Effect of green tea extract in reducing the formation of acrylamide from bread baking process

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, ‘Effect of green tea extract in reducing the formation of acrylamide from bread baking process’, contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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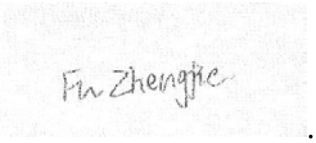
Signed: ……  …….

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ANOVA Analysis of Variance

DPPH 2,2-diphenyl-1-picryl-hydrazyl

EC (−) epicatechin

ECG (−) epicatechin-3-gallate

EGC (−) epigallocatechin

EGCG (−) epigallocatechin-3-gallate

FAO the Food and Agriculture Organization

GC (+) gallocatechin

GC-MS Gas Chromatography Mass Spectrometry

GI Glycaemic index

GTE Green Tea Extract

HPLC-UV High-Performance Liquid Chromatography with UV/Visible Detector

IARC the International Agency for Research on Cancer

LC-MS Liquid Chromatography Tandem Mass Spectrometry

LOAEL Lowest Observed Adverse Effect Level

NOAEL No Observed Adverse Effect Level

ROS Reactive Oxygen Species

SD Standard Deviation

SEM Scanning Electron Microscope

TEAC Trolox Equivalent Antioxidant Capacity

TPA Texture Profile Analysis

TRC Toronto Research Chemicals

USDA United States Department of Agriculture

WHO the World Health Organization

Abstract

Bread is one of the most famous foods worldwide. It is consumed as a staple food for many Western countries and contains high content of carbohydrates. Nowadays, an increasing number of people prefer healthy foods. Tea antioxidants have drawn increased attention in recent years because of their potential health benefits, not only as an antioxidant agent but also as antiarteriosclerotic, anticarcinogenic, and antimicrobial agents. In this project, a new bread product with added green tea extract (GTE) was developed with reduced acrylamide content. The objectives of this research were to investigate the effect of GTE addition on reducing acrylamide formation in white bread loaf and whether GTE addition has any effect on changing the physicochemical properties of the bread. My hypothesis was that the fortification of bread with GTE will reduce the acrylamide content with improved physicochemical property. In my study, bread formulations with GTEs at 3.3 g, 6.6 g and 9.9 g per kg of flour were developed. All of the fortified bread samples showed significant decrease in acrylamide content and moisture content with the addition of GTE. The acrylamide content has been reduced from 52.19 $\mu g.L^{-1}$ to 32.69 $\mu g.L^{-1}$. There was no significant difference between the control and the GTE added samples except cohesiveness from texture analysis. The crust of the GTE added bread was significantly increased ($p < 0.05$) in lightness L* (53.92 to 65.85) and yellowness b* (25.64 to 27.12), while decreased in redness a* (9.36 to 9.14). Contrasting result was found in crumb according to the L*, a*, b* colour system.

Overall, the results showed that the incorporation of GTE inhibited the acrylamide formation and changed the physicochemical properties of bread. The outcome of the

present study will have great health implication on the use of GTE to develop functional foods with anti-cancer potential and be helpful to the development of healthier food products in the future.

1 Introduction

Bread, as a traditional staple food of Western countries, has become increasingly popular worldwide (Cauvain, 2003). The crust of bread is usually golden brown with white crumbs. In 2002, the University of Stockholm and the National Food Administration reported that acrylamide is produced when starchy foods (e.g. potatoes) were cooked at high temperature. Later, the International Agency for Research on Cancer (IARC) classified acrylamide as 'probably carcinogenic to humans' at same year and reported that it will cause damage to the nervous system when exposed to high levels. Numerous research activities concerning the methods of analysing acrylamide, origin of acrylamide in foods, its health risk to humans, and mitigation of acrylamide in food have been published (Claus, Carle, & Schieber, 2008)

GTE contains lots of antioxidants. One of the important features of GTE is that it can inhibit angiogenesis and tumour cell invasiveness to a certain extent, which demonstrates its cancer preventative property. The aim of this research was to explore whether GTE bread could inhibit the formation of acrylamide during the baking of bread. The objective of this project was to study the effects of GTE on the physicochemical property of fortified breads and thereby help to develop a healthy bread product with low acrylamide content.

This thesis is composed of five parts. Chapter two is a literature review summarising the past studies of acrylamide and fortified breads including their physicochemical properties. The materials and methodology used in the project, including the experimental design and statistical data analysis are shown in Chapter three. In Chapter four, results obtained from the study are presented and discussed. The conclusion of this project, including the main findings and suggestions for future related research, is summarised in Chapter five.

2 Literature Review

In this chapter, a brief introduction of bread (2.1), fortification of bread (2.2) and Green tea extract (2.3) are reviewed. The toxic effect and formation of acrylamide (2.4 to 2.5) are reviewed. The method to reducing the acrylamide in bread (2.6) and analysing the acrylamide (2.7) were also reviewed.

2.1 Bread

Bread is one of the staple foods for the world population (Cauvain, 2003). Due to its ease of preparation, low cost and high nutritional values, bread is well accepted worldwide. Bread is usually made by kneading wheat-flour into dough and baking in an oven with high temperature. Bread not only provides daily energy for humans, but also plays a critical role in supplying nutrients for the human body. It is rich in carbohydrates and a good source of wheat proteins (e.g. gluten), amino acids (lysine), vitamins (particularly group B) and minerals, such as magnesium, calcium and iron (Isserliyska, Karadjov, & Angelov, 2001). Across different cultures, bread differs in appearance, taste, texture, ingredients and processing methods. For example, Baguette for France, Hot cross buns for England, Pretzel for Germany, Danish bread for Demark and so on. According to United States Department of Agriculture (USDA, 2014), one slice (25g) of commercial white bread loaf contains: 1 g of fat, 15 g of carbohydrate, 2 g of protein and high content of sodium (170mg). Bread loaf can be structurally divided into two parts, crust and crumb. The crust formed on the surface of the dough is from

baking, and it is darker in colour with hard and crisp texture depending on the type of bread and the way it is baked. The commercial bread is baked using jets that direct steam towards the surface of the bread to help produce a desirable crust. The white bread loaf is classified to be high in glycaemic index (GI), which typically lies in 79 ± 5 range according to the Brand Miller classification (Hettiaratchi, Ekanayake, & Welihinda, 2009).

The term ‘bread’ in this thesis refers to ‘white bread loaf’, which is typically made with high grade flour from wheat, water, salt, sugar, shortening and dry yeast.

2.2 Fortification of bread

The word “fortification” was firstly defined by the Codex Alimentarius Commission (1987) as the addition of one or more essential nutrients to a food for the purpose of preventing or correcting the deficiency of one or more nutrients. Examples of micronutrients added to wheat flour include iron to reduce the iron-deficiency anaemia, thiamine to reduce beriberi, and niacin to reduce pellagra (Backstrand, 2002). Bread, as a traditional staple food, provides a significant portion of nutrients required for energy, metabolism and well-being. However, with the increasing demand of healthy and functional foods, improvements in the nutritional value of bread have been attempted with the addition of extra ingredients. These are summarised in Table 1.

Table 1. A table summarising value-added breads and the changes in physicochemical and nutritional properties.

Type	Added ingredients	Physicochemical properties	Nutritional properties	References
White bread	14,16 and 18% Sesame seed protein	Loaf volume decreased from 250 ml to 233 ml.	Protein content increased from 12.4% to 18.6% Minerals content marked increased (Cu, Zn, Fe, Mn, Mg, Na, Ca, K, P). Total essential amino acids content increased from 35.2 to 37.5 per 16 g nitrogen.	(El-Adawy, 1995)
White bread	0,5,10, and 15% Matured soursop flour	Moisture content increased from 26.65% to 32.49% Hardness increased from 446 g to 889 g	Increase in protein content from 4.39% to 11.68%. Decrease in fat content from 5.63% to 4.14%.	(Zabidi & Yunus, 2014)
White flat bread	5, 15 and 25% Sprouted red kidney bean	N/A	1% increase in protein content and 12% decrease in protein digestibility was seen in 25% formula	(Viswanathan & Ho, 2014)
White pan bread	0-5g/100g omega-3; 0-0.1g/100g rosemary extract	Firmness increased from 5.5 N to 12.32 N Lightness (L*) decreased from 80.84 to 78.11	N/A	(de Conto, Oliveira, Martin, Chang, & Steel, 2012)
White bread	3, 6% pectin; 3% pectin and fruit phenolic extract	Dough weight increased from 848 g to 876g Moisture content increased from 31.0% to 39.3% Up to 50% of colour variation (∆E) was found	Antioxidant activity increased from 0 to 8 $mg.kg^{-1}$, detected by Folin-Ciocalteu assays	(Sivam et al., 2011)
White bread	1,3,5, and 7%	Firmness increased from 5.0 N to 9.0 N.	Antioxidant content increased by more than 50% compared to the control by DPPH (2,2-diphenyl-1-picryl-	(Das, Raychaudhuri, & Chakraborty,

Type	Added ingredients	Physicochemical properties	Nutritional properties	References
	coriander leaf powder	Crumb moisture increased from 39.0%to 40.5%.	hydrazyl) radical scavenging assay research	2012)
White Bread	300mg,600mg,1g grape seed extract	Lightness decreased from 71.35 to 62.13. Redness increased -0.58 to 3.39. Yellowness increased from 17.19 to 20.15. No significant difference in hardness.	Stronger antioxidant activity (from 1 to 7 nmol.mg^{-1} bread was detected by trolox equivalent antioxidant capacity (TEAC) assay	(Peng et al., 2010)
White Bread	0, 0.3, 0.4, 0.5 and 0.6% guava protein isolate	Hardness decreased from 10.2 N to 2.20 N. Cohesiveness decreased from 0.53 to 0.41. Lightness (L*) increased from 62.65 to 67.41.	Protein content did not increase when 0.3% protein isolate was added	(Perez-Rocha et al., 2015)
Wheat Bread	15, 17, 19, 21% pumpkin seed products (raw, roasted, autoclaved, germinated, fermented, pumpkin protein concentrate and pumpkin protein isolate)	Moisture content increased from 9.60% to 10.31%.	Protein content increased from 13.50% to 21.19% Total essential amino acid content increased from 35.28 g.16 g nitrogen^{-1} to 40.82 g.16 g nitrogen^{-1} Protein digestibility increased from 72.51 to 81.72.	(El-Soukkary, 2001)
Wheat Bread	5, 10, 15 and 20% Tilapia Fish Protein Flour	Moisture content increased from 25% to 29.1%. No significant difference in sensory result.	Protein content increased from 9.08% to 18.01%.	(Adeleke & Odedeji, 2010)

From Table 1, it can be seen that the most of value-added breads were designed for increasing the nutrition value of bread, particularly protein and total essential amino acid contents. Some of those products were acceptable for consumers as confirmed by sensory test. El-Soukkary (2001) reported that bread with different pumpkin products has not changed the acceptability of bread of its flavour and colour. The addition of 20% tilapia fish protein flour in bread also showed no significant difference in sensory attributes by Friedman and Levin (2008). Peng et al. (2010) reported that bread with different levels of grape seed extract added did not make significant alteration in quality attributes (sweetness, porosity, astringency and stickiness). However, all of the breads with added antioxidant or phenolic compounds have shown strong antioxidant activity. It was reported that the main characteristic of antioxidants is trapping the free radicals. The free radicals may oxidize the proteins, lipid or DNA and initiate degenerative disease such as heart disease. Hence, increasing the antioxidant activity in food is good for health.

Research from (Sivam et al., 2011) has shown that thermal processing can decrease the antioxidant activity of the phenolic compounds and antioxidant added to bread while Peng et al. (2010) reported greatly enhanced total antioxidant capacity of bread when antioxidant and phenolic compounds were added as additive. Peng et al. (2010) pointed out that there was no significant difference in hardness of bread samples with or without the addition of grape seed extract. They also tested colour for their bread sample and reported that the lightness of bread decreased from 71.35 to 62.13 with grape seed extract has been largely changed. (refer to Table 1). Das et al. (2012) found about 50% increase in antioxidant content in coriander leaf powder bread with higher moisture and firmness. These findings have confirmed that the addition of antioxidants to bread

results in greater antioxidant capacity regardless of thermal processing and resulted in a darker crumb colour (depend on the type of antioxidant). In the current project, green tea extract containing antioxidant was chosen as antioxidant to be added to bread. From the literature, it was hypothesised that green tea extract would help to increase the antioxidant activity of bread.

2.3 Teavigo (Green Tea Extract)

Teavigo (DSM, 5009227) is a caffeine-free, highly purified extract from the leaves of green tea (*Camellia sinensis*). It is a fine, off-white to pale pink coloured powder, and composed of a minimum of 94% EGCG. EGCG is the major polyphenolic catechin in green tea which accounts for 50 to 80%. The beneficial effects of green tea are attributed to polyphenolic compounds in green tea, particularly catechins, which make up 30% of the dry weight of green tea leaves. There are several polyphenolic catechins in green tea: (−)epicatechin (EC), (−) epicatechin-3-gallate (ECG), (−) epigallocatechin (EGC), (−) epigallocatechin-3-gallate (EGCG), (+)catechin, and (+) gallocatechin (GC) (Zaveri, 2006). 200 to 300 mg of EGCG is present per brewed cup of green tea. However, different types of catechins have different effects. The most common GTE usually is a brown coloured powder and contains 10 to 60% EGCG, about 30% other catechins such as ECG and 2 to 5% caffeine. Compared with the common GTE, Teavigo GTE has higher amount of content active substance (EGCG) and it is free of caffeine. The structure of EGCG is shown in Figure 1. EGCG has three aromatic rings linked with a pyran ring. Since it contains 8 hydroxyl groups on its aromatic rings, the transfer of hydrogen atom or single-electron transfer reactions can easily occur and enable EGCG to possess strong antioxidant activity (Min & Kwon, 2014).

Historically, green tea has always been consumed by Japanese and Chinese for centuries, and it is probably the most consumed beverage besides water (Zaveri, 2006). Green tea has attracted great attention in regards to the benefits in health, from weight loss to cancer (Zaveri, 2006; Zhao Liping & Wanfang, 2007). Recently, the trend of fortification of bread using GTE has risen (Table 2). As shown in Table 2, Sharma and Zhou (2011) have shown that EGCG has high retention levels of about 83% in biscuit

and bread after baking which means EGCG are relative stable in bread and biscuit. Lu, Lee, Mau, and Lin (2010) reported that the GTE could strongly enhance the antioxidant activity. These findings have confirmed that the GTE could be used as a useful antioxidant in food system with high recovery. In addition, the colour of food product with the addition of GTE has significantly changed compared with the control. The lightness and redness of all bread samples were largely decreased with the addition of GTE. The hardness of bread with 5% $g.kg^{-1}$ GTE increased while no difference was seen in bread with 2 g of microencapsulated GTE power. However, the sensory results of these GTE fortified bread have been reported to be unsatisfactory with most of the GTE bread showing lower degree of sweetness due to the bitterness arising from normal GTE. To date, application of GTE fortification in bread remains largely unexplored. As mentioned in section 2.4, the α-amylase and glucosidase contribute to the formation of acrylamide since they contains carbonyl group. Goh et al. (2015) investigated *in vitro* digestibility of GTE-fortified baked and steamed bread and pointed out there is an inhibitory effect by catechins on the enzymatic activity of pancreatic α-amylase and glucosidase during the digestion. Hence, it could confirm the catechins could actually indirectly inhibit the acrylamide formation. Pasrija, Ezhilarasi, Indrani, and Anandharamakrishnan (2015) tried to improve the bread quality, in terms of hardness and flavour, by adding microencapsulated green tea polyphenols in dried or freeze powder form. Moreover, they showed that the fortification of bread with GTE can improve the quality characteristics of bread along with the functionality, for example, enhancement in taste and antioxidant activity on bread. Similar results were found by Wang, Zhou, and Isabelle (2007). They tested the texture and sensory property of the GTE fortified bread, and demonstrated that the brightness and sweetness decreased with the addition of GTE, whereas the hardness (2.40 to 3.35), stickiness (2.02 to 2.82) and astringency (0.24 to 1.19) increased.

Animal studies have proved that EGCG is the strongest antioxidant compound in catechins. The antioxidant activity of EGCG is 20 times stronger than that of the Vitamins E and 6 times stronger than superoxide dismutase. The most recent anticancer studies have shown that EGCG could exert anticancer activity when combined with conventional therapies. Also, its protective role against cancer therapy side effects has been proposed, Lecumberri, Dupertuis, Miralbell, and Pichard (2013) reviewed the effect of EGCG as adjuvant in cancer therapy and pointed out that the use of EGCG could enhance the effect of conventional cancer therapies through additive, synergistic effects or through the amelioration of deleterious side effects such as immunosuppression and myelosuppression.

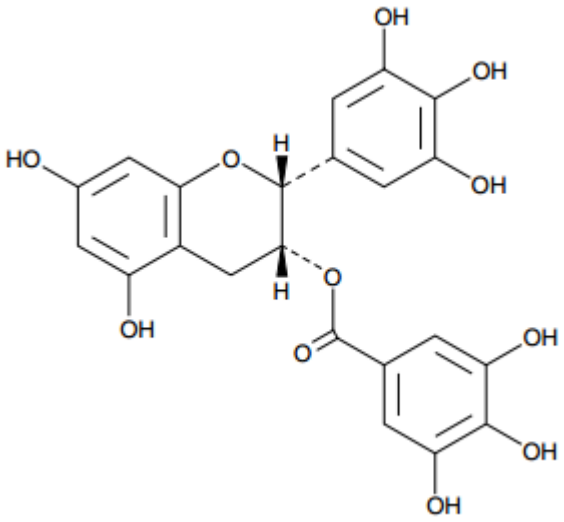

Figure 1. The structure of EGCG (Sigma-Aldrich, 50299)

Many scholars have shown that EGCG has beneficial and preventable effects of EGCG on anti-aging (Esposito et al., 2002), cardiovascular or neurodegenerative disease (Higdon & Frei, 2003), anti-HIV (Nance & Shearer, 2003) Parkinson's disease, Alzheimer's disease (Mandel & Youdim, 2004) and type 2 diabetes (Wu et al., 2004).

EGCG can inhibit tumour incidence and multiplicity in different organ sites such as liver, stomach, skin, lung, mammary gland and colon by altering the expression of cell cycle regulatory proteins, activating killer caspases, and suppressing oncogenic transcription factors and pluripotency maintaining factors. When combined with conventional therapies, the main function of EGCG in therapies has been as a chemo-sensitizer and a radio-sensitizer. The cancer-preventive effects of EGCG are widely supported by results from cell culture, clinical studies (Singh, Shankar, & Srivastava, 2011), epidemiological and animals studies (Minxing & Yaling, 2013).

Although the anti-cancer mechanism of EGCG is complex and various, recent studies have gradually changed direction from inducing apoptosis in cancer cells, blocking their cycles and inhibiting their proliferation of the enzymes associated with cell apoptosis and its mRNA expression and signalling pathways (Minxing & Yaling, 2013).

Similarly, Min and Kwon (2014) have shown that EGCG promotes anticancer effects by modulation of multiple processes including: inhibition of carcinogen activity, tumorigenesis, proliferation, and angiogenesis, and induction of cell death. They proposed that EGCG is associated with modulation of reactive oxygen species (ROS) production. Although EGCG has a dual function of antioxidant and pro-oxidant potential, EGCG-mediated modulation of ROS production is reported to be responsible for its anticancer effects. This conclusion is widely supported by (Lambert & Elias, 2010; Muzolf-Panek et al., 2008; Yang et al., 2000) which used in clinical treatment against cancer cell line. Zhao & Shao (2007) pointed out that EGCG has multiple phenolic hydroxyl groups which can provide protons and remove the lipid-free radicals effectively. They also clarified that it can break off the chain reaction of lipid oxidation, and prevent lipid peroxidation. The recent pre-clinical anticancer studies have shown

that EGCG, as the major phenolic compound in green tea, could exert anticancer activity when combined with conventional cancer therapies. Also, its protective role against cancer therapy side-effects such as immunosuppression and myelosuppression has been proposed (Singh et al., 2011). Until now, the clinical studies examining the effect of fortification using of GTE studies are still scarce. Some studies described that the anticancer effect of EGCG may be inhibited when used together with other anticancer drugs, such as Bortezomib and nelfinavir (Golden et al., 2009).

Overall, it has been confirmed that the EGCG in GTE could be used as either an antioxidant in food to enhance the antioxidant activity or an adjuvant of anticancer drug enhance the effect of conventional cancer therapies.

Table 2. A table showing the effects of GTE fortification in food

	Addition of GTE	Change of colour and texture	Other effects	References
Stability of GTE Bread	50, 100, and 150 mg per 100 g of flour	Lightness decreased to 7% in 150mg per 100 g of flour formula.	Retention levels of EGCG were from 83%.to 86%	Wang and Zhou (2004)
GTE Bread	1.5g and 5g/kg	Lightness decreased from 77.41 to 63.99 Yellowness decreased from 12.78 to 10.54 respectively, Redness increased from -0.81 to 2.18. Hardness increased from 2.40 to 3.35.	Sweetness intensity decreased from 2.24 to 1.70 Astringency intensity increased from 1.24 to 1.83	Wang et al. (2007)
GTE Sponge cake	substituted 10%, 20%, and 30% of wheat flour	Hardness increased from 173.7-327.3. Brightness decreased from 71.32 to 35.71. Yellowness decreased from 18.09 to 15.45. Redness increased from -1.18 to 2.39.	No significant difference in moisture content. Antioxidant activity increased, the EC_{50} value was decreased from 14.83 to 8.89. Sweetness results decreased from 5.8 to 4.7 out of 10	Lu et al. (2010)
GTE Biscuit	150, 200, and 300 mg per 100 g of flour	N/A	Relative stability of green tea catechins in bread was ranked as caffeine, CG (catechin gallate) > EC > ECG > GCG > EGCG > EGC.	Sharma & Zhou, (2011)
GTE Bread	2 g of microencapsulated powder (with 60%-76% EGCG) in 100 g of wheat flour	Lightness decreased from 65.7 to 50.7. Yellowness increased from 12.21 to 14.4. Redness increased from -0.11 to 3.91 No significant difference in texture parameters.	Increased 30% of moisture content Taste results decreased from 8.8out of 10 to 7 out of 10	Pasrija et al. (2015)
GTE Bread	0.45%, 1%, and 2%	N/A	Decrease in readily digestible starch from 8.3% to 14.9% for crust and 5.4% to 14.8% for crumb. 2% GTE reduce the glycaemic response from 530 mg/g to 400 mg/g	Goh et al.(2015)

Note: EC50 value: the effective concentration at which the antioxidant activity reached 50%.

2.4 Acrylamide and its toxic effect in human health

Acrylamide is an unsaturated amide which is white, odourless crystalline solid toxic chemical compound with Rat Oral LD50 of 170mg.kg^{-1} (Control & Prevention, 2012). It has molecular weight of 71.1g.mol^{-1}. The boiling point and melting point of acrylamide is 192.6 °C and 84.5 °C respectively. It is readily soluble in water, 2155 g.L^{-1} at 30°C, and polar solvents such as acetone, methanol, and ethanol but soluble not in non-polar solvents. Acrylamide is usually used in the production of polyacrylamides, which is used in water treatment, paper processing and electrophoretic separations in bioscience fields. About 0.2% of monomers of acrylamide are used in food packaging adhesives, paper, and paperboard; to wash or peel fruits and vegetables. (Cheng, Kao, Shih, Chou, & Yeh, 2009). Acrylamide is used in the synthesis of dyes, in co-polymers for contact lenses, and in the construction of dam foundations, tunnels, and sewers (Habermann, 2002). It is stable under room temperature and pressure but may decompose or polymerize when heated or exposed to ultraviolet light. It contains an a,b-unsaturated amide system that reacts with nucleophilic compounds via a Michael addition. The major site of reaction is sulfhydryl groups contained in proteins and amino acids. Formation of acrylamide is further reviewed in section 2.5.

The International Agency for Research on Cancer (IARC) classified acrylamide as group 2A ‘probably carcinogenic to humans’ and indicated that it will cause damage to the nervous system when exposure to high levels (NOAEL 0.5 mg.kg^{-1} body weight per day). The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have also warned that the unintentional acrylamide in certain foods might be of public health concern as it has been shown to cause cancer in animals

(Cheng et al., 2009). Acrylamide can be absorbed through the digestive tract from consumption of foods, through the respiratory tract by inhaling of acrylamide and through the mucous membrane in the skin by a physical contact (Ping, 2011).

Claus, Mongili, Weisz, Schieber, and Carle (2008) studied the impact of formulation and technological factors of acrylamide content in wheat bread and bread rolls and pointed out that the formulation and processing technology can strongly influence the acrylamide content in the baked products. Estimated daily intake of acrylamide through foods ranges from 0.5 to 0.6 $\mu g.kg^{-1}$ body weight per day for adults and 0.4 to 1.26 $\mu g.kg^{-1}$ for children and adolescents (Wilson, Rimm, Thompson, & Mucci, 2006). Table 3 shows the acrylamide content in some food products (Ahmad,2007). As shown in Table 3, potato crisps have the highest content of acrylamide (752 $\mu g.kg^{-1}$). This is expected since potatoes contain large amount of starch and are processed by frying in high temperature. The concentration of acrylamide in bread varies and depends on the type of bread. The mean concentration of breads and rolls is 446 $\mu g.kg^{-1}$, which was calculated from taking an average of 192 samples, collected from 24 different countries. Negoita et al. (2016) reported that the concentration of acrylamide in bread ranged from 5 $\mu g.kg^{-1}$ and 1,987 $\mu g.kg^{-1}$.

Table 3. A table showing the quantity of acrylamide in selected food products (Ahmad, 2007).

Food Source	**Mean concentration of acrylamide found ($\mu g.kg^{-1}$)**
Breads and rolls	446
Breakfast cereals	96
Cereal-based processed products (all)	366
Cereals and cereal-based products	343
Cereals and pasta (processed: toasted, fried, grilled)	123
Cereals and pasta (raw and boiled)	15
Coffee (brewed), ready-to-drink	13
Coffee (ground, instant or roasted, not brewed)	288
Pastry and biscuits (cookies)	350
Pizza	33
Potato baked	169
Potato chips (French fries)	334
Potato chips, croquettes (frozen, not ready-to-serve)	110
Potato crisps (chips)	752
Potato purees/mashed/boiled	16

(Friedman, 2003) reported that two main hazard effects of acrylamide on health are carcinogenicity and neurotoxicity. However, the neurotoxicity effect will only occur at a very high level (Lowest-observed-adverse-effect level (LOAEL) of 2 $mg.kg^{-1}$ body weight per day), which cannot be reached by dietary intake from food. (Friedman, 2003) also reported that the carcinogenic and genotoxic effects of acrylamide were found in *in vivo* and *in vitro* studies. Bull et al. (1984) reported that acrylamide is able to cause skin cancer in mice, and promote the development of lung adenoma. Similar results were seen by others. (Rice, 2005) reported that the tumours were observed from mice exposed to a high level (75 $mg.kg^{-1}$) of acrylamide. Woo et al. (2007) indicated that the water containing 0.02% acrylamide on rats with neural toxicity, and can cause the scrotum.

Paulsson et al (2005) have reported the influence of polymorphic enzymes in acrylamide and its metabolite glycidamide and reported that acrylamide would not effect on the blood dose with the addition of ethacrynic acid or laurylamine. Dybing et al. (2005) reported that 0.06% of people can develop cancer due to the intake of acrylamide rich foods. K. M. Wilson et al. (2006) reported that acrylamide and glycidamide induce chromosomal breaks and point mutations, which contributes to cancer.

Michels, Rosner, Chumlea, Colditz, and Willett (2006) found that the people who eat French fries once a week during their childhood have 1.27 of relative risk (RR) value on breast cancer. In addition, Pelucchi et al. (2003) indicated that the people who seldom have fried potatoes have a lower risk of breast cancer. This research has been confirmed by Wilson et al. (2009). They also reported that the fried potatoes contributed to about 10% of total dietary acrylamide intake. Based on the review of relevant literature for both human and animal studies by Wang and He (2011), there is no relevance between the dietary intake of acrylamide and breast cancer, brain cancer, bladder cancer, prostate cancer, lung cancer, digestive tract cancer in human epidemiology. Recently, Dong and Yu (2012) reported that the intake of food containing acrylamide is associated with cancer on a series of organs, including the breast, endometrial, ovarian, prostate, esophagus, stomach, colorectal. Until now, the link between dietary intake of acrylamide and human cancer is still under debate and it remains as ‘‘potentially carcinogenic to humans’’. Acrylamide levels in foods should be kept as low as possible for safety of consumers.

2.5 Formation of acrylamide in food

Acrylamide in foods is formed when certain starchy foods, for example bread and French fries are cooked at high temperatures of 121.11 ℃. The University of Stockholm and the National Food Administration reported 2002 that acrylamide is not naturally found in raw foods. Acrylamide in food is usually produced when starchy foods (e.g. breads and potatoes) are cooked at high temperature. Acrylamide in foods is predominantly formed in the Maillard reaction with a complex mechanism. It is formed during a series of reactions between an amino acid, primarily asparagine, and reducing sugars (Figure 2) (Nature, 2002) reported that asparagine plays a key role in the production of acrylamide by asparagine pathway. (Lingnert et al., 2002) reported the formation of acrylamide can be linked with Maillard reaction. The asparagine reacted with reducing sugars generates significant amounts of acrylamide when pyrolysed at temperatures greater than 120℃ (no more than 185℃). Casado, Sánchez, and Montaño (2010) found pH value could largely influence the acrylamide formation in olive juice. The acrylamide content will increase when pH at 3 to 6 and then linear decrease when pH 6 to 9. According to (Wilson et al., 2006), the formation of acrylamide begins at a temperature around 120 ºC and peaks between 160 ºC and 180 ºC. Frying, roasting, and baking are typical cooking methods where acrylamide is produced. The Maillard reaction gives thermally processed food its desirable flavour and golden brown colour. It is comprised of mainly three reaction phases:

1. The carbonyl group of a reducing sugar reacts with the amino group of an amino acid and produce N-substituted glycosylamine and water.
2. The unstable glycosylamine undergoes Amadori rearrangement, forming ketosamines.

3. Ketosamines follow Strecker degradation and form diacetyl, aspirin, pyruvaldehyde and other short-chain hydrolytic fission products (Zhang, 2003).

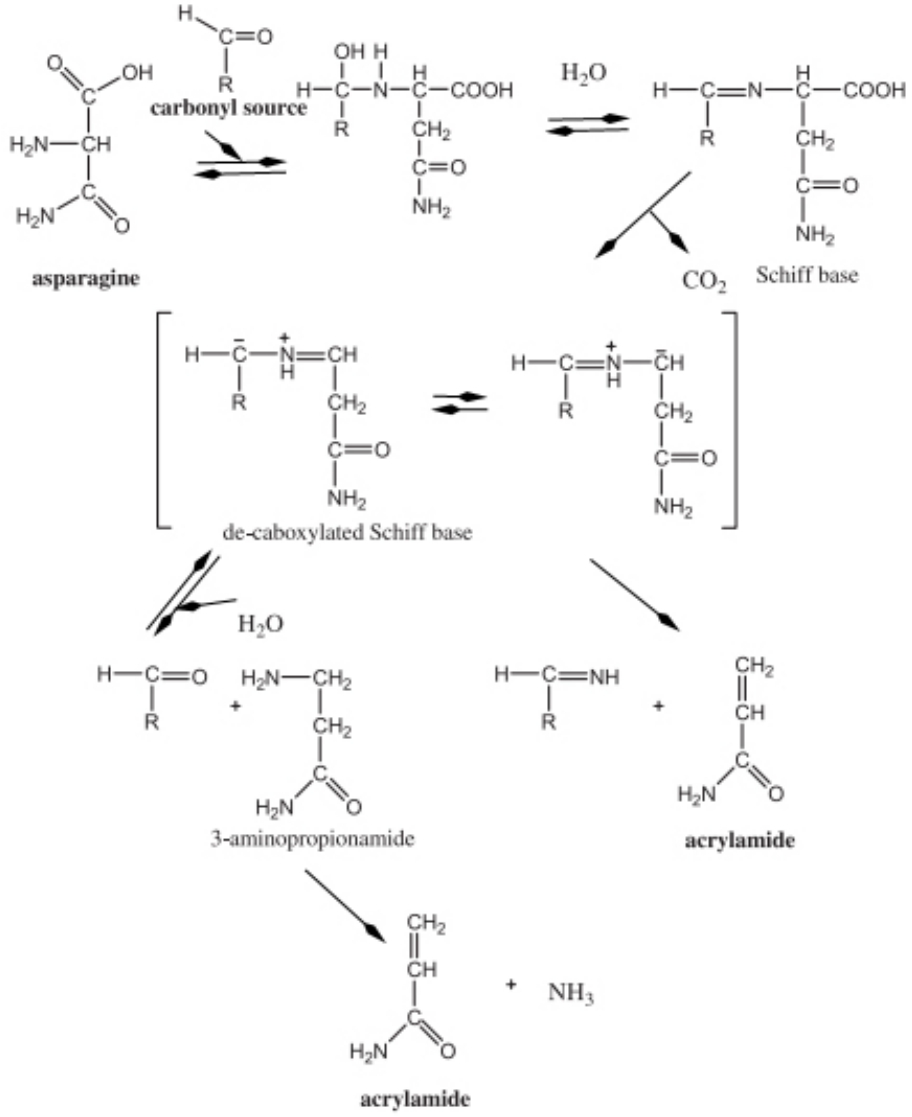

Figure 2. Schematic diagram illustrating the mechanism of acrylamide formation through Maillard reaction (Zyzak et al., 2003)

As shown in Figure 2, the R-amino group of free asparagine firstly reacts with a carbonyl source and forms a Schiff base. The Schiff base decarboxylates, which is

facilitated by delocalization of negative charge, form a product that can react one of two ways. It can be hydrolysed with water to form 3-aminopropionamide that can further degrade by the elimination of ammonia to form acrylamide when heated. The decarboxylated Schiff base may decompose directly to form acrylamide by elimination of an imine.

Yet, the exact mechanism outlining the formation of acrylamide from the Maillard reaction is not fully understood. The formation of acrylamide from asparagine and reducing sugars in the Maillard reaction is still considered the major route of formation to date (Claus, Carle, et al., 2008).

2.6 Reducing the acrylamide in bread

Bread baking is a complex process involving physical and chemical changes. Bread colour develops late in baking, simultaneously with crust formation, and arises from chemical reactions such as the Maillard reaction and sugar caramelization which produce acrylamide. Recently, numerous studies have started to validate methods of analysing acrylamide. Zyzak et al. (2003) tried to find out the formation mechanism of acrylamide in heated food. Stadler and Scholz (2004) reported the method of analysing acrylamide and the potential strategies of control. Claus et al. (2008) reviewed the formation of acrylamide and the way to mitigate acrylamide in cereal food.

As reveiwed in section 2.5, the formation of acrylamide is influenced by many factors, including processing temperature and time (Claus, Carle, et al., 2008), presence of free amino acid (Bråthen, Kita, Knutsen, & Wicklund, 2005), pH (Graf et al., 2006) and concentrations of proteins (Rydberg et al., 2003). Many attempts have been made to reduce acrylamide from foods by different ways. (Friedman & Levin, 2008) and (Anese, Suman, & Nicoli, 2009) have reviewed the main mitigation strategies to reduce the acrylamide content through dietary intake. These include agronomical (i.e., selection of raw materials with low sugar and asparagine contents) and technological interventions (pretreatments like dipping, process, and formulation changes). The major pathways to mitigaite the formation of acrylamide in foods are described in the following sections:

2.6.1 Substitution of an ingredient to reduce the formation of acrylamide

Reducing sugar is one of the key components for Maillard reaction to take place, which is associated with the final concentration of acrylamide and the development of brown colour (Lingnert et al., 2002). When the content of reducing sugar is lowered, the rate of Maillard reaction was slowed ,and results in a decrease in the browness of the baked goods. As mentioned in section 2.4, the acrylamide content in foods is largely influenced by the reaction between asparangie and reducing sugar. The less asparangie and reducing sugar produced less carbonyl-group and inhibited the Maillard reaction. Hence, it can be interpreted as the low browness seen in baked food means low amount of acrylamide is present. (Heenan, Dufour, Hamid, Harvey, & Delahunty, 2008) have shown that consumers judged the freshness of bread by the bread colour. The darker colour of bread has shown consumers to doubt its freshness and shelf-life. Hence, substitution of reducing sugars may represent a feasible way of reducing acrylamide.

The total or partial replacement of reducing sugars with sucrose, which is a non-reducing sugar, resulted in a significant reduction in acrylamide in bakery products, which was observed by (Amrein, Schönbächler, Escher, & Amado, 2004; Claus et al., 2008). Vass, Amrein, Schönbächler, Escher, and Amado (2004) used surcose to substitute invert sugar syrup in wheat crackers and reduced the acrylamide content by 60%. Similar results were observed when sucrose was replaced by invert sugar syrup and honey in ginger bread (Amrein et al., 2004). These findings have shown that lack of reactive carbonyls (fructose and glucose) will decrease the rate of Maillard reaction and produce less acrylamide.

Ammonium carbonate and ammonium bicarbonate, which are commonly used as leavening agents in baked products, have been reported to contribute towards the formation of acrylamide (Friedman & Levin, 2008). Amrein et al. (2004) reported that the addition of 1.6 g ammonium bicarbonate per 100 g of dough promoted the formation of acrylamide (880 $\mu g.kg^{-1}$) and browning in gingerbread. Graf et al. (2006) found that the biscuit prepared with ammonium bicarbonate always contained high amount of acrylamide (160 $\mu g.kg^{-1}$). The reaction pathway of this enhancement is that ammonium released from ammonium bicarbonate and reacts rapidly with the carbonyl group of glucose and fructose due to its nucleophilic character. The imines formed allow generation of glucosones and, further on, hydroxyethanal, erythrose, and glyoxal by retro-aldol reaction, which are much more reactive with asparagine to form acrylamide (Thomas M Amrein, Andres, Manzardo, & Amadò, 2006). The total replacement of ammonium carbonate and/or ammonium bicarbonate with the corresponding sodium salts have been shown to reduce the formation of acrylamide by up to 70% (Thomas M. Amrein et al., 2004; Graf et al., 2006; Sadd, Hamlet, & Liang, 2008).

According to (Amrein et al., 2004), the use of the sodium hydrogen carbonate reduced the final acrylamide concentration by nearly 70% in potato products. This has been explained as a pH reduction by De Vleeschouwer, van der Plancken, van Loey, and Hendrickx (2006), that the sodium hydrogen carbonate has a lower pH value than that of the ammonium carbonate and/or ammonium bicarbonate. And this contributes to partial inhibition of the Maillard reaction and therefore the formation of acrylamide. They also reported that acrylamide could also be reduced one third by combining sodium hydrogen carbonate and citric acid. Unfortunately, the flavour and taste arose from the substitution of ammonium carbonate with sodium hydrogen carbonate and

ammonium bicarbonate were considered not acceptable by the consumers (Claus, et al., 2008). Further research warrants for a combinational effect of the ingredient substitution on the formation of acrylamide to produce baked goods with acceptable sensory attributes.

2.6.2 Addition of an ingredient to reduce the formation of acrylamide

Addition of polyvalent cations salt, in the form of sodium chloride or calcium chloride, has been shown to reduce the formation of acrylamide in baked goods (Lindsay & Jang, 2005). As discussed in section 2.4, the first step of acrylamide production is the dehydration of the N-glycosyl compound between the carbonyl and α-amino group of asparagine which will form a Schiff base. The presence of Ca^{2+}or Na^{+} has an impact on this step and prevent the formation of the Schiff base which restricts the formation of acrylamide (Gökmen & Şenyuva, 2007). The research from (Kolek, Šimko, & Simon, 2006) reported that the addition of 1% NaCl solution in asparagine/D-glucose model system has reduced the formation of acrylamide by 40%. This result is in agreement with other studies: (Gökmen & Şenyuva, 2007) compared the effect of mono- and divalent cations (Na^{+} and Ca^{2+}) on the formation of acrylamide in a fructose–asparagine model system as well as in potato strips and found that with the use of 2Mm Ca^{2+}, the formation of acrylamide can be completely inhibited. They also suggested that the addition of an equimolar amount of Na^{+} decreased acrylamide formation by approximately 70%. (Claus, et al., 2008) reported that when 2% NaCl was used in wheat bread, the acrylamide content was decreased to 10 $\mu g.kg^{-1}$ while at higher NaCl levels, the acrylamide content significantly increased, which was ascribed to an inhibition of the yeast growth by the salt. In addition, (Sadd et al., 2008) pointed out

that dipping of potato strips in 0.1 M NaCl or $CaCl_2$ before frying resulted a significant decrease (up to 65% as shown on Table 4) in acrylamide formation, where the latter being more effective . However, most of these methods were only of scientific interest since the crackers, breads and potatoes with salt concentrations above 3% are not considered to be acceptable by the consumers. Table 4 summarises the effect of salt addition in acrylamide formation in more details.

Table 4. A table summarising the effect of salt addition (Na^+ or Ca^{2+}) in acrylamide formation.

Cation	Food	Cation addition **(% w/w)**	Acrylamide reduction (%)	References
Na^+	Crackers	2	42, 64, 56(a)	(Levine & Smith, 2005)
Na^+	Crackers	5	65, 53, 46(a)	(Levine & Smith, 2005)
Na^+	Dipping water for potatoes to be fried	0.23	43	(Sadd et al., 2008)
Na^+	Dipping water for potatoes to be fried	0.23	40, 54, 58(b)	(Gökmen & Şenyuva, 2007)
Na^+	Bread rolls	1	None	(Claus, et al., 2008)
Na^+	Bread rolls	2	25	(Claus, Mongili, et al., 2008)
Ca^{2+}	Crackers	1	60	(Mestdagh, De Wilde, Delporte, Van Peteghem, & De Meulenaer, 2008)
Ca^{2+}	Sweet biscuits	2	60	(Mestdagh et al., 2008)
Ca^{2+}	Dipping water for potatoes to be fried	0.4	79, 92, 95(b)	(Gökmen & Şenyuva, 2007)
Ca^{2+}	Dipping water for potatoes to be fried	0.4	93	(Sadd et al., 2008)

Note: (a) Reductions seen at 15, 20, and 30 min of baking time at 180℃, respectively.

(b) Reductions seen at 15, 30, and 60 min of dipping times at room temperature, respectively.

The addition of consumable acids is also a very simple but efficient method to inhibit the acrylamide formation in bakery products. It has been shown that the concentration of acid is inversely related in a linear way to the formation of acrylamide with no negative effects on sensory properties (e.g. taste, flavour, smell). Jung, Choi, and Ju (2003) reported that the use of 0.2% , 1% and 2% of citric acid could reduce up to 82.2% reduction of acrylamide in corn chips and French fries. Graf et al. (2006) reported that the addition of 1.95 $g.kg^{-1}$ tartaric acid in biscuit could reduced the acrylamide content by about one-third. Baardseth et al. (2006) reported that potato rods fermented with latic acid could reduced 71% of acrylamide level in French fries. Other studies reported that acrylamide mitigation also can be achieved by adding amino acids or protein-based ingredients to food, which may influence the Maillard reaction pathway.

From the literature, glycine was considered the most effective for inhibiting the formation of acrylamide above all amino acids (Bråthen et al., 2005). They revealed that reductions of up to 70% in acrylamide was seen in potato derivatives when glycine of 0.01M to 0.05M was used. The higher the concentration of glycine used, the more the reduction of acrylamide was seen. However, (Sadd et al., 2008) showed contrasting results in baked products, where lower acrylamide reductions (around 15–20%) were achieved by adding lysine and cysteine to the dough before baking. (Low et al., 2006) reported another option in reducing the acrylamide formation. They used a combination of 0.39 % (w/w) glycine or soy protein hydrolysate acidified with 0.39 % w/w citric acid which have resulted in a better acceptability in sensory attributes (flavour and taste) and lower concentration of acrylamide (up to 64%) in potato cakes model.

2.6.3 Addition of antioxidants to reduce the formation of acrylamide

(Zhang & Zhang, 2007a) indicated that acrylamide formation can be effectively reduced by the addition of antioxidants. Rydberg et al. (2003) reported that potatoes with 0.3, 1.7, and 8.3% (w/w) of ascorbic acid added could largely reduce the acrylamide content from 3100 $\mu g.kg^{-1}$ to 50 $\mu g.kg^{-1}$. (Levine & Smith, 2005) also observed the reduction in acrylamide content by adding 3% ferulic acid to crackers. The effect of flavonoids on the formation of acrylamide has also been studied. (Fernandez, Kurppa, & Hyvonen, 2003) reported that the addition of a flavonoid-rich spices mix could effectively reduce acrylamide levels by up to 50% in potato chips. (Becalski et al., 2004) observed a 25% decrease in the acrylamide content of fried potato slices when 5 g of rosemary herb which contain high amount of polyphenol) was homogenised in the olive oil for frying. (Zhang & Zhang, 2007b) demonstrated that addition of 0.1 $g.kg^{-1}$ and 1 $g.kg^{-1}$ of two types of flavonoid-rich extracts (antioxidant of bamboo leaves and extract of green tea) could significantly decreased the formation of acrylamide by 82.9% in fried bread stick.

Cheng et al (2009) found that naringenin could scavenge the amino group of asparagine-derived intermediates in its flavonoid structure and divert them from the pathways that leads to acrylamide formation. Recently, (Cheng, Chen, Zhao, & Zhang, 2015) also reported that tricin, apigenin and luteolin could also effectively reduce acrylamide formation. They reported that the rates of inhibition of acrylamide formation induced by tricin, apigenin and luteolin (4 to 11 $mol.L^{-1}$) ranged from 8.7% to 52.9% in a Maillard reaction system, made of potato powder. Antioxidants act mainly through two alternative reaction mechanisms: single electron transfer pathway and hydrogen

atom transfer pathway. So the antioxidant capacity is dependent on the total number of phenolic hydroxyl groups of antioxidant. The inhibition of acrylamide formation was correlated with increased numbers of phenolic hydroxyl groups on the antioxidant (Cheng et al., 2015). Moreover, Constantinou and Koutsidis (2016) examined the formation of acrylamide in Maillard reaction systems. They pointed out that when 0.1, 0.5 and 1.0 $\mu mol.g^{-1}$ of caffeoylquinic acids, caffeic acid, epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG) are added, they can significantly reduce the formation of acrylamide by up to 60%. Antioxidants added to foods act as active agents in preventing the generation of reactive carbonyl groups, trapping the key intermediates of Maillard reaction, participating in the precipitation of asparagine, reacting with acrylamide via Michael addition reactions and eliminating the formation of acrylamide as their oxidised forms (Jin, Wu, & Zhang, 2013)

Overall, the extent of reduction in acrylamide formation may differ across the different types of antioxidants used and the type of food matrix that they are applied to. Yet, the research examining the effect of antioxidant on the formation or reduction of acrylamide in foods remains scarce. Further studies in examining the pathways and/or mechanisms involved in the inhibition of acrylamide formation from the Maillard reaction are required. A combinational approach of examining the effect of antioxidants on the resultant properties of the foods, for example, physicochemical, nutritional and sensorial properties, should be taken to determine the applicability of adding antioxidants to the baked goods.

2.7 Methods of analysing acrylamide in food

In the literature, analytical instruments, including gas chromatography mass spectrometry (GC-MS), liquid chromatography tandem mass spectrometry (LC-MS-MS), high-performance liquid chromatography with UV/visible detector (HPLC-UV) have been used to quantify acrylamide.

GC-MS is one of the most widely used analytical instruments to detect and quantify acrylamide in foods. It requires small sample volumes and provides a higher degree of specificity than traditional GC. It is useful for identification of the volatile and thermally stable compounds (i.e caffeine in tea, thermally stable with a boiling point of 178 ℃). The non-volatile samples such as acrylamide must be chemically derived prior to analysis. Figure 3 shows a schematic diagram of the GC-MS unit.

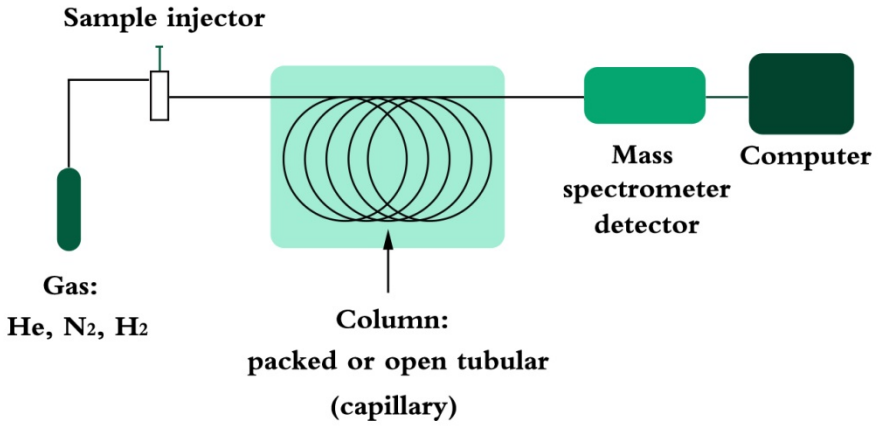

Figure 3. A schematic diagram of a typical GC-MS unit.

In GC-MS, the sample is injected into a stream of carrier gas (usually Nitrogen or Helium). The sample is carried through a packed capillary column and then separate based on their ability to distribute themselves between the mobile and stationary phases. The MS detector will then create and detect charged ions. A signal is sent to computer to provide a chromatogram. Since acrylamide present in foods is trace and bound with other compounds, it must be extracted and treated prior to analysis through GC-MS. The pre-treatment procedure is relatively complex for GC-MS compared to LC-MS and LC-MS-MS. Commonly used extraction agents are water, methanol with water and other organic reagents. (Biedermann et al., 2002) used propanol-water and ketone to extract acrylamide in roasted potatoes. They commented that enhancement in extracting acrylamide from fat particles was seen with the use of organic solvent and it made the process at ease to concentrate the extracted acrylamide for further analysis. Owing to the thermal instability of acrylamide, it needs to be chemically derived to improve the sensitivity and specificity of GC detection methods.

As early as in 1976, Hashimoto and others first reported on adding hydrogen bromide, potassium bromide or elemental bromine into the pre-treatment sample to create the brominated derivatives of acrylamide (Hashimoto, 1976). Later, in 2002, Nemoto and others reported a way to improve the efficiency of derivative by adding sodium bromide and bromine in acidic medium (Nemoto, Takatsuki, Sasaki, & Maitani, 2002). Until now, bromination is still being used as the main derivatization method, which usually is based on 1:1 ratio of KBr or $CaBr_2$ solution to the sample, to produce 2,3-dibromopropionate amide and 2-bromo- acrylamide.

Table 5. A table summarising the use of GC-MS in acrylamide quantification for different food products

Instrument	Sample pre-treatment	Sample extraction	Samples	Detection limit	References
GC	1:1 Sample mixed with saturated bromine water (KBr)	1:5 extracted with ethyl acetate. 100% efficiency	River water, sea water, sewage effluent	1 μg kg^{-1}	(Hashimoto, 1976)
GC-MS	Mixed 3 ml acetonitrile and 20 ml hexane	Propanol, water and ketone	Roasted potatoes	20 μg.kg^{-1}	(Biedermann et al., 2002)
GC-MS	1:1 Sample mixed with saturated bromine water (KBr)	Homogenised with 40mL deionized water. Centrifuge 2600rpm for 10 min 92.3% efficiency	Potato chips, snacks	30 μg.kg^{-1}	(Nemoto et al., 2002)
GC-MS	1:1 Sample mixed with saturated bromine water (KBr)	Homogenised with 400mL deionized water Centrifuge at 20000rpm for 20 minutes	Japanese food (popcorn, potato chips, cookie)	40 μg.kg^{-1}	(Ono et al., 2003)
GC-MS	1:1 saturated bromine water ($CaBr_2$),	ethyl acetate/hexane (4:1, v/v)	Cereal-based products	5 μg.kg^{-1}	(Pittet, Périsset, & Oberson, 2004)
GC/Ion-Trap MS	1:1 Sample mixed with saturated bromine water (KBr)	Oasis HLB cartridge (Waters, Milford, MA, USA) connected in tandem to an Oasis MCX cartridge (Waters).	Fried gluten, instant noodles, and twisted cruller	5 μg.kg^{-1}	(Wei-Chih et al., 2006)
GC-MS	Polymeric ionic liquid fibre	Solid-phase microextraction	Brewed coffee and coffee powder	0.5 μg L^{-1}	(Cagliero, Nan, Bicchi, & Anderson, 2016)
GC/MS/MS	1:1 Sample mixed with saturated bromine water (KBr)	400 μL of ethyl acetate and 40 μL of triethylamine	Different types of Bread (round bread, white bread hypoglucidic bread etc)	Not mentioned	(Negoita et al., 2016)

As shown Table 5, all of the food sample, have been treated with the bromination for GC-MS analysis. However, food is a complex system with protein, fat, carbohydrate. The efficiency of brominated derivatives of acrylamide is actually depended on the types of food. The papers in Table 5 have not mentioned efficiency of their bromination which may cause a lower final concentration of acrylamide. In addition, the bromine water is a toxic volatile chemical.

LC-MS is a more advanced analytical chemistry technique for detecting the acrylamide in food. The LC-MS system consists of a LC device, a mass analyser and a computer. LC separates the sample components and then introduces them to the MS. The MS creates and detects charged ions. The computer records the signals from the detector and gives a chromatogram map (Technologies, 1998). A schematic diagram of the Agilent LC-MS unit is presented in Figure 4.

Ono et al. (2003) used both GC-MS and LC-MS-MS to quantify the acrylamide in processed Japanese foods. From their results, it can be found that LC-MS-MS method produced smaller relative standard deviation values which mean more reliable and reproducible results can be obtained. Recently, an improved LC-MS-MS method was validated for analysing acrylamide in foods by (Cheng et al., 2009). They reported that the combination of tandem cartridge and a long column worked well to minimise interferences and improve detection limit when analysing the acrylamide.

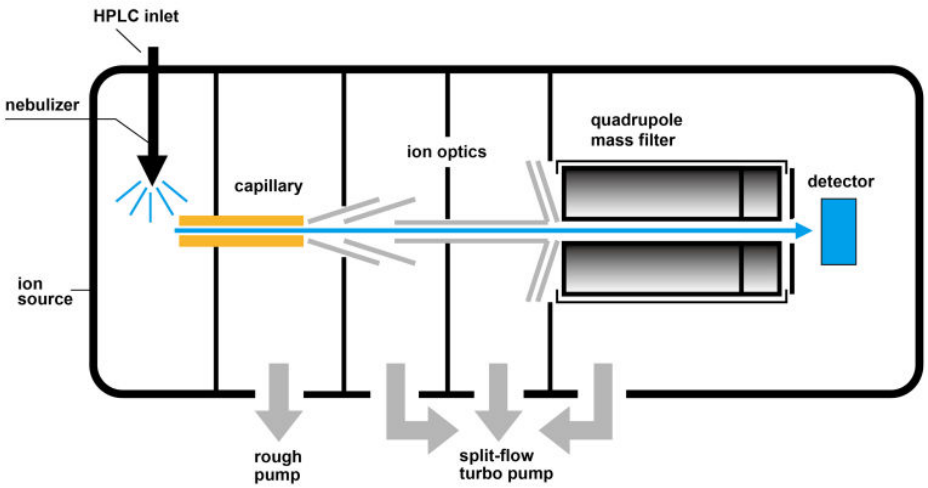

Figure 4. A schematic diagram of the GC-MS unit (modified from Agilent, 1998).

Similar to HPLC, LC-MS utilizes a compound's intrinsic affinity for both a "mobile phase" and a "stationary phase". A pump is used to provide a solvent flow to dissolve the sample. The nebulizer transfers the sample solution to atomization phase and mix with solvent. Once the sample is in the mobile phase, it goes through a capillary column in the system. The mixture compounds in the sample is separated by their own affinity to the coated particles in the column. After the sample components are separated, the target ions pass through a mass detector. The mass detector gives a response in the form of "Retention time", which refers to the time it takes for a compound to pass from the injector to the detector, then a signal from the detector is sent to the computer to compare with the reference material in database and for analysis the concentration (Agilent, 1998).

As shown in Table 4 and Table 5, the LC-MS method is able to analyse more kinds of food samples than GC-MS. The detection limit of acrylamide in LC-MS is normally below 10 $\mu g.kg^{-1}$ while for the GC-MS, it is about 10 to 20 $\mu g.kg^{-1}$. Compared with GC-

MS, LC-MS has easier and non-toxic sample extraction and preparation procedure. GC-MS is limited to samples that are thermally stable and easily volatilized. Non-volatile samples such as peptides and carbohydrates, can be analysed by GC only after they have been made more volatile by a suitable chemical derivatization which is quite time-consuming (Harvey, 2000). For GC-MS, the acrylamide needs to be derivatized (or brominated), which makes the compound is unstable and hard to grasp, while the acrylamide can be directly measured at room temperature using LC-MS. This also overcomes the thermal instability issue of acrylamide, thereby providing a more convenient and rapid analysis (Ping, 2011). However, everything has two sides: The triple quadrupole mass spectrometers are expensive and the widely used cheaper single stage LC-MS are not sensitive enough for the direct analysis of acrylamide. Also, the costs of the column and mobile phase are high. Moreover, the poor recovery efficiency of acrylamide during the extraction and purification of a sample matrix have not been resolved.

LC-MS method determines the amount of acrylamide by the ionic strength and the relative retention time which has a high sensitivity in the identification of the chemical structure with 20 to 50 $\mu g.kg^{-1}$ detection limit (Rosén & Hellenäs, 2002). Rosén and Hellenäs (2002) were the first to analyse acrylamide in cooked foods using LC-MS-MS. They purified the extracted sample many times and used deuterium-labelled acrylamide-d3s as the internal standard and operated in the positive electrospray mode. Their method was developed and improved by several scholars (Andrzejewski, Roach, Gay, & Musser, 2004; Cheng et al., 2011; Cheng et al., 2009; Jezussek & Schieberle, 2003; Kim, Hwang, & Lee, 2007; Pittet et al., 2004; Takatsuki, Nemoto, Sasaki, & Maitani, 2003). Moreover, (Cheng et al., 2011) used LC-MS-MS method to test eleven kinds of

potato chips and indicated that the variety of potato would influence the concentration of acrylamide in it. Until now, LC-MS method is becoming more and more popular and well validated. The table below sums the representative LC-MS methods used to analyse in different kinds of food.

Table 6. A table summarising LC-MS methods for acrylamide analysis in different food products.

Method	Sample extraction	Food samples	Detection range	References
LC-MS/MS	40 ml Mill-Q Water Centrifuge at 3600g for 10 minutes	Mashed potatoes, crispbread and potato crisps	25 to 2000 $\mu g.kg^{-1}$	(Rosén & Hellenäs, 2002)
LC-MS	50 ml of distilled water. Homogenized for 30 s	Potato chips, crispbread and butter cookie	4.0 $\mu g.kg^{-1}$	(Jezussek & Schieberle, 2003)
LC-MS/MS	150 mL Mill-Q Water	Japanese foods (popcorn, potato chips and cookie)	0.8 $\mu g\ L^{-1}$	(Ono et al., 2003)
LC-MS/MS	shaking with 9 mL of water for 20 min	Instant Coffee	10 $\mu g.kg^{-1}$	(Andrzejewski et al., 2004)
LC-MS	Distilled water Centrifuge at 5000 rpm for 10 min at -5℃	Turkish foods (Crackers, potato chips, biscuits, cakes, baby foods, corn chips, cookies, breakfast cereals, breads and grilled vegetable)	15–20 $\mu g.kg^{-1}$	(Şenyuva & Gökmen, 2005)
LC-MS	Mill-Q Water Centrifuged	Rice, bread, potato chips, biscuits, candy, and coffee	2 $\mu g.kg^{-1}$	(Kim et al., 2007)
LC-MS/MS	Oasis HLB cartridge (Waters) tandem to an Oasis* MCX cartridge	Fried potato, snack food, baked breakfast food, bread, coffee and tea drinks	3 $\mu g.kg^{-1}$	(Cheng, Kao, Shih, Chou, & Yeh, 2009)
LC-MS/MS	Mixed with 10mL acetonitrile, 5mL hexane, 10mL Mill-Q water. Centrifuge at 10000 rpm for 10 minutes	Potato chips	25 to 1600 $\mu g.kg^{-1}$	(Cheng Jianghua et al., 2011)
LC-MS/MS	Agilent Bond Elut QuEChERS Extraction kit for acrylamides (p/n 5982-5850)	French fries	Up to 3mg. kg^{-1}	(Al-Taher, 2012)

3 Material and Methods

In this chapter, the procedure of bread sample preparation, extraction and LC-MS quantification analysis are shown in details in 3.1 and 3.2. The methods of analysis for moisture, colour, texture and scanning electron microscopy were shown in 3.3, 3.4, 3.5 and 3.6, respectively. The statistical analyses are shown in 3.7.

3.1 Preparation of bread samples

The main ingredients of bread, including high grade flour, white sugar, iodised table salt (Cerebos), active dry yeast (Edmonds) and shortening (Chefade) were purchased from a local New Zealand supermarket. Distilled water was used to make dough. DSM Teavigo® green tea extract was purchased from Pharmachem Laboratories. The GTE powder from Teavigo® contained 94% of EGCG, maximum of 5% other catechins and free of caffeine. The Teavigo powder was stored in a -20 °C freezer until use. The 'Green tea extract' or 'GTE' used in the thesis refers to DSM Teavigo® green tea extract.

The standard formula used for making the control bread sample is shown in Table 6. The weighed ingredients were mixed together using an automatic bread maker (Breville®, Baker's Oven). For all of the samples, active dry yeast was added at the last, directly to the final dough. Yeast was mixed with distilled water at room temperature (20~24℃) and stirred with a glass rod for 10 minutes. The bread maker had pre-set

controlled functions for mixing and kneading the bread dough. The total time it took to make dough was 30 minutes including 10 minutes for the first knead and 20 minutes for the second knead. Then the bread dough was taken out the bread maker and it was kneaded by hand for about 5 minutes. After that, the dough was put into a proofer (LineMiss XL195-B, Unox UK Ltd) for 1 hour, which was set at 40 ºC, for medium humidity. The bread dough was baked for 20 minutes at 200 ºC and another 20 minutes at 180 ºC in a conventional oven. After baking, the bread was cooled down at room temperature for 2 hours. Once it was cooled, the crust of bread was shaved using a scalpel. Crust was freeze dried in a freeze dryer (Christ Alpha2-4 LD-Plus) at -80 ºC for 24 hours. Freeze dried crust samples were wrapped in zip-lock bags and stored in a desiccator for further analyses. The fresh crumb samples were divided to two portions: one portion was blended by coffee blender (Breville®) and put into the crucibles and petri dishes for moisture and colour test, respectively. The other portion was chopped into 1cm x 1cm x 1cm cubes for texture analysis.

Table 7. A table summarising the amount of ingredients used for preparing the bread samples.

Sample	Flour (g)	Dry yeast (g)	Distilled water (g)	Sugar (g)	Salt (g)	Shortening (g)	GTE (g)
Control	1000	10	620	40	20	30	0
3.3GTE	1000	10	620	40	20	30	3.3
6.6GTE	1000	10	620	40	20	30	6.6
9.9GTE	1000	10	620	40	20	30	9.9

3.2 Quantification of acrylamide with LC-MS

3.2.1 Reagents and Chemicals for LC-MS analysis

All reagents used in this project were either Optima or LC/MS grade. Acetonitrile (Sigma-Aldrich 34967), Hexane (Sigma-Aldrich 270504), formic acid (Sigma-Aldrich 94318-50ML-F) and acrylamide (Sigma-Aldrich A9099) were purchased from Sigma-Aldrich. Acrylamide-d3 (A191302) was bought from Toronto Research Chemicals (TRC) Inc. The Milli-Q water was produced by Purite Fusion Milli-Q water purifying machine.

3.2.2 Extraction of acrylamide from the bread crust

The freeze dried bread crust sample was ground into powder by a coffee blender (Breville®, BCG200). After that, 1 g of the ground bread powder was weighed and placed into a 50 mL centrifuge tube from the Agilent Bond Elut QuEChERS Extraction kit (Figure 5). The internal standard (acrylamide-d3) was added to the centrifuge tube 100 $\mu g.L^{-1}$ as the final concentration. Then, 5 mL of Hexane was added to the tube and mixed using a vortex. 10 mL of Milli-Q water and 10 mL of acetonitrile were added followed by the Agilent Bond Elut QuEChERS extraction salt mixture. The sample tube was subjected to a vortex for 1 minute and centrifuged at 5000 rpm for 10 minutes.

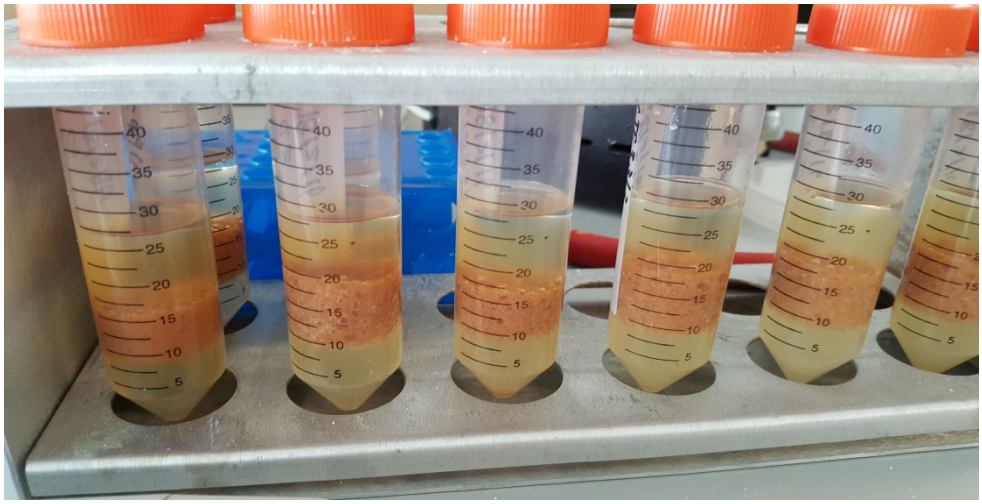

Figure 5. A photo of Agilent Bond Eultxtraction QuEChERS kit loaded with different bread samples.

After centrifuging, the hexane layer was discarded and 1 mL of acetonitrile extract was transferred to a 2 mL Agilent Bond Elut QuEChERS AOAC Dispersive SPE tube. The tube contained 50mg of PSA and 150mg of anhydrous $MgSO_4$. The tubes were subjected to a vortex for 1 minute and then centrifuged at 5000 rpm for 2 minutes. 250

µl of the supernatant was transferred using a pipette to a 1.5 mL vial. Acetonitrile was evaporated with Centrivap centrifugal vacuum concentrator (LABCONCO, USA). 100 µl of Mill-Q water was added and mixed using a vortex for 1 minute and centrifuged at 10000 rpm for 2 minutes. 50 µl of the supernatant was taken and transferred into the 100 µl glass insert in vial and stored in a -20℃ freezer until LC-MS analysis.

3.2.3 Equipment and Material

The acrylamide analysis was carried out by Agilent 1200 Series HPLC system equipped with Agilent 6460 Triple Quadrupole LC/MS system. Separation of the chemicals was performed on a Hypercrab column (2.1 mm × 100 mm, particular 3 µm). The bread extraction and clean-up were achieved with QuEChERS Extract Tubes for Acrylamides (Agilent Technology, AGI-5982-5850) and QuEChERS AOAC Dispersive SPE kit (Agilent Technology, AGI-5982-5022). All of the data were processed by Agilent Mass Hunter Quantity Analysis software.

3.2.4 Preparation of standard stock solutions and calibration

Acrylamide standard stock solution (1.00 $g.L^{-1}$) was prepared by dissolving 100 mg of acrylamide in 100 mL of Milli-Q water. It was stored at 4 °C in a fridge until use. The internal standard acrylamide-d3 stock solution (1.00 $g.L^{-1}$) was made by dissolving 10 mg of acrylamide-d3 in 10 mL of acetonitrile. This was stored in -20℃ freezer until use. All working solutions were prepared daily by serial dilution in Milli-Q water. The concentrations of the standard working solutions were 31.25 $\mu g.L^{-1}$, 50 $\mu g.L^{-1}$, 75 $\mu g.L^{-1}$, 125 $\mu g.L^{-1}$, 250 $\mu g.L^{-1}$, 375 $\mu g.L^{-1}$ and 500 $\mu g.L^{-1}$ respectively. The calibration curves were linear when forced through the origin with correlation coefficients (R^2) close to unity.

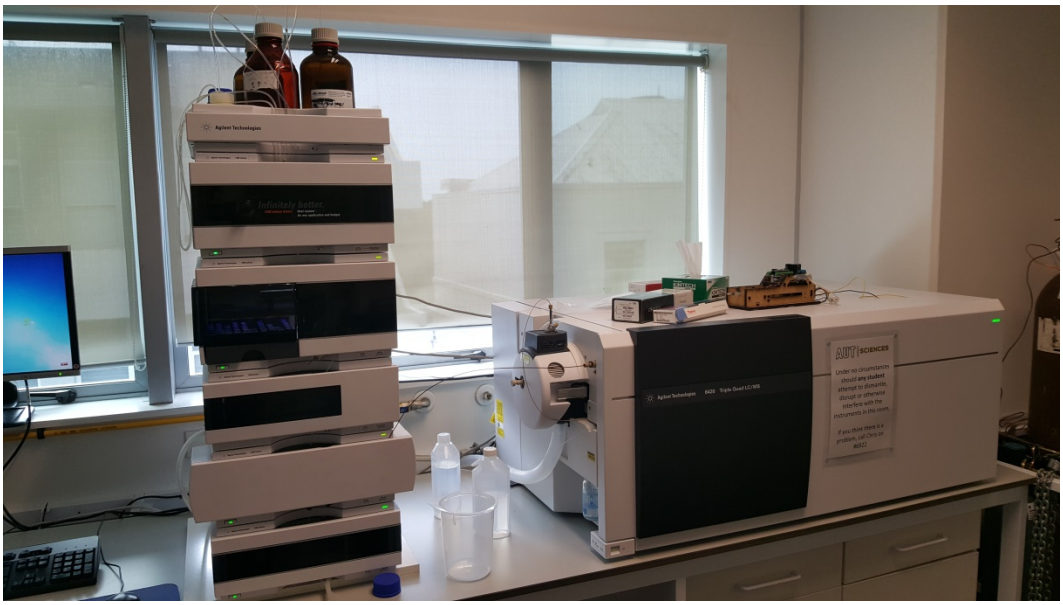

Figure 6. A photo of Agilent 6420 Triple Quad LC/MS used in this project.

3.2.5 LC/MS conditions

The LC-MS was operated in positive electrospray mode. The Hypercarb column (2.1 x 100 mm, particle size 3 µm, Thermo Scientific, USA) was maintained at 30 °C. The mobile phase was comprised of 0.1% v/v formic acid and the flow rate was maintained

at 0.3 mL.min^{-1}. The injection volume for each sample was 5.00 µL. The capillary voltage was 1.5 kV. The cone voltage was 20 V. The source temperature was 120 °C and the gas temperature was 325 °C. For the transitions, m/z 72 (Acrylamide) and m/z 75 (Acrylamide-D3), m/z 55.2 (Acrylamide) and m/z 58.2 (Acrylamide-D3) were used with the collision energies all set to 20V. The column was washed for a minimum of 20 minutes with 50:50 methanol/acetonitrile after 12 samples or at the end of daily operations. Nitrogen gas was used at 6 L.min^{-1}.

3.3 Moisture analysis

Moisture content of the bread sample (crust and crumb) was measured using oven drying method according to AOAC 950.46B (Solids, 2005). Fresh bread samples were used for the moisture analysis to avoid any water loss during the storage. Crucibles were cleaned and pre-dried at 105 °C to constant weight. The weight of each crucible was recorded prior to loading the samples. 10 g of each bread sample was cut into small pieces and spread out in the crucibles to facilitate the evaporation process. All samples were dried in a convection oven (SANYO Electric Biomedical Co. Ltd, Japan) at 105 °C until a constant weight was achieved. Crucibles were placed in a desiccator until they were completely cooled down to room temperature. The weight of the crucibles with dried samples was measured. All samples were analysed in triplicate, and measurements were taken in triplicate per sample. Moisture content was obtained from the formula below:

W1= Weight of sample before drying W2= Weight of sample after drying.

$$\text{Moisture (g/100g)} = \frac{W_1 - W_2}{W_1} \times 100$$

3.4 Colour of crust and crumb

A Hunter lab (45/0, Colourflex) colour meter was used to measure the crumb and crust colour of bread samples. Before measuring, the Chroma meter was calibrated with a white plate (L* 93.94, a* -0.95, b* 0.94) and checked for recalibration during measurements. Each of the bread crust samples and bread crumb samples was ground into fine powder and placed into a petri dish. This was placed on top of the Chroma lens and the lid was closed. The colour was measured three times per sample. Colour values were recorded as L* (0 = black, 100 = white), a* (-a* = greenness and +a* = redness), and b* (-b* = blueness, +b* = yellowness). The parameter of the cylindrical coordinates C* (Chroma) was calculated using the formula from (Minolta, 1994).

$$C^* = (a^2 + b^2)^{½}$$

3.5 Texture profile analysis of bread crumb

Texture Profile Analysis (TPA) was used to measure the texture of bread crumb in terms of hardness, springiness, cohesiveness, chewiness, gumminess and resilience. The bread crumb texture was analysed for its hardness, springiness, cohesiveness, chewiness, gumminess and resilience. The Hardness was found by the peak force of the first compression cycle and expressed in kg. Cohesiveness was calculated as the ratio of the area under the second curve (Area 2) to the area under the first curve (Area 1).

Springiness was calculated as a ratio of time recorded between the start of the second area and the second probe reversal to the time recorded between the start of the first area and the first probe reversal. Resilience was calculated as a ratio of the area during the withdrawal of the first penetration (Area 5), divided by the area of the first penetration (Area 4) (Stable Micro system, 2005).

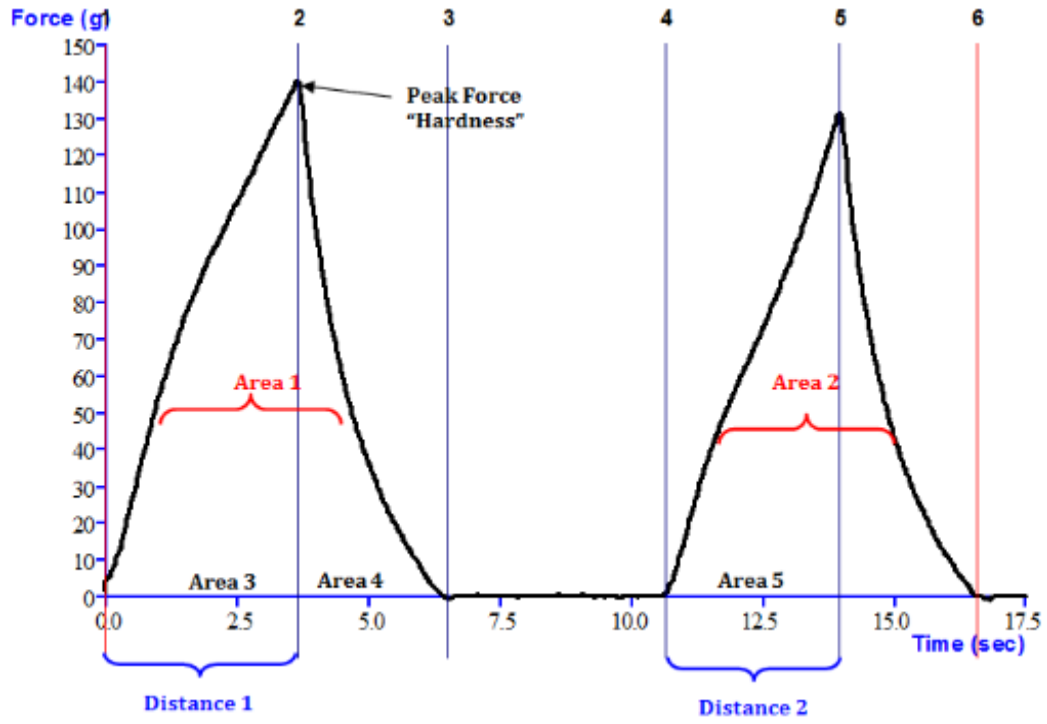

Figure 7 A plot of texture profile analysis calculation for texture expert software.

All samples were tested on the day they were baked to avoid staling. The bread samples were cut into slices with a bread knife. The central slice was used to perform textural analysis. TPA was performed using a Stable Micro Systems T.A.X.T plus Texture analyser (Surry, UK) equipped with a Film Support Rig (HDP/FSR) on a heavy duty platform (HDP/90). Each bread sample was cut into a 1 cm^3 cube. The cube was taken from the middle of each loaf to evaluate the crumb texture. Each bread sample was placed centrally on the base plate and beneath the p/50 cylindrical aluminium probe (50

mm) for a consistent flat surface. The probe was calibrated according to the manufacturer's instruction every 5 runs. It was programmed to press the sample at a speed of 20 $mm.s^{-1}$ with two cycles separated with a 5 s delay. The strain required for 50% compression was recorded using the following settings: pre-test speed: 10.0 $mm.s^{-1}$, test speed: 0.5 $mm.s^{-1}$, post-test speed: 10.0 $mm.s^{-1}$, time 30 s; load cell: 50 kg; trigger force: auto. Measurements were repeated 5 times per sample. All the data were processed and calculated by Texture Exponent 32 Stable Micro System with Simplifed TPA macro.

3.6 Scanning electron microscopy (SEM) for bread crumb

Scanning electron microscope (SEM) is a high-tech electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The bread crumb samples were first cut into size of about 10mm x 10mm x 3mm slice using a scalpel and freeze dried by using a freeze dryer (ALPHA 2-4 LD-plus) for 24h to completely dehydrate the water in bread sample. The interior surface of each crumb sample was exposed to platinum sputtering by an ion sputter coater (Hitachi E-1045) for 100 seconds with 25 mA current. The prepared samples were then viewed and photographed using analytical scanning electron microscope (SU-70, Hitachi, Japan) at an accelerating voltage of 5 kV.

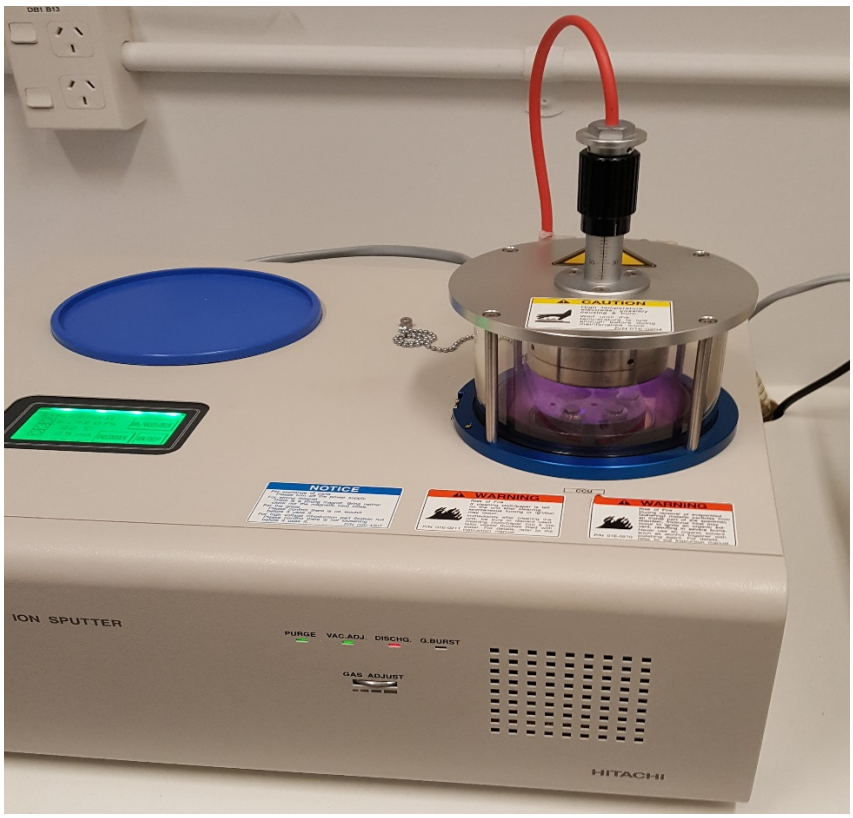

Figure 8. The ion sputter coater for SEM (Hitachi E-1045).

Figure 9. The Hitachi SU-70 analytical Scanning Electron Microscope.

3.7 Statistical analyses

Each measurement analysed at least in triplicate. Results were expressed by means of values ± standard deviation. The instrumental results were tested by one way analysis of variance (ANOVA) for significance and in case of significance ($p < 0.05$), mean separation was accomplished by Tukey's (HSD) test at 5% significance level. All statistical analyses were performed using the Statistical Analysis System XLSTAT version 2014.5.03 (Addinsoft, New York, NY, USA).

4 Results and Discussion

In this chapter, the acrylamide content analysed by LC-MS in bread are presented and discussed in 4.1. After that, the moisture, texture, colour and SEM data are shown and discussed in 4.2, 4.3, 4.4, and 4.5, respectively.

4.1 Quantification of acrylamide with LC-MS

Literature on validation of LC-MS method for quantification and identification of acrylamide in bread remain scarce compared to the analysis of other types of foods. The current method was developed for a faster, cheaper and more accurate method than GC-MS. Compared with the literature, the Agilent method (appendix) and the method from Cheng et al. (2009) were chosen as the main body method. Although their research was more focused on French fries, fried potato and snack food, baked breakfast food with different internal standard and column, they were helpful for the method development in this project. Since the internal standard C_{13}-acrylamide works similar with the acrylamide-D_3, the internal standard C_{13}-acrylamide in this project was replaced with acrylamide-D_3 to reduce the cost. Also, the column was changed from Atlantics dCI8 and AQASIL C18 to Hypercarb since the internal standard has been changed. (Takatsuki et al., 2003) demonstrated the use of four different columns under similar condition with this project and found that the limit of quantitation as 30$\mu g.L^{-1}$. The limit of the quantification in this project was about 20$\mu g.L^{-1}$, which is acceptable when compared with literature (refer to Table 6).

The condition of LC-MS was slightly changed to the specific for bread testing to prevent the matrix effect or peak shift during the experiment. (Refer to chapter 3). For optimisation, the conditions of LC-MS have been changed many times. For example, the mobile phase was changed from acetic acid and methanol to 0.1% formic acid, the flow rate was changed from 0.5 $mL.min^{-1}$ to 0.25 $mL.min^{-1}$ and then finally to 0.3 $mL.min^{-1}$ to have a quick and a clear peak out. The extraction procedure was optimised during the project. The Agilent QuEChERS extraction kit with SPE clean-up process was firstly used as the main method. However, due to a use of different internal standard and column, the result of this method has shown matrix effect and split peak problem, hence it was discarded in this project. The extraction procedure was modified from Agilent method to include one more clean-up step. Acetonitrile was evaporated and then refilled with Milli-Q water. The samples were all centrifuged and only the supernatant was taken for the injection to make sure the sample was defatted and all the matrix affecting the ionization of acrylamide was removed in MS analysis. Similar sample extracted method was used by (Cheng et al., 2011; Kim et al., 2007; Takatsuki et al., 2003). (Cheng et al., 2011) used the same amount of chemicals used in the Agilent manual and used nitrogen gas to dry the acetonitrile layer and then refilled with Milli-Q water while others (refer to Table 6) just mixed their sample with deionized water since the column they used had solid extraction function. Compared with their sample preparation methods, the acetonitrile layer in this project was dried by a centrifuge evaporator and the sample was finally dissolved in Milli-Q water phase and injected by an auto sampler. Also, there was no great difference on LC-MS settings when compared to Cheng et al.(2011); Kim et al.(2007) and Takatsuki et al.(2003).

As shown in Figure 11, the shape of the acrylamide peak was smooth with no interference and no matrix effect was observed. This is likely to be due to the extra clean-up procedure. During the project, the Agilent method did not work properly and some polysaccharides or proteins were either getting trapped on the 0.2μm filter before the column, or they blocked the column, during the extraction. After adding the clean-up procedure, large sized molecules including protein and carbohydrate in the bread sample were removed. In addition, there was a column washing procedure for every 12 samples to prevent the sample build-up in the column to improve and maintain the peak shape of acrylamide and acrylamide-d3 during the project. Combined with the sample cleaning procedure, the regular washing procedures have made the baseline stable.

The retention time of acrylamide in LC-MS are normally 6-8 minutes (Cheng Jianghua et al., 2011; Jezussek & Schieberle, 2003; Takatsuki et al., 2003). Compared with those methods, the acrylamide peak in this project was observed earlier and separated well at about 3 minutes with the use of the Hypercab column. The internal standard peaks were stable and clear (Figure 10). In addition, it was found that during the experiment that both of acrylamide standard and the bread samples were degraded to about half of the initial concentration after 2 weeks of storage which means all of the samples must be analysed as soon as possible once they were ready. The reason of this maybe that the solution with trace concentration is unstable, even when stored at -20℃ freezer. Moreover, the sample preparation time of sample took about 1.5 hours for each run. Compared with the Agilent manual, the samples in this project needed to be evaporated by the centrifuge evaporator first and this took 40 minutes, and then refilled with Milli-Q water before injection. However, compared with the method used by Cheng et al. (2011), the current method used in this project was still a faster way to analysis. Overall,

this method gave an easy, quick, affordable way to quantify the content of acrylamide in bread and provided a repeatable result. It would be advantage to enhance the lower limit of quantification of acrylamide with further optimisation of the method used in this study.

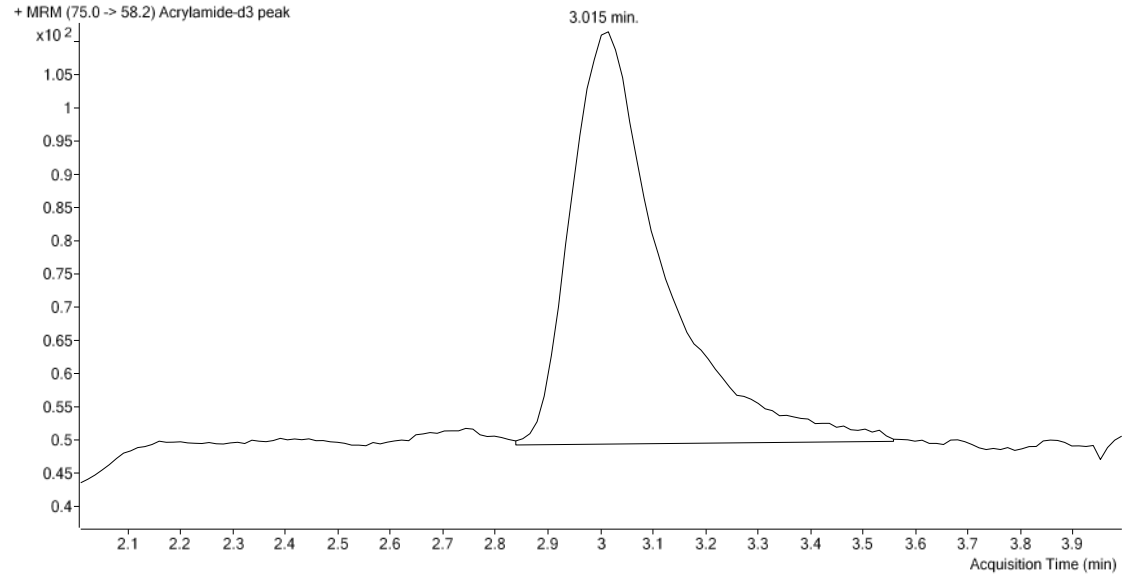

Figure 10. A chromatogram showing a peak of acrylamide-d3 (Internal standard) of the sample under m/z (75-58.2) using Agilent 6420 Triple Quad LC-MS.

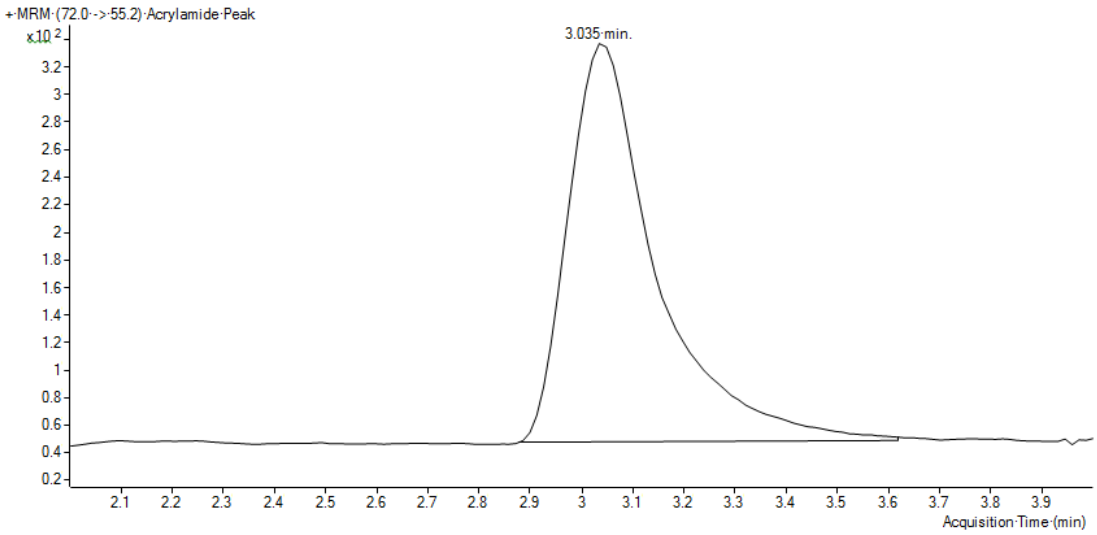

Figure 11. A chromatogram showing a peak of acrylamide of the sample under m/z (72-55.2) using Agilent 6420 Triple Quad LC-MS.

The calibration curve was prepared by using acrylamide-d3 as an internal standard. The calibration curve was linear in the range of 31.25 to 500 µg.$L^{-1}$of acrylamide with a correlation coefficient R^2 of 0.9968. During the project, the standard curve was prepared with the bread crumb to remove the matrix effect of bread. The standard curve was obtained by plotting the relative responses of analyte (peak area of acrylamide/peak area of internal standard (acrylamide-d3) to the relative concentration of analyte (acrylamide)/concentration of internal standard (acrylamide-d3). The standard curve is shown in Figure 12.

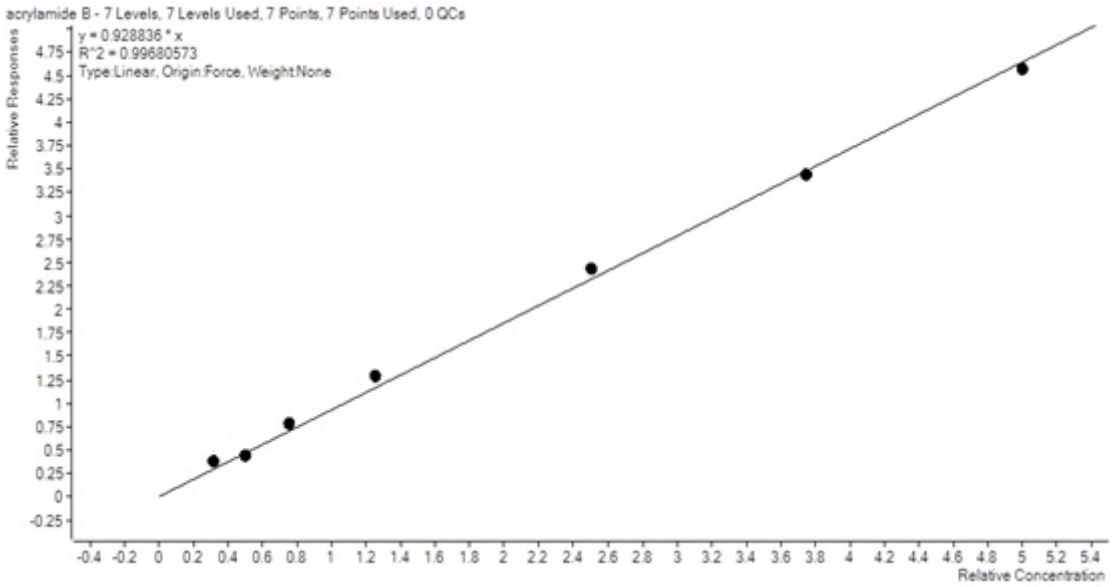

Figure 12. the calibration curve of acrylamide using acrylamide-d3 as internal standard.

Table 8. A table summarising results of the acrylamide content in the GTE added bread

Sample	Concentration of Acrylamide ($\mu g.L^{-1}$)
Control	52.19 ± 4.58^{a}
3.3 GTE	36.41 ± 8.54^{b}
6.6 GTE	34.29 ± 5.97^{bc}
9.9 GTE	32.69 ± 6.25^{c}

Data represent mean ± standard deviation (SDS) from 5 independent analyses.

Different superscript letters in the column are significantly different using one-way ANOVA and Tukey's (HSD) test ($p<0.05$).

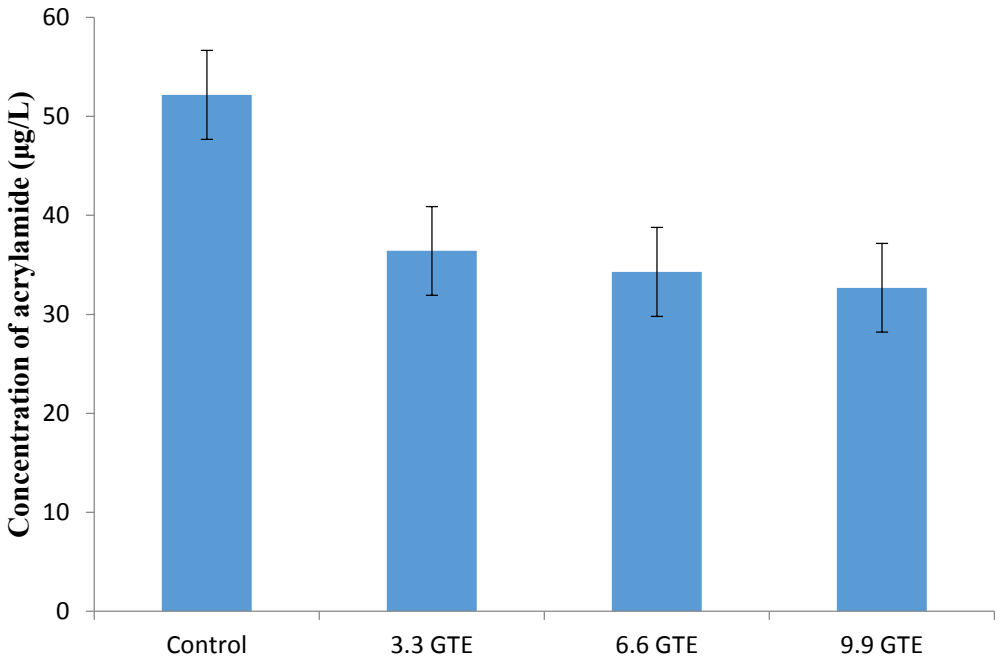

Figure 13. A plot of acrylamide concentration found in four bread samples

Data are presented as mean ± SD. Error bars indicate SD.

From Table 8, it can be seen that the acrylamide content in the bread crust was significantly reduced ($p < 0.05$) with the addition of GTE. The control bread crust sample contained the highest amount of acrylamide, 52.19 $\mu g.L^{-1}$, which was in agreement with Zhang (2003) who reported the range as 10 to 130 $\mu g.L^{-1}$, Takatsuki et al. (2003) for 53 $\mu g.L^{-1}$ and Negoita et al. (2016) for 5 $\mu g.kg^{-1}$ to 1,987 $\mu g.kg^{-1}$. However, the samples with different ratio of GTE have shown different results. As shown on Table 8, a large decrease in the acrylamide content from 52.19 $\mu g.L^{-1}$ (control) to 36.41 $\mu g.L^{-1}$ (by 30.24%) was seen in 3.3 GTE sample while for the 6.6 GTE and the 9.9 GTE, the acrylamide content was decreased by 34.30% and 37.36%, respectively. Since the heating time and baking conditions were kept constant in this project, it could be confirmed that GTE has inhibited the formation of acrylamide as an antioxidant. These results have shown that the GTE inhibited formation of the acrylamide as an antioxidant during the baking process by scavenging the free radical. The exact mechanism of how the GTE interferes with acrylamide formation is not clear. Reductions in acrylamide with the addition of antioxidants have been shown by others in the past in many types of bread (refer to section 2.6.3). Peng et al. (2010) added grape seed extract to bread and found that catechins and proanthocyanidins in grape seed extract were able to scavenge the intermediate dicarbonyls (such as methylglyoxal, glyoxal), which may indirectly decrease the formation of acrylamide. Lu et al. (2010) tested the effectiveness of antioxidant properties (scavenging ability, antioxidant activity) and pointed out that the addition of GTE could greatly enhanced the antioxidant properties of the sponge cakes and could be used in gram levels in food. Pasrija et al (2015) also reported that GTE encapsulated powder added to breads has shown high polyphenol content with high antioxidant activity.

It has been reported by many scholars that acrylamide content in foods may result from the differences in raw materials and processing conditions such as temperature and heating time. For example, Rydberg et al. (2003) demonstrated the relationship between the temperature and heating time in potato strips. They found the acrylamide will first increased exponentially with time at 200℃ and then decrease because acrylamide degrades at high temperature.

The standard deviations of the LC-MS result were relative high (up to 8.54μg.L^{-1}). This may have been caused by pipetting of small volume of samples - the pipettes used in this study were not calibrated every time prior to use and direct pipetting, instead of reverse pipetting was used. Overall, it can be seen from the result that the acrylamide in bread was largely inhibited by adding GTE. Further studies should be conducted on how to totally eliminate the acrylamide in different food and consumer testing should be done to investigate the acceptable range of GTE addition to bread.

4.2 Moisture content of bread crumb

Table 9. the result of moisture content %

Sample	Moisture %
Control crumb	42.74 ± 0.19^{c}
3.3 GTE crumb	41.39 ± 1.00^{ab}
6.6 GTE crumb	42.32 ± 0.59^{bc}
9.9 GTE crumb	40.45 ± 1.23^{a}

Data represent mean ± standard deviation (S.D.) from 9 independent measurements. Mean values with different superscripts (a-c) within the same column are significantly different ($p < 0.05$) on Tukey's (HSD) test

As shown in Table 9, moisture of the four bread crumb samples ranged from 40.45% (9.9 GTE) to 42.74% (Control). The moisture content in bread samples decreased ($p < 0.05$) with the addition of GTE. (Hayashi, Ujihara, & Kohata, 2004; Ujihara & Hayashi, 2015) have reported on the formation of complexes between catechins and caffeine in water. According to their studies, EGCG has a strong affinity to caffeine and resulted in forming a complex in water. However, the GTE used in this project contains high percentage of EGCG (at least 94%) and is caffeine free. Thus, it is unlikely that caffeine and catechins have formed a complex in water. The decrease of moisture content in bread sample could have been caused by the large self-association constant of GTE

powder in water. The main component of GTE, EGCG, has a large self-association constant (10.1) which means it can easily form self-assembled dimers in water. Due to this self-association, the water binding ability of GTE may have been suppressed loading to a decrease in moisture with an increase in GTE. EGCG dimers are known to provide multiple intermolecular interactions, such as aromatic/aromatic interaction, CH/π interactions, OH/O interaction and OH/π interaction. The binding abilities have been reported to be influenced by multiplicity of intermolecular interactions that remain effective in aqueous media. The OH/O interaction and OH/π interaction have been proved to be weakened in aqueous media (Nishio, 2011; Salonen, Ellermann, & Diederich, 2011). However, due to the presence of van der Waals forces, the hydrophobic group remains effective in aqueous media. The aromatic/aromatic or CH/π interaction in EGCG also remains effectiveness in aqueous environment. (Ujihara & Hayashi, 2015). Thus, the self-assembled dimers of EGCG lowered the binding ability of GTE in bread.

Also, Wang and Zhou (2004) studied the stability of GTE during the bread making process and pointed out that GTE can be easily converted to their corresponding epimers under the high temperature (from 82℃-215℃) and high pH (>5) conditions during the baking process. Pasrija et al. (2015) reported that microencapsulated GTE (freeze dried) with maltodextrin could drop the moisture content of bread. All these literatures are in agreement with the result in this project. Similar result have also be found in other food, Giroux et al. (2013) reported GTE with concentrations of 1 to 2 g L^{-1} can decrease the 1.9% of moisture of cheddar cheese. Han et al. (2011) reported that the cheese with 0.5 $mg.mL^{-1}$ of EGCG and catechins added resulted in a decrease in the moisture content from 71.48 to 67.30 and 69.53 respectively.

In addition, combined with the result from LC-MS, the moisture content of bread is highly correlated with the concentration of acrylamide. The more the GTE was added to the bread, the lower the moisture content in bread was observed and the lesser acrylamide was formed. This finding is supported by Ahrné, Andersson, Floberg, Rosén, and Lingnert (2007). They analysed the effect of bread temperature and water content on acrylamide formation during baking of white bread and indicated that the acrylamide concentration would decrease at very high temperatures (above 200℃) and lower water contents. However, there is a lack of explanation of the reason that caused the decrease of moisture content in all of those papers. Future studies on examining the structure of GTE bread by NMR analysis would be helpful to understand the mechanism of how GTE act as an antioxidant in bread.

4.3 Texture

Instrumental texture evaluation is important when developing new products (Dubost et al., 2003). The texture of fortified bread was determined as hardness, cohesiveness, springiness, gumminess, chewiness, and resilience by using Texture profile analysis (TPA). The results of TPA are shown in Table 10.

Table 10. A table showing the results of texture parameters from TPA

Sample	**Control**	**3.3 GTE**	**6.6 GTE**	**9.9 GTE**
Hardness (g)	13.46 ± 2.67^{a}	13.85 ± 0.57^{a}	14.42 ± 1.15^{a}	19.68 ± 3.99^{a}
Springiness	0.39 ± 0.04^{a}	0.38 ± 0.11^{a}	0.31 ± 0.05^{a}	0.40 ± 0.12^{a}
Cohesiveness	0.55 ± 0.01^{c}	0.47 ± 0.07^{b}	0.47 ± 0.03^{b}	0.37 ± 0.02^{a}
Gumminess	7406.73 ± 1670.95^{a}	6567.96 ± 2035.30^{a}	7694.95 ± 2356.43^{a}	7205.80 ± 1316.99^{a}
Chewiness	2834.00 ± 427.99^{a}	2100.54 ± 564.58^{a}	2345.81 ± 576.02^{a}	2731.82 ± 208.54^{a}
Resilience	0.25 ± 0.02^{a}	0.24 ± 0.07^{a}	0.22 ± 0.04^{a}	0.17 ± 0.02^{a}

Note: Data represent mean ± standard deviation (S.D.) from 5 independent measurements.

Mean values with different superscripts (a-c) within the same row are significantly different ($p < 0.05$) on Tukey's (HSD) test

The hardness is commonly used as an index to determine bread quality because it is related to the bite force (Bourne, 2002). The texture of bread mostly depends on starch gelatinization. The swollen starch granules in bread dough are promoted to stretch into elongated forms which allow gas to expand during the dough formation (Pasrija et al., 2015). Compared to the control, there were no significant differences in all texture parameters of the bread samples except for cohesiveness. In this project, the breads with GTE were slightly harder than the control. However the difference was statistically insignificant. Similar result was found in the study by Pasrija et al. (2015). They added microencapsulated of green tea polyphenols in their bread and found no significant difference in the hardness of bread. Indeed, bread with GTE had higher moisture retention. The EGCG present in GTE compete with native wheat starch granules in the dough for water and result in higher hardness of bread. Also, EGCG suppress the activity of the amylase, which confined the yeast activity and give bread a smaller loaf volume with harder and denser texture (Wang et al., 2007; J. Zhang & Kashket, 1998).

This mechanism is explained and indirectly proved by many researchers; Wang, Zhou, Yu, and Chow (2006) used two types of GTE which contains 73% and 60% EGCG to incorporate the bread and noticed a 40% increment in hardness. Wang et al. (2007) reported the hardness of white bread increased with the addition of 5.0g.kg^{-1} GTE. Lu et al. (2010) made the sponge cake with 10-30% GTE of flour substitution and found the cake became harder with increasing levels of GTE.

During the experiment, the cohesiveness significantly decreased with the addition of GTE, which means the crumb of bread with GTE had lower moisture content. Cohesiveness quantifies the internal resistance of food structure. It shows the strength of the internal bonds in the sample which is calculated by the ratio of the second compression to the first compression during the texture analyser. As discussed in the moisture content at section 4.2, GTE has a large self-association constant in water and it is unstable under high temperature (180-220℃) during the bread baking. The EGCG lose its binding during that process and directly proved that the cohesiveness of bread will decreased with the addition of GTE bread. Lu et al. (2010) added GTE to the sponge cake and got similar results, the cohesiveness is decreased from 0.79 to 0.62. However, most of the literature only looked at the hardness profile when determine the bread quality and literatures about cohesiveness are relatively less. Thus, the addition of GTE will not significantly modify the texture of bread. This is desirable that the bread sample with GTE has the usual texture properties and it will not influence the acceptability of bread by the consumers. Since the exact mechanism of how GTE change the bread structure is unknown, further studies should put emphasis on not only the texture parameters, but also the bread structure and the porosity of the bread to explore the GTE effect on other ingredient.

4.4 Colour

Generally, colour is defined as the most important visual attribute in the perception of product quality. Consumers tend to associate colour with flavour, safety, storage time, nutrition and level of satisfaction due to the fact that it correlates well with physical, chemical and sensorial attributes of food quality (Pedreschi, León, Mery, & Moyano,

2006). Research from Scanlon, Roller, Mazza, and Pritchard (1994) has shown that the colour of foods such as potato chips is an extremely important criterion for the potato processing industries and consumers. For example, the acceptable range of lightness (L* value) of bread crust is between 54 and 62 (Therdthai, Zhou, & Adamczak, 2002). Zanoni, Peri, and Gianotti (1995) indicated that the Maillard reactions and caramelisation make the colour of bread surface brown. As discussed in section 2.4, the GTE inhibits the Maillard reaction and caramelisation and gives the GTE bread crust a lighter colour with higher L* and b* values (shown in Table 11). The Table 11 shows the colour parameters of crumbs and crusts of bread supplemented with GTE.

Table 11. Colour parameters of crumbs of bread supplemented with GTE at different level and GTE powder.

	Colour Parameters			
	L*	**a***	**b***	**C**
Control Crust	53.92 ± 0.73^{a}	9.36 ± 0.33^{a}	25.64 ± 0.31^{a}	60.44
3.3 GTE Crust	57.26 ± 0.42^{b}	10.5 ± 0.19^{b}	28.76 ± 0.37^{c}	64.93
6.6 GTE Crust	60.92 ± 0.70^{c}	9.67 ± 0.17^{c}	28.37 ± 0.27^{c}	67.89
9.9 GTE Crust	62.09 ± 0.79^{d}	9.14 ± 0.35^{a}	27.12 ± 0.78^{b}	68.38
Control Crumb	65.85 ± 0.36^{A}	0.67 ± 0.14^{A}	15.29 ± 0.48^{A}	67.61
3.3 GTE Crumb	61.54 ± 0.26^{B}	1.93 ± 0.08^{B}	10.42 ± 0.2^{BC}	62.45
6.6 GTE Crumb	59.14 ± 0.77^{A}	3.06 ± 0.10^{C}	10.19 ± 0.45^{C}	60.09
9.9 GTE Crumb	61.35 ± 0.39^{B}	3.43 ± 0.06^{D}	10.72 ± 0.15^{B}	62.37
GTE powder	82.36 ± 0.12	6.72 ± 0.06	6.29 ± 0.31	82.88
p value	< 0.0001	< 0.0001	< 0.0001	

Note: Data represent mean ± standard deviation (S.D.) from 9 independent measurement.

Mean values with different superscripts (a-c, A-C) within the same column are significantly different ($p < 0.05$) on Tukey's (HSD) test

The GTE used in this project was in fine powder form with a light pink colour, which was expected to cause a colour change in bread. As shown in Table 11, the colours of the crumb and crust were all affected significantly with the addition of GTE for

lightness (L*), redness (a*) and yellowness (b*) ($p < 0.05$). When observed with bare eyes, the control bread crumb sample was slightly more yellow and brighter than all the sample with GTE (Figure 15). Bread crumb samples containing GTE were darker with reddish brown colour compared to the control sample. These were seen as lower L* values and higher a* values. In contrast, the control bread crust sample was dark yellow colour while all of the crust samples with GTE have shown light yellow and brighter colours. The observations were in with the finding from the Hunterlab colour meter for the L*, a*, b* values shown in Figure 14.

For the Crumb, the bread with more GTE added had the L* and b* values reduced and a* value increased, which means the brightness was decreased and the redness was increased. The L* and b* values of GTE samples were decreased down to 10.20% and 33.36% respectively, while the a* value of the 9.9 GTE samples was increased up to 2.42 times than the control. The lightness (L* value) of crumb samples was significantly decreased as the levels of GTE increased and the lowest value was seen at 6.6 GTE while the redness (a* value) of the sample was significant increased with the increasing ratio of GTE. The yellowness (b* value) of sample was first decreased as the GTE was added as 3.3 $g.kg^{-1}$ flour and then keep stable around 10 with different amount of GTE. Also, the results in Table 11 show that the GTE powder alone has very high L*, a* and b*value as 82.36 (L*), 6.72 (a*) and 6.29 (b*) respectively. These results have shown that the addition of GTE in bread gave the bread crumb a dark pink with slight yellow colour which was expected, considering the colour of the GTE. After baking, the oxidation of GTE can took place and the GTE powder turned into the dark pink colour which made the bread crumb colour darker and redder. These results are supported by Wang and Zhou (2004) and Wang et al. (2007). They pointed out that the bread with

GTE (up to 1.5g.kg^{-1}) decreased in brightness and increased in red colour. However, these were accepted by consumers when compared with the commercial unfortified bread. Thus, the colour change of bread crumb has resulted from the addition of GTE. The pale pink colour of GTE is changed to dark red during the baking since the GTE was involved in caramelisation and oxidised by reducing sugars.

It is worth to note that the L* value of the bread crust. The higher L* value, the lighter the bread colour. L* value of all GTE bread crust samples in this project were higher than that of the control, although it looked slightly greyish when compared to the control sample, by bare eyes (Figure 16). The lightness of the bread was significantly increased linearly with increasing level of GTE. The colour of bread crust has been proved to be associated with Maillard reaction and caramelisation due to the presence of reducing sugars in bread. Since the sample was added with GTE as antioxidant, the higher L* value result was shown in bread crust sample which means GTE enhanced lightness of bread crust. The reason of this might due to the scavenging free radicals of EGCG. Wang and Zhou (2004) reported that tea catechins can react with wheat protein and develop a cross-linking structure in bread dough by broken the disulfide bonds in the dough. As shown in Figure 2, the presence of EGCG in bread indirectly inhibits the formation of acrylamide. Although the exact mechanism of GTE work in Maillard reaction is not clear, the GTE has shown to inhibit the Maillard reaction and thereby the formation of acrylamide. Based on the study by (Wang & Zhou, 2004), the L* value of the GTE bread in this research is ranged from 57.26 to 62.09 which fell in the range of market products, such as whole meal bread and fibre enriched bread, which means that the appearance of GTE fortified bread would be acceptable by the consumers. The b* value of the crust sample was significant increased from 25.64 to 28.72 with the

addition of 9.9 $g.kg^{-1}$ of flour GTE. This means the yellowness of the bread crust with GTE is higher than the control bread. The a* value did not show any increase or decrease in trend as the other two values did. Nevertheless, the L*, a* and b* values were all significantly different ($P < 0.001$) between the control and the bread with GTE at the two levels. Similar results have been published by Jusoh, Chin, Yusof, and Rahman (2009) and Shittu, Raji, and Sanni (2007).

When combined with LC-MS data obtained in the current experiment, a strong positive correlation was found between colour (Chroma) and acrylamide concentration: the higher the Chroma value in bread, the lower the acrylamide content. The Chroma value (C) is an indicator of saturation. It was calculated from L*, a* and b* values for simplify the interpretation of three dimensional colour values. The C value was plotted against the L* value to give a two dimensional plot of bread samples in Figure 17. The saturation of bread crust and crumb samples ranged from 60.09 (6.6 GTE Crumb) to 68.38 (9.9 GTE Crust). It is quite clear that the Chroma was increased in the crust sample and decreased in the crumb sample which means that redness (a*) and yellowness (b*) affected the saturation as well as lightness (L*). These results are supported by (Ahrné et al., 2007) and (Surdyk, Rosen, Andersson, & Åman, 2004). Ahrné et al. (2007) reported that the colour and acrylamide concentration are well correlated, but only up to the Chroma value of 65 while the Chroma from 6.6 GTE and 9.9 GTE crust sample in this project were out of the reported range. Future studies should investigate the acceptability in appearance (colour) for the GTE bread to confirm whether it is acceptable for the market.

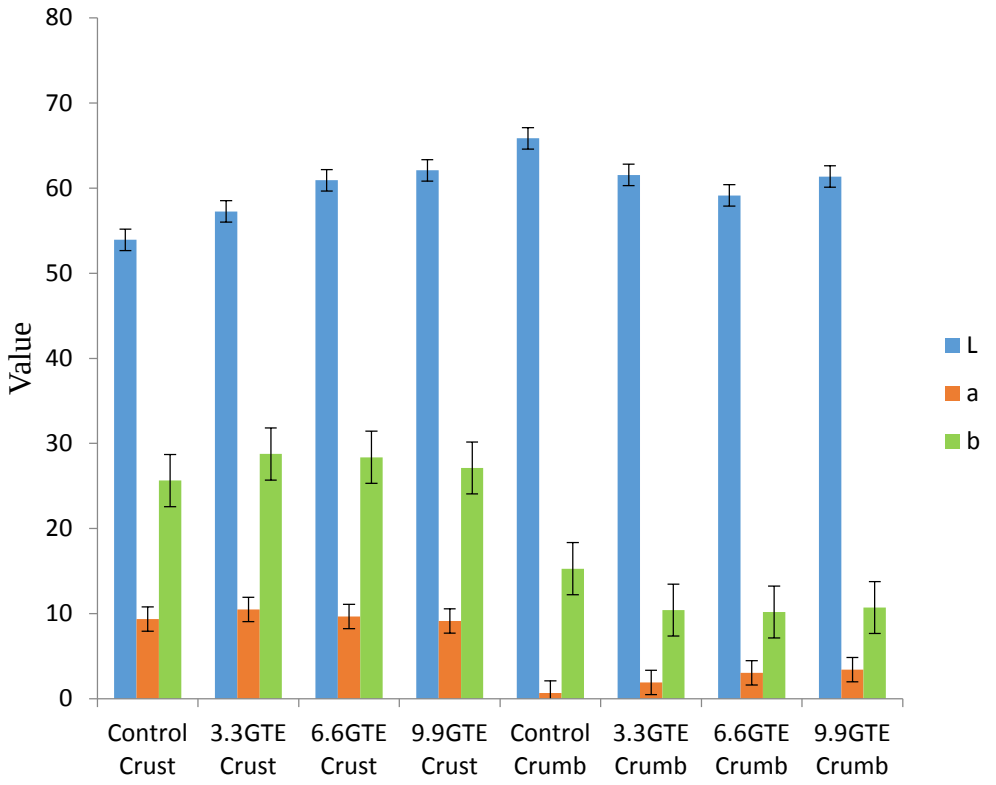

Figure 14. L* a* b* values of fortified bread crust and crumb samples.

Data are represented as mean with standard deviation (SD). Bar chart indicate mean, Error bars indicate SD.

Figure 15. A photograph of the four different kinds of bread crumb samples. The level of GTE addition is shown on the photograph.

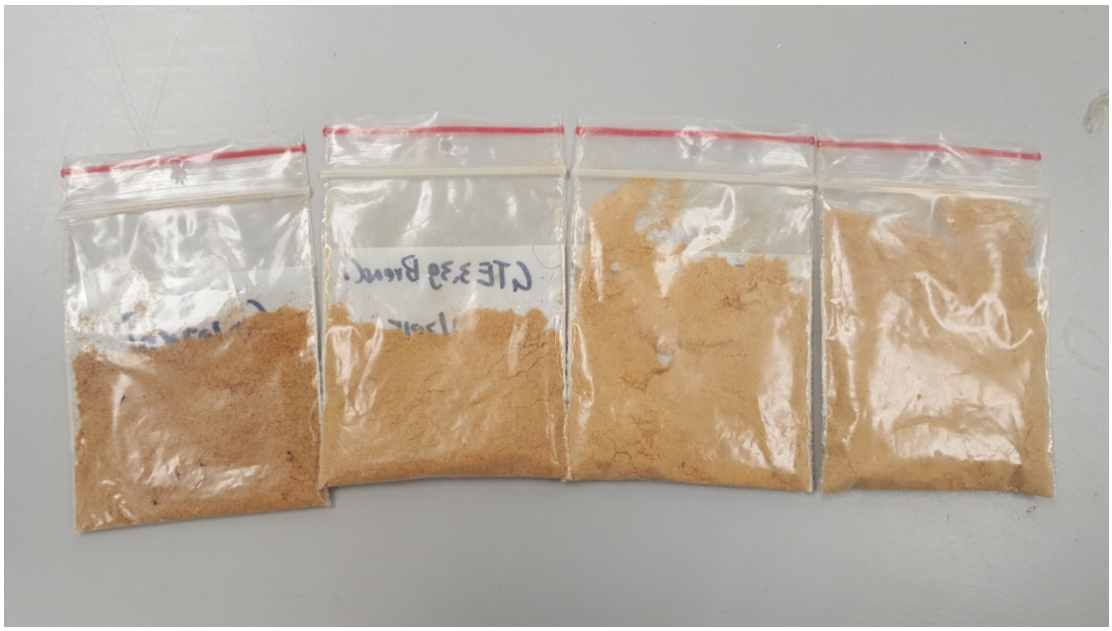

Figure 16. A photograph of four different kinds of bread crust samples (from left to right: control, 3.3 GTE, 6.6 GTE, 9.9 GTE).

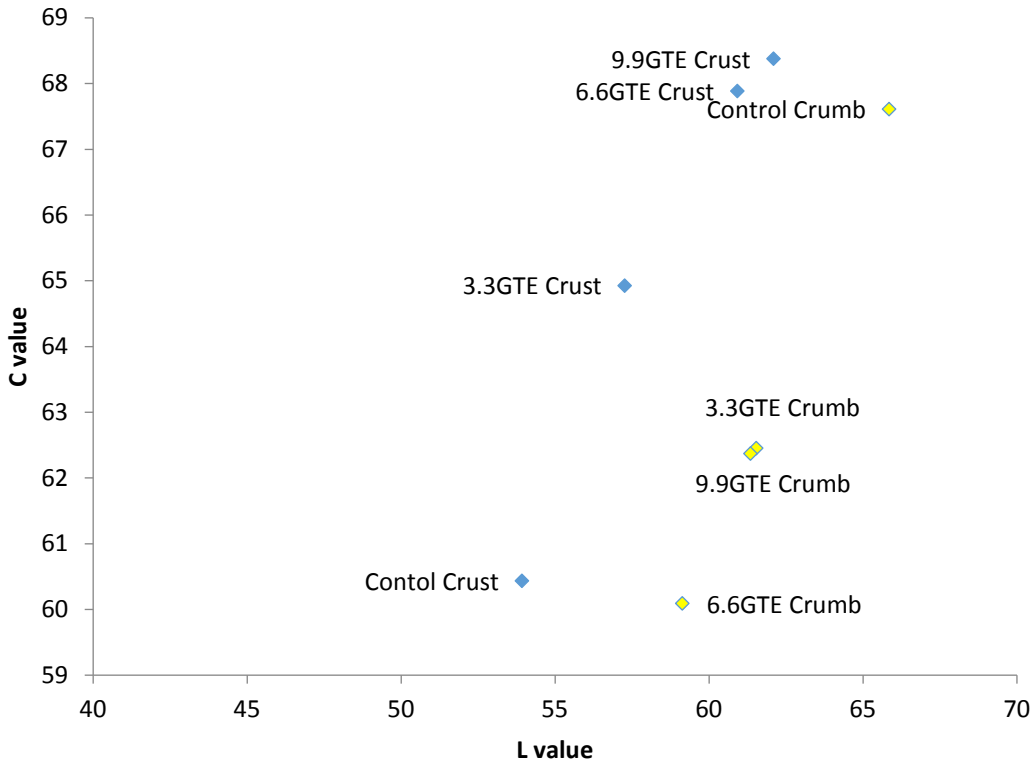

Figure 17. A plot of Chroma (C) against Lightness (L*) of the control and GTE added bread samples. Crusts are plotted in blue colour. Crumbs are plotted in yellow colour.

4.5 Scanning Electron Microscope (SEM)

Mosharraf, Kadivar, and Shahedi (2009) reported that the ingredients determined the structure of a final food product and play an important role in developing the function food. SEM has been proved as an extremely useful investigative tool for examination of the rheological properties and structure of food (Aranyi & Hawrylew, 1969); Prabhasankar, Indrani, Rajiv, and Rao (2003) reported the porosity of bread using SEM and presented that the bread contains a gluten network at a molecular level.

Micrographs of bread samples with different levels of GTE and the control are shown in Figure 18. It can be found that the control bread crumb looked like an open sponge with interconnected cavities. The starch granules were discontinuously distributed on the surface of the control sample. The cavities were about 250 μm in diameter and looked rugged (Figure 18). This may be due to the gelatinisation of starches. Kim, Morita, Lee, and Moon (2003) pointed out that gelatinization could cause covering of bread surface and make the bread to have has a few gas vacuoles with coarse surface. Also, starch granules have been confirmed to mostly remain unchanged in the structure during baking (Khoo, Christianson & Inglett, 1975).

However, from Figure 18 and Figure 19, it can be seen that the GTE fortified bread samples had smaller starch granules than the control and more densely distributed granules in the samples. The small starch granules enmeshed with each other, which made the surface of bread with GTE relatively smooth and not bumpy when compared with the control. The cavity size in these GTE breads decreased compared with the control and ranged from 50 μm to 150 μm in diameters. This phenomenon may be due to the specific property of EGCG. By combining the SEM images with the moisture and texture data in the current study, it is clear that the more rugged the structure seen in the GTE bread sample, the lesser the moisture content and cohesiveness. As reviewed in section 4.1, the EGCG has a large self-association constant and self-assembles to its dimers during the bread making process. Later, some of the EGCG may have degraded and lost its antioxidant activity due to the high temperature baking. The reactions like degradation and oxidation during the bread baking finally lower the water binding capacity and then decreased the moisture content of bread.

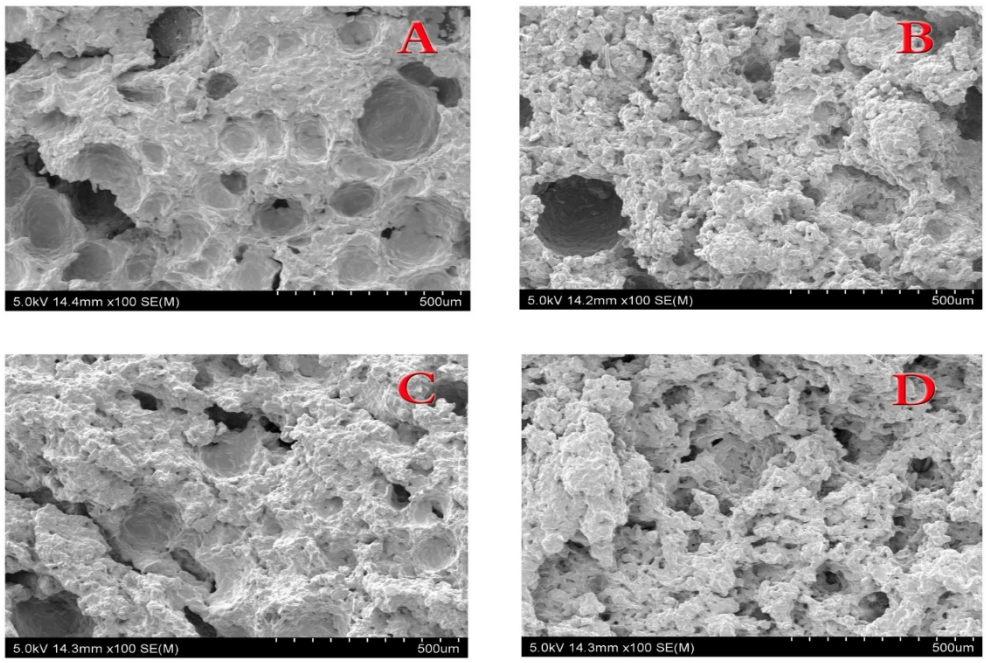

Figure 18. SEM pictures of four kinds of bread crumb samples with magnification of X100 (A: Control, B: 3.3 GTE, C: 6.6 GTE, D 9.9 GTE).

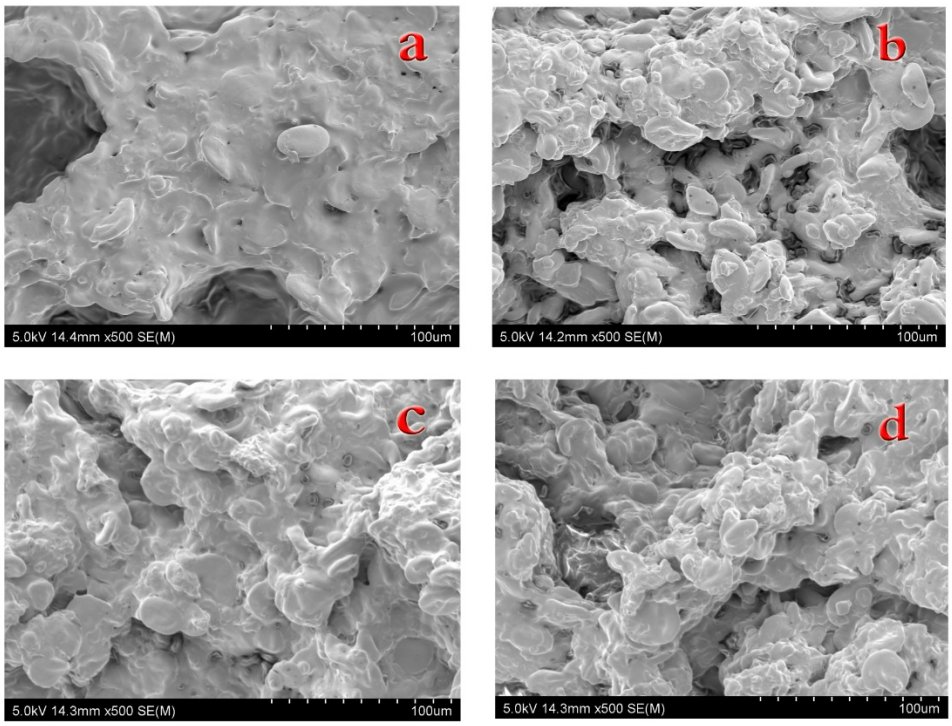

Figure 19. S SEM pictures of four kinds of bread crumb samples with magnification of X500 (a: Control, b: 3.3 GTE, c: 6.6 GTE, d:9.9 GTE).

5 Conclusion

In this project, GTE was supplemented to bread as an antioxidant to inhibit the formation of acrylamide in bread. This was the first investigation to investigate the extent of reduction in acrylamide formation during baking with the use of low range of GTE addition and to explore whether the GTE influence the texture and colour parameters and interact with other ingredients (e.g., starches and fibre). 3.3 $g.kg^{-1}$, 6.6 $g.kg^{-1}$ and 9.9 $g.kg^{-1}$ of GTE powder were added into white bread loaves as shown in Table 7. Acrylamide was analysed using LC-MS and the method was optimised for determination of acrylamide in bread for faster, cheaper and more efficient results. The supplementation of GTE significantly reduced up to 37.36% of the acrylamide content compared to the control due to its antioxidant ability. The moisture content decreased down to about 6% with the addition of GTE, which may be related to its high water binding ability and the OH/O interaction or OH/π interaction of itself. The texture parameters of the fortified bread (hardness, springiness, gumminess, and chewiness) were not significantly affected by the addition of GTE. Moreover, addition of 9.9 GTE significantly increased the lightness (L*) and the yellowness (b*) of bread crust and decreased the redness (a*) for 22%, 6% and 2% respectively, while contrasting results were found in the bread crumb. These may have been caused by the pale pink colour of GTE powder. The Chroma values (indicator of saturation) were increased up to 13% for the crust and decreased down to 8% with the addition of 9.9 GTE. These results may be due to the colour of GTE used in this project. SEM was used to investigate the structure of the GTE added bread samples. Smaller starch granules and cavities were observed in GTE fortified bread samples compared with the control. This may due to the increased GTE content and insufficient gelatinization of starch.

The mechanism of how the GTE acts in the acrylamide formation in food is still not clear. A full understanding of the asparagine pathway of Maillard reaction is required in the future study. The interaction of GTE such as OH/O interaction and OH/π interaction during the bread dough formation should also be investigated. The sensory properties and nutrition content of GTE bread should also be analysed for a commercial potential.

Overall, GTE has successfully reduced the acrylamide content in bread. The structure and appearance of the bread have changed with the addition of GTE. The GTE fortified breads may inhibit starch gelatinization and the asparagine pathway in Maillard reaction as well.

The data from this study will be useful to develop the GTE added foods in the future. The GTE fortified bread gives a positive implication in preventing the cancer such as breast, endometrial, prostate, stomach, colorectal in human. With the continuous development of catechins analytical and extract techniques, GTE food certainly has the potential to become a commercial healthy anti-cancer food product in the future global market.

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Appendix

Appendix 1. The LC-MS condition from Agilent method

Equipment and Material

The analysis was performed on an Agilent 1200 Series HPLC system equipped with an Agilent 6460 Triple Quadrupole LC/MS system (Agilent Technologies, Inc., CA, USA). Separation of the chemicals was performed on a reversed phase C18 column (2.1 mm × 150 mm, 3 µm). The data was processed by Agilent MassHunter software.

Extraction and cleanup were achieved with an Agilent Bond Elut QuEChERS Extraction kit for acrylamides (p/n 5982-5850) and an Agilent Bond Elut QuEChERS AOAC Dispersive SPE kit (p/n 5982-5022), Agilent Technologies (CA, USA).

Instrument Conditions:

Table 1. LC/MS/MS Conditions

Column	Reversed C-18 column, 2.1 mm × 150 mm, 3 µm
Column temperature	30 °C
Isocratic mode (%B)	2.5% methanol/97.5% of 0.1% formic acid
Flow rate	0.2 mL/min
Injection volume	10 µL
Run time	7 min
Post run time	3 min
Mass Spectrometer	Positive electrospray ionization mode with jet stream technology
Capillary voltage	4000 Volts
Nozzle voltage	500 V
Sheath gas temperature	325 °C at 5 L/min
Drying gas temperature	350 °C at 11 L/min

Appendix 2. the LC-MS method of Cheng et al. (2009)

MATERIALS AND METHODS

I. *Internal Standard and Standard Solution Preparation*

$^{13}C_3$-acrylamide (1 mg/mL, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was used as an internal standard. It was transferred to a 100 mL volumetric flask and made up to the volume with methanol as an internal standard stock solution. Ten milligrams of AA (99.9%, J. T. Baker, Phillipsburg, NJ, USA) was dissolved in deionized water and diluted to 100 µg/mL of AA standard stock solution. Calibration curve was established using the standard solution with 100 ng/mL of $^{13}C_3$-acrylamide combined with the AA standard solutions at five concentrations between 5 and 1000 ng/mL.

II. *Sample Preparation*

Sample was prepared according to the methods reported by Andrzejewski *et al.*[10] and Roach *et al.*[20]. Some modifications including solid phase extraction cartridge usage, and eluent volume were examined to improve the detection limit. Two columns (Atlantics dC18 and AQASIL C18 column) with different lengths were evaluated for better chromatographic separations by eliminating possible interferences.

Sample was pulverized by a food processor and weighed into a 50 mL centrifuge tube (i.e. around 1 g for bread, cake, and potato chips samples; 0.1 g for instant coffee samples; 1 mL for drink samples). Test portions were spiked with 1 mL (1 µg/mL) of $^{13}C_3$-acrylamide as an internal standard. The test portions were then diluted with 9 mL (8 mL for drinks samples) of deionized water. The mixture was homogenized for 3 min, shaken at high speed on a horizontal shaker for 20 min. The homogenate was centrifuged (Sorvall® RC-5B, Du Pont Instruments Inc., Germany) at 10,000 × g for 20 min at 5°C. A glass pipette was used to transfer 5 mL of the clarified aqueous to be filtrated through a syringe filter (Nylon membrane, pore size: 0.45 µm, filter size: 25 mm). The filtrate (1.5 mL) was subjected to Oasis® HLB cartridge (Waters, Milford, MA, USA) gravitationally pass through and was followed with another 0.5 mL of deionized water. The eluent was discarded. The sample solution loaded Oasis® HLB cartridge was then connected in tandem to an Oasis® MCX cartridge (Waters, Milford, MA, USA). The tandem cartridges were further eluted with 3 mL of deionized water. The eluent was collected in a 10 mL test tube and reduced to about 0.5 mL by a gentle steam of

III. *LC/MS/MS Analysis*

LC-MS/MS analysis was carried out using a Waters 2695 Separations Module HPLC (Waters Corp., Milford, MA, USA) coupled to a Micromass Quattro Premier (Waters Corp., Milford, MA, USA) triple-quadrupole mass sptectometer equipped with an electrospray source and Masslynx version 4.0 software for separation, detection and quantification. The analytical column was a AQUASIL C18 column (5µm, 2.1 mm × 250 mm) (Thermo Hypersil-Keystone, Thermo Electron Corp., Walthan, MA, USA) maintained at 30°C. The mobile phase was 10% methanol with 0.1% formic acid and the flow rate was maintained at 0.2 mL/min. The injection volume was 20 µL. The AA was analyzed using electrospray ionization in positive ion mode. Multiple reaction monitored mode (MRM) was acquired with the characteristic fragmentation transitions *m/z* 72 > 55 ($[M+H-NH_3]^+$), 72 > 54 ($[M+H-H_2O]^+$), 72 > 44 ($[M+H\text{-ethene}]^+$), 72 > 27 ($[M+H\text{-formamide}]^+$) for AA and *m/z* 75 > 58 for $^{13}C_3$-acrylamide (Figure 1). MS/MS conditions were as follows: capillary voltage, 2.5 kV; cone voltage, 20 V; source temperature, 125°C; desolvation gas temperature, 250°C; desolvation gas flow, 900 L/h; cone gas flow, 100 L/h nitrogen. The argon collision gas pressure was adjusted to 6.86×10^{-3} mbar for MS/MS. The collision energy was varied and optimized for each MRM transition. The transitions monitored for AA were *m/z* 72 > 72 at 5 V, 72 > 55 at 13V, 72 > 54 at 11V, 72 > 44 at 10V, and 72 > 27 at 25V. The transitions monitored for $^{13}C_3$-acrylamide were *m/z* 75 > 75 at 5 V, 75 > 58 at 13V, and 75 > 29 at 25V. Four identification points (IP = 4) were achieved for analysts identification in the criteria of European Commission Decision 2002/657/EC[21]; we selected one precursor ion (*m/z* 72) and two transition ions (*m/z* 72 > 55 and *m/z* 72 > 54) to monitor the analysis. The MRM transitions *m/z* 72 > 55 and *m/z* 75 > 58 were acquired for the quantification of AA concentration as shown in Figure 2. The ion ratio of *m/z* 54/55 and 55/72 were 8.3 and 11.8%, respectively. The dwell time for each transition was 0.3 s with 0.02 s of interchannel and interscan delay.

IV. *Quantification*

AA was quantified using a linear calibration curve with standard solutions of AA dissolved in water at concentrations ranging between 5 and 1000 ng/mL with 100 ng/mL $^{13}C_3$-acrylamide as an internal standard. AA in samples was determined from calibration curve constructed by plotting the peak area ratios (*m/z* 55 and *m/z* 58) against the concentrations of AA.