKM ^C | 94 ± 7 | 73 ± 7 | 56 ± 9 |
| Water Control ^D | 62 ± 8 | 60 ± 6 | 57 ± 4 |

Values (mM Iron Sulfate equivalent for FRAP assay) present means ± SD. Values followed by the same letter in the same column did not show statistically significant difference ($p > 0.05$) by pairwise Tukey's test 95% confidence. Results has shown for crocin bleaching assay as means ± SD which is the amount of antioxidant unit/l. GT is saliva after taking plain green tea. FFT is saliva after ingestion green tea with full fat milk, SKM is saliva after drinking green tea mixed with skimmed milk.

Table 9: Salivary total phenolic content after 1, 2, and 3 hours of ingestion of 200 ml of green tea and green tea with adding 10 ml of full fat milk and skimmed milk, water was control. (n = 6 independent experiments).

| Method | Time (h) | | |
|----------------------------|----------|-------------|----------|
| | 1 | 2 | 3 |
| TPC | | | |
| GT ^A | 420 ± 43 | 376.0 ± 41 | 346 ± 26 |
| FFM ^A | 420 ± 40 | 366.12 ± 23 | 330 ± 46 |
| SKM ^B | 344 ± 51 | 325.6 ± 31 | 280 ± 28 |
| Water Control ^C | 285 ± 25 | 280.1 ± 29 | 281 ± 28 |

Values present as a mean ± SD mM gallic acid equivalent. Values followed by the same letter in the same column did not show statistically significant difference ($p > 0.05$) by pairwise Tukey's test 95% confidence. GT is saliva after taking plain green tea. FFT is saliva after ingestion green tea with full fat milk, SKM is saliva after drinking green tea mixed with skimmed milk.

Table 10: Salivary antioxidant capacity before and after consumption of 200 ml of green tea measured by five assays.

| Methods | Salivary antioxidant capacity | |
|---------|-------------------------------|--------------------------|
| | Baseline | After |
| TEAC | 111 ± 11 ^a | 163 ± 24 ^b |
| DPPH | 164 ± 64 ^a | 659 ± 101 ^b |
| FRAP | 252.8 ± 22 ^a | 527.9 ± 104 ^b |
| CBA | 72.16 ± 4 ^a | 131 ± 8 ^b |
| TPC | 303.2 ± 17 ^a | 508 ± 68 ^b |

Values are expressed as mean ± SD for Antioxidant capacity unit/l for CBA, for the DPPH assay is DPPH radical quenched mM, for the TEAC assay is ABTS radical quenched mM, TPC: gallic acid mM equivalent, for the FRAP assay is Iron sulphate mM equivalent. Those content same letter is not significantly difference ($p > 0.05$) by pairwise Tukey's test 95% confidence. GT is saliva after taking plain green tea. FFT is saliva after ingestion green tea with full fat milk; SKM is saliva after drinking green tea mixed with skimmed milk. GT is saliva after taking plain green tea. FFT is saliva after ingestion green tea with full fat milk, SKM is saliva after drinking green tea mixed with skimmed milk.

Table 11: Salivary antioxidant capacity before and after consumption of 200 ml with addition of 10 ml full fat milk to green tea in five different assays

| Methods | Salivary antioxidant capacity | |
|---------|-------------------------------|-------------|
| | Baseline | After |
| TEAC | 103 ± 13 | 168 ± 22 |
| DPPH | 117 ± 22 | 406 ± 232 |
| FRAP | 208 ± 7.7 | 436 ± 43.8 |
| CBA | 86 ± 17.2 | 175 ± 3.9 |
| TPC | 437 ± 13.8 | 607 ± 129.7 |

Values are expressed as mean ± SD. Values are expressed as mean ± SD antioxidant capacity unit/l for CBA, for DPPH assay is DPPH radical quenched mM, TEAC assay is ABTS radical quenched mM, TPC is gallic acid mM equivalent, FRAP assay is Iron sulphate mM equivalent. Values of baseline and after ingestion were significantly difference ($p < 0.05$) by Tukey's test 95% confidence

Table 12: Salivary antioxidant capacity before and after consumption of 200 ml with addition of 10 ml skimmed milk to green tea in five different assays

| Methods | Salivary antioxidant GT with SKM | |
|------------------------|----------------------------------|-----------------------|
| | Baseline | After |
| TEAC | 77 ± 19 ^a | 144 ± 24 ^b |
| DPPH | 68 ± 16 ^a | 315 ± 33 ^b |
| FRAP | 250 ± 19 ^a | 267 ± 18 ^b |
| Crocin bleaching assay | 58 ± 8 ^a | 140 ± 18 ^b |
| TPC | 357 ± 29 ^a | 504 ± 73 ^b |

Values are expressed as mean ± SD. Values are expressed as mean ± SD antioxidant capacity unit/l for CBA assay, for DPPH assay is DPPH radical quenched mM, TEAC assay is ABTS radical quenched mM, TPC is gallic acid mM equivalent, FRAP assay is Iron sulphate mM equivalent. Values of baseline and after ingestion were significantly difference ($p < 0.05$) according to pairwise Tukey's test 95% confidence.

Table 13: Salivary antioxidant capacity and total salivary phenolic content after consumption of 200 ml of hot water (control). Measured by FRAP, TEAC, CBA, and DPPH assay.

| Methods | Salivary antioxidant activity | |
|---------|-------------------------------|---------------------------|
| | Baseline | After |
| TEAC | 46 ± 14 ^a | 54 ± 3 ^a |
| DPPH | 10 ± 3 ^a | 12 ± 0.7 ^a |
| FRAP | 216.3 ± 22 ^a | 201.6 ± 52 ^a |
| CBA | 62.15 ± 7.8 ^a | 62.52 ± 8 ^b |
| TPC | 306.3 ± 31 ^a | 286.7 ± 16.6 ^a |

Values are expressed as mean ± SD. Values are expressed as mean ± SD antioxidant capacity unit/l for CBA assay, for DPPH assay is DPPH radical quenched mM, TEAC assay is ABTS radical quenched mM, TPC is gallic acid mM equivalent, FRAP assay is Iron sulphate mM equivalent. Following the same letter in the same column is not significantly difference (P > 0.05) by Tukey's test 95% confidence.

Chapter 6

Antioxidant activity of Spices

Introduction

During the past few years, the study of antioxidant activity of essential oils of spices in food had increased in importance. Consequently, many papers have reported on the biological effects of spices. They play may an important role in preventing and treating illnesses in the human body, as antioxidants, anti-ulcerous, anti-inflammatory, antimicrobial and antiviral agents (2009, Lu et al. 2011).

Since the beginning of the last century, butyld hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are in widespread use for the extension of the shelf life of food stuffs because they are powerful synthetic antioxidants, however they are believed to possess carcinogenic activity (Lindberg Madsen and Bertelsen 1995) and (Thomas et al. 2010). Thus, research for natural replacement to synthetic antioxidants has been very popular because it has been proven that many plant materials are an excellent sources of antioxidants and herbs and spices have been long used as colouring agent and in folk medicines. In addition, spices also have flavouring properties and uses in cosmetics and perfumery (Hossain et al. 2011).

Spices can retard oxidation and its degrading effects, thereby extending the shelf life of food. Oxidation is a major problem of food spoilage causing rancidity. In the past decades, there has been an increasing interest in using spices in both the food industry and in scientific research because of their high

antioxidant content including vitamins, minerals, carotenoids, flavonoids, etc. As stated by a photochemical database, some plants contain up to 40 different antioxidants (such as tea 36, thyme 32, soybean 42, onion 32, organo 34). According to this database, walnut, cocoanut, guava and betelnut have the highest antioxidant contents (Milan 2006).

The oxidative degradation of lipids is one of the main factors limiting the shelf-life of food products. In recent years, several undesirable disorders have been detected as side-effects of using commonly used synthetic antioxidants. As an alternative to such synthetic additives, natural extracts have been used to control oxidative rancidity. Also honey, could potentially be used in small amounts in fat-containing food systems to prevent or delay some types of chemical deterioration that occur during the storage, as it has antioxidant activity (Viuda-Martos et al. 2009) and (Ruiz-Navajas et al. 2011). Antioxidant Dietary Fibre (AODF) has been defined as fibre containing significant amounts of natural antioxidants associated to the fibre matrix with a set of characteristics (Saura-Calixto 1998). Plant extracts oils (i.e. rosemary) have been successfully used to reduce lipid oxidation in meat products, including crispy bacon (Tan and Kuri, 2006), burgers and sausages (Aleson-Carbonell et al. 2005) and meatballs (Fernández-López et al. 2005).

Estévez and Cava (2004) and Estévez et al. (2007) also reported contradictory effects when the antioxidants were used in a food product matrix that already contained natural antioxidants, such as alpha-tocopherols (Estévez and Cava 2004). This may be due to the interaction between different antioxidant compounds. The activity of plant phenolics could be reduced due to their oxidation, and subsequent prooxidant activity of the oxidation products that

could act promoting oxidative reactions as it may be the case in systems with high oxidative instability (Huang and Frankel 1997). Other compounds in the matrix may play a role on the oxidation reactions, as do fatty acid profiles. These effects have been reported to be concentration-dependent. However, studies on the effect of antioxidant activity of tomato extracts, showed no effect of lycopene and β -carotene concentrations, but the effect appeared when these interacted with small amounts of tomato-occurring compounds (Strazzullo et al. 2007).

Objective:

The objective of these experiments were:

1. To assess the antioxidant activity on lipid of seven different oleoresin spice extracts (fennel, clove, cardamom, cinnamon, ginger, anise, and black pepper) using the Rancimat test under set conditions at (100-120 °C, 20 l air/h).
2. To determine the phenolic content and the antioxidant capacity including DPPH radical scavenging activity, and Trolox equivalent antioxidant capacity.

Material and methods

These are outlined in chapter 2.

Natural extracts

Cinnamon, cloves, black pepper, ginger, fennel, anise, vanilla, and cardamom extracts were purchased from (Kalsec Mildenhall, UK) aquaresin (water dispersible) and oleoresin (oil soluble) for all extracts.

Statistical analysis

Analysis of variance (ANOVA) two-way followed by pairwise Tukey's test 95% confidence was applied to the data to determine significantly differences (p value < 0.05) by using Minitab version 16.

Measurement of the antioxidant capacity of spice extracts

Rancimat method

Experiments were performed with a Rancimat 679 (Metrohm, Herisau, Switzerland) to determine the oxidative stability index, which is the widest used method to indicate the susceptibility against oxidation of edible oils and fats under accelerated condition (Velasco et al. 2009).

Procedure: The dry and wet section of the Rancimat instrument were turned on with the air set at 20 l/h and the temperature set at 120 °C one hour before starting the experiment. Spice extracts (100 µl) at different concentrations (1, 10, 50, and 100%) g/l were added to 3.25 g of vegetable oil and mixed well, and then 3.25 ± 0.01 g of the mixture was introduced to the reaction tube and a glass ring inserted into each tube prior to the reaction tube attachments being secured to the tubes with the aid of ground glass joint clamps. When the set temperature was reached the tube were placed into the cavities on the heating block and then connected to the measuring vessels by joint clamps. The reaction tubes were then connected to the air supply and the air rate adjusted to the specific value. The sudden increase of water conductivity indicated the end of induction period (IP) due to dissociation of volatile acids. Vegetable oil without extract added was used as a blank sample. Three replicates of these measurements were carried out.

The antioxidant activity index (AAI) was calculated by the measurement of induction time of blank (pure oil) and oil with spice extracts according to Viuda-Martos et al. (2010) using via the following formula:

$$\text{AAI} = \text{induction period of vegetable oil with spice extracts} / \text{induction period of plain vegetable oil}$$

Results and Discussion

Many chemical and physical parameters could increase the rate of the reaction which causes rancidity of fats and oils, such as temperature, oxygen partial pressure, and metal catalysis. Oxidation of lipids is the most fundamental reaction which can cause deterioration of fats and oils, leading to loss of nutritional value, the development of unpleasant flavors, and consumer rejection of food products. Thus, it is necessary to choose the best conditions to increase the shelf-life of food products. The Rancimat method is an easy, rapid, and common method used by the food industry to determine the antioxidant capacity of lipids; results can be obtained in a few hours instead of weeks and months. An antioxidant activity index higher than one indicates the prevention of lipid oxidation by the spice extracts. The higher the values represent more antioxidant capacity.

The antioxidant activity of seven different spices extracts (ginger, black pepper, clove, cinnamon, cardamom, fennel, and anise) at four different concentrations (1, 10, 50, and 100 g/l) were tested in vegetable oil. Figure 9 illustrate the relationship between antioxidant activity index and spices at different concentrations. Table 19 shows the related antioxidant activity index (AAI) of vegetable oil with spice extracts added. By this method all spices have significantly increased the oxidation time of vegetable oil ($p < 0.05$), among

them ginger exhibited the highest activity at the four different concentrations that is due to diarylheptanoids, zingerone, and gingerol which is isolated from ginger (Kikuzaki et al. 1994), followed by black pepper > clove = cardamom = cinnamon > anise = fennel. Similar results was found for the antioxidant activity of cinnamon by Singh et al. (2004) as well as Bozan et al. (2003) who investigated antioxidant activity of cinnamon extracts in sunflower oil at 100 °C by the Rancimat method. In another investigation, Murcia et al. (2004) studied antioxidant capacity of seven spices (cinnamon, anise, mint, vanilla, nutmeg, ginger, and licorice) compared to common food artificial antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate (PG). They found nutmeg, propyl gallate, ginger, and licorice can delay oxidation of oils (sunflower, corn, and olive) and fats (margarine and butter) by using the Rancimat method. The activity of black pepper may due to piperine, phenolic amide, ascorbic acid, feruperine, falavonoids, and ferulic (Peter 2000) and also biflorin, gallates, its isomer eugenol and eugenyl acetate isolated from cloves (Viuda-Martos et al. 2009) and (Lindberg Madsen and Bertelsen 1995). Furthermore, Ho et al. (1992) studied antioxidant activities in lard by the Rancimat method, and reported that polyphenol extracts from green tea were greater than those from black tea or semi-fermented tea. Generally, the antioxidant properties of spices due to may substance such as falavonoids, carotenoids, minerals, phytoestrogen, terpenoids, polyphnolic compounds, and some vitamins (Viuda-Martos et al. 2009) and (Viuda-Martos et al. 2010). Also Singh et al. (2004) have demonstrated that the water and ethanol extracted of pepper have antioxidant activity. These activities, due to the phenol amide compound and acidic fraction of extracts. Furthermore, Gülçin et al. (2004) have reported that the aqueous and ethanolic clove extracts both show high

antioxidant activity when evaluated by different tests; free radical scavenging, reductive potential, superoxide anion radical scavenging, and metal chelating activities. Heat processing (i.e. frying) could also reduce the levels of natural and added antioxidants in fats, and this seems to be dependent on the saturation level and heating regimes, as studied Barrera-Arellano et al. (2002) for tocopherols and by Gomez-Alonso et al. (2003) for phenolic compounds. The matrix may then play a relevant role on the antioxidant-prooxidant activity, as it may influence interactions of compounds and possibly interfere with reaction kinetics.

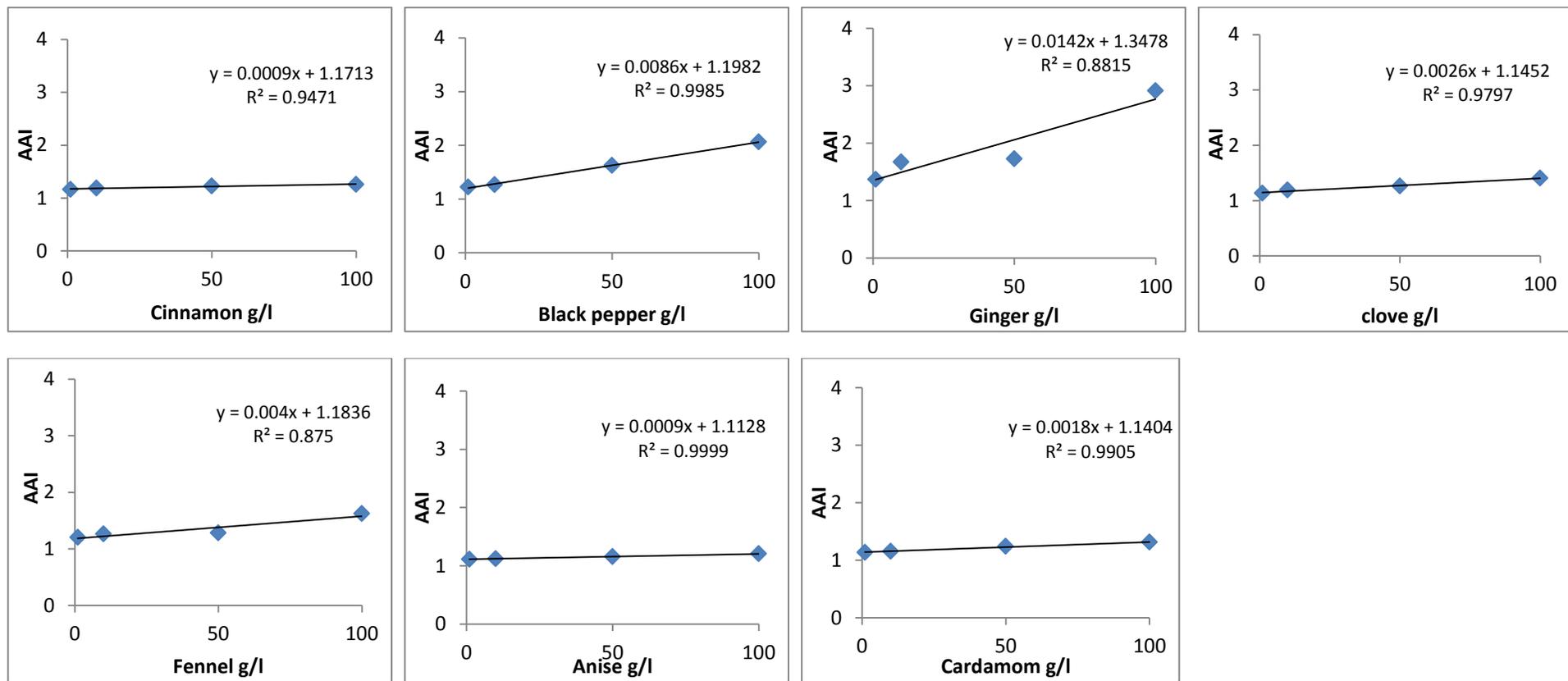


Figure 8: The relationship between the antioxidant activity index (AAI) of seven spice extracts at different concentration in vegetable oil determined with the Rancimat method

Table 14: Antioxidant activity of Fennel, Anise, Cinnamon, Clove, Black pepper, Cardamom, and Ginger essential oils at different concentrations (1, 10, 50, 100 g/l) measured by Rancimant method.

| Spice extract | 1 g/l | 10 g/l | 50 g/l | 100 g/l |
|---------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Fennel ^D | 1 ± 0.02 ^a | 1.1 ± 0.06 ^a | 1.14 ± 0.0 ^a | 1.45 ± 0.1 ^a |
| Anise ^{CD} | 0.1 ± 0.02 ^a | 1 ± 0.03 ^a | 1.05 ± 0.0 ^a | 2.0 ± 0.1 ^a |
| Cinnamon ^{CD} | 1 ± 0.08 ^a | 1 ± 0.02 ^{ab} | 2.0 ± 0.1 ^{bc} | 1.12 ± 0.1 ^c |
| Black pepper ^B | 1.2 ± 0.06 ^a | 1.2 ± 0.03 ^a | 1.6 ± 0.1 ^b | 2.0 ± 0.1 ^b |
| Clove ^C | 1.03 ± 0.1 ^a | 1.1 ± 0.1 ^{ab} | 1.15 ± 0.1 ^b | 1.27 ± 0.1 ^c |
| Cardamom ^C | 1.0 ± 0.06 ^a | 1 ± 0.2 ^{ab} | 1.13 ± 0.1 ^c | 2.0 ± 0.1 ^c |
| Ginger ^A | 1.2 ± 0.1 ^a | 1.5 ± 0.08 ^a | 1.6 ± 0.17 ^a | 2.7 ± 1 ^b |

Values followed by the same capital letter within the same column are not significantly different ($p > 0.05$) according to Tukey's test. Values followed by the same small letter within the same line are not significantly different ($p > 0.05$) when analysed by pairwise Tukey's test 95% confidence.

Phenolic compounds are potent antioxidants in plant extracts. The Folin–Ciocalteu’s method was used to measure the amount of total phenolic content of the spice extracts. The content of total phenols was expressed as gallic acid equivalents. During the reaction process, a problem was observed with fennel and anise extracts due to the formation of turbidity after adding the reagent. This was subsequently solved by preparing a ‘dummy’ Folin–Ciocalteu reagent. Samples were assayed with both Folin–Ciocalteu’s reagent and with the ‘dummy’ reagent. The results were then subtracted to correct for the turbidity.

All extracts were significantly different from each other ($P < 0.05$) according to Tukey’s test 95% confidence. The highest significant results were found in anise extracts in contrast to cinnamon, which showed very low level of phenolics. Total phenolic content of different concentration of extracts fennel, cinnamon, vanilla, anise, ginger, black pepper, and clove are presented in Table 20. In a recent study, vanilla exhibited the highest antioxidant activity among spices (cinnamon, anise, mint, nutmeg, ginger, and licorice) in the peroxidase based assay (Murcia et al. 2004). The main phenolics in anise are quercetin, luteolin and apigenin glycosides whereas cardamom contains phenolic acids such as *p*-coumaric acid, protocatechuic acid, and caffeic acid. It has not been reported that there are any polyphenols in ginger but zingerone and gingerol possess a monophenolic moiety which is why the Folin-Ciocalteu’s reagent gives a reaction (Thomas et al. 2010). Study by Lu et al. (2011) reported the highest level of total phenolic content in galangal and the lowest in white pepper. According to recent reports, a positive relationship between total phenol and antioxidant capacity was found in many plants (Gülçin et al. 2004), (Viuda-Martos et al. 2010), (Hinneburg et al. 2006), (Lu et al. 2011), and (Viuda-Martos et al. 2009).

Table 15: Total phenol content of spice extracts gallic acid equivalent mM at different concentrations (0.01, 0.02, 0.04, 0.06, 0.08 g/l)

| Species extracts | Concentrations g/l | | | | |
|---------------------------|--------------------------|--------------------------|-------------------------|-------------------------|--------------------------|
| | 0.01 | 0.02 | 0.04 | 0.06 | 0.08 |
| Anise ^A | 0.15 ± 0.00 ^a | 0.4 ± 0.00 ^b | 1.6 ± 0.01 ^c | 2.8 ± 0.00 ^d | 4.44 ± 0.05 ^e |
| Black pepper ^B | 0.1 ± 0.00 ^a | 0.2 ± 0.00 ^b | 0.3 ± 0.00 ^c | 0.4 ± 0.02 ^d | 0.60 ± 0.01 ^e |
| Cinnamon ^C | 0.1 ± 0.00 ^a | 0.1 ± 0.00 ^b | 0.2 ± 0.00 ^c | 0.2 ± 0.00 ^d | 0.24 ± 0.00 ^e |
| Cloves ^D | 0.3 ± 0.00 ^a | 0.4 ± 0.00 ^b | 0.7 ± 0.00 ^c | 1.0 ± 0.00 ^d | 1.31 ± 0.00 ^e |
| Fennel ^E | 0.3 ± 0.00 ^a | 0.5 ± 0.00 ^b | 1.2 ± 0.01 ^c | 2.0 ± 2.04 ^d | 3.33 ± 0.01 ^e |
| Ginger ^F | 0.1 ± 0.00 ^a | 0.15 ± 0.00 ^b | 0.2 ± 0.00 ^c | 0.3 ± 0.31 ^d | 0.38 ± 0.00 ^e |
| Vanilla ^G | 0.2 ± 0.00 ^a | 0.2 ± 0.00 ^b | 0.4 ± 0.00 ^c | 0.6 ± 0.6 ^d | 0.68 ± 0.00 ^e |

Values followed by the same capital letter within the same column are not significantly different ($p > 0.05$) according to Tukey's test 95% confidence. Values followed by the same small letter within the same line are not significantly different ($p > 0.05$) when analysed by pairwise Tukey's test 95% confidence.

The radical scavenging capacities of spice extracts determined by the TEAC and DPPH assay which both are involved an electron transfer reaction Table 21, shows the effect of concentrations of each spices required for scavenging radicals and the value of scavenging by using Beer-Lambert law. Ginger displayed significant highest scavenging ($p < 0.05$) followed by black pepper while cinnamon exhibited the lower activity by the TEAC assay. Similar result reported by Viuda-Martos et al. (2010). In contrast, the TEAC assay was used to determine the antioxidant activity of spices and artificial food antioxidant. Cinnamon showed a highest scavenging activity, which approximately equal to propyl gallate, among seven different spices (cinnamon, anise, mint, vanilla, nutmeg, ginger, and licorice), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) (Murcia et al. 2004). Nakatani (1986) identified all phenolic amides from black pepper and reported that they have a potential antioxidant activity. Also showed that feruperine possess a significant antioxidant activity as high as BHA and BHT.

By the DPPH assay, ginger showed significantly high scavenging ($p < 0.05$) and cinnamon had lower scavenging than black pepper. That is associate with the phenolic content, Ginger had higher phenolic content than black peppers, and cinnamon had lower phenolic content than black peppers. Similar studies have reported by Schwarz et al. (2001) and Lu et al. (2011) Galangal and cinnamon showed the highest antioxidant capacity among 19 spices consumed in China, that measured by different assays (DPPH, TEAC, and FRAP), which associate with the highest total phenolic content. Badei Adel et al. (2002) reported that cinnamon extracts and powder have potential antioxidants against peroxidation in cookie fats. Cinnamon and mint were showed inhibition of oxidation as tested by the lipid peroxidation assay (Murcia et al. 2004). In another research by

Kikuzaki et al. (2001) cardamom were fractionate into three fractions the dichloromethane extract, water-soluble fractions, and the ethyl acetate-soluble. Antioxidant capacity and radical scavenging activity of red, yellow, and green peppers examined by using DPPH assay and β -carotene/linoleic acid assay, among them red pepper from methanol extract showed higher level of DPPH radical scavenging activity, while green pepper in the phenolic extracts showed higher (Viuda-Martos et al. 2009). Bonanni et al. (2007) reported rosemary showed highest antioxidant capacity measured by DPPH assay whereas oregano by ORAC test, parsley exhibited the lowest activity in both assays. Many compounds have been isolated in spices. For instance, pimeatol is present in all spices; engenol and gallants in cloves; carnosic acid, carnosol, rosamariquinone and rasmanol in sage and rosemary; Flavonoides, phenolic amides and ascorbic acid in black pepper; gingerol and zingerone in ginger (Thomas et al. 2010).

In addition, tea, clove, rosemary and sage have been reported to decrease lipid oxidation as much as synthetic antioxidant in cooked meat (Kong et al. 2010). Yoo et al. (2008) have investigated 17 herbs and observed high levels of DPPH radical scavenging activity inhibited H_2O_2 –induced oxidative stress, catalase capacity and enhanced superoxide dismutase in the some herbs. Hinneburg et al. (2006) tested nine different spices, among them basil and laurel had the highest antioxidant capacity. Essential oils from herbs and spices despite their use as a greater flavor also contain antioxidants thus, may serve as natural antioxidants in foods. Also, Shan et al. (2007) investigated in vitro antibacterial activity of 46 medicinal herbs and spices, and have found a high positive relationship between the total phenol of the extracts and their antibacterial activity.

In future experiments, it would be better to investigate the effectiveness of the extracts in different food systems. Our results showed that spices extracts can act as an antioxidant, that is needed to be investigated to understand the exact mechanisms.

Conclusion

In a view of four different methods (Rancimat, total phenol content, DPPH, and TEAC) used for spices have an antioxidant activity therefore, using natural antioxidants will continue in the future. It is necessary to study interaction, chemical information, and their changes in more detail. Thus scientist will look for new herbs to investigate antioxidants, which should be the objective of future research. Moreover, total phenol content and antioxidant capacity of spice extracts were different from each other. Among spice extracts clove appeared to have a highest level total phenolic content that is associate with the highest level of phenolic content. Furthermore ginger showed high scavenging activity of ABTS^{•+} radical cation but low total phenol content.

Table 16: Antioxidant capacities of black pepper, ginger, and cinnamon at different concentrations (0.01, 0.02, 0.04, 0.06, 0.08 g/l) determined by TEAC and DPPH assays

| Species extracts | Concentrations | | | | |
|---------------------------|-------------------------|-------------------------|--------------------------|---------------------------|---------------------------|
| | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 |
| TEAC | | | | | |
| Black pepper ^A | 46 ± 5 ^a | 112 ± 8 ^{ab} | 182 ± 66 ^{cb} | 255 ± 17 ^{cd} | 288 ± 4 ^d |
| Ginger ^B | 372 ± 18 ^a | 418 ± 22 ^b | 476 ± 13 ^c | 545 ± 6 ^d | 557 ± 13 ^d |
| Cinnamon ^C | 25 ± 5 ^a | 80 ± 11 ^b | 139 ± 15 ^c | 190 ± 23 ^d | 244 ± 6 ^e |
| DPPH | | | | | |
| Cinnamon ^A | 0.2 ± 0.04 ^a | 0.2 ± 0.04 ^a | 0.33 ± 0.01 ^b | 0.4 ± 0.003 ^{bc} | 0.45 ± 0.003 ^c |
| Ginger ^B | 103 ± 103 ^a | 237 ± 166 ^{ab} | 473 ± 98 ^{cb} | 627 ± 58 ^c | 1028 ± 124 ^d |
| Black pepper ^C | 37 ± 16 ^a | 41 ± 12 ^{ab} | 131 ± 73 ^{ab} | 185 ± 80 ^{bc} | 316 ± 58 ^c |

Values represents as (mean ± SD) that the amount of radical quenched mM and not sharing a capital common letters was significantly different ($p < 0.05$) in a column. Values followed by the same small letter in the same rows were not significantly difference, when analysed by pairwise Tukey's test 95% confidence.

General discussion

Oxidation is the interaction between oxygen molecules and all different substances as they are contact. That causes the loss of electrons. Many biological processes are dependent on oxidation reactions. BHT, BHA, PG, and TBHQ are synthetic antioxidants which are usually used in food industry to prevent oxidation. Thus, during the past two decades tendency towards using natural antioxidants increased instead of synthetic ones. It has been reported that tea has a potential antioxidant capacity and also is a widely consumed beverage in the world next to water which. From the experiments performed it was observed by assays (FRAP, DPPH, CBA, TPC, and TEAC) that the brewed green tea after 2 hours had a potential antioxidant activity and decreased afterwards. It has been reported that the harvesting time can have an effect on the polyphenols (Demeule et al. 2002) and other factors such as soil for tea cultivation, and the exposure of the sun may influence the catechin content of green tea (Kumamoto and Sonda 1998). According to the changes during brewing, catechin (Green tea) can be classified into two groups, EC and EGC are time dependent catechins, and a second group is time/ temperature dependent such as EGCG, ECG, and GCG (Labbé et al. 2006).

Tea preparing methods are different in the world according to countries and traditions. In the UK, tea is usually consumed with milk. In this study, we tested the hypothesis that the addition of milk may have an effect on the antioxidant capacity of green tea. From the results that were obtained by five experiments (FRAP, DPPH, TEAC, TPC, CBA) was found that the milk (Full fat, semi-skimmed, and skimmed) was significantly decreased antioxidant capacity of green tea. From the experiments performance, it is clear that full fat and semi-

skimmed milk had little effect. The big effect was with skimmed milk, this could account for the titration behavior with skimmed milk. Decreasing levels of green tea antioxidant after adding milk is due to the interaction between phenolics in tea and protein in milk covalently or non-covalently, which lead to precipitation of protein. It is either a multisite interaction in which several phenolic bound to one protein molecule, or multidentate interaction, in which one phenolic is bound to several protein molecules. Also, the degree of masking of antioxidant capacity depend on the polyphenol composition and the milk (Sharma et al. 2008) and (Arts et al. 2002). Also, It has been suggested that these interactions involve the formation of hydrogen bond and hydrophobic interaction between protein (carboxyl group) and phenolic compound (hydroxyl group) (Spencer et al. 1988).

There is a growing demand of plant-derived food additives in the human diet due to the negative effect of synthetic food additives on human health. Spices are natural plant products which have been used as flavoring and folk medicines for a thousand of years. On the basis of the results of the present study in vitro, it is clear that plant extracts have a potential antioxidant activity in vitro that is due to phenolic content.

Saliva is the first biological medium which can be used to interact with external materials that are taken by humans. The most interesting finding of the current study was the demonstration of measuring antioxidant activity of saliva in vivo after digestion of green tea and the effect of milk on green tea. From the results that we obtained the consumption of a cup of green tea has increased salivary antioxidant capacity and after 1 hour has reached a peak value and then decreased to the baseline. In summary, the results of antioxidant capacity in

human saliva depends on the method employed as well as the condition of the assay. Thus saliva could be used as a new way to measure antioxidant capacity and it is much easier to obtain than the blood. In summary, our results indicated that milk in vitro and in vivo significantly decrease antioxidant capacity of green tea. This needs more research to understand the mechanisms.

General conclusion

Green tea has been reported to be a rich source of antioxidants. This study has highlighted a number of parameters such as matrix (milk with different level of fats), infusion time, and concentration which may affect the antioxidant capacity of green tea by using DPPH, TEAC, TPC, FRAP, and CBA. Overall antioxidant potential of green tea infusion reached peak after two hours, and the addition of milk significantly decreased the antioxidant capacity of green tea. However, this effect was much greater with skimmed milk if compared to full fat milk.

The results obtained from determination of oxidative rancidity (stability index), total phenolic content, DPPH and TEAC assays to evaluate antioxidant capacity, showed that the spice extracts (fennel, clove, cinnamon, cardamom, ginger, black pepper, and anise) can be used as a good source of natural antioxidants.

In vivo tests indicate that saliva could be used as an alternative way to measure antioxidant capacity in humans.

Future work

The following work could be carried out as a direct continuation of this study:

1. The effect of different matrices on antioxidant capacity of green tea, for example, fat quantity and emulsion types.
2. To study the salivary antioxidant capacity of milk after ingestion of different types of milk with different fat levels.
3. Salivary antioxidant capacity kinetics in vivo, after ingestion of plain green tea.
4. Study the effect of matrix to optimize antioxidant effect
5. Study interaction of green tea with spices after ingestion of a single cup of in human fluids (plasma, serum, and saliva).

Appendices

Table 17: Total phenolic content GAE mM of green tea and green tea mix with milk (different fat levels) at different concentrations (n = 6 independent experiments)

| Tea samples | Concentrations g/l | | | | |
|-------------------|--------------------|------------|-------------|------------|-------------|
| | 1 | 2 | 4 | 6 | 8 |
| GT ^A | 7.9 ± 0.2 | 14.1 ± 0.2 | 26.40 ± 0.9 | 38.9 ± 1.5 | 48.44 ± 1.6 |
| FFM ^A | 8.1 ± 0.4 | 14.3 ± 0.6 | 26.93 ± 1.5 | 38.3 ± 1.1 | 47.75 ± 2 |
| SSKM ^B | 5.4 ± 0.3 | 13.2 ± 0.5 | 25.47 ± 1.1 | 37 ± 2.1 | 47.58 ± 2.1 |
| SKM ^C | 4.3 ± 1.6 | 6.7 ± 1 | 12.31 ± 2 | 23.2 ± 3.3 | 37.18 ± 1.8 |

Results showed as Mean ± StDv of Gallic acid mM equivalent (GAE). Tea samples followed by the same letter in the same column did not show statistically significant difference (p > 0.05) by Tukey's test 95% confidence. Gt is plain green tea, FFM is green tea mixed with green tea, SSKM is green tea mixed with semi-skimmed milk, and SKM is green tea with skimmed milk.

Table 18: Scavenging activity of plain green tea, green tea mix with full fat milk, semi-skimmed milk and skimmed milk measured by TEAC assay. (n = 6 independent experiments)

| Tea samples | Concentration (g/l) | | | | |
|-------------------|---------------------|----------|----------|---------|----------|
| | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 |
| GT ^A | 57 ± 11 | 153 ± 11 | 313 ± 49 | 429 ± 3 | 509 ± 19 |
| FFM ^B | 75 ± 11 | 127 ± 18 | 223 ± 2 | 282 ± 3 | 360 ± 14 |
| SSKM ^C | 52 ± 4 | 99 ± 18 | 192 ± 10 | 244 ± 2 | 309 ± 12 |
| SKM ^C | 41 ± 9 | 93 ± 19 | 185 ± 8 | 237 ± 2 | 294 ± 10 |

Results shown as a (mean ± SD) which were ABTS radical cation quenched mM. Concentrations were significant different. Tea samples followed by the different letter in the same column showed statistically significant difference (p < 0.05) using Tukey's test 95% confidence. GT is plain green tea, FFM is green tea with full fat milk, SSKM is green tea with semi-skimmed milk, SKM is green tea with skimmed milk.

Table 19: Scavenging activity of plain green tea and green tea with adding 4 ml of full fat, semi-skimmed , and skimmed milk in different concentration measured by DPPH assay. (n = 6 independent experiments)

| Tea samples | Concentrations g/l | | | | |
|-------------------|--------------------|----------|----------|-----------|-----------|
| | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 |
| GT ^A | 259 ± 40 | 516 ± 29 | 874 ± 82 | 1186 ± 48 | 1397 ± 52 |
| FFM ^B | 181 ± 32 | 318 ± 24 | 634 ± 42 | 869 ± 33 | 1094 ± 68 |
| SSKM ^C | 202 ± 23 | 333 ± 20 | 558 ± 50 | 749 ± 44 | 930 ± 56 |
| SKM ^D | 74 ± 21 | 151 ± 23 | 348 ± 30 | 560 ± 35 | 746 ± 16 |

Results presented as mean ± SD of DPPH radical quenched mM. Tea samples followed by the same letter in the same column did not show statistically significant difference ($p > 0.05$) in Tukey's test 95% confidence. Statistically shown activity were significantly increase by increasing concentration. GT is plain green tea, FFM is green tea with full fat milk, SSKM is green tea with semi-skimmed milk, SKM is green tea with skimmed milk.

Table 20: antioxidant activity of plain green tea and green tea with adding of whole milk, semi skimmed, and skimmed milk measured by FRAP assay. (n = 6 independent experiments)

| Tea samples | Concentrations (g/l) | | | | |
|-------------------|----------------------|----------|----------|------------|----------|
| | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 |
| GT ^A | 14 ± 2 | 23.4 ± 1 | 41 ± 1 | 60.5 ± 0.8 | 70.5 ± 4 |
| FFM ^B | 12 ± 1 | 23.7 ± 4 | 37.5 ± 2 | 55.6 ± 5 | 66.9 ± 5 |
| SSKM ^C | 8.6 ± 3 | 17 ± 2.5 | 31.3 ± 3 | 50 ± 6 | 65 ± 12 |
| SKM ^C | 8.7 ± 0.5 | 16.7 ± 1 | 27 ± 4 | 46.4 ± 3 | 58 ± 4 |

Results presented as mean ± SD as Iron sulphate equivalent mM. Tea samples followed by the same letter in the same column did not show statistically significant difference ($p > 0.05$) analysed by Tukey's test 95% confidence. GT is plain green tea, FFM is green tea with full fat milk, SSKM is green tea with semi-skimmed milk, SKM is green tea with skimmed milk.

Table 21: antioxidant capacity of green tea and green tea with full fat milk, semiskimmed milk, and skimmed milk determined by crocin bleaching assay (n = 6 independent experiments).

| Tea samples | Concentrations g/l | | | |
|-------------------|--------------------|----------|----------|----------|
| | 0.2 | 0.4 | 0.6 | 0.8 |
| GT ^A | 699 ± 75 | 801 ± 41 | 841 ± 15 | 867 ± 27 |
| FFM ^B | 247 ± 126 | 458 ± 94 | 551 ± 76 | 561 ± 52 |
| SSKM ^C | 149 ± 36 | 378 ± 61 | 523 ± 53 | 540 ± 24 |
| SKM ^D | 117 ± 19 | 336 ± 14 | 392 ± 9 | 402 ± 6 |

Results presented as mean ± SD of antioxidant unit/l. Tea samples that do not share the same letter in the same column are significantly different according to Tukey's test 95% confidence. GT is plain green tea, FFM is green tea with full fat milk, SSKM is green tea with semi-skimmed milk, SKM is green tea with skimmed milk

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