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Determination of acrylamide content in selected Saudi Arabian foods and estimation of the associated dietary exposure

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Determination of Acrylamide in Saudi Infants Food by Using LC-MS

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List of abbreviations

CHO	Carbohydrate
CV	Coefficient of variation
IS	Internal standard
FDA	Food and drug administration
JECFA	Joint FAO/WHO committee on food additives
WHO	World health organization
ND	Not-detectable
LOD	Limit of detection
LOQ	Limit of quantity
FSA	Food standards agency
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
mAU	Milli-absorbance units
CI	Confidence interval
DAD	Diode array detector
Kg-bw	Kilogram body weight
min	Minute
sec	Second
m/z	Mass-to-charge ratio
S/N	Signal to noise ratio
TIC	Total-ion-current
RT	Retention time
HMF	Hydroxymethylfurfural
AOAC	Association of official agricultural chemists
SPE	Solid phase extraction
IRMM	Institute for reference materials and measurements

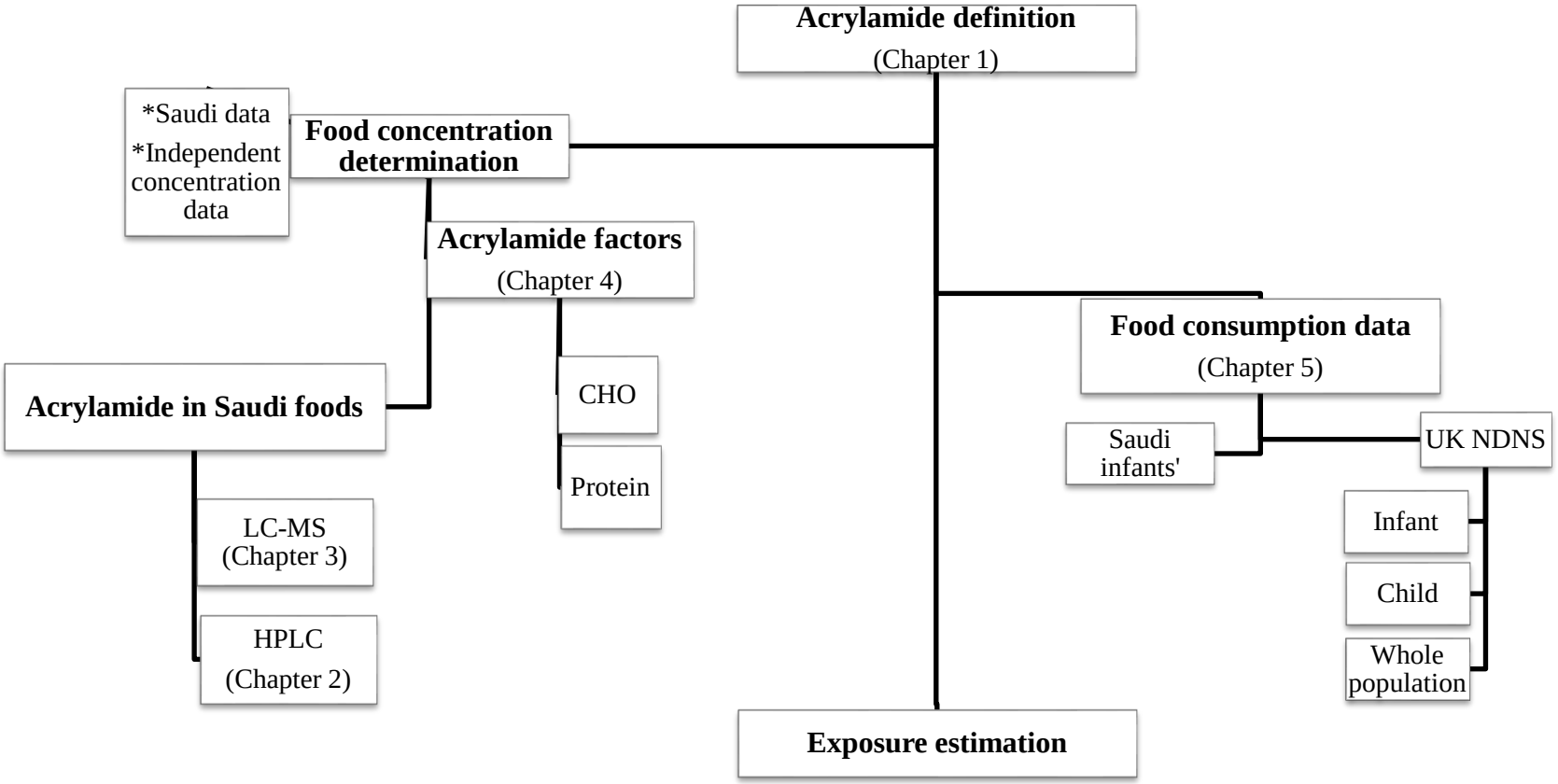
Abstract

In 2002, the Swedish National Food Administration and the University of Stockholm reported that certain foods which are processed at high temperatures may contain high levels of acrylamide. Acrylamide is synthesized via the Maillard reaction from the amino acid asparagine and a reducing sugar. It has been proven from animal experiments that acrylamide is a toxic compound and it is a probable carcinogen and a mutagen for humans. Acrylamide has been found in starchy foods such as French fries, bread crust, and baked cereal foods. Some of these foods are commonly consumed by weaning infants and children. For the first time, this thesis estimated the acrylamide exposure of Saudi infants by determining the acrylamide content in foods commonly consumed by Saudi infants using liquid chromatography mass spectrometry (LC-MS) and estimating exposure using deterministic approach. Additionally, the thesis estimated the exposure to acrylamide of the UK infants, children and whole population by using published acrylamide composition and dietary information from the National Diet and Nutrition Surveys of 1992-3 (SN3481) and 2011-12 (SN6533) using deterministic and probabilistic approaches. Acrylamide levels were analysed in 16 foods commonly consumed by Saudi infants using high-performance liquid chromatography (HPLC), however, the resolution of the acrylamide peak was not sufficient to allow quantification. LC-MS was consequently used. The food samples were extracted with water after adding 2 µg/mL of internal standard C_{13} acrylamide. The extract was cleaned-up with solid phase extraction (SPE) cartridges. The LC-MC was set in positive mode at m/z 72 and 75 for acrylamide and acrylamide C_{13} respectively. Acrylamide levels in most foods were found to be lower than the limit of detection (LOD) (17.2 µg/kg). Saudi infants had a median exposure of 0.22 µg/kg-bw/day. The exposure in British infants, children and whole population were ranged between 0.43 to 1.07 µg/kg-bw/day. Most groups –except Saudi infants- have an upper percentile that exceeds the tolerable daily intake (TDI) cancer value (2.6 µg/kg-bw/day). Overall, results show Saudi infants are at low risk of acrylamide-related disease, but 25% of UK infants, children and whole population may be at risk and should reduce consumption of acrylamide containing foods.

Key words:

Acrylamide, Saudi infant, NDNS, HPLC, LC-MS and TDI.

Chapter 1: General introduction



Flow chart for thesis plan.

1 Chapter 1: General introduction

1.1 *Introduction*

Thermal treatment of foods stimulates biological, physical and chemical modifications. This may change the food nutritionally, sensorially and texturally. Typical example of domestic and industrial processing methods are boiling, frying, steaming, baking and roasting.

The Maillard reaction is one of the chemical reactions caused by heat treatment which may affect the food's nutrients and lead to the formation of mutagenic and carcinogenic components. Acrylamide is a component formed by the Maillard reaction which is potential carcinogenic to humans (Van Boekel *et al.*, 2010).

Childhood food consumption affects individual health in the future (Baker-Henningham *et al.*, 2004). Infant and children populations are a sensitive sub-group due to their high consumption to low body weight ratio and reduced capacity for detoxification putting them at a higher risk of the toxic effects of acrylamide (European Food Safety Authority, 2011a, Bongers *et al.*, 2012). This study is focused on estimating Saudi infants' exposure to acrylamide and comparing to the exposure in UK infants, children and whole population.

1.2 *The Maillard reaction*

The Maillard reaction is a complex series of non-enzymatic reactions (chemical reactions), occurring in the presence of a reducing sugar (carbonyl containing compounds) and a free amino compound (amino acid or other amino compound), which react together to produce brown compounds under specific conditions. The Maillard reaction depends on the type and concentration of the reagent, time, temperature, moisture level and pH. At least 15 different components are formed through the reaction of one type of reducing sugar with a specific amino acid. For example, a glucose with glycine reaction gives 24 types of Maillard reaction products (MRPs) (Nursten, 2005). Low moisture content, high temperature (more than 100 ºC) and pH between 4 -7 (in the pH range of most food) are important for the Maillard reaction to occur (Fernandez-Artigas *et al.*, 2001) (these factors will

be explained later when discussing acrylamide formation). High temperatures during food processing such as baking, frying, grilling and roasting, or storing at room temperature for a long time should also be considered as factors leading to the Maillard reaction occurring (Nursten, 2005).

1.2.1 Mechanisms

The Maillard reaction was first reported in 1912 by Louis-Camille Maillard, but the essential stage of the reaction was designed by Hodge in 1953 (cited in Coultate, 2009). In the first phase of the Maillard reaction, the reducing sugar and the amino compound react reversibly to produce N-substituted glucosylamine. This may quickly rearrange via the 1,2-enaminol to form the so-called Amadori compound. In the next phase, at pH below 5, the amino compound decomposes to leave a 3-deoxyaldoketose. This can dehydrate to give hydroxymethylfurfural (HMF). At a pH above 7 the Amadori compound breaks down, which may result in a number of end-products such as maltol, isomaltol and some other α-dicarbonyl compounds. These compounds contribute to the flavour of many food stuffs (Coultate, 2009).

The Maillard reaction produces components such as melanoidins which are associated with brown colour, aldehydes and ketones which are linked with flavours, and aromas, and potential health impact components such as acrylamide (Coultate, 2009). These products are important for many food products such as forming the flavour of chocolate and cereal-based foods (Fernandez-Artigas *et al.*, 2001).

1.2.2 The role of the Maillard reaction in food

1.2.2.1 Sensory quality

The Maillard reaction enhances the sensory qualities (aroma, flavour and colour) of food, particularly in food processes such roasting coffee, baking bread, toasting cereals and cooking of meat (Thomas *et al.*, 2004, Mottram and Wedzicha, 2007). The Maillard reaction is associated with brown colour (melanoidin), which occurs and develops during food processing and storage (Wang *et al.*, 2011). Flavour is

developed in the intermediate and last stages of the Maillard reaction (Ames, 1990). Food texture is influenced by the Maillard reaction via protein cross-linking (Gerrard, 2002).

1.2.2.2 Nutrition quality

A wide range of Maillard reaction products occur that have significant importance for the nutritional value of foods. (Thomas *et al.*, 2004, Mottram and Wedzicha, 2007). The Maillard reaction decreases the nutritional quality of food because of the use of reducing sugar and amino compound to form Maillard products (Somoza, 2005). For example, when the Maillard reaction takes place in milk, the quality of the milk decreases due to the amino acids in the milk, especially lysine, reacting with lactose, which leads to a loss of important amino acid, a change in the milk's colour to brown and unacceptable milk flavours (Thomsen *et al.*, 2005, Coultate, 2009). Mineral bioavailability can be affected by Maillard reaction products which are able to chelate with minerals. The study of Delgado-Andrade *et al.* (2011) found that when adolescent males were exposed to a diet rich with Maillard reaction products, negative effects in terms of phosphorus absorption occurred.

1.2.2.3 Health effects

Studies show that Maillard reaction products may be carcinogenic, which could be a particular issue for infants due to their sensitivity, something which is due to them having a low body weight and small organs. There are some products that are considered potentially carcinogenic to humans such as HMF and acrylamide (Capuano and Fogliano, 2011) (Table 1-3 shows an epidemiological study of acrylamide and cancer). Heterocyclic aromatic amines (HAAs) were classified as highly mutagenic and carcinogenic compounds (Felton *et al.*, 1997)

Advanced glycation end products (AGEs) were found accumulated in the renal area of renal failure patients (Hartog *et al.*, 2007). Also, AGEs may increase vascular complication in diabetes patients due to the interaction of these compounds with cell receptors (Marchetti, 2009).

There are some advantages of Maillard reaction products as they can act as antimutagenic compounds, antioxidants and antibiotics (Thomas *et al.*, 2004, Mottram and Wedzicha, 2007). A positive health effect of the Maillard reaction is potentially as an antioxidant, as found in the study of Zeng *et al.* (2011) when they heated d-psicose and l-lysine at 120 ºC and pH 9.0 for up to 8 hours. The melanoidins, which are linked to brown colour, were found to have an antioxidant effect (Hayase *et al.*, 2008).

1.3 *Acrylamide*

1.3.1 Introduction

Although the chemical properties of acrylamide are not new, the discovery of its presence in food is a relatively new finding. It was used to produce water soluble polyacrylamides (used for clarifying drinking water), acrylamide gel is used in biotechnology laboratories, in cosmetic additives (creams, body lotion and shampoo), in food packaging (paper or paperboard), and in cigarettes (International Agency for Research on Cancer, 1994b). In 2002, the Swedish National Food Administration (SNFA) and University of Stockholm published a list of certain foods that are processed at high temperatures and that contain high levels of acrylamide (Mottram *et al.*, 2002a). This announcement led to world-wide attention focusing on acrylamide research in relation to food due to its known toxic effect. Following these findings, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) undertook a comprehensive review of data on the occurrence of acrylamide from many countries, mainly in Europe and North America. The outcome of this review was that the main contributing to acrylamide food groups were identified as potato-based products, cereal-based products and coffee (Toda *et al.*, 2005). The International Agency for Research on Cancer (IARC) classified acrylamide as a 'probable human carcinogen' and it was defined as a compound with the potential to cause a range of toxic effects (International Agency for Research on Cancer, 1994a). Regarding the IARC (2010), acrylamide was classified as being in carcinogenic group 2A (limited evidence in humans and sufficient evidence in animals).

Chapter 1: General introduction

Tareke *et al.* (2002) showed that acrylamide is found in a range of cooked foods, such as French fries, bread crust, cereal and baked goods, with the highest content and range of 50 to 4000 μg/kg in carbohydrate-rich foods and a range of 5-50 μg/kg in protein-rich foods. Acrylamide is taken into the body via different routes: 1) inhalation via cigarette smoking, 2) ingestion by eating cooked rich-carbohydrate food and 3) skin absorption via cosmetic products (Carere, 2006).

In studies in 2002, the formation of acrylamide in foods was demonstrated to take place during Maillard reactions involving the free amino acid asparagine and reducing sugars such as glucose, fructose, maltose and lactose, as well as sucrose under high temperatures (Mottram *et al.*, 2002a, Stadler *et al.*, 2002).

The mutagenic and carcinogenic properties of acrylamide are related to its metabolite, glycidamide. It was proven from animal experiments that glycidamide is a toxic compound and it is a carcinogen and mutagen for humans (Zhang *et al.*, 2005b, Lindhauer and Haase, 2006).

Factors affecting acrylamide formation are present at many points from farm to table. Therefore, guidance to reduce the acrylamide formation should be provided to farmers, manufacturers and food service operators. FDA recommends that manufacturers be aware of acrylamide levels in their products, which is essential for determination of the effectiveness of acrylamide reduction techniques (Food and Drug Administration, 2013) .

To evaluate the acrylamide risk to humans, dietary assessment methods should be used to determine exposure. Estimation acrylamide exposure requires laboratory analysis and statistical analysis to determine the contents within foods and combine with individual consumption. Acrylamide determination in foods needs to be extended to foods consumed around the world, not just those in Western diets. Acrylamide exposure varies by country depending on the staple food (Dybing *et al.*, 2005).

Acrylamide exposure depends on both acrylamide content and food consumption. Thus, consuming a great quantity of foods low in acrylamide content, such as

bread, can be a source of acrylamide exposure (increasing the total exposure of acrylamide) (Matthys *et al.*, 2005).

1.3.2 Acrylamide chemistry

The acrylamide (2-propenamide) chemical formula is C_3H_5NO (Figure 1-1). The three-carbon and amide group of acrylamide molecule comes from the asparagine and reducing sugar (Amrein *et al.*, 2003). Acrylamide is a small hydrophilic molecule of molecular weight 71.08 g/mol. It is an odourless solid and its colour ranges from colourless to white. Acrylamide is soluble in a number of polar solvents, such as acetone, acetonitrile and water. The solubility of acrylamide in water is 216 g/100 mL at 30 °C. The acrylamide melting point is around 84–85 °C and the boiling point of acrylamide is 125 °C. It exhibits both weak acidic and basic properties. Acrylamide is also a highly reactive molecule which can react through ionic and free radical mechanisms. It is found in free form in food (Food and Agricultural Organization and World Health Organization, 2002a, Girma *et al.*, 2005).

The high polarity and low molecular weight of acrylamide makes the quantification of acrylamide in a food matrix more difficult for extraction and determination. Also, acrylamide can be hydrolysed and rearranged to acrylic and methacrylic acid (Paleologos and Kontominas, 2005).

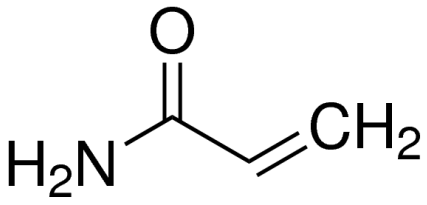


Figure 1-1: Acrylamide structure (C_3H_5NO).

1.3.3 Acrylamide in food

Cereals and potato contribute towards a third of the individual daily intake of calories of most people around the world (Petersen and Tran, 2005). Acrylamide is found in a wide range of food products which are rich in carbohydrate and asparagine, with low water content and which undergo a high temperature process (deep frying, baking or roasting, but not boiling), with the major contribution originating from cereal-based products, potato-products and coffee. Among cereal products, the main contribution is from pastries, biscuits, breads and processed cereal products (Dybing *et al.*, 2005, Zhang *et al.*, 2005b).

Bent *et al.* (2012) and Svensson *et al.* (2003) have shown that there is no acrylamide in boiled or raw foods in limit of detection (LOD) and limit of quantification (LOQ) 5 and >30 µg/kg food respectively. Toda (2005) highlights how acrylamide levels in different foods show a wide range of variation. Table 1-1 shows the content of acrylamide (µg/kg) in different food items according to the European Food Safety Authority (EFSA) (2013).

Table 1-1: Content of acrylamide in different food items according to EFSA 2007-12.

Food item	Mean content (µg/kg)
French fries ready to eat	600
Potato crisps	1000
Bread	80-150
Biscuits	500
Breakfast cereal	200-400
Roast coffee	450
Instant coffee	900
Baby food (not cereal foods)	50-80
Baby food (biscuits & rusks)	200
Baby cereal	50

(European Food Safety Authority, 2013)

The acrylamide content varies within the same food product group. In French fries and potato crisps group, acrylamide level has been found to range from 172 to 930

µg/kg (Williams, 2005). The variability is due to differences in the source of raw materials, processing conditions and the procedure used for analysis (Williams, 2005, El-Assouli, 2009). Stadler *et al.* (2002) and Amrein *et al.* (2003) point out that potato cultivars have different levels of potential acrylamide formation because of different contents of reducing sugar and asparagine. Also, they show that potato has a varied range of glucose and fructose contents compared with asparagine.

There are many aspects to acrylamide in food that need to be understood, such as formation, bioavailability, accumulation and toxicity.

1.3.4 Mechanism of acrylamide formation

The Maillard reaction in the presence of asparagine is the main pathway for acrylamide formation in starchy food. The suggested pathway for the formation of acrylamide is via Strecker degradation of the amino acid asparagine in the presence of dicarbonyl products (such as reducing sugar) from the Maillard reaction (Mottram *et al.*, 2002a). Stadler *et al.* (2002) have suggested that N-glycoside formation could be favoured in food processing matrices which contain reducing sugar and free amino acid. The combination of high temperature and water loss may lead to the potential of acrylamide formation (Amrein *et al.*, 2003). According to Zhang *et al.* (2005b), understanding the mechanism and its conditions make reducing the acrylamide produced during the heating process possible. According to Xu *et al.* (2014) asparagine with dicarbonyl products (Figure 1-2) form a Schiffbase or a corresponding Schiffbase which change to 3-aminopropionamide as the last step before acrylamide formation.

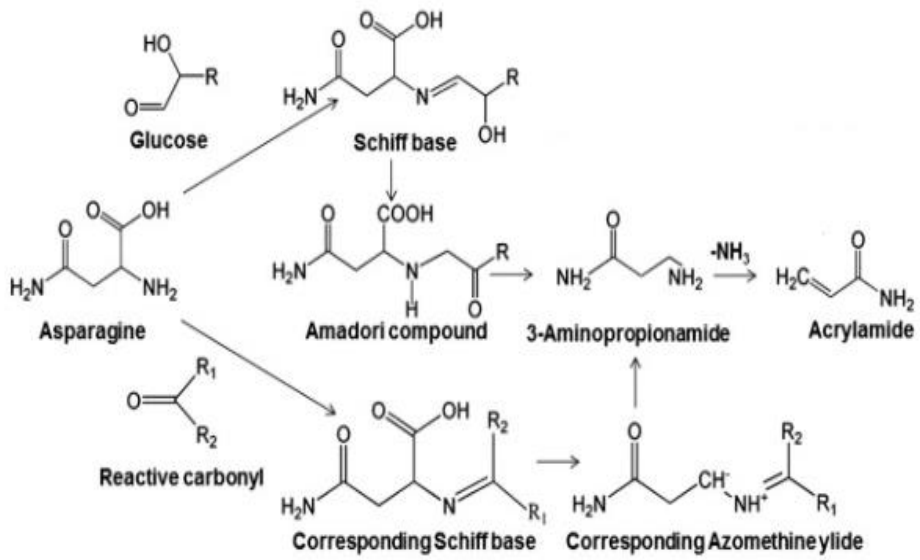


Figure 1-2: Major pathway of acrylamide formation in food (reaction with asparagine with glucose or reactive carbonyl) adapted from Xu *et al*. (2014).

1.3.5 Factors affecting acrylamide formation

There are many factors affecting the formation of acrylamide, such as the amount of reducing sugar and asparagine, the ratio of amino acid and reducing sugar, the heat processing method (baking time) used, heating time, heating temperature, pH, and storage conditions for raw and prepared foods (Stadler *et al.*, 2002, Rydberg *et al.*, 2003, Amrein *et al.*, 2004). Figure 1-3 shows some factors affecting acrylamide formation in rich-carbohydrate foods.

1.3.5.1 Cultivars and growing conditions

A study by Claus *et al.* (2006) found that content of free asparagine in nine wheat cultivars and two rye cultivars ranged between 5-25 and 41-44 mg/100 g flour respectively. Whereas the reducing sugar content in these cereals ranged between 0.4-0.9 and 1.1- 1.2 g/100 g flour in the nine wheat and two rye cultivars respectively.

Stadler *et al.* (2002) and Amrein *et al.* (2003) point out that potato cultivars have different levels of potential acrylamide formation because of different contents of

reducing sugar and asparagine. Also, they show that potato has a varied range of glucose and fructose content compared with asparagine. A study by Robert *et al.* (2004) shows that the sugar type affects the amount of acrylamide formed in food. The concentration of glucose and fructose in potato tubers were positively correlated with acrylamide formation.

Bent *et al.* (2012) conclude the products high in asparagine and reducing sugar such as whole wheat and white bran, have higher acrylamide content comparing with white wheat. Surdyk *et al.* (2004) showed asparagine had a very strong relationship to acrylamide content. Adding asparagine (0.7 g/100 g) in wheat flour increased the acrylamide content from ND to 6000 µg/kg of crust. The study results showed a significant effect in adding asparagine, whereas, adding fructose had no significant effect. These results were in agreement with Mustafa (2010) who studied the relationship between added asparagine and fructose in acrylamide content in rye crispbread.

According to the Food Drink Europe acrylamide toolbox (2011), the acrylamide level can be reduced in cereal food products by minimizing reducing sugars, and by selecting a potato or wheat variety with a low amount of reducing sugar. A study by Sanny *et al.* (2012) shows how acrylamide formation in French fries and crisps potato can be reduced by using a potato low in asparagine and reducing sugars. French fries were found to have a low content of asparagine and reducing sugars, at 928 and 322 µg/kg in order.

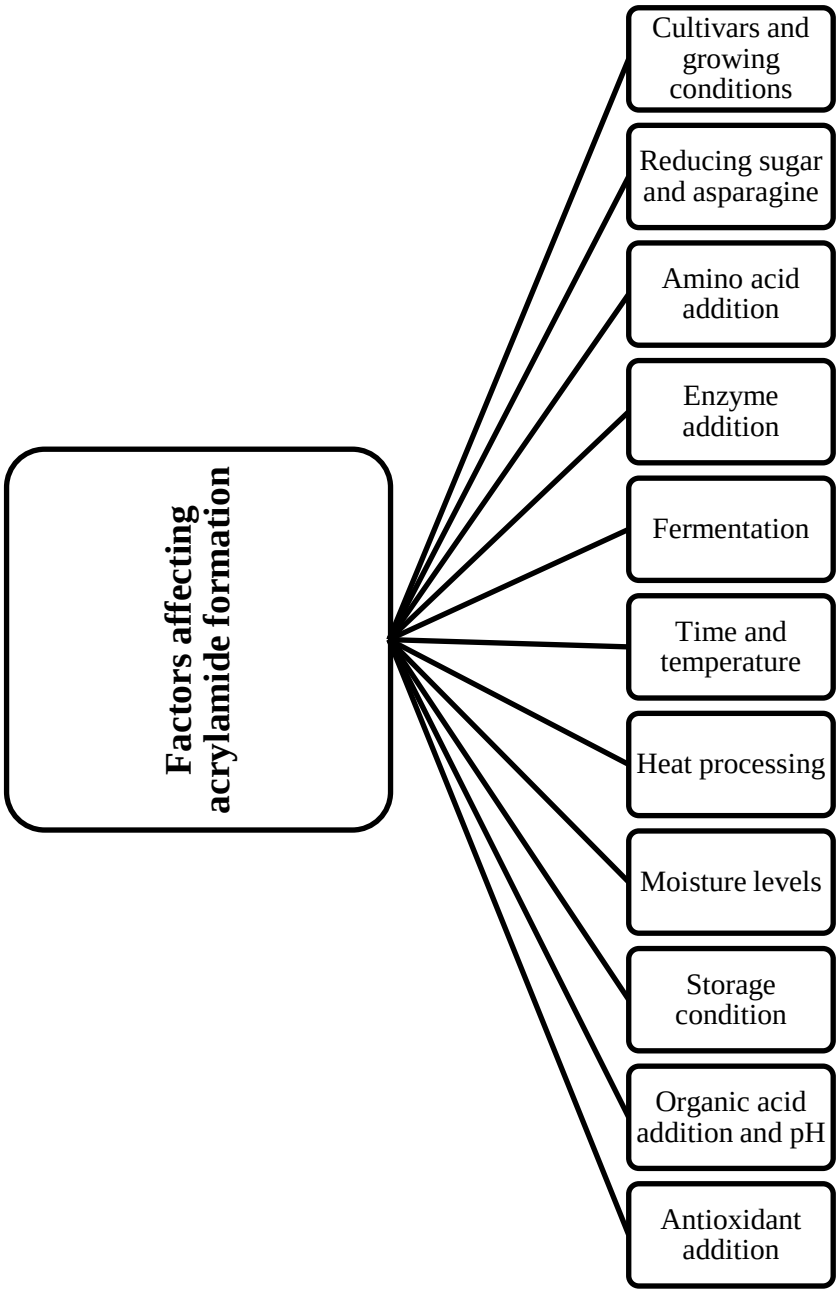


Figure 1-3: Scheme for factors affecting acrylamide formation in rich-carbohydrate foods.

1.3.5.2 Reducing sugar and asparagine

According to Mottram *et al.* (2002a) asparagine reacts with reducing sugar to form acrylamide in a dry system and buffer, and there is a variation in acrylamide level based on the type of amino acid. Table 1-2 shows the differences in acrylamide level occurring in two different conditions and amino acid types. Asparagine without sugar was found not to form any acrylamide in a study by Mottram *et al.* (2002a). A study by Stadler *et al.* (2002) found that acrylamide was formed when they mixed different amino acids (asparagine, glycine, cysteine and methionine) with equal amounts of D-fructose, D-galactose, lactose or sucrose. Also, they found heating sugar alone did not form acrylamide.

The ratio of amino acid and reducing sugar is important for acrylamide formation. The asparagine percentage in potato, wheat and rye of the total amino acid were found to be 40%, 14% and 18%, with 940, 167 and 173 µg acrylamide formed per one kilogram of potato, wheat and rye respectively (Mottram *et al.*, 2002a). A mixture of asparagine and fructose formed acrylamide in lower temperatures (125 ˚C) earlier than those containing glucose (140 ˚C), corrolating with the sugar's melting point (Robert *et al.*, 2004). A study by Surdyk *et al.* (2004) showed that increasing the asparagine content by spiking the dough with asparagine before baking led to a strong increase in the level of acrylamide, whereas, an increase in fructose had no effect. A study by Sanny *et al.* (2012) showed that replacing reducing sugars with sucrose could lower acrylamide. Therefore, reducing the free asparagine can be a way to control acrylamide formation (Yaylayan *et al.*, 2003).

Granby *et al.* (2008) studied the correlation between acrylamide and asparagine by determining the amount of asparagine in flour, dough and bread and the acrylamide content in bread. They also added different amounts of asparagine in wheat and rye to study the change in asparagine content and correlated this with acrylamide content in toasted wheat and rye bread. The asparagine level was 0.14 and 0.76 g/kg in wheat and rye flour respectively. They added different amounts of asparagine to the flour from 0.08 to 46 g/kg and they found the asparagine content decrease after toasting the bread (around 60%). The acrylamide content increased dramatically when asparagine content was increased, for example, 0.14, 0.22, 0.37

and 0.60 g/kg asparagine in medium toasted bread formed 11, 26, 46 and 130 µg/kg acrylamide and a reduction in asparagine content to 0.01, 0.03, 0.08 and 0.19 g/kg respectively was noted. The acrylamide content in hard toasted bread was 15, 34, 65 and 161 µg/kg respectively.

Table 1-2: Changes in acrylamide level under varying conditions.

Acrylamide level (mg/mol of amino acid)	Conditions (185 ˚C)	Sugar	Amino acid
221	Phosphate buffer	Glucose	Asparagine
>0.5		Glucose	Glycine, cysteine and methionine
0.5-1			Glutamine or aspartic acid
25	Dry mixture	Glucose	Asparagine
>0.5			Glutamine or aspartic acid

1.3.5.3 Amino acid addition

Acrylamide formation can be reduced by adding other amino acids which may compete with asparagine in specific amounts provide they do not affect the flavour (Food Drink Europe, 2011). Wedzicha *et al.* (2005) showed in a food model system that the concentration of amino acid and the proportion to asparagine is an important factor for acrylamide formation. Adding amino acid (cysteine) to the dough reduces acrylamide formation. Furthermore, glycine and glutamine can be used to reduce acrylamide formation in wheat bread (Brathen *et al.*, 2005, Fink *et al.*, 2006).

1.3.5.4 Enzyme addition

Asparagine can be reduced in potato and cereal by adding the asparaginase enzyme, which through hydrolysis leads to aspartic acid and ammonia. This addition was described in a study by Zyzak *et al.* (2003) as a method to reduce acrylamide formation in starchy foods. According to Amrein *et al.* (2004), adding asparaginase enzyme to bakery products results in a decrease in asparagine content by 75% then acrylamide formation by 55%.

1.3.5.5 Fermentation

Fermentation of dough using yeast reduces acrylamide formation in yeast-leavened products. Fredriksson *et al.* (2004) suggest that an extension in the fermentation time of wheat bread dough leads to a reduction in acrylamide formation in bread and bread rolls, as a result of the asparagine being consumed by yeast. According to Claus *et al.* (2008), sourdough (lactic acid bacteria and yeast) fermentation reduces the acrylamide level in crisp bread by 75% and this is a result of a drop in the pH (from 6.0 in the control to 3.7 in their study).

1.3.5.6 Time and temperature

Baking and microwaving time and temperature have an effect on acrylamide formation in potato and cereal foods. The control of important processing parameters; such as; heating temperature and heating time, is one of the main ways to reduce acrylamide (Stadler *et al.*, 2002).

Yuan *et al.* (2007) found that an increase in treatment time led to an increase in acrylamide contents. However, Erdogdu *et al.* (2007) showed that a longer pre-treatment time in a microwave at 850 W reduced acrylamide formation by around 36% to 60%, with a frying temperature of 150 °C and 190 °C respectively. They also reported no acrylamide formation in microwaved potato strips without any frying.

Surdyk *et al.* (2004) found that around 99% of acrylamide in wheat bread, formed on the bread's surface (crust). High temperatures and longer baking times led to a decrease in the moisture level of the bread crust. For these reasons toasting bread has been found to increase acrylamide formation (Ahn *et al.*, 2002).

1.3.5.7 Heat processing

Cooking methods such as baking, frying and toasting are the food processing methods that increase acrylamide content in starchy foods the most. Ahn *et al.* (2002) showed how grilled, deep fried, shallow fried, oven cooked, boiled and raw potatoes have different levels of acrylamide, at 1510, 420, 530, 230, >10 and >10 µg/kg respectively. These results were obtained using GC-MS and LC-MS

methods on the same samples, with similar results. Also, oven cooked French fries contain four times more acrylamide than fried French fries, at 12,800 and 3500 µg/kg respectively. Also, they showed that the French fries' shape (surface area) effects the acrylamide level, as chipped potato contained higher levels than potato wedges, at 2800 and 420 µg/kg respectively.

Rydberg *et al.* (2003) showed how an increase in microwaving time from 100 to 150 sec increased acrylamide level from 47 to 4400 µg/kg in pre-prepared potato strips. Granby *et al.* (2008) studied the correlation between acrylamide, toasted bread and asparagine in wheat and rye. Their results showed that toasting levels are important in terms of acrylamide formation. A high level of asparagine (0.6 g/kg) in untoasted bread did not lead to the formation of a detectible level of acrylamide (<5 µg/kg), whereas medium and hard toasting increased acrylamide level to 130 and 161 µg/kg.

Bent *et al.* (2012) showed, when analysing acrylamide in Caribbean foods, that there was no acrylamide in boiled foods. Roasting foods (such as peanut cake) which need high temperatures (320 ºC or more) show an increase in acrylamide level and the amount of food can increase the roasting time, which increases the acrylamide level further (no data was provided).

Frying pre-treated potato at different temperatures, namely 150, 160 and 170 ºC, was shown to increase acrylamide level to 87, 233 and 760 µg/kg respectively with potato water treatment, and 6, 6 and 30 µg/kg respectively with potato blanching treatment (Heat-generated Food Toxicants, 2007).

Acrylamide can be decreased by 40% and 60% using a steam technique in traditional baking in the last 5 minutes, as well as using infrared radiation heating. Whereas frying potato in a vacuum (50 mm Hg) reduces acrylamide level from 760 to 267 µg/kg respectively at the same temperature (170 ºC) (Heat-generated Food Toxicants, 2007).

1.3.5.8 Moisture levels

Moisture levels affect acrylamide formation and elimination; for instance, food low in moisture with heat was found to lead to acrylamide formation (Amrein *et al.*, 2004). In food with very high moisture levels, such as boiled food, acrylamide formation was found to decrease (Mestdagh *et al.*, 2006).

1.3.5.9 Storage condition

Storage conditions may affect acrylamide level in the raw matrix because of carbohydrate hydrolysis which increases the reducing sugar, although it does not change asparagine and other amino acid contents. For example, storing potato at a temperature lower than 10 °C increases the acrylamide level during cooking (Grob *et al.*, 2003). Also, a study by Robert *et al.* (2004) found that glucose, fructose and sucrose contents increased during cold storage because of sugar hydrolysis, leading to increased acrylamide levels during cooking processes, whereas asparagine and other amino acid contents do not change during storage. The Food Drink Europe acrylamide toolbox (2011) suggests controlled tuber storage conditions can minimize reducing sugar level.

Acrylamide reduction in cereal foods during storage time was studied by Stadler (2005) who studied acrylamide stability in different foods stored in different temperatures for different periods of time. The results showed that acrylamide in cereal products were stable for 12 months. However, Andrzejewski *et al.* (2004) found that the acrylamide level decreased (40%-65%) after storing coffee at room temperature for 6 months. Whereas storing the same batch at -40 °C in a sealed container did not significantly affect the acrylamide content.

1.3.5.10 Organic acid addition and pH

Lowering pH by adding a controlled amount of citric or ascorbic acid leads to a reduction in acrylamide formation, but it may affect the colour and flavour of the food (Cook and Taylor, 2005). Studies have shown the effect on pH reduction of adding a consumable acid (lactic, tartaric, citric or hydrochloric acids) to a biscuit model, as well as the decrease in acrylamide level (Levine and Smith, 2005, Graf *et al.*, 2006).

1.3.5.11 Antioxidant addition

Antioxidants may reduce acrylamide formation. A study by Hedegaard *et al.* (2008) found there was a decrease in acrylamide formation in bread by 62% when they added 1% aqueous rosemary extract, suggesting rosemary is an inhibitor of acrylamide formation. Also, treating French fries with olive oil or corn oil and adding rosemary decreases the acrylamide content by 25% (Becalski *et al.*, 2003). Zhang (2007) studied the effect of antioxidants on fried bread sticks. The results showed a reduction in acrylamide level by 83% and 78% for bamboo leaves and extract of green tea respectively (1 g/kg).

1.3.6 Efforts to reduce acrylamide levels

Since the discovery of acrylamide in food in 2002, it has still not been determined whether there is any risk from consuming acrylamide. This makes it problematic in using regulations for reducing acrylamide exposure and acrylamide levels in food and drink. Nevertheless, industry has tended to reduce acrylamide levels in foods, but there is no single solution for this reduction. Changes in processing conditions (heating temperature or time) to reduce acrylamide formation may lead to changes in product quality; for example, a changed flavour, a decrease in nutrients or a decrease in food safety (Food Drink Europe, 2011).

Food Drink Europe (2011) have published a 'toolbox' for reducing acrylamide levels in food produced by industry. Reducing acrylamide content can lead to lowering exposure estimates and thus the potential risk to a population. Recommendations for pre-prepared products advise the consumer to follow guidance such as; 1) cook to a golden yellow colour, 2) cook at a maximum of 175 °C, 3) reduce the cooking time and 4) do not overcook, as these can all help to reduce the formation of acrylamide (Food Drink Europe, 2011).

Based on the factors affecting acrylamide formation (section 1.2.5) and according to FDA (2013), to reduce acrylamide formation several factors should be controlled, such as follows: 1) control reducing sugar content by selecting potato varieties low in reducing sugar, avoid low temperatures from harvest to processing and use a treatment to decrease reducing level (blanching and dipping); 2) lower

asparagine content by choosing wheat varieties low in asparagine and use yeast fermentation to reduce it in wheat; 3) decrease processing temperature (>175 ºC) and increase the time by increasing the thickness of potato chip slices or bakery items and 4) use colour as an indicator by frying, baking or toasting food to a golden yellow colour.

Most mitigation measures have only been tested in laboratories or on a pilot scale. Also, it is not clear if levels of acrylamide reduction in a laboratory can be achieved in food processing (Capuano and Fogliano, 2011).

It would be beneficial to reduce acrylamide levels in foods that contribute to population exposure, especially infants as they are a sensitive group with high exposure to acrylamide in foods such as bread, biscuits and potato products (Food Drink Europe, 2011).

1.3.7 Acrylamide metabolism

Acrylamide is absorbed through all routes of exposure. Absorption is most rapid and complete by the oral route in all species. Also, it is absorbed by the mucosae, skin and lungs (if inhaled). Around 25% of acrylamide can be absorbed from the gastro-intestinal tract in 24 hours (Dearfield *et al.*, 1988). It is widely and rapidly distributed in the body through the blood due to hydrosolubility to all tissues, as shown experimentally in rats, mice, dogs and pigs (Southgate *et al.*, 1978). Acrylamide and glycidamide are equally distributed in body tissues (Toda *et al.*, 2005). Acrylamide can be found in many organs, such as the liver, heart, brain and kidneys (Abramsson-Zetterberg *et al.*, 2005), and in breast milk, it is also easily transferable to the new-born infant through the placenta (Sorgel *et al.*, 2002).

Acrylamide is accumulated and persists in red blood cells (Dearfield et al., 1988). In children a high level of acrylamide in the liver may be anticipated because children's under-developed liver cannot carry as high a burden as an adult's liver (Miller et al., 1982). In the intestine acrylamide can bind with protein, reducing the level of acrylamide in the blood (Schabacker et al., 2004).

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Acrylamide and its metabolites (glycidamide) can accumulate in the body when they bind to protein in nervous system tissues or haemoglobin in blood. (Exposure to Environmental Hazards, 2003). Regarding acrylamide accumulation, FAO/WHO (2006) reported that DNA adducts have been found after repeated dosing of acrylamide or glycidamide in mice's liver, lung, testis and kidney, whereas in rats the finding was in the liver, thyroid, testis, mammary gland, bone marrow and brain. Doerge *et al.* (2005) show acrylamide and glycidamide accumulation in mice and rats. The rates were 0.94 and 0.91 µM/hour for acrylamide and glycidamide respectively. Crofton *et al.* (1996) pointed the accumulation rate of acrylamide and glycidamide was related to the dose rate.

Consumed acrylamide is taken into circulation and excreted mainly with urine. From a dose of 0.9×10^3 µg of acrylamide taken by a male, 60% was recovered from urine in 72 hours (Fuhr *et al.*, 2006). The half-life in blood is approximately 2 hours and in tissues approximately 5 hours (Dybing *et al.*, 2005).

Unsaturated double bond (alpha and beta) in acrylamide imparts of its activity due to interacts with proteins (Schabacker *et al.*, 2004). Acrylamide is metabolised in the body to glycidamide, a reactive compound formed through epoxidation of the double bond of acrylamide (Lignert *et al.*, 2002). The cytochrome P450 2E1 enzyme (CYP 2E1) is responsible for oxidizing acrylamide to glycidamide (Sumner *et al.*, 1999). Glycidamide is important for evaluation because it has a major role as an acrylamide carcinogenic in rodents (Tardiff *et al.*, 2010). According to a FAO/WHO report (2006), the conversion of acrylamide to glycidamide is different between species, the lower conversion being in humans, rats then mice, in order. In mice it was found that 50% of acrylamide oxidized the DNA by CYP 2E1 to glycidamide (Jägerstad and Skog, 2005). Bergmark *et al.* (1991) found more than 50% of acrylamide was converted to glycidamide in vivo (Spraque-Dawley rats).

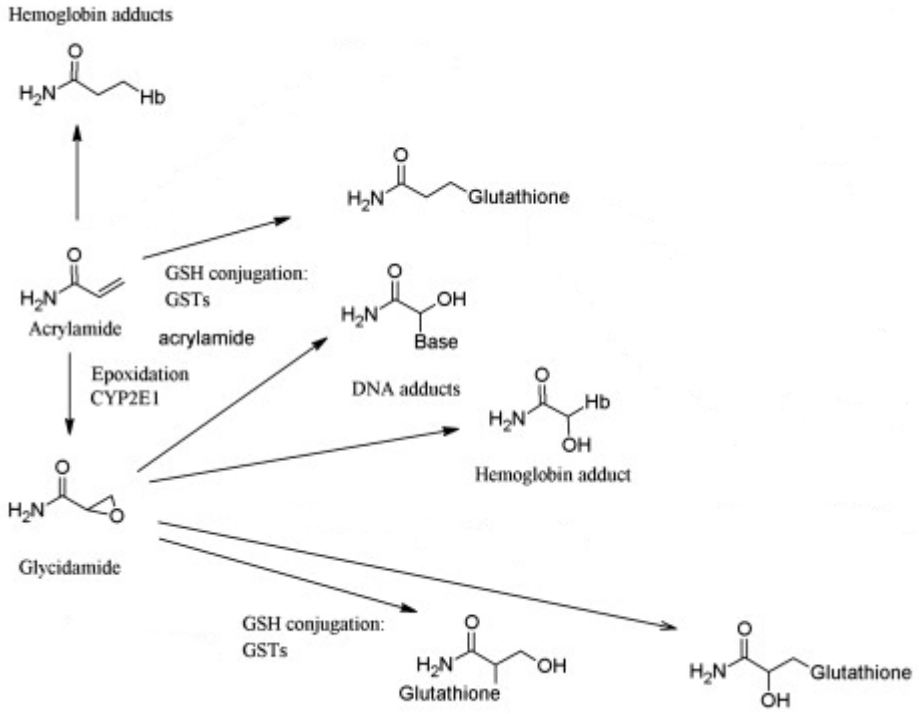

Figure 1-4: Proposed metabolism of acrylamide adapted from Capuano and Fogliano (2011).

Acrylamide and glycidamide are detoxified by glutathione (GSH) conjugation and hydrolysis respectively. The excretion of acrylamide and glycidamide in humans is via the urine N-acetyl-S(2-carbamoylethyl)cysteine which is a degradation of the conjugated glutathione (Carere, 2006). The glutathione conjugation of glycidamide is a major pathway in rodents, but it is very low in humans (Fennell *et al.*, 2005)

Sweeney *et al.* (2010) compared human and rat metabolism of acrylamide in the blood. They found that the value of 0.1×10^3 µg/kg of acrylamide in rats is equal to a human dose of 0.02×10^3 µg/kg and 0.13×10^3 µg/kg of acrylamide and glycidamide respectively. According to FAO/WHO report (2006), acrylamide absolute bioavailability range from 23% to 48% in rodents for a dose of 0.1×10^3 µg/kg-bw (from food) over a 30 min period of time.

Capuano and Fogliano (2011) summarised acrylamide metabolism (Figure 1-4). Acrylamide epoxides to glycidamide and both of them conjugate to gluthione or haemoglobin adducts. However, glycidamide also metabolises to DNA adducts.

1.3.8 Acrylamide toxicology

Acrylamide and glycidamide form adducts with protein including haemoglobin (reactive compounds) (Paulsson *et al.*, 2001). Haemoglobin (Hb) adducts are formed from the reaction of acrylamide and protein. These adducts are formed at the site of sulfhydryl (SH) groups and amino groups (N-terminal amino acids (α-NH_2)) (Carere, 2006, Klaunig, 2008). Carere (2006) studied acrylamide and glycidamide-haemoglobin adducts in rodents and adults and suggest that glycidamide formation is lower in humans than in rodents. Vikstrom *et al.* (2012) found the adducts of haemoglobin acrylamide and glycidamide in humans compared to rats were about five and two times higher respectively.

The formation of acrylamide adducts with DNA is very slow (Gamboa da Costa *et al.*, 2003). Glycidamide has the ability to form DNA adducts, which increase the risk of genotoxicity and cancer (Tornqvist, 2005).

According to the Food Standard Agency (2005) and International Agency for Research on Cancer (IARC) (1994a), the acrylamide dose which causes cancer in laboratory rats is 1000 times higher than the human exposure level for acrylamide. That is because the glycidamide percentage from dietary acrylamide exposure in humans is only 25% compared to 50% for rats and mice. This finding means there are less reaction products of glycidamide in humans for example, less DNA and haemoglobin adducts (Southgate *et al.*, 1978).

Acrylamide is toxic at high oral doses. The lethal dose (LD_{50}) values in rats range from 1.7×10^3 to 203×10^3 µg/kg-bw (European Commission, 2002). However, Manson *et al.* (2005) found that the acrylamide LD_{50} in rats and mice through the oral route is around 107×10^3 to 251×10^3 µg\kg-bw. Whereas FAO/WHO (2006) reported that human LD_{50} is less than 150×10^3 µg\kg-bw for oral dose, and the acute toxic value for a single oral dose was reported to be $>100\times10^3$ µg\kg-bw. The No Observable Adverse Effect Level (NOAEL) for acrylamide neuropathy as

stipulated by FAO and WHO consultation is 0.5×10^3 µg\kg-bw/day, and the NOAEL for fertility changes is four times higher than for peripheral neuropathy and 2000 fold greater than estimated dietary exposures. Also, the NOAEL estimate for reproductive toxicity is 2×10^3 to 5×10^3 µg/kg-bw/day, but no reproductive toxicities have been reported in humans (Food and Agricultural Organization and World Health Organization, 2002a). The NOEL for acrylamide neurotoxic effect was estimated in 2002 (Food and Agricultural Organization and World Health Organization, 2002a) to range from 0.2×10^3 to 10×10^3 µg /kg-bw/day, which is higher than dietary exposure.

The Tolerable Daily Intake (TDI) is the amount of chemical consumed daily from contaminant food or drink that is safe for humans throughout their whole lifetime. It is calculated on the basis of laboratory toxicity data (Benford, 2000). The TDI estimation of acrylamide for neurotoxicity was 40 µg/kg-bw/day, whereas for cancer it was 2.6 and 16 µg/kg-bw/day for acrylamide and glycidamide respectively, based on studies on rats (Tardiff *et al.*, 2010).

Margins of exposure (MOE) were used by JECFAS to estimate the risk posed to humans. The MOE value was calculated by dividing the toxicity estimate from animal experiments by the estimated exposure from food. The lower result of the MOE shows greater public concern. In a FAO/WHO report (2006), to represent the mean exposure of the whole population the value of 1 µg/kg-bw/day was compared with a benchmark dose (BMD) value of 30 µg/kg-bw/day, the MOE for induction mammary tumours in rats is 300. The respective MOE was 75 when representing the high level exposure of acrylamide of 4 µg/kg-bw/day. The cancer risk in humans was in the range of 1-4 $\times10^4$ for the thyroid, central nervous system and mammary gland for mean and high levels of consumption (Food and Agricultural Organization and World Health Organization, 2011).

Acrylamide exposure as a daily occurrence may result in by chronic exposure over a lifetime despite relatively short half-life, potentially causing health risks. It has been stated in Svensson *et al.* (2003) that a lifetime risk of dietary acrylamide exposure could be compared to the lifetime risk of ionizing radiation. Long-term exposure to acrylamide may lead to damage of the nervous system to some extent

in both humans and animals. It may also be a probable genetic and reproductive toxin with mutagenic and carcinogenic properties, as found in experimental mammalians both in *in vitro* and *in vivo* studies (Dybing and Sanner, 2003, Zhang *et al.*, 2005b). Acrylamide oral exposure has acute (short-term) and chronic (long-term) effects, such as nervous system damage (United State Environmental Protection Agency, 2000).

Pedersen *et al.* (2012) studied the association between acrylamide and low birth weight and head circumference of infants delivered by 1101 pregnant women from Europe. Food data was collected by food frequency questioner (FFQ) and the results showed a positive association with low birth weight and head circumference.

Risk assessment estimates of cancer in humans from acrylamide exposure are usually based on linear extrapolation from carcinogenicity data and long-term bioassays in rodents (Petersen and Tran, 2005). Mucci and Wilson (2008) studied the acrylamide level exposure and the risk of cancer in four different countries (Sweden, Italy, the Netherlands and the United States). The study showed there is no association between high exposure of acrylamide and the risk of cancer in the large bowel, kidney, bladder, oral cavity and pharynx, esophagus, larynx, ovary and breast. However, the possible risk of acrylamide exposure to human health has been discussed in many other studies, as shown in Table 1-3. These epidemiological studies have illustrated the correlation between acrylamide consumption and cancer. There are studies with conflicting results regarding the association between acrylamide and ovarian and kidney cancer (positive and negative association). Acrylamide can be passed via the blood and diffuse around the body and for this reason it may cause cancer in the following: 1) the gastric system (colorectal, gastric, esophageal, oral cavity, oro-hypopharynx, larynx and thyroid) due to consumption; 2) the respiratory system may be affected by inhalation of acrylamide; and 3) the endocrine system may be affected due to excretion from the body (Carere, 2006).

Although there is significant evidence supporting the view that acrylamide is genotoxic and carcinogenic *in vivo* and *in vitro*, epidemiological data has not yet

shown a relationship between acrylamide dietary exposure and an increase in cancer risk for humans, as shown in Table 1-3. This result may change if studies were to consider biomarkers of exposure instead of dietary estimation (Olesen *et al.*, 2008). There are several reasons that could explain the disagreement in results of epidemiological studies: 1) relative cancer risk is so low even at high exposure levels that no epidemiological studies can detect the effect, even in well-designed epidemiological studies (Mucci and Adami, 2005), 2) there could be an inaccurate estimation of acrylamide exposure, (using dietary consumption and not acrylamide biomarker although it precautionary principles applied underestimation is unlikely, 3) acrylamide levels in the same food are highly variable depending on the season, cooking method and preparation method, 4) people change food brands much of the time which may lead to a change in acrylamide exposure levels, 5) there may be variations in acrylamide levels in batches of the same food brand, 6) the instrument used to record food consumption may be another source of underestimation of acrylamide exposure, 7) comparing human exposure with animal exposure cannot be avoided and introduce uncertainty due to species sensitivity of acrylamide bioavailability and the effect of other food content on acrylamide absorbance and 8) there is no standard analytical method for determination of acrylamide in food or haemoglobin (Hogervorst *et al.*, 2008a, Mucci and Wilson, 2008, Xu *et al.*, 2014). The amount of acrylamide in the same food may also vary depending on the equipment used. Combining a dietary consumption method with acrylamide biomarkers could be a more accurate approach to studying acrylamide genotoxicity and its carcinogenic nature.

The strengths of epidemiological studies are that they directly report human exposure of acrylamide from food and the risk of cancer and/or other risks. Also, they tend to avoid uncertainties from extrapolating data from animals due to the higher doses and exposure compared with humans (1000-10,000 times) (Mucci and Wilson, 2008).

These studies show the importance of acrylamide analysis in food and estimating the consumption in all age groups, especially those of sensitive age.

Table 1-3: Summary of literature showing the association of acrylamide in humans using dietary methods for acrylamide measurement.

Population	Dietary assessment method (items)	Acrylamide exposure (mean µg/kg-bw/day)	Association between dietary exposure of acrylamide and cancer risk (cancer type)	Reference
Swedish with cancer of the large bowel, bladder, kidney and healthy controls, numbered 591, 263, 133 and 538 respectively (-).	FFQ (188)	0.30	No association (large bowel, bladder and kidney).	(Mucci *et al.*, 2003)
The Netherlands Cohort based on diet and cancer (NLCS) included 2,589 women aged 55-69 years in 1986 then follow up after 11.3 years. 327, 300 and 1,835 cases of endometrial, ovarian and breast cancer respectively (1986-2000).	FFQ (150)	0.26	Positive association with P< 0.01 (postmenopausal endometrial and ovarian) and no association (breast).	(Hogervorst *et al.*, 2007)
5000 participants from NLCS aged 55-69 years. 2190, 563, 349 and 216 cases of colorectal, gastric, pancreatic and esophageal cancer respectively (1986-2000).	FFQ (150)	0.30	No association (colorectal, gastric, pancreatic and esophageal).	(Hogervorst *et al.*, 2008b)
5000 participants aged 55-69 years from NLCS. 339, 1210 and 2246 cases of kidney cell, bladder and prostate cancer respectively (1986-2000).	FFQ (150)	0.30	No association (bladder and prostate) and positive association (kidney cell) in group of men and women.	(Hogervorst *et al.*, 2008a)
216 participants with brain cancer from NLCS aged 55-69 years (1986-2000).	FFQ (150)	0.30	No association (brain).	(Hogervorst *et al.*, 2009a)
2649 participants with lung cancer from NLCS aged 55-69 years (1986-2000).	FFQ (150)	0.30	No association (lung).	(Hogervorst *et al.*, 2009b)

FFQ: Food frequency questionnaire, DQ: Dietary questionnaire

Continued:

Population	Dietary assessment method (items)	Acrylamide exposure (mean µg/kg-bw/day)	Association between dietary exposure of acrylamide and cancer risk (cancer type)	Reference
430 participants from NLCS aged 55-69 years. 101, 83, 180 and 66 cases of oral cavity, oro-hypopharynx, larynx and thyroid cancer respectively (1986-2000).	FFQ (150)	0.32	No association (oral cavity, oro-hypopharynx, larynx and thyroid).	(Schouten *et al.*, 2009)
1084 women with breast cancer (aged 35-69 years) from a UK women cohort study, followed up for a median of 11 years.	FFQ (217)	0.30	No association (breast).	(Burley *et al.*, 2010)
325,006 women from the European Prospective Investigation into Cancer and Nutrition (1992-1998).	DQ (-)	0.40	No association (epitheal ovarian cancer).	(Obón-Santacana *et al.*, 2014)
301,113 women from the European Prospective Investigation into Cancer and Nutrition (1992-1998).	DQ (-)	0.40	No association (endometrial cancer).	(Obon-Santacana *et al.*, 2014)
477,308 participants from the European Prospective Investigation into Cancer and Nutrition (1992-1998).	DQ (-)	0.45	No association (pancreatic cancer).	(Obón-Santacana *et al.*, 2013)

FFQ: Food frequency questionnaire, DQ: Dietary questionnaire

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The results of acrylamide studies *in vivo* and *in vitro* are illustrated in Tables 1-4 and 1-5 respectively. The acrylamide with *in vivo* and *in vitro* have different effects including damaging nerves, reducing fertility, increasing mortality rate and causing tumours. For example, nerves were damaged in cats fed with 15×10^3 µg/kg-bw/day for 4-6 weeks. In El-Assouli's (2009) study acrylamide genotoxicity was investigated by feeding rats by oral gavage with 0.4×10^3 µg/kg-bw/day of potato crisps (Lays') extraction for 30 days. The results did not show any effect of acrylamide on the rats' DNA. Rats fed with acrylamide extracted from food for a long period of time (106 weeks) with low doses ($0.5\text{-}10\times10^3$ µg/kg-bw) or a short time (10 days) with high doses (10, 15, or 20×10^3 µg/kg-bw) led to an increase in mortality rate. Also, rats fed with high doses of acrylamide for a short period of time had reduced fertility, sperm, and weight, as shown in Table 1-4.

In *in vitro* studies, the effect of acrylamide was shown to give potential of forming breast cancer and cellular transformation. A positive result from *in vitro* studies was that acrylamide binds with protein which reduces acrylamide levels in the blood, as shown in Table 1-5.

Tables 1-3, 1-4 and 1-5 show the differences between human studies and *in vivo* and *in vitro* studies. Epidemiological studies estimate acrylamide exposure (long term), whereas in *in vivo* and *in vitro* studies acrylamide exposure in specific periods (acute high doses) is studied. Also, acrylamide fed in *in vivo* and *in vitro* studies was higher compared with human consumption based on body weight.

As shown in Table 1-3, all studies show acrylamide exposure was less than human LD_{50} ($>150\times10^3$ µg\kg-bw), which means there is no risk of death from acrylamide exposure. Whereas the animal exposure in Table 1-4 was lower than human LD_{50} in most studies except study of Szczerbina *et al.* (2008) which shows high dose lethal for the larvae.

Table 1-4: Summary of publications that show the effect of acrylamide exposure on health outcomes *in vivo* with different species of rats, mice, cats and Musca, with different acrylamide doses for different numbers of days.

Population	Acrylamide dose $\times 10^3$ µg/kg-bw/ (day)	Acrylamide effects	Reference
Rats (long-Evans hooded)	0, 5, 15, 30, 45 or 100 (5 days)	Reduce fertility and absence of sperm.	(Sublet *et al.*, 1989)
Rats (Sprague-Dawley)	0, 5, 10, 15, or 20 (10 days)	Increased mortality rate and decrease body weight in groups consuming $\leq 10\times 10^3$ µg/kg /day.	(Wise *et al.*, 1995)
Rats (Fischer 344)	0, 0.1, 0.5 0r 2 for male; 0, 1 or 3 for female (106 weeks)	Increased mortality rate and variety of tumour type (thyroid and mammary glands) with high doses.	(Friedman *et al.*, 1995)
Rats (long-Evans)	0, 5, 15, 30, 45 or 60 (5 days)	Reducing in weight, fertility and pregnancy at $>15\times 10^3$ µg/kg-bw/day.	(Tyl *et al.*, 2000)
Rats (Sprague-Dawley)	0.4 (30 days)	Did not induce any DNA break.	(El-Assouli, 2009)
Rats (Sprague-Dawley)	0, 5, 15 or 30 (4 weeks)	Toxicological effect on reproductive system	(Ma *et al.*, 2011)
Mice (Albino Swiss) **Rats (Sprague-Dawley)**	0, 3, 15 or 45 (7 days). 0, 2.5, 7.5 or 15 (7 days)	High dose reduced body weight.	(Field *et al.*, 1990)
Mice (CD-1 & C57 BL/6J)	20, 60 or 100 (7 weeks)	High dose reduced body weight, led to severe neuropathy and 100% mortality within 2 weeks.	(Teal and Evans, 1982)
Mice (CD-1)	0, 3, 15, or 45 (7 days)	Reduced foetal weight with higher acrylamide doses.	(George *et al.*, 1998)
Musca (housefly)	82, 164 or 246 (2 days)	Higher doses of acrylamide were lethal for the larvae.	(Szczerbina *et al.*, 2008)
Cats (-)	15 (4-6 weeks)	Damage to the sympathetic nervous system.	(Post and Mcleod, 1977)

Table 1-5: Summary of publications that show the effect of acrylamide exposure on health outcomes *in vitro* with different acrylamide doses for different numbers of days.

Population	Acrylamide level and time			Outcome	Reference
	mM	µg/L	time		
Syrine hamster embryo (SHE) cell morphological	0.01 to 10	0.1 to 71.0	7 days	Acrylamide induced cellular transformation.	(Park *et al.*, 2002)
Human epithelial Caco-2 cell	1 and 10	7.1 to 71.0	90 min	Acrylamide bound to dietary protein, which reduced the acrylamide uptake through Caco-2.	(Schabacker *et al.*, 2004)
Non-tumorigenic breast cell (MCF-12-A)	0.001, 0.01, 0.1or 0.5	0.0, 0.1, 0.7 or 3.6	1 day	Acrylamide induced changes similar to the early stages of breast cancer development.	(Lyn-Cook *et al.*, 2011)

1.3.9 Acrylamide biomarkers

The ratio of acrylamide to glycidamide in urine can be considered as a measure of the extent of conversion of acrylamide to glycidamide, thus reflecting the latest internal exposure and being a biomarker for acrylamide consumption (Fuhr *et al.*, 2006). Haemoglobin adducts of acrylamide and glycidamide reflect exposure to acrylamide over the last four months (lifetime of the protein) and have been used as a biomarker of long-term exposure to acrylamide (Hagmar *et al.*, 2001, Lignert *et al.*, 2002, Friedman, 2003). DNA adducts can be a biomarker of acrylamide biologically active (internal dose) (Kirman *et al.*, 2003, Walker *et al.*, 2007, Young *et al.*, 2007).

Vesper *et al.* (2008) found difference in acrylamide exposure, determined by using haemoglobin, between European countries ranging between 15 to 623 and 8 to 377 pmol/g for haemoglobin acrylamide and haemoglobin glycidamide respectively.

A study by Olesen *et al.* (2008) investigated the association between acrylamide consumption and breast cancer by using acrylamide and glycidamide haemoglobin as biomarkers, and 374 breast cancer cases and 374 controls (aged 50-64 years) from a cohort of postmenopausal women consuming Danish diet (1993-1997). They found no association between acrylamide and breast cancer. In another study, Olsen *et al.* (2012) found study the same association using 420 postmenopausal women with breast cancer from a Danish cohort study (2001-2009), again, they did not find an association.

Figure 1-5 summarises the acrylamide metabolism and biomarker. Acrylamide and glycidamide biomarkers are divided into short exposure, such as urine and DNA, and long exposure (haemoglobin adducts).

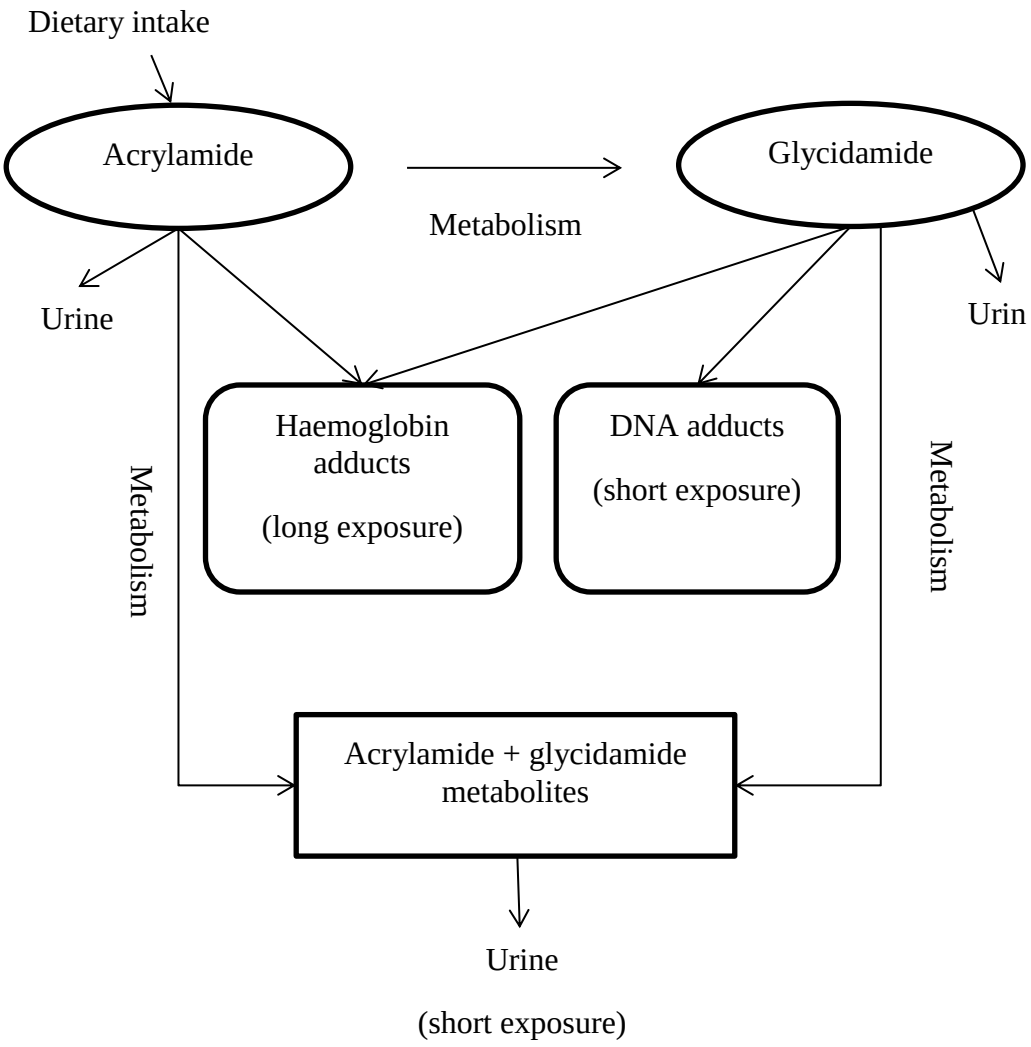


Figure 1-5: Scheme of acrylamide metabolism and biomarker for determination of long and short term exposure.

1.3.10 Measuring acrylamide

There is no AOAC (Association of Official Agricultural Chemists) method for acrylamide determination, so several extractions and analytical methods have been used to measure acrylamide level in the food matrix.

Chromatographic methods such as gas chromatography mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and liquid chromatography

mass spectrometry (LC-MS) have been used to determine acrylamide levels. GC-MS has been used to analyse acrylamide with or without derivatisation. The molecule is normally brominated to form a derivative which has improved properties for GC separation and detection. Also, liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) has been used (Croft *et al.*, 2004, Zhang *et al.*, 2005b). There have been many studies that have analysed acrylamide in food using LC-MS, GC-MS and HPLC (see Table 2-2).

The different methods used to determine acrylamide levels include use of a variety of extraction and clean up procedures for different food matrices. Water has been the most used solvent for acrylamide extraction and with clean up steps by centrifugation and/or solid phase extraction (SPE) cartridges required before analysis (as will be explained further in Chapter 2). The detection of acrylamide is more difficult with a food matrix that has low levels of acrylamide (Govaert *et al.*, 2006).

Owen *et al.* (2005) showed that laboratories have a large variation for a range of matrices. The variation in acrylamide levels between laboratories will affect the estimation of exposure levels and so it is recommended that acrylamide be quantified in foods of the target population where possible.

El-Assouli (2009) analysed acrylamide in a selection of foods (23 types) using GC-MS. The range of acrylamide was found to vary from non-detectable to 2200 µg/kg for French fries, potato crisps, bread, biscuits, breakfast cereal and coffee. The study found that the level of acrylamide in Pringles potato crisps is 930 µg/kg. However, the study did not provide the limit of detection or quantification. Another study in the Kingdom of Saudi Arabia (SA) by El-Ziney *et al.* (2009) analysed acrylamide in some traditional foods, infant milk and cereal based foods such as bread (2) biscuits (1) and infant cereal (1), using LC-MS/MS. This study is different to both of the former studies due to analysing a higher number and variety of different of food types consumed by Saudi infants, as will be shown in Chapter 3.

There is no ideal method for analysing acrylamide in food samples and no specific sampling method to reduce variations in acrylamide levels. For this reason two methods were developed to analyse acrylamide levels in Saudi food samples using HPLC and LC-MS.

1.4 *Acrylamide exposure*

FAO/WHO (2006) estimates acrylamide mean exposure at a national level (17 countries) to be between 0.3 and 2.0 µg/kg-bw/day for the whole population. Also, they estimate acrylamide exposure of the 90th to 97.5th percentile consumer to be in the range of 0.6 to 3.5 µg/kg-bw/day, although, the highest exposure was 5.1 µg/kg-bw/day (this result was based on limited data obtained by analysing part of the diet). WHO and FAO estimated acrylamide exposure by using Monte Carlo statistical techniques.

Exposure to acrylamide among children (1.5-4.5 years) was shown to be between 1.0 and 1.8 µg/kg-bw/day (Food and Agricultural Organization and World Health Organization, 2002a, Food Standards Agency, 2005). Children in general take two or three times more than adults based on body weight. This may be because of a combination of children's high calorie intake relative to body weight as well as their higher consumption of certain foods rich in acrylamide, such as French fries and potato crisps (Food and Agricultural Organization and World Health Organization, 2002a).

Fohgelberg *et al.* (2005) estimated the acrylamide exposure for Swedish infants in their first year of life to be in the range 0.04 to 1.20 µg/kg-bw/day, based on analyses of breast milk and infant formula. In the Netherlands, the mean infants' exposure to acrylamide is 0.48 µg/kg-bw/day (Konings *et al.*, 2003).

The acrylamide contribution of each individual product varies between countries depending on food habits, and many other factors such as age group, gender, region, season and ethnicity (Food and Agricultural Organization and World Health Organization, 2002a). FAO/WHO (2006) reported that main food contributors to acrylamide total exposure in most countries were 16%-30% French fries, 6%-64% potato crisps, 13%-30% coffee, 10%-20% pastries and sweet

biscuits, and 10%-30% bread. According to FAO/WHO (2002a), the acrylamide level in bakery food such as bread is low, but high consumption of it increases the exposure of acrylamide. Also, toasted bread is commonly eaten for breakfast, which means the acrylamide level is higher (Ahn *et al.*, 2002, Becalski *et al.*, 2003). Among German infants, bread accounts for about 18%-46% of acrylamide exposure due to their high consumption of bread (Hilbig *et al*., 2004).

1.4.1 Acrylamide dietary assessment methods

The first step of acrylamide estimation is identifying dietary assessment methods. The methods used to measure individual consumption of food are divided into two groups 1) records of individual quantities of daily consumption, such as 24-hour recalls and dietary records and 2) methods including dietary history and food frequency questionnaires (FFQs), which provide information on the eating habits of individuals. The use of household measurements such as cups and tablespoons, or food models and photographs of food can help with the recording of food consumed in terms of portion size over one or more days. The data should be collected non-consecutive days; for example, two days during weekdays and a day at the weekend. After collecting data from participants, these data can be converted to calculate consumption of acrylamide by using a food consumption database (Becalski *et al.*, 2003, Dybing *et al.*, 2005).

Twenty-four-hour recall methods asses actual consumption in the last 24-hour period by the same individual and can be done over several days in order to have sufficient data to enable estimation of mean consumption values. This method was used to estimate acrylamide exposure as a validation method for an FFQ (Bongers *et al*., 2012). The FFQ method involves lists of foods for use in specifying how frequently each food item or group is consumed during a specified time period (Pelucchi *et al.*, 2011, Bongers *et al.*, 2012). A FFQ considers usual consumption (long term exposure) which reduces the variability of acrylamide exposure from day to day. This is in contrast with 24-hour recall and dietary consumption. The best approach is to combine the two different methods into a specially designed FFQ and 24-hour dietary consumption or diet record (Dybing *et al.*, 2005). Among the Saudi infants a 24-hour recall was used for data collection and diet history was

used to validate the results (Thaiban, 2006). A dietary record method (weighed food record) is the preferred method in the UK to estimate the consumption of usual foods such as in the National Diet and Nutrition Survey (NDNS) (will explain in Chapter 5). The individual records the amount of food and beverages consumed over a day or more. The food is measured by using a scale or household measure (Hilbig *et al*., 2004). El-Ziney *et al.* (2009) studied the consumption of acrylamide in 50 Saudi adults (18-30 years old) by using a 7-day record, and 50 Saudi mothers of infants were asked about the type of milk and food brands they fed to their infants in the first year. Most studies have used dietary records as the food assessment method for 2 to 7 days, or the 24-hour method for 3 to 6 days (as shown in Table 1-6). The present study is novel because it will estimate infant's exposure from actual food consumption data taken in Saudi Arabia and actual acrylamide content in those foods consumed was measured, as will be shown in Chapter 5.

Wilson *et al.* (2009) used Hb adducts of acrylamide and glycidamide to validate a FFQ in the Nurses' Health Study 2 cohort (1989-2000). The FFQ contained more than 130 food items. Blood samples were provided two to three times from 296 women over 1-3 years. The results showed that around 30% of women were in the same classification for the FFQ and acrylamide and glycidamide adducts. The study concluded that the FFQ had errors such a limited food list and imperfect participant recall for food consumption and portion size. Also, they showed how the different methods led to different acrylamide levels being recorded in different periods of time; for example, the FFQ measured acrylamide exposure over a year, whereas the Hb-adducts measured acrylamide exposure, absorbance and metabolism over four months.

1.4.2 Acrylamide dietary composition

Dietary acrylamide exposure is a worldwide problem due to the large variety of staple foods that contain or may contain acrylamide. Population exposure occurrence varies between countries due to different levels in food and different dietary and processing practices; accordingly, contributions to exposure may be assigned to a particular food group, such as potato or cereal products (Dybing *et*

al., 2005). Owen *et al.* (2005), analysed a range of variations of acrylamide level in different laboratories for a range of matrices. This variation in acrylamide level affects the estimation of acrylamide exposure.

Table 1-6 shows the estimated daily exposure of acrylamide through diet in different countries. European countries (Sweden, the Netherlands, the UK and France) and other countries such as Turkey and Egypt estimate the acrylamide consumption in different age groups by using one or more food assessment methods. The table also illustrates the main food contributors to acrylamide level.

Most research has been concerned about the study of whole populations aged 1-97 years old or a specific age group such as adults and teenagers. There are only five studies in which absolute acrylamide exposure has been evaluated from aged one year. Only three studies have estimated infant's (1-3 years) consumption of acrylamide, in Turkey, the Netherlands and Finland (as shown in Table 1-6). In each study the foods found to contribute to acrylamide consumption have been different depending on the country and age group. However, French fries, potato crisps, bakery items and coffee have been common in most studies.

A study by Boon *et al.* (2005) estimated the dietary exposure of acrylamide in the Dutch population. Acrylamide contents used in the estimation used Institute for Reference Materials and Measurements (IRMM) database. Consumption data were reported for 6250 people from the whole population aged from 1 to 97 years old in the Dutch National Food Consumption Survey (1997-1998). The dietary exposure estimate for the Dutch population (1-97 years old) was 0.5 µg/kg-bw/day. A study by Konings *et al.* (2003) also estimated the dietary exposure of acrylamide in the Dutch population. Acrylamide contents used in the estimation were analysed using a variety of Dutch foods

There are several factors related to selection of data on acrylamide levels in food, including food representation, industrial food processing, food preparation (catering and home), analytical quality, measure of variability and statistical consideration (Dybing *et al.*, 2005).

Chapter 1: General introduction

The European chronic dietary exposure estimation is based on the assumption that a person might consume mean amounts of several different foods but only one or two at a high level (European Commission, 1998).

Table 1-6: Summary of literature on acrylamide dietary exposure in different age groups and countries

Country and organisation	Participants	Food assessment methods	Age group (year)	Acrylamide mean daily exposure (µg/kg-bw/d)	Observation	Consumption foods (acrylamide contribution)	Reference
Dutch, the third National Food Consumption Survey (NFCS) (1998).	6250	2-day dietary records.	1-97	0.48, 0.71 and 1.04 for the whole population, 7-18 and 1-6 years respectively.	The children's consumption of acrylamide was two times higher than the whole population. A small percentage of the whole population had a long-term consumption four times higher than the mean.	French fries (31%-46%) and crisps (18%-23%) respectively.	(Konings *et al.*, 2003)
Sweden, Swedish National Food Administration Survey (1997-1998).	1200	7-day recorded.	18-74	0.50.	The individual consumption in the 5^{th}, 95^{th} and 100^{th} percentile were 0.15, 1.03 and 1.42 µg/kg-bw/day.	Bread and coffee (N.A).	(Svensson *et al.*, 2003)
Dutch, the Dutch National Food Consumption Survey (1997-1998).	6250 of which 530 were young children.	24-hour recall for 7 days.	1-97 and 1-6	0.5 and 1.1 for the whole population and young children respectively.	-	Potato products (N.A).	(Boon *et al.*, 2005)

Continued:

Country and organisation	Participants	Food assessment methods	Age group (year)	Acrylamide mean daily exposure (µg/kg-bw/d)	Observation	Consumption foods (acrylamide contribution)	Reference
Sweden, Swedish infants.	-	-	First year	0.04-1.2.	The mean acrylamide exposure during the first year from the breast and formula milk varied because of the length of breast feeding and the type of milk and infant food.	Commercial infant food then gruel (N.A).	(Fohgelberg *et al.*, 2005)
Finland, Finnish adolescents.	341	7-day dietary records.	13-18	0.19 to 1.09	-	French fries and bread (-)	(Matthys *et al.*, 2005)
Egypt, Alexandrian population from different age groups.	822	Interviewed and 24-hour recall for 3-days.	3-50	Over all mean was 1.75.	The highest and lowest mean daily exposures were 3.82 and 0.49µg/kg respectively.	French fries 57.2% and 32.6% in children less than 6 years and more than 50 years old.	(Saleh and El-Okazy, 2007)
The Netherlands cohort study on diet and cancer 1986.	5000	-	55-69	0.30	Men had a slightly lower acrylamide exposure then women at 0.29 and 0.32 µg/kg-bw/day respectively.	-	(Hogervorst *et al.*, 2008b)
Turkey, Turkish toddlers.	302	Questionnaire and 24-hour recall for 3days.	1-3	1.43	No significant difference between boys and girls.	Bread (-)	(Brisson *et al.*, 2014)

- (-) Indicates number not supplied

Continued:

Country and organisation	Participants	Food assessment methods	Age group (year)	Acrylamide mean daily exposure (µg/kg-bw/d)	Observation	Consumption foods (acrylamide contribution)	Reference
The UK and Ireland, the National Diet and Nutrition Survey (2002).	-	7-day food record.	18-64	0.56 and 0.59 for UK and Irish adults respectively.	The highest level of acrylamide dietary exposure in UK and Irish adults was 1.29 and 1.75 µg/kg-bw/day respectively.	Bakery products and bread (+ 25% and 33%) in the UK and Ireland respectively.	(Mills *et al.*, 2008)
France, French Second National Consumption Survey (2005-07).	33,662	7-day food record.	3-17 (children) 18-79 (adult)	0.43 and 0.69 for adults and children respectively.	The 95th percentile was found to be 1.02 and 1.80 µg/kg-bw/day for adults and children, in order.	French fries (44.8% and 60.8%) for adults and children respectively	(Sirot *et al.*, 2012)
European adults.	37,000	24-hour recall for a day.	35-74	0.19-0.67	Great differences between countries. Acrylamide exposure is higher in northern European countries	-	(Matthäus *et al.*, 2004)
Finland, the National FINDITE Survey (2007)	2038 + 1514	24-hour recall for 6 days.	25-74 (adult) 1,3 and 6 (children)	1.01, 0.87 and 0.44 for 3, 6 and 25-44 years respectively.	3 year old children had the highest exposure amount.	Coffee and starch-rich casseroles (-).	(Hirvonen *et al.*, 2011)

- (-) Indicates number not supplied

1.4.3 Exposure estimates

Acrylamide exposure estimates combine dietary consumption data with acrylamide content in food, and then the estimation is compared with reference values to evaluate the risk level. Estimating acrylamide consumption is a high priority for government and industry due to formation during the heating of food in industry, retail, catering and at home (Dybing *et al.*, 2005). Individual consumption of acrylamide needs to be expressed using body weight (bw). Young people's exposure to acrylamide is higher compared with adults in various countries, as shown in the study and in Table 1-6. Also, FAO/WHO (2002a) indicates that children's exposure to acrylamide is two to three times higher than adults based on body weight. This is because children consume more food containing acrylamide per kg of body weight than other age groups.

Using analytical data to estimate acrylamide consumption is difficult because, as Dybing *et al.* (2005) suggest, some food samples do not contain acrylamide and others contain acrylamide but the contents cannot be detected (ND). Fresh foods or boiled foods, for example fruits, vegetables, rice and milk, contain levels of acrylamide below the limit of quantification (LOD) (less than 30 µg/kg food). Therefore, these foods are usually not used for calculating the consumption of acrylamide in diets (Svensson *et al.*, 2003).

According to Dybing *et al.* (2005), the is no linear relationship between food consumption and acrylamide content in food. This disruption can provide different levels of accuracy. For combining data there are many methods, such as the following: 1) point estimation is a simple way to multiply single levels of consumption by single levels of contamination (screening tool); and 2) other points can be mean consumption multiplied by mean contamination using Monte Carlo for combining levels of food consumed by levels of chemicals in food.

Turkish children (1-3 years) have a higher exposure rate compared with other children, as shown in Table 1-6 (Brisson *et al.*, 2014). Fohgelberg *et al.* (2005) estimated the acrylamide exposure of Swedish infants (first year) by analysing breast milk and infant formula, it range between 0.04 and 1.20 µg/kg bw/d. The dietary exposure estimates for the Dutch population (1-97 years old) were 0.48, 0.71

and 1.04 µg/kg-bw/day for the whole population, those aged 7-8 and those aged 1-6 years respectively. The main food groups that contributed to total exposure were potato crisps (31%-46%) and French fries (18%-23% %) (Konings *et al.*, 2003).

1.5 *Aims*

Estimating consumer exposure to acrylamide is a high priority for governments and industry. This is because of the potential carcinogenic and toxic effects of acrylamide.

The aim of this study is to determine the acrylamide level in food consumed by Saudi infants (≤ 2 years) in the laboratory and using this data to estimate acrylamide exposure in Saudi infants. Additionally, the acrylamide exposure in the UK's infant, children and whole population will be estimated by using NDNS consumption data and published acrylamide levels in foods. For this reason, more specific aims of this study are to:

1. develop methods for acrylamide quantification in extracted starchy food samples using HPLC-UV and LC-MS,
2. determine the acrylamide level of starchy foods consumed by Saudi infants,
3. analyse the effect of high temperature on acrylamide levels in infant cereal and bread loaf,
4. determine total soluble sugar and protein levels in selected infant cereals,
5. estimate the acrylamide exposure of Saudi infants and compare with UK exposure levels in infants,
6. compare different age groups' exposure of acrylamide from the UK NDNS SN6533: (combined) of the rolling programmed (2008/2009 – 2011/12) and the earlier study SN3481 (1992-93).

1.6 *Objectives*

The specific objectives of the study are to:

1. optimise an analytical methodology for the analysis of acrylamide in 47 starchy foods consumed by Saudi infants.

2. estimate dietary exposure to acrylamide using consumption data from Saudi infants' survey and the UK's National Diet and Nutrition Survey (NDNS).
3. estimate the population's potential exceedance above the TDI limit established for cancer.
4. find the relative contributions of total acrylamide exposure from food groups established in the exposure assessment.

1.7 *Hypotheses*

1. The acrylamide level in baked and fried foods consumed by Saudi infants is high.
2. There is no acrylamide in infant cereals consumed by Saudi infants.
3. Infant cereals contain a sufficient amount of reducing sugar and protein for acrylamide formation during storage or microwaving.
4. Saudi and UK infants' exposures to acrylamide are high.
5. The UK children population's exposure to acrylamide has decreased from 1992 to 2011.

2 Chapter 2: Optimisation of acrylamide quantification by high-performance liquid chromatography (HPLC-UV)

2.1 *Introduction*

These are popular in Saudi Arabia and are consumed regularly by weaning infants. According to Paleologos and Kontominas (2005) quantitative analysis of acrylamide in foods is difficult because: 1) its low molecular weight and water solubility make quantification difficult by chromatography, 2) low acrylamide levels in food which may be close to limit of detection and 3) the complex food matrix may lead to interference with acrylamide analysis which may remain even after clean up.

In order to determine the exposure of Saudi infants to acrylamide from foods (Chapter 5), it was necessary to determine the levels of acrylamide in the foods they consumed.

2.1.1 Acrylamide extraction from foods

In the extraction process, several factors need to be considered, such as extraction solvent, extraction temperature and extraction time. It was determined that different extraction solvents and matrices led to variation in acrylamide analysis results between laboratories, with acrylamide recovery ranging from 60% to 95% of acrylamide (Wenzl *et al.*, 2003, Wang *et al.*, 2008).

Water at room temperature is commonly used to extract acrylamide from many food matrices because of the chemical properties of acrylamide, such as it being hydrophilic and having a small molecular weight. Methanol, water, or hot water (80 °C) have all been used to extract acrylamide (Rosen and Hellenas, 2002, Tateo and Bononi, 2003, Paleologos and Kontominas, 2005). However, some methanol mixtures may lead to shorter retention time (Zhang *et al.*, 2005b).

Shaking the sample at high speed on horizontal shaker or mixing with a vortex mixer improves the sample extraction (Wenzl *et al.*, 2003). The extraction time is also an important factor in acrylamide extraction. A long extraction time may lead to

the acrylamide decomposition. Kim *et al.* (2011) found the optimal extraction time was 20 min from most matrices.

Fatty food may need a defatting step before acrylamide extraction. Lowering the temperature to 0 °C before centrifuging at 15,000 g will solidify the oil layer, which can then simply be removed by filtration (Wang *et al.*, 2008). A water extraction at room temperature for 20 min, following by defatting by cooling and centrifugation was chosen as the initial extraction method in this study.

2.1.2 Acrylamide sample clean-up

Acrylamide in high content has been found in processed starchy foods for example, French fries, potato crisps and breakfast cereal (Food and Agricultural Organization and World Health Organization, 2002b). The starch and oil in these foods make acrylamide analysis difficult because of the high level of endogenous matrix which can bind with acrylamide (Tareke *et al.*, 2002). Therefore, a sample clean-up step is necessary to prevent starch and oil from interfering with analysis by Chromatographic methods such as gas chromatography mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS). Also, as a result of using water as the solvent in acrylamide extraction, many co-extraction components such as proteins, amino acids and sugars are found in the extract and need to be removed using a clean-up step (Tareke *et al.*, 2002). The clean-up step has been shown to improve signal to noise ratio in the sample when LC-MS technique was used (Roach *et al.*, 2003). Good accuracy and high recovery of sample extraction can be achieved with an effective, reasonable and well-designed clean-up procedure. Most procedures consist of a combination of two or three cartridges with filtration and centrifugation to avoid blockage of the analytical instrument.

Filtering the extract using a filter membrane (0.45 µm Nylon or PVDF) is a necessary step to reduce the endogenous matrix before the clean-up step (Wenzl *et al.*, 2003). Solid phase extraction (SPE) is a system designed for fast, selective sample preparation and purification prior to chromatographic analysis. SPE cartridge can be used to separate hydrophobic compounds from the hydrophilic analytes. The SPE cartridge sorbent retains matrix interferences when the sample is loaded on to

it. After that, a series of washes are used to elute analytes of interest from the cartridge by using specific solvents. The clean-up procedure reduces sample enrichment therefore a concentration step usually follows SPE extraction (Wenzl *et al.*, 2003, Wang *et al.*, 2008).

The SPE cartridge sorbent needs to be conditioned with organic solvent such as methanol. Then, the SPE cartridge needs to equilibrate with a low strength solvent such as water before the extract is loaded to the cartridge. The sorbent should not be dry during the clean-up; otherwise the sample cannot enter the hydrophobic surface of the sorbent (Waters, 2009). The most important role during the clean-up procedure is the type of SPE cartridge used. Most cartridges used in acrylamide clean-up are a combination of Oasis HLB and Oasis MCX or Bond Elute or Oasis MAX (the common cartridges used are shown in Table 2-1), (Zhang *et al.*, 2005b, Govaert *et al.*, 2006). Ultrasonicating or heating the sample mixture during the extraction procedure may generate small particle components. If a clean-up procedure is then carried out using SPE cartridges, these particles can saturate the SPE cartridge and reduce their efficiency of recovery (Wenzl *et al.*, 2003). Sample dilution may decrease sample detection sensitivity (Mavungu, 2009). Analyte breakthrough happens when unwanted analytes are not retained by the cartridge sorbent. That occurs when the solution loaded is highly organic, the sorbent is over loaded by the matrix component, or the flow rate is fast, so there is not enough contact time of the analyte with the sorbent. To avoid these problems, the sample should either be diluted, the right SPE size chosen or the vacuum adjusted (Waters, 2009). An Oasis HLB and MCX cartridges were activated with methanol then water, followed with loading the sample then water. These conditions were chosen initially in this study.

Table 2-1: Types and properties of SPE cartridges used in the clean-up step for acrylamide analysis.

SPE cartridges	Properties
Oasis HLB (reversed phase)	Strongly hydrophobic with unique hydrophilic-lipophilic balance reversed phase sorbent for acids, bases and neutrals. Stable in organic solvents.
Oasis MCX (ion exchange)	Waters patented mixed-mode, reversed-phase/strong ion cation-exchange sorbent for bases, stable in organic solvents.
C_{18} (reversed phase)	Hydrophobic, silica-based phase used to adsorb analytes of hydrophobicity from aqueous solutions.

(Waters, 2009)

2.1.3 HPLC-UV methodology for acrylamide determination

For acrylamide separation and quantification HPLC-UV chromatography has been used by several groups. The components of extracted mixtures are allowed to pass through a hydrophobic stationary phase (column C18 or X-Bridge) in a polar mobile phase such as acetonitrile, methanol and water. HPLC-UV is an excellent analytical technique for both hydrophilic and hydrophobic molecules because of its good resolution for very closely related molecules and the simplicity of manipulating chromatographic selectivity by simple changes in elution solvent characteristics (Aguilar, 2004). Some studies using HPLC-UV as the analytical method for acrylamide determination are shown in Table 2-2 where the LOD, food sample, column, solvent and other parameters are shown.

2.1.4 Columns

High polarity components, such as acrylamide, make retaining and separating difficult using reverse phase HPLC. These difficulties are because the analyte passes through the column unretained, and so co-elute at the beginning of the chromatographic run. However, some columns are designed for this type of challenging separation such as C18 and dC18 columns (silica based columns) (Zhang *et al.*, 2005a). The most common column used in HPLC and LC-MS for acrylamide analysis is a C18 column as shown in Table 2-2. A C18 column was initially chosen in this study. The increase in column temperature from room temperature to higher temperature results in loses in peak shape and retention time.

For that reason, the oven temperature was setup in the method to be 25 °C (Zhang *et al.*, 2005a). In the binary gradient program the solvent increased after acrylamide separation, lowering the column pressure and conditioning the column for next sample analysis. For the column to equilibrate the mobile phase should be allowed to run for at least 20 to 90 min before the start of a sample run (Kim *et al.*, 2007). The conditions used by Wang (2008) which is increasing the solvent (high concentration) percentage after acrylamide separation, then decreasing to zero for conditioning the column for next sample analysis was initially chosen as the starting conditions in this study.

2.1.5 Solvents

As acrylamide is very polar, it is difficult to choose an appropriate mobile phase to achieve good analyte elution with a reasonable retention time. Acrylamide is stable in acid but unstable in base conditions and is light sensitive (Kim *et al.*, 2007).

Acrylamide was satisfactorily separated on a column from other components extracted from food samples when 4% acetonitrile in water was used as a mobile phase (Wang *et al.*, 2008). Also, 4% acetonitrile in water produced minimum interference from other peaks at the shorter ultraviolet detector (UV) wavelength (210 nm) in HPLC-UV detection (Kim *et al.*, 2007). Table 2-2 shows the most commonly used solvents, solvents concentrations and UV wavelengths in acrylamide determination. An acetonitrile was chosen as the initial solvent in this study with two different concentrations 4% and 100%.

2.1.6 Aim

The specific aim of this chapter is to develop method for acrylamide quantification in extracted starchy food samples using HPLC-UV.

Table 2-2: Analytical methods available for acrylamide sensitivity and detection (food sample, solvents, column, gradient and retention time).

Instrument	LOD (µg/kg)	Food sample	Solvent	Column	Gradient	RT (min)	Reference
LC-MS/MS	10	Coffee	0.5% methanol in water.	Hydro-RP 80 (250 mm×2.0 mm).	Isocratic (m/z 72-55), flow rate: 0.05 mL/min.	7	(Andrzejewski *et al.*, 2004)
LC-MS/MS	10	Various	50% acetonitrile in 1% acetic acid and 100% acetonitrile.	100 CN (250 mm × 4 mm).	Gradient (100% B at 5-10 min) (m/z 72-55), flow rate: 0.7 mL/min.	3.6	(Hoenicke *et al.*, 2004)
LC-MS	10	Various	Acetonitrile1.8% and acetic acid 0.2% in water.	Polar-RP 80A (150 mm×2.1 mm).	m/z 73, flow rate: 0.5 mL/ min.	6	(Murkovic, 2004)
GC-MS	6	Cereal	NA.	ZB-WAX (30 mm × 0.25 mm).	m/z 70, 149 and 151.	11.3	(Pittet *et al.*, 2004)
LC-MS/MS	1	Infants milk and foods in jars	10% methanol/0.1% formic acid in water.	Atlantis dC18 column (210 mm × 1.5 mm).	Isocratic, (m/z 72-55), flow rate: 0.2 mL/min.	3.3	(Mestdagh *et al.*, 2008)
HPLC	15	Various	Acetonitrile 20% with 0.01M sulphuric acid (A) and acetonitrile 80% (B).	Aminex HPX-87H (300 mm ×7.8 mm).	Isocratic (UV 200 nm), flow rate: 0.6 mL/min.	18	(Paleologos and Kontominas, 2005)
LC-MS	10	Various	0.01 mM acetic acid in 0.2% of formic acid in water and 0.2% acetic acid in acetonitrile (98:2, v/v).	Inertsil ODS-3 (250 mm×4.6mm).	Isocratic, (m/z 72-55), flow rate: 0.6 mL/min.	6	(Senyuva and Gokmen, 2005)
LC-MS/MS	10	Various	0.1% acetic acid.	C18 (300 mm×3.9 mm).	Isocratic (m/z 72-55), flow rate: 0.6 mL/min.	8.19	(Govaert *et al.*, 2006)

NA→ not applicable

Continued:

Instrument	LOD (µg/kg)	Food sample	Solvent	Column	Gradient	RT (min)	Reference
HPLC	6	Chines fast food	Acetonitrile 4% (A) and pure acetonitrile (B).	Altima C18 (150 mm ×2.1 mm).	Gradient (20% B at 10-12 min) (UV 210 & 225 nm) flow rate: 0.1 mL/min.	7	(Wang *et al.*, 2008)
LC–MS/MS	25	Various	Water with 0.1% acetic acid.	µ-Bondapak C18 (300 mm×3.9 mm).	Isocratic (m/z 72-55), flow rate: 0.3 mL/min.	8	(Arisseto *et al.*, 2007)
GC-MS	10	Various	NA.	DB-1701 (30 mm × 0. 53 mm).	m/z 70, 149, and 151.	7.4	(Bent et al., 2012)
LC-MS/MS	25	Various	Water with 0.1% acetic acid.	Altima HP C18 amide (250×2.1 mm).	Isocratic, (m/z 72-55), flow rate: 0.2 mL/min.	4.6	(Douny *et al.*, 2012)
LC-MS/MS	25	Infant food	0.1% formic acid in mixture of water and methanol (9:1, v/v).	Hypercarb (150 mm×2.1 mm).	Isocratic (m/z 72.1-55.2), flow rate: 0.35 mL/min.	2.5	(Constantin *et al.*, 2014)
HPLC	8	Chines food	Acetonitrile 10% with 0.1 formic acid (A) and pure acetonitrile (B).	Hypersil ODS-C18 (250 mm ×4.6 mm).	Gradient (20% B at 15-17 min) (UV210 nm), flow rate: 0.4 mL/min.	8.9	(Wang *et al.*, 2013)

NA→ not applicable

2.2 *Materials used for acrylamide analysis*

2.2.1 Reagents

- Acrylamide (99%) HPLC (Sigma, UK, cat. No. A8887),
- Acetonitrile for HPLC and LC-MS grade (VWR; UK, cat. No.83640-320). 4% acetonitrile was prepared by dissolving 40 mL into 960 mL of Millipore water,
- Methanol LC-MS grade, 95% (v/v) (VWR, UK, cat. No.83638-320),
- A Millipore Elix water purifying system (Milli-Q Advantage A10, Millipore UK Ltd, UK) was used to provide ultrapure, nuclease free water (≥ 18.2 MΩ).

2.2.2 Consumables

- Oasis HLB 60 mg, 3.5 mL extraction cartridges (Waters, UK, part. No.WAT094226),
- Oasis MCX 60 mg, 3.5 mL extraction cartridges (Waters, UK, part. No. 009232153A),
- Syringe filters 0.45 and 0.2 μm PVDF (17mm) (Avonchem limited, UK, cat No. SF-20100 and SF-2050),
- Syringe with needle (21 g x 38 mm, 2 mL), (UK, Cat No 613-2001, 17193L),
- Micro test tube EZ, with cap, 2 mL (Bio-Rad UK, Cat No 223-9430),
- Falcon tubes, polypropylene 15 and 50 mL (VWR; cat No 188271, 18010 L and 227261, 18040 L),
- Amber glass auto-sampler vials with septum caps and screw cap (Chromacol, UK, batch No. 0697-216219),
- Pipette tips (10, 1000 μl- 5 mL) (Starlab Ltd, Milton Keynes, UK).

2.2.3 Equipment

- Megafuge 16R centrifuge (Thermo Scientific, UK),

- A low pressure evaporator (Genevac EZ-2, Genevac Ltd, Ipswich, UK),
- Shaker incubator (Orbet incubate 100),
- Manifold SPE cartridge (Waters, UK.),
- Ultrasonic (Decon FS3000, Ultrasonic limited),
- Moulinex Blender with Mill (LM2211 Uno),
- High performance liquid chromatography (HPLC-UV) (Shimadzu, UK limited) that consisted of the following components:
  - DGU-20AS degasser for removal of air from the solvent line,
  - Two 20AD-XR high pressure pumps for transfer of solvents through the system,
  - SIL-20AC-XR autosampler for automated and accurate sample injections,
  - SPD-M20A diode array detector for wavelength monitoring between 200 and 600 nm,
  - CTO-20AC column oven used to maintain the temperature of the column for stable retention times,
  - LabSolution program version 5.53, Shimadzu Corporation.
- Analytical column (X Bridge Amide, 4.6×150 mm i.d., 3.5 µm particle size) (Waters, Ireland, part no. 186004869),
- Analytical column (Phenomenex C18-Silica, 4.6×250mm5µm particle size) (Phenomenex, USA, part No.00G4435E0).

2.2.4 Food sampling

The food samples for this study were chosen based on our previous work Al-Theeban (2006), which detailed the daily consumption of foods among 150 infants in Jeddah in the Kingdom of Saudi Arabia. Thirty two food samples were collected from supermarkets in Jeddah in the winter of 2011. The analytical survey compared

bread, rice, oats, pasta (noodles, macaroni), popcorn, potato crisps, breakfast cereal, infant biscuits and infant cereals. The samples were prepared before extraction. The bread, potato crisps, breakfast and biscuit samples were ground, whereas the rice, oats, pasta and popcorn samples were cooked and then homogenised for 3 min. Moulinex (LM2211) was used to grind and homogenise the samples. All samples were kept at -18 °C until analysis.

2.2.4.1 Bread

Table 2-3: A description of the bread samples used in the study (name, brand, place of purchase and ingredients).

Cereal name	Brand	Place of purchase	Main ingredients
White loaf	Al-rashed food company	Danube Jeddah, KSA	Wheat flour, sugar, yeast, milk, vegetable oil & salt.
White loaf	L'usine		
White loaf	Aljazei		
Samoli	Danube		
Samoli	L'usine		
Samoli	Aljazei		
White Arabic bread	Danube		
White Arabic bread	Al-raya	Al-raya, Jeddah, KSA	Wheat flour, sugar, yeast, vegetable oil & salt.
White Arabic bread	Othaim	Othaim, Jeddah, KSA	
Brown Arabic bread	Othaim		
Brown Arabic bread	Danube	Danube, Jeddah, KSA	
Brown Arabic bread	Al-raya	Al-raya, Jeddah, KSA	
Croissant	Tasbeera	Othaim, Jeddah, KSA	Wheat flour, vegetable oil & sugar.
Croissant	Al-raya bakery	Al-raya, Jeddah, KSA	
Croissant	Danube bakery	Danube Jeddah, KSA	

The Saudi infants consumed many types of bread. The bread crusts of different types of bread and bakery items may have different levels of browning due to variation in production method and ingredient variation; in other words, different levels of acrylamide. Due to this, all types of bread consumed by Saudi infants were chosen from three different bakeries or factories. The main ingredients in all bread types were wheat flour and sugar. The names of the samples, places of purchase and ingredients are shown in Table 2-3.

2.2.4.2 Biscuits and cakes

Four and three samples of biscuits (infant biscuits and biscuits) and cake consumed by Saudi infants were chosen respectively from the most consumed brands, and from the same type of biscuit and cake three different products were purchased from local markets in Jeddah. Wheat flour and sugar were found to be the most common ingredients in biscuits and cake, as shown in Table 2-4.

Table 2-4: A description of the biscuits and cake samples used in the study (name, brand, place of purchase and ingredients).

Sample name	Brand	Place of purchase	Main ingredients
Infant' biscuits	Hero baby biscuits	Othaim, Jeddah, KSA	Wheat flour, sugar, milk, vegetable oil, salt minerals & vitamins.
Biscuits	Bahlsen		Sugar, wheat flour, egg, milk, vegetable oil, salt minerals & vitamins.
Biscuits	Members Bambini		
Infant' biscuits	Farley's Rusks	Danube, Jeddah, KSA	Wheat flour, sugar, oil, minerals & vitamins
Marble cake	Danube		Wheat flour, sugar, eggs, vegetable oil& salt.
Marble cake	Americana	Banda, Jeddah, KSA	
Marble cake	Sarita		Wheat flour, sugar, eggs, vegetable margarine butter, milk & salt.

2.2.4.3 Cereals

In this study, a number of cereals identify as consumed by Saudi infants were analysed. These cereals comprised infant cereals (5) and breakfast cereals (3), as shown in Table 2-5. The samples were found to be made basically from wheat, oats,

maize or rice, milk and different forms of sugar, such as honey, dates and sucrose, as shown in Table 2-5. Infant and breakfast cereals were found to be consumed by adding hot water or milk.

Table 2-5: A description of the cereal samples used in the study (name, brand, place of purchase and ingredients).

Cereal name	Brand	Place of purchase	Main ingredients
Cerelac wheat	Nestle	Danube Jeddah, KSA	Wheat-based, milk, iron & vitamins
Cerelac honey & wheat			Wheat-based, honey & milk.
Cerelac dates & wheat			Wheat-based, milk, dates, sucrose, date syrup, vegetable oil & dates flakes.
Cerelac rice			Rice, milk powder, iron, minerals & vitamins.
Oats	Heinz		Oats flour (40%), milk powder, sugar, oil & vitamins.
Cornflakes	Nestle country	Othaim, Jeddah, KSA	Maize, sugar barley malt, salt & vitamins.
Cornflakes	Kellogg's		
Cornflakes	Briiggen		

2.2.4.4 Potato crisps and popcorn

Table 2-6: A description of the potato crisps and popcorn samples used in the study (name, brand, place of purchase and ingredients).

Sample name	Brand	Place of purchase	Main ingredients
Potato Crisps	Lay's	Banda, Jeddah, KSA	French fries, oil & salt.
Potato Crisps	Albatal		
Potato Crisps	Pringles		Dehydrated potatoes, oil & salt.
Popcorn	Al-batal, ready to eat	Danube, Jeddah, KSA	Popcorn, oil, cheese flavor & salt.
Popcorn	Al-alali, prepared on hob		Popcorn & oil.
Popcorn	Butterkist, prepared on microwave r kist		Popcorn, oil & salt.

Three different types of potato crisps and popcorn of different brands were purchased from local supermarkets in Jeddah, as shown in Table 2-6. The popcorn selection comprised, namely factory made (ready to eat), prepared on a hob and prepared in a microwave, as per the packaging's instructions. Most of the samples did not need preparation. However, popcorn was made as per the product's instructions, such as on a hob or in a microwave.

2.2.4.5 Noodles, macaroni, oats and rice

Foods in this group were purchased based on the most consumed brand names in Saudi Arabia. All samples were prepared by boiling in water. The sample weights and water amounts were of the same ratio as those given in the preparation guidelines. The main ingredient in noodles and macaroni is wheat flour. Table 2-7 shows the sample names, brands, places of purchase and ingredients. The number of samples for each food amounted to three different brands. Samples were prepared as basic recipes, Rice, macaroni, oats and noodles being boiled in water until they became soft.

Table 2-7: A description of the noodles, macaroni, oats and rice samples used for the study (name, brand, place of purchase and ingredients).

Sample name	Brand	Place of purchase	Main ingredients
Noodles	Andomie	Banda, Jeddah, KSA	Wheat flour, vegetable oil & salt.
Noodles	Toya		
Noodles	Mamee		
Macaroni	Goody		Durum wheat pasta.
Macaroni	Alalali		
Macaroni	Perfetto		
Oats	Quaker		White oats.
Oats	Alalali		
Oats	Captin oats		
Rice	white swan		White rice (Egypt).
Rice	Alwalimah		White rice (India).
Rice	Two girls		White rice (USA).

2.3 *Acrylamide determination: analytical methods*

2.3.1 Acrylamide standard preparation

Acrylamide solution concentration 1 mg/mL was prepared, then a series of different acrylamide concentrations (µg/mL) were prepared from the stock, as shown in Table 2-8. All standards were stored in Falcon tubes at 4 °C for further use. The maximum storage time for the working standards and internal standards was two months.

A calibration curve was constructed by plotting peak area of acrylamide eluted from the column against the concentration of acrylamide, calculated from the concentration of the solution and the injection volume. An equation, y = mx+c (as shown in Figure 2-4), was used to relate the chromatography peak area with the mass eluted. Acrylamide peaks (from food samples) were identified by comparing the retention times with that of the known standard. In situations where an unknown peak was suspected to be that of acrylamide, the samples were spiked with acrylamide standard. Enlargement of the concerned peak then confirmed the identity of the acrylamide.

Table 2-8: The series of different acrylamide concentrations (µg/mL) prepared from the stock solution (10 µg/mL) through dilution in water.

Acrylamide concentration (µg/mL)	Acrylamide solution 10 µg/ mL (mL)	Millipore water (mL)
0.0	0.0	10.0
0.05	0.05	9.95
0.1	0.1	9.9
0.2	0.2	9.8
0.3	0.3	9.7
0.4	0.4	9.6
0.5	0.5	9.5
1.0	1.0	9.0
2.0	2.0	8.0
3.0	3.0	7.0

2.3.2 Sample extraction

Two grams of solid ground samples were accurately weighed into separate 50 mL Falcon centrifuge tubes and 20 mL of Millipore water was added. For recovery purposes, samples were prepared similarly with 2 mL of 1 µg/mL acrylamide standard added to 18 mL of water. All of the capped tubes were shaken horizontally for 20 minutes in a shaking incubator (Orbet incubate 100) at room temperature. The tubes were centrifuged at 5000× g for 15 min at 5 °C. A pipette was inserted through the top oil layer to the aqueous layer, avoiding the bottom solids with the pipette tips, and 6 mL of the aqueous was taken. The aqueous solution was centrifuged at 5000× g and 0 °C for 30 min to precipitate further oil residue in the aqueous sample solution. Then 4 mL aliquot of clarified aqueous layer was promptly removed and filtered through a 0.45 µm PVDF syringe filter. An Oasis HLB SPE cartridge and an Oasis MCX SPE cartridge were used to remove a number of early eluting co-extractives. A solid phase extraction manifold (SPE) (Waters, Milford, Massachusetts, USA) was used with running water as a vacuum source. HLB and MCX SPE cartridges were initially conditioned with 2 mL of methanol, followed by 2 mL of water; the methanol and water portions were discarded. The filtered extract was allowed to successively pass through both SPE cartridges and the cartridge was then washed with 1 mL of Millipore water. The sample and water was collected. The extract was then evaporated to ~1 mL on a rotary evaporator (Genevac LTD; model EZ-2 MK2; UK) (solvent programme setup as lamp off, aqueous). The resulting pellet was re-diluted with Millipore water and adjusted to 1.5 mL, mixed using a vortex. The aqueous extract was filtered through a 0.2 µm PVDF syringe filter to a 2 mL amber glass vial. All clean sample extracts were stored at 4 °C until future use. The summary of final extraction and clean-up are shown in Figure 2-1 and 2-2 respectively. Figure 2-3 shows different sample mixtures where 1 is the residue and the oil layer and 2 is the same tube with clear aliquot.

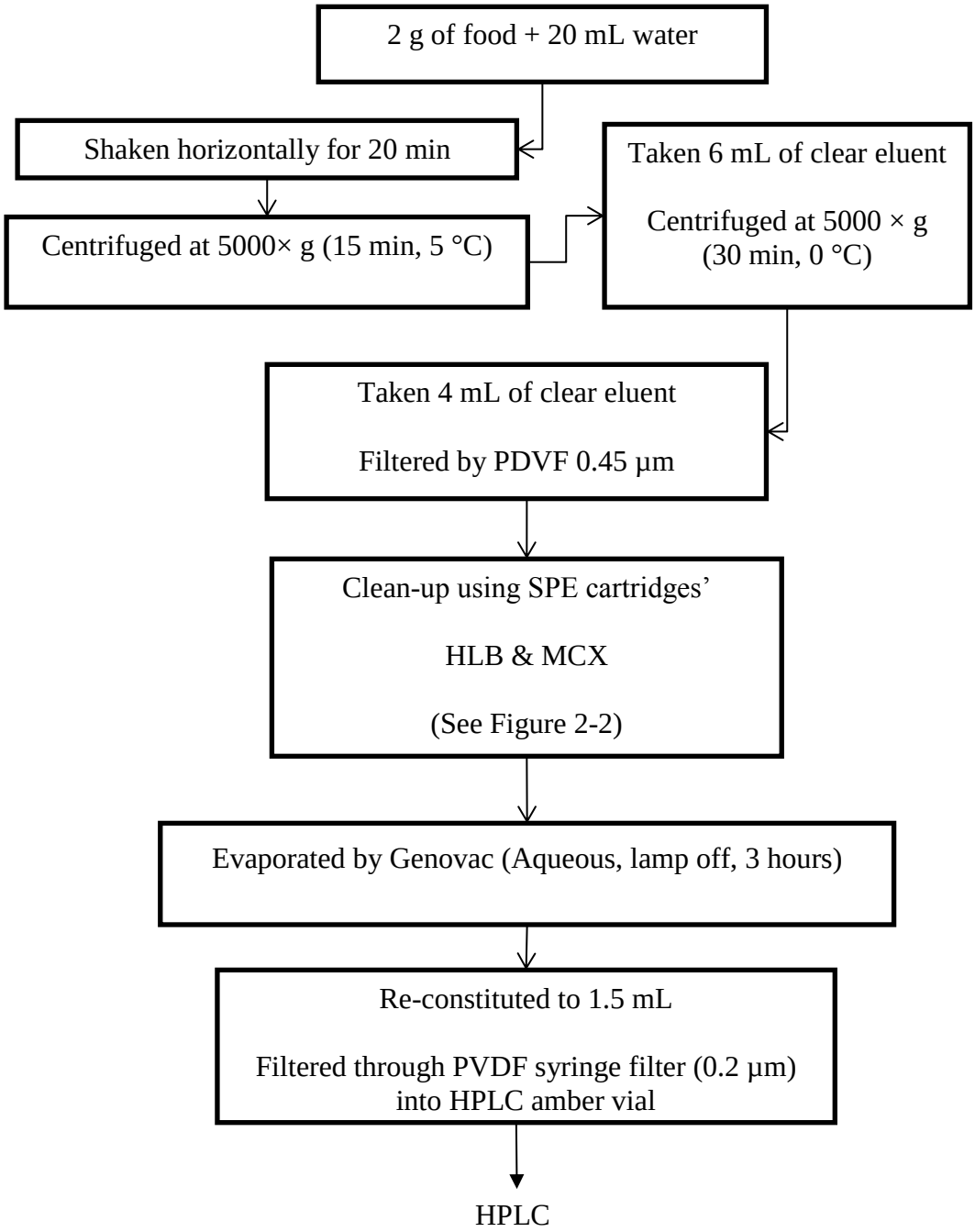


Figure 2-1: Final extraction and clean-up of acrylamide treatment methods before HPLC analysis.

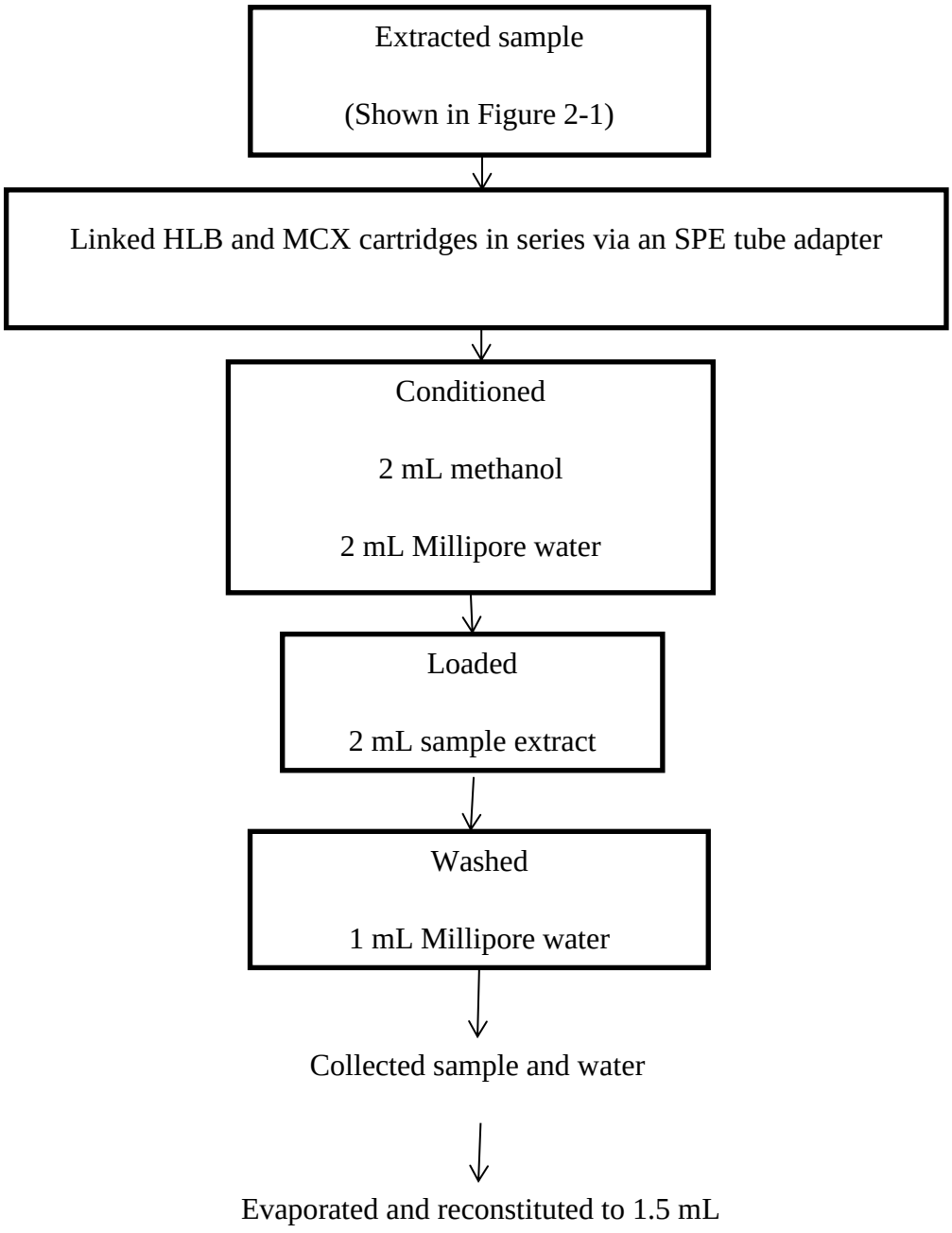


Figure 2-2: Final SPE cartridges, Oasis HLB and Oasis MCX clean-up method.

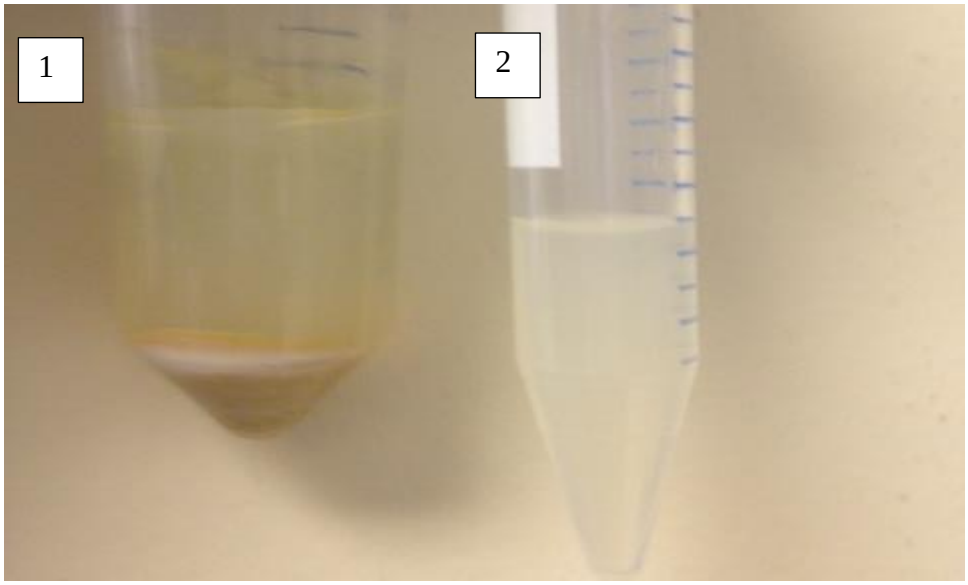


Figure 2-3: Cerelac sample extracted with water after the first centrifugation (5 °C) tube number 1 shown the residue and the oil layer and after the second centrifugation (0 °C) tube number 2 shown removed of the residue and the oil layer.

2.3.3 Modification of extraction procedure

2.3.3.1 Increase in the amount of aqueous layer taken for determination

Taking a clear aqueous layer from 10 mL of centrifuged sample was difficult. For that, the amount of the sample and water were increased to be 2 g of the sample in 20 mL of water, which made collecting the clear aliquot (reduced unwanted oil or residue) easier. After the second centrifuge step, 4 mL were taken from the 7 mL. The 4 mL of extraction was divided into 2 mL for clean-up.

2.3.3.2 Dilution of sample before HPLC-UV analysis.

As a result of having a great peak area for acrylamide in the HPLC-UV analysis, several dilutions in water were made to reduce the peak area and to improve acrylamide analysis in the samples. For that, the sample was diluted 2 and 10 times with water and it was analysed at the same time to test the dilution factor effect.

2.3.3.3 Optimisation of the clean-up

The Oasis HLB SPE cartridge and Oasis MCX SPE cartridges were used to attempt to remove a number of early eluting co-extractives such as carbohydrate, protein fat mineral, vitamin and other MRPS (99%). Different methods and solvents were used to condition the SPE cartridge for clean-up the acrylamide in food extraction.

Method A

The method by Wang *et al.* (2008) method was used to condition the Oasis HLB and Oasis MCX SPE cartridges.

Method B

This method was adapted from method A in order to test whether reducing the solvent amount would affect the acrylamide clean-up. The SPE cartridges HLB and MCX were connected in a series via an SPE tube adapter. To condition the SPE cartridges HLB and MCX, 2 mL of methanol followed by 2 mL of water were used, then discarded. The filtered extract (2 mL) was allowed to successively pass through both SPE cartridges, then it was washed with 1 mL of Millipore water. Sample and water were collected as before.

Method C

The method by Riboldi *et al.* (2014) was used to condition the Oasis HLB and Oasis MCX SPE cartridges.

Method D

A clear extraction sample was used without clean-up by SPE cartridge. The sample extraction was filtered through a PDVF filter 0.2 µm pore size (Wenzl *et al.*, 2003).

2.3.4 HPLC–UV analysis

The calibration standards and samples extracts were injected via a 20 µL injection volume loop. The oven column temperature was set at 25 °C and a wavelength set at a range from 210 to 254 nm. Also, the samples were extracted as described in section 2.3.2. To choose a method for acrylamide analysis of foods, several elution methods were tested to find the optimal method. The binary gradient and isocratic

programs were used to optimise the acrylamide separation. Different mobile phases were used (formic acid, methanol and acetonitrile) at different concentrations (0.05, 0.1, 4 and 10%) and at different flow rates (0.3 and 0.5 mL/min). According to Longhua *et al.* (2012) , acrylamide peak separation was better when the flow rate volume increased from 0.1 to 0.5 mL/min. Roach *et al.* (2003) showed the effect of column temperature peak resolution. They tested three different temperatures: 26, 35 and 40 °C. Their analysis data showed that 25 °C is the most appropriate temperature for quantitation of food products.

A fresh 2 L of solvent was prepared and ultrasonically degassed for 20 min for every batch run, due to the fact that old solvent is less active and solvents with gas can increase the instrument pressure. Prior to the sample runs, air bubbles were purged from the solvent lines by pumping the solvent through the lines for 10 min and through the autosampler for 25 min. The solvent was then allowed to run through the lines for 10 min to get rid of the old solvent before placing the column. The column was placed in a column oven set at 25 °C and checked to see whether there was any leakage. If the pressure was observed as being too high, the suction filter was removed and sonicated in 10% propanol with water (v/v) for 30 min to remove any dirt that may have been responsible for the blockage. If the pressure remained high this meant the lines needed to be washed with 50% methanol with water (v/v). After the wash, the solvent was allowed to pass through the lines for 30 min to reduce the pressure (Thermo, 2004).

The autosampler needle and injection ports were washed before each run, twice with methanol and water 50% (v/v) and twice with water so as to clean any residue from the previous run. Also, it was washed once with water between sample runs to clean the needles from the previous injection. The column was washed with acetonitrile and water 50% (v/v) for 15 min. After that (at the end of each day) the line and column was washed with 50% methanol and water (v/v) for 15 min. Acetonitrile 50% was used for rinsing the needles between sample injections. Isopropanol 10% was used for the pumps. The HPLC detector was set to monitor absorbance had to be 210 nm for each run.

Columns used

An X-Bridge Amide column (Waters 4.6×150 mm 3.5 µm particle size) was used to analyse acrylamide in HPLC-UV in all methods. A C18 column (Phenomenex 4.6×250 mm 5 µm particle size) was used with the method 6 to test the column affect. The X-Bridge column was broken during the analysis which led to tray the C18 column.

2.3.4.1 Optimisation isocratic methods

Method 1

The method by Waters (2009) was used with X-Bridge.

Method 2

The method by Cavalli *et al.* (2003) was used with X-Bridge column.

Method 3

The method by Clarke *et al.* (2002) was used with X-Bridge column.

2.3.4.2 Optimisation binary gradient methods

To choose the optimal method for acrylamide determination, several HPLC-UV gradient methods were used. These methods used 0.5 mL/min as a flow rate and acetonitrile in different concentration as solvent A and acetonitrile 100% as solvent B.

Method 4

The method by Wang *et al.* (2008) was used with X-Bridge column.

Method 5

A method from method 4 was used which consisted of elution of sample with 10% acetonitrile in Millipore water (v/v) as solvent A, and 100% acetonitrile as solvent B with X-Bridge column. The method was run for 24 min with 0% B for 10 min. It was then increased to 20% for 10 to 17 min, then decreased to 0% after that for 7 min.

Method 6

A method adapted from method 4 was used which consisted of elution of sample with 4% acetonitrile in Millipore water (v/v) as solvent A, and 100% acetonitrile with X-Bridge and C18 column. The method was run for 40 min with 0% B and this was increased until it reached 80% after 20 min, remaining as 80% for 5 min (20 to 25), after which it was decreased gradually to 0% B (at 25 min) and kept at 0 for 15 min. This method was used as initial method in this study.

2.3.5 Calculation of acrylamide content

2.3.5.1 Calculation of acrylamide content in food

To calculate the amount of acrylamide in the sample, find the X value by using the equation for straight line in the calibration curve and the area as Y value, then the content of acrylamide is multiplied by 20 (mL of water) (equation 2-1). This calculation gives how many µg in 2 g, which needs to be multiplied by 500 to find the content of acrylamide by µg/kg (equation 2-2).

$$\boldsymbol{Amount\ of\ acrylamide\ in\ the\ sample}\ (\mu\mathbf{g\ in\ 2\ g}) = \mathbf{X\ value} \times \mathbf{20}$$

Equation 2-1

$$\boldsymbol{Amount\ of\ acrylamide\ in\ a\ kilogram}\ (\mu\mathbf{g\ in\ kg}) = \mu\mathbf{g\ in\ 2g} \times \mathbf{500}$$

Equation 2-2

2.3.5.2 Limits of detection and quantification

The limit of detection (LOD) is the smallest amount of an analyte that is required for reliable determination, identification or quantitation (signal greater than background noise). LOD and limit of quantification (LOQ) were calculated in accordance with Food Standards Agency (2002), LOD was calculated based on the standard deviation (SD) of the intercept and the slope (S) of the calibration curve; as SD multiplied by 3.3 divided by S, whereas LOQ was calculated as standard deviation multiplied by 10 divided by slope. LOD and LOQ were established as µg/kg by using equation 2-3 and 2-4 respectively:

$$\boldsymbol{LOD = 3.3 \times (SD|S)}$$

Equation 2-3

$$\boldsymbol{LOQ = 10 \times (SD|S)}$$

Equation 2-4

Where SD is standard deviation and S is the curve slope (European Commission, 2002).

2.3.6 Statistical analysis

Microsoft Excel 2007 was used to generate a calibration curve, which was used to quantify the level of acrylamide within food samples as the coefficient of variation (CV) associated with the result. Also, it used to calculate the LOD and LOQ of the HPLC.

2.4 *Results*

2.4.1 Calculation of acrylamide content

2.4.1.1 Initial calibration curve of acrylamide

The HPLC-UV method 6 was used to determine acrylamide concentration in the standard using the X-Bridge column. The calibration curves were obtained by plotting the peak area or peak height against acrylamide concentrations. As it has been shown in previous studies, the acrylamide level can vary from being non-detectable to being around 4000 µg/kg in food. For that, the lowest and highest standard concentrations used were 0.05 and 5 µg/mL. A linear response was obtained with a correlation coefficient higher than 0.999 for peak area and height, as with (R^2 = 0.9998 and 0.9998) in order, as shown in Figure 2-5. The error bars indicate the precision of triplicate injections. When all the different standard concentrations of acrylamide were plotted on a graph, they came constantly to the same retention time, as shown in Figure 2-4.

The acrylamide standard peak was found at 4.6 or 10 min with X-Bridge and C18 respectively. In the chromatographs there are many peaks. These peaks may be

acrylamide, injection, solvent or other component peaks. To choose the acrylamide peak, a comparison with different concentrations was made as shown in Figure 2-4. In samples the acrylamide peak was chosen based on the retention time.

2.4.1.2 Limits of detection and quantification

The LOD and LOQ for the HPLC-UV was 3 and 11 µg/kg respectively. The coefficient of variation (CV) obtained for 6 analyse was 3.0% for the intraday precision of the method.

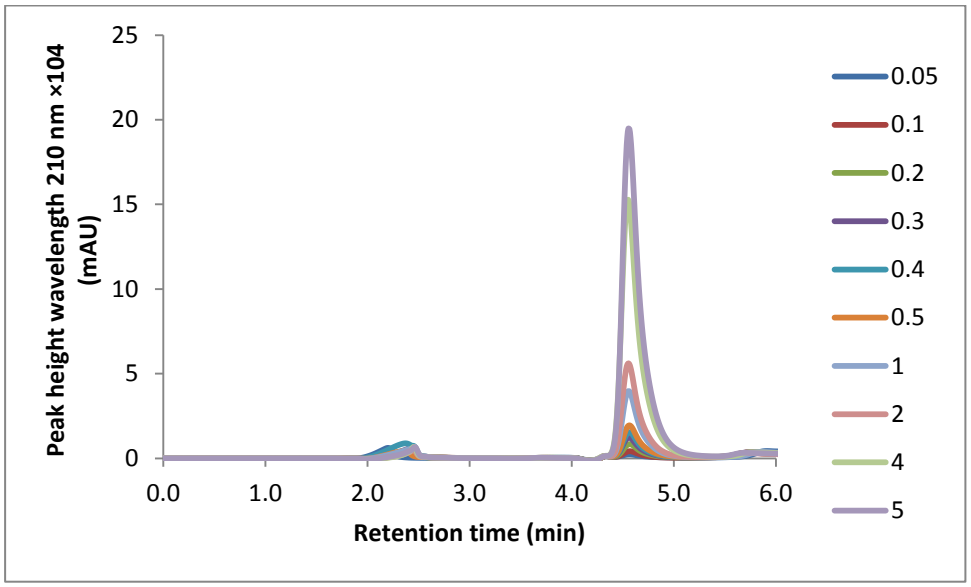


Figure 2-4: Acrylamide standards HPLC-UV chromatograph at a wavelength of 210 nm. Different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 4 and 5 µg/mL of acrylamide) were used. Separation method 6 was used with the X-Bridge column.

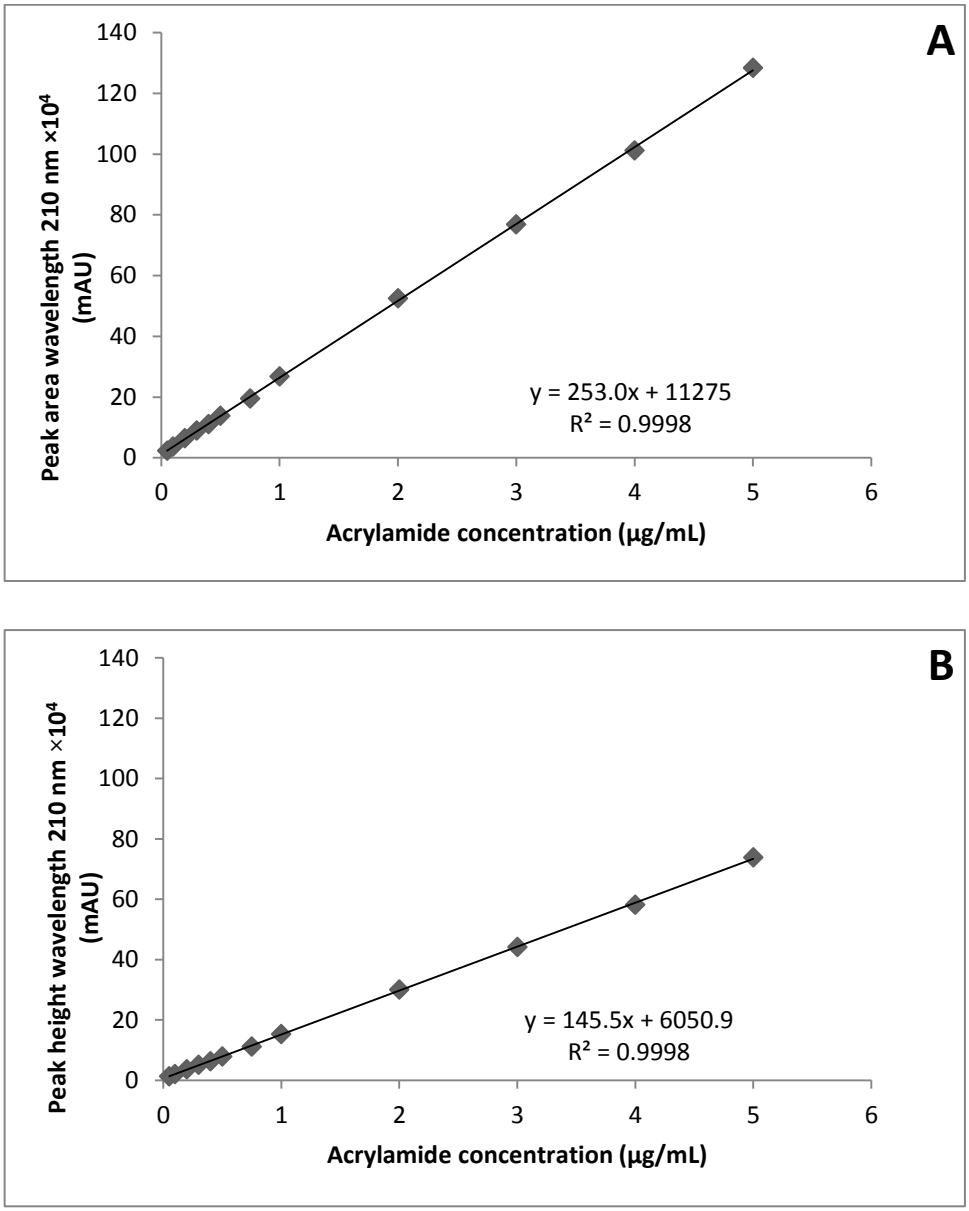


Figure 2-5: Acrylamide standard curves, peak area (A) and peak height (B) from 0.05 to 5 µg/mL. Using the HPLC-UV method (6) at wavelength 210 nm, with the X-Bridge column.

2.4.1.3 Calculation of acrylamide content in food sample

The peak area and height standard slope equations were used to find the acrylamide content in the food sample. This was then multiplied by the dilution factor. For example, the Cerelac wheat area reading was 2,638,663 mAU*min; this reading was used as the Y value in a standard curve equation to find the value of X. After the X value was multiplied by the dilution factor [(×20) (×500)], the final result was 26,386,630 µg/kg (26,4 g/kg) acrylamide, which is much higher than is expected and an indicator that the acrylamide peak cannot be resolved and separated by HPLC due to likely interference from other food components. Results are not shown due to overestimation of acrylamide.

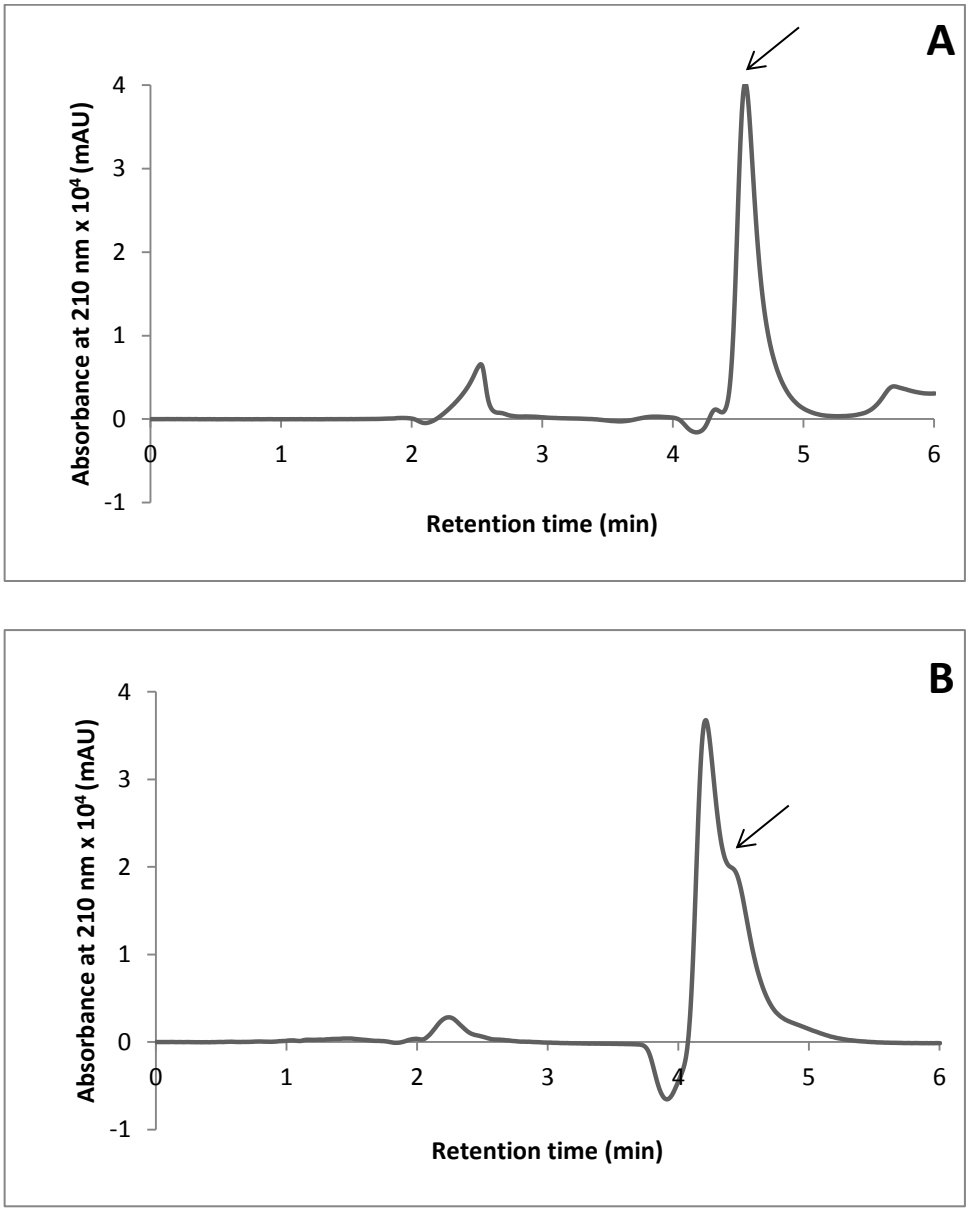


Figure 2-6: HPLC-UV chromatography of acrylamide standards (1 µg/mL) at detection wavelength 210 nm. Separation method 4 (A) and 5 (B) were used with the X-Bridge column (arrow indicates the acrylamide peak).

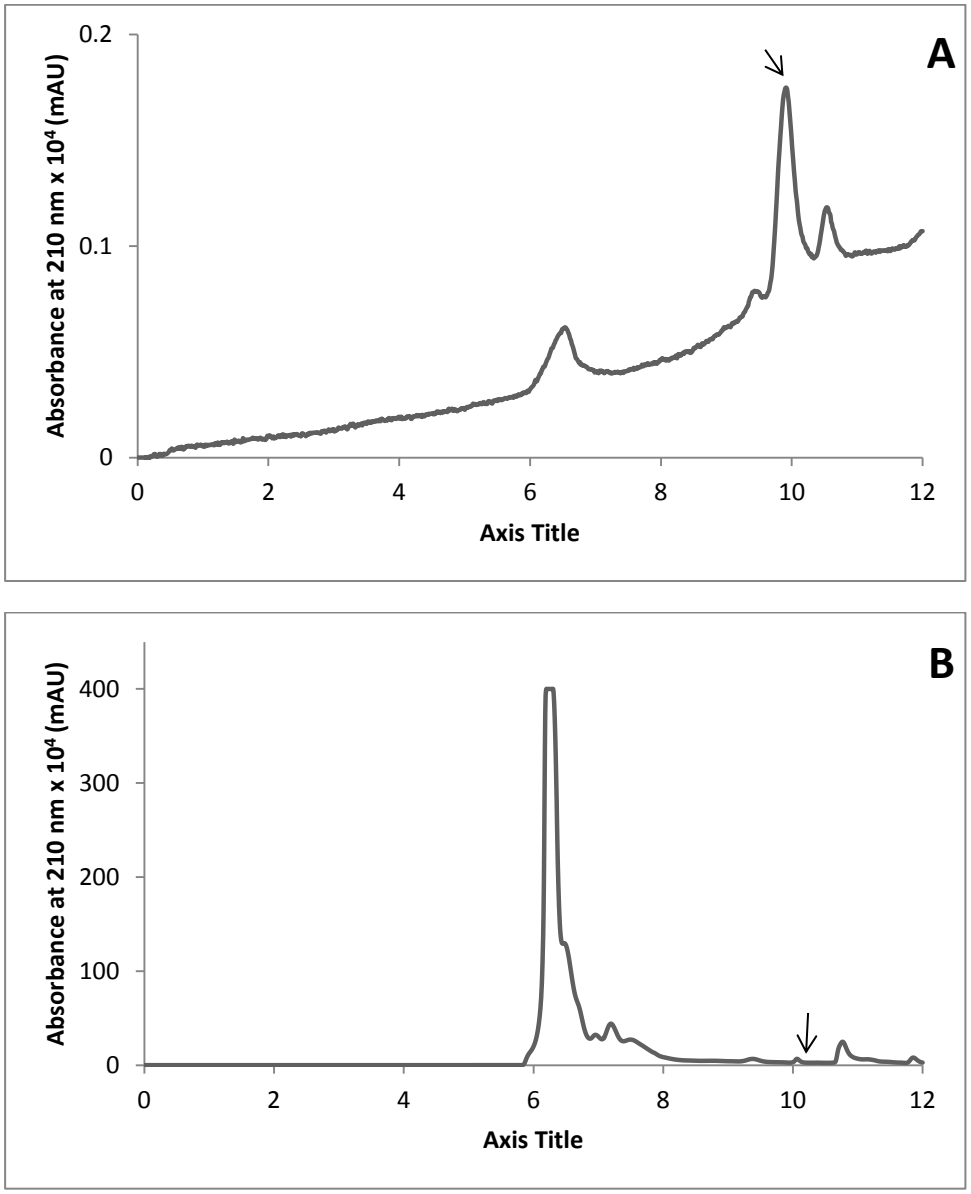


Figure 2-7: HPLC-UV chromatography of acrylamide standard 0.05 µg/ml (A) and potato crisps. Separation method 6 was used with the C18 column (arrow indicates the acrylamide peak).

2.4.2 Extraction

During sample extraction, increasing the amount of the sample and water to be 2 g of the sample in 20 mL of water, made collecting the clear aliquot easier for two centrifuge steps.

2.4.3 Clean up

Acrylamide samples with four different clean-up methods were analysed using HPLC-UV. The samples' HPLC-UV results showed there were no differences between clean-up methods because the acrylamide peak could not be resolved. Different acrylamide clean-up methods did not change or improve acrylamide peak separation. Method D could only be used with clear sample extraction because filtration of the extract through the filter PDVF 0.2 µm was impossible with most of the samples which shows the importance of the clean-up step.

Following on from the HPLC-UV analysis results, clean-up method C was used as the initial method because it required less solvent and time for conditioning of the SPE cartridge compared with methods A and B, and methods A and C had given similar results. Figure 2-8 and 2-9 show the results from the different clean-up methods.

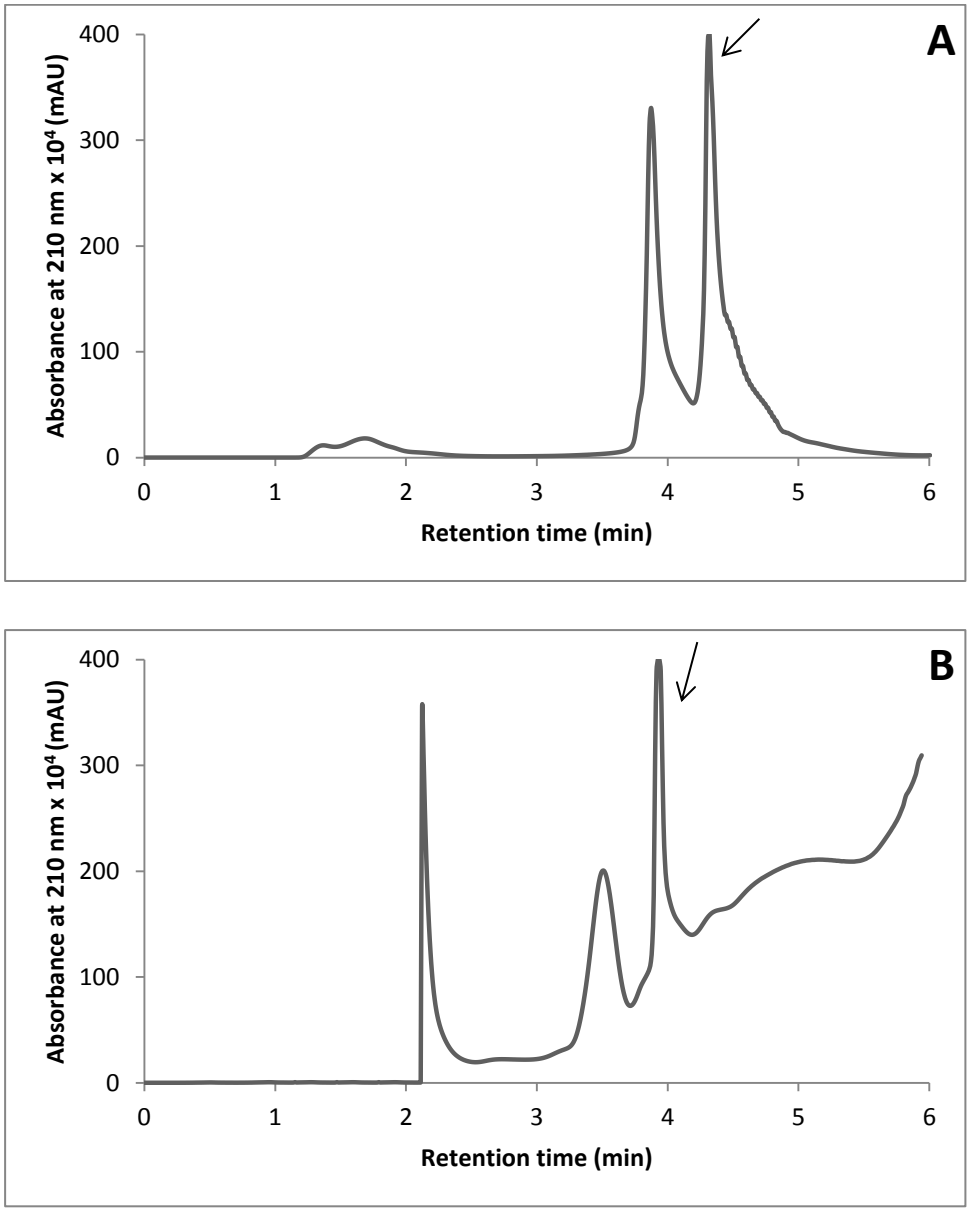


Figure 2-8: HPLC-UV chromatography of Cerelac cereal with different SPE clean-up methods method A (A) and method B (B) at detection wavelength 210 nm. Separation method 6 was used for both with the X-Bridge column (arrow indicates the acrylamide peak).

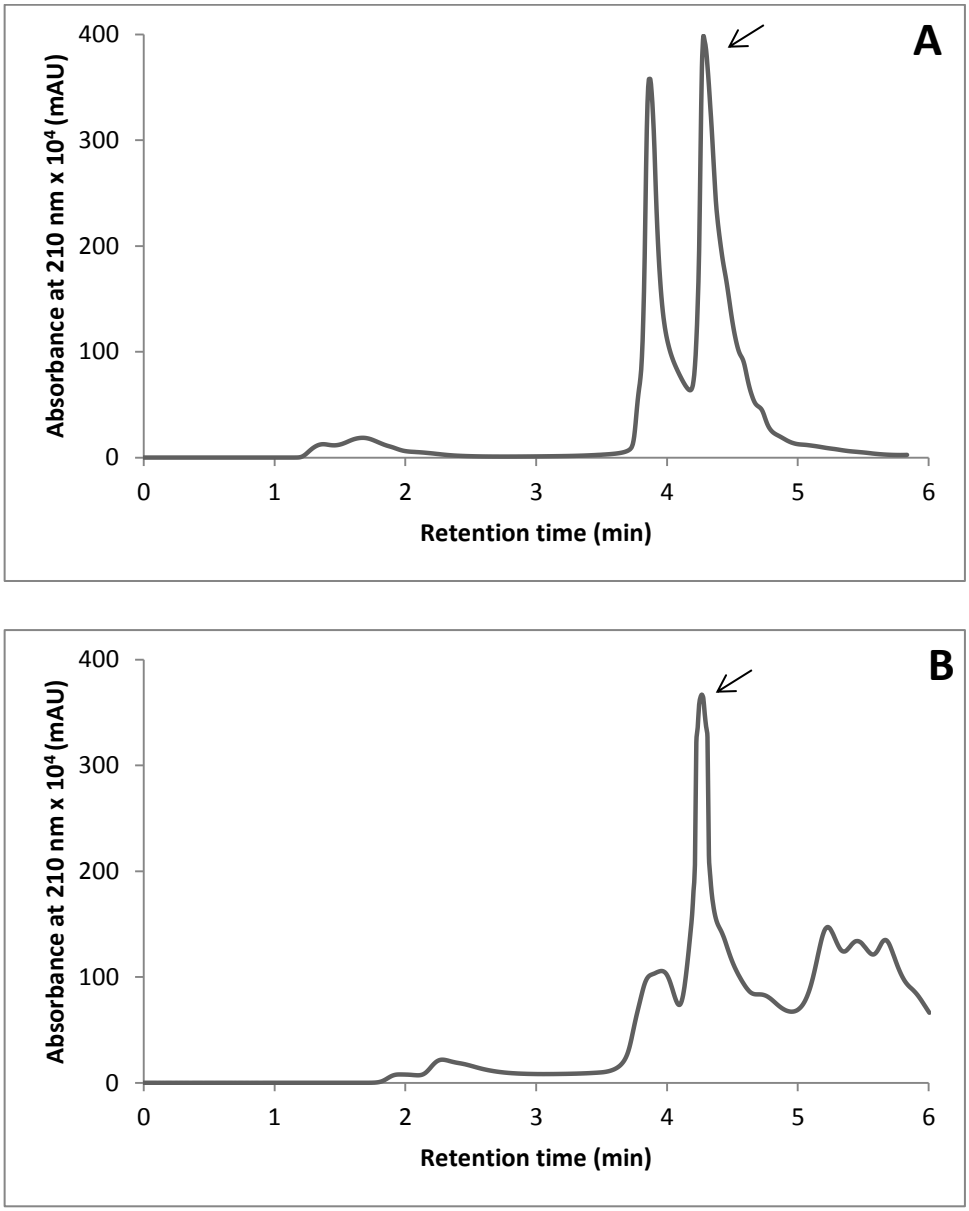


Figure 2-9: HPLC-UV chromatography of Cerelac cereal with different SPE clean-up methods method C (A) and method D (B) at detection wavelength 210 nm. Separation method 6 was used with the X-Bridge column (arrow indicates the acrylamide peak).

2.4.4 Acrylamide separation by HPLC–UV

2.4.4.1 Wavelength

The wavelength in the HPLC-UV analysis was chosen to be 210 nm, because at this wavelength the interfering peaks were less, as shown in Figure 2-10. This finding was similar to that of Wang *et al.* (2008) and other studies which have used closer wavelengths, from 200 to 215 nm (Paleologos and Kontominas, 2005).

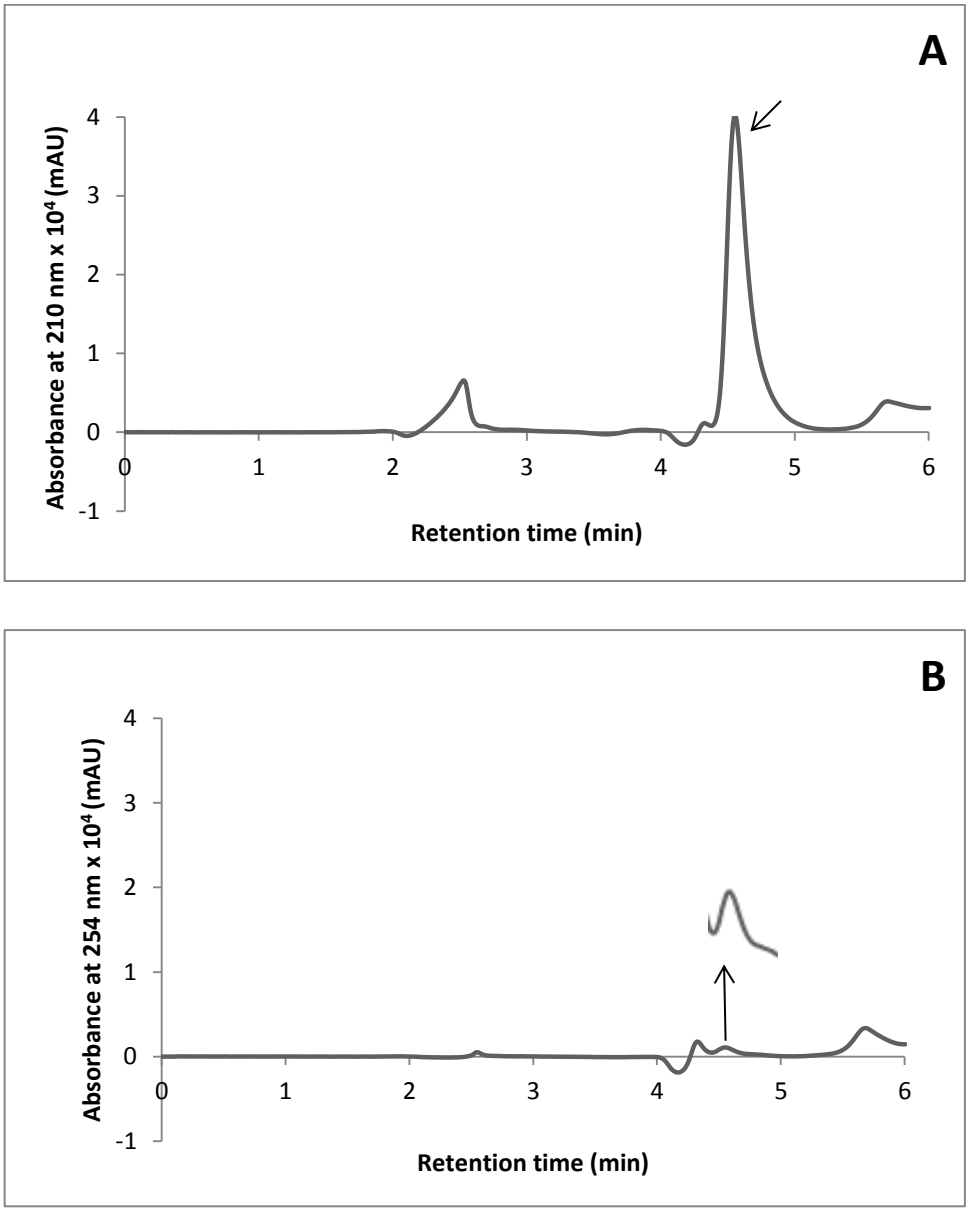


Figure 2-10: HPLC-UV chromatography of acrylamide standards (1 µg/mL) at detection wavelength 210 (A) nm and 254 (B) nm. Separation method 6 was used with the X-Bridge column (arrow indicates the acrylamide peak).

2.4.4.2 Isocratic methods

In general, with the change of solvents, flow rate in the isocratic methods, the acrylamide peak was not well separated in methods 1 and 3. The change in flow rate from 0.3 to 0.5 did not show any improvement in the peak. However, in method 2 the acrylamide peak with methanol was with a high interference background and so a binary gradient was then chosen as shown in Figure 2-11 and 2-12.

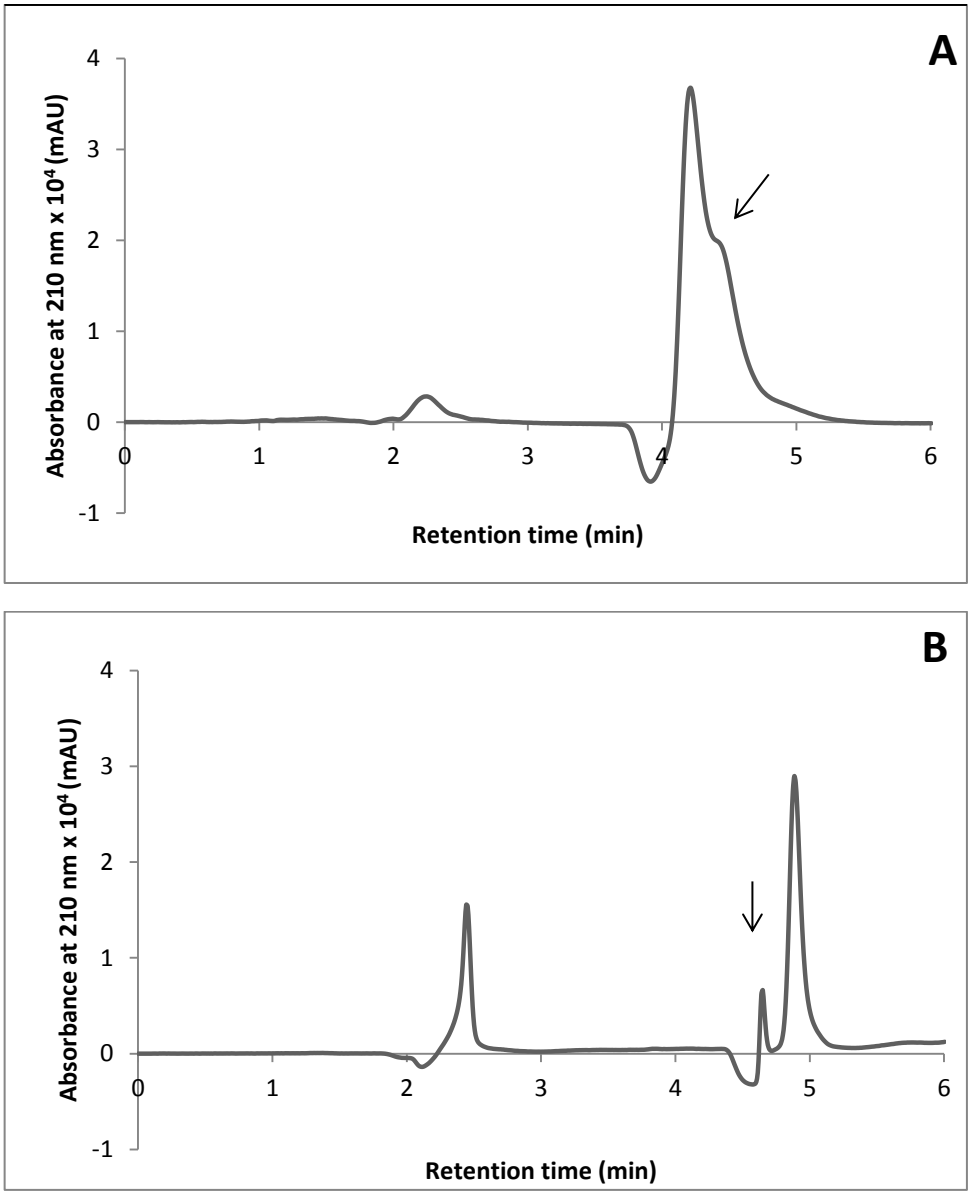


Figure 2-11: HPLC-UV chromatography of acrylamide standards (0.5 µg/mL) at detection wavelength 210 nm. Separation method 1 (A) and 2 (B) were used with the X-Bridge column (arrow indicates the acrylamide peak).

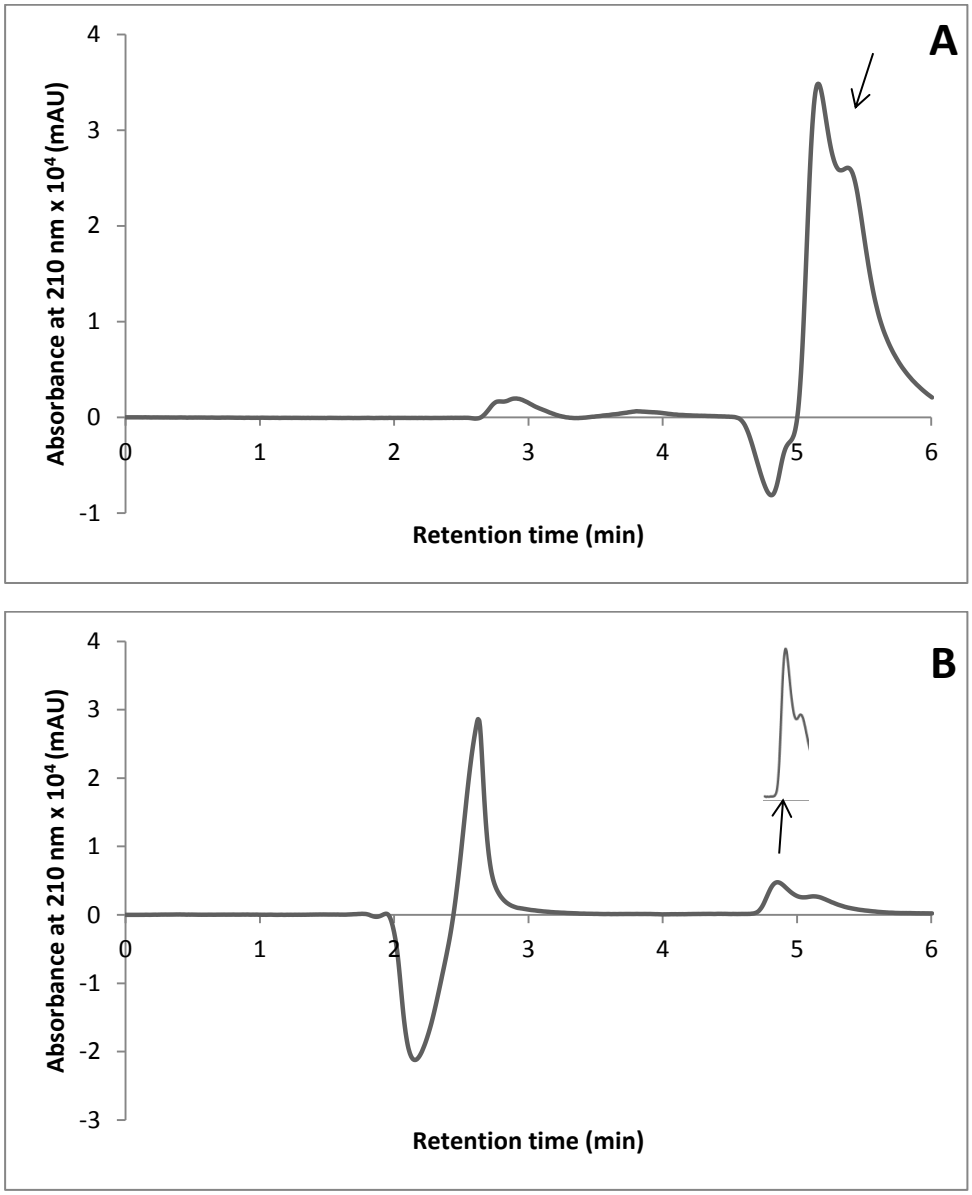


Figure 2-12: HPLC-UV chromatography of acrylamide standards (0.5 µg/mL) at detection wavelength 210 nm. The flow rate 0.3 mL/min (A) and flow rate was 0.5 mL/min (B). Separation method 3 was used with the X-Bridge column (arrow indicates the acrylamide peak).

2.4.4.3 Binary methods

With the binary gradient methods, finding a separate acrylamide peak in chromatography was not easy. All gradient methods showed an acrylamide peak but it was not satisfactory when the increase of B solvent concentration started at 10 min. When acetonitrile 4 and 100% was used as the solvent, the acrylamide peak was separated with acrylamide standard, which seems to suggest that acetonitrile solvent has a good effect on acrylamide separation. The optimal method for acrylamide determination was to use the X-Bridge or C18 column with acetonitrile 4% (A) and acetonitrile 100% (B), an injection volume of 20 µL and a flow rate of 0.5 mL/min for the X-Bridge and 0.4 mL/min for the C18 column. The gradient method started with 0% B solvent then increased to reach 80% at 20 min. It remained at 80% for 5 min from 20 to 25 min then dropped to zero in 5 min, remaining at 0% for 10 min. The methods results are shown in Figures 2-6 and 2-7.

It is difficult to choose an appropriate mobile phase to achieve good peak separation in the acrylamide analysis with acceptable retention time because of acrylamide's high polarity (Zhang *et al.*, 2005b). Different solvents and concentrations were used in the HPLC-UV analysis depending on the method used, such as Millipore water with acetonitrile, formic acid or methanol.

The study by Wang *et al.* (2008) used methanol and acetonitrile with water in different concentrations and it was concluded that both solvents have a similar elution effect on acrylamide. Wang *et al.* (2008) found that the use of 4% acetonitrile as a mobile phase was satisfactory for separation of food sample interferents, whereas 1, 5, and 10% methanol with 0.1% of acetic acid led to unsatisfactory separation. These results were similar to the HPLC-UV analysis findings. However, the results were not reliable because the HPLC-UV did not give acceptable results. Acetonitrile solvent improved the peak shape, shortened the retention time and improved instrument performance (Paleologos and Kontominas, 2005, Wang *et al.*, 2008).

2.4.5 Acrylamide analysis by HPLC-UV

The results of the acrylamide analysis of foods using the HPLC-UV were overestimated because the acrylamide peak could not be resolved. The different samples being cleaned and diluted with water did not improve the acrylamide peak separation when it was analysed by the HPLC-UV.

Figure 2-6 (A) shows the good separation of acrylamide standard using method 4 which used acetonitrile with water 4% (v/v) as solvent A and 100% as solvent B. There was an increased time for solvent B to reach an 80% level, from 10 to 12 min of run time, and this was followed by a drop to zero. However, the gradient method was changed to improve the acrylamide peak separation. However, the binary gradient method 6 has the best acrylamide peak separation.

The analysis of acrylamide standard (A) and acrylamide in the food sample (B) (Cerelac wheat) showed an unresolved acrylamide peak for the sample compared with the standards, as shown in Figure 2-13. According to Hilbig *et al.* (2004), infant cereals are low in acrylamide (≤131 μg/kg), but the acrylamide sample peak was not clearly separated, thus the acrylamide determination was difficult. However, the various methods and columns showed that the acrylamide peak was being overestimated and the results were not acceptable in the case of all samples because the acrylamide peak could not be resolved. To ensure the overestimation came from the extraction enhancement or from the HPLC-UV methods, the same vials were run through the same method in the LC-MS; the results were within the acrylamide range in foods, as will be described in Chapter 3.

The X-Bridge and C18 column were used to quantify the acrylamide standard, although C18 had a slightly better separation with the HPLC-UV. This may be the result of acrylamide peak retention times, which were 4.6 and 10 min for the X-Bridge and C18 respectively. According to Zhang *et al.* (2005b), a C18 column provides ideal balance in terms of retention time for polar and non-polar compounds in reversed-phase chromatography and it gives a greater peak shape. The C18 column was used in further experiments (Chapter 3).

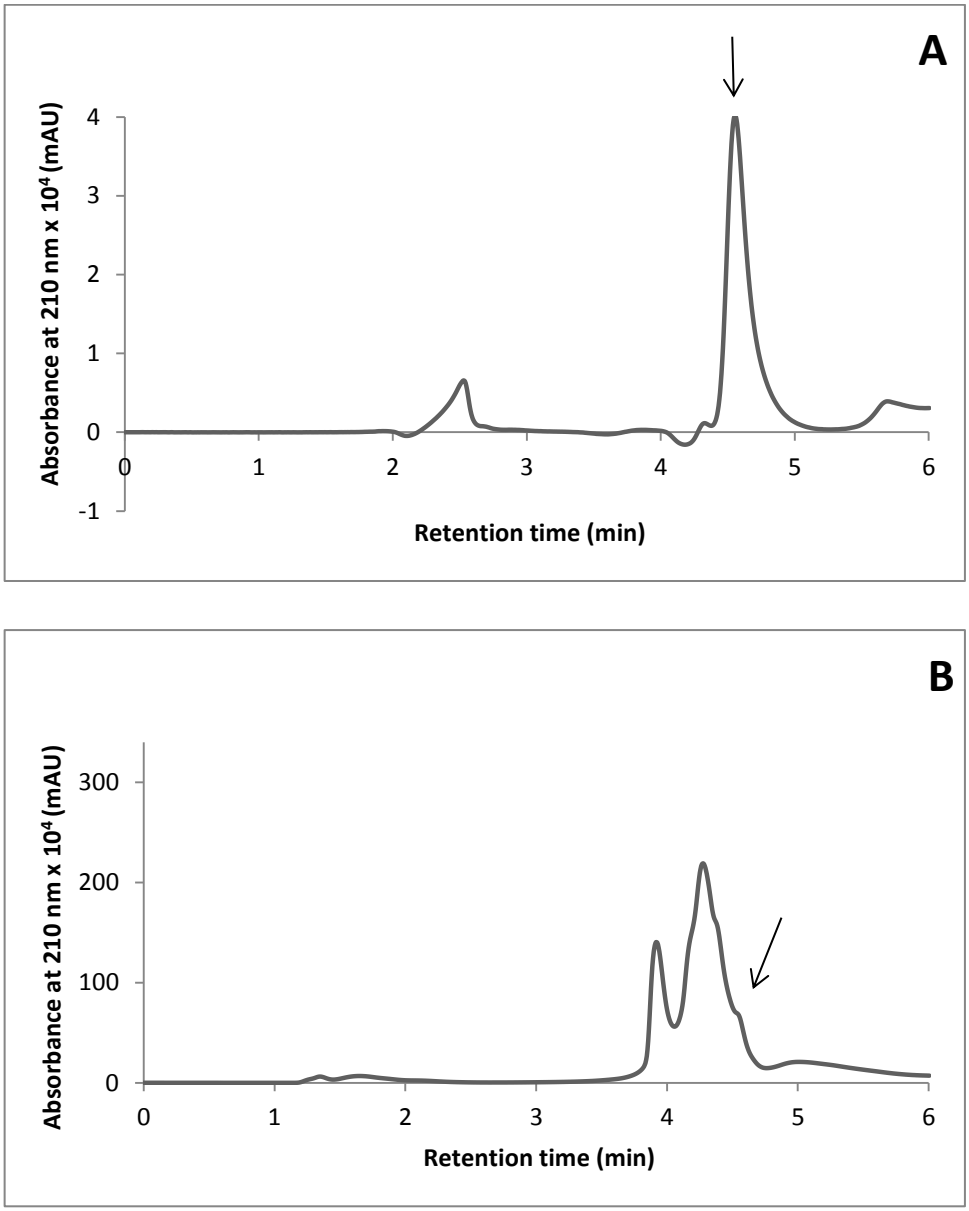


Figure 2-13: HPLC-UV chromatography of acrylamide standard 0.5 µg/mL (A), Cerelac wheat (B) at a detection wavelength of 210 nm. Separation method 6 was used with the X-Bridge column (arrow indicates the acrylamide peak).

2.5 *Discussion*

Since the discovery of acrylamide in food, many analytical methods have been developed for determining the acrylamide content in foods. Several studies have been conducted to develop extraction procedures for removal of acrylamide from food matrices. Today, after 12 years of work on this area, no method has been announced as the optimal method for analysis and extraction.

In this study, acrylamide was extracted from foods such as infant cereal, potato crisps, bread, biscuits and cake using water for solvent extraction. Water was used for acrylamide extraction due to the fact that acrylamide is soluble and stable in water (Wang *et al.*, 2008). Regarding extracted acrylamide from food samples 2 g of each sample were used as the sample size. Roach *et al.* (2003) found that the sample size (1 or 4 g) did not affect the acrylamide results when analysed by LC-MS. Similarly, using 2 g of a sample instead of 1 had no effect on the acrylamide results in this study. Agitating the sample with water horizontally for 20 min was done to extract the acrylamide from the sample because it dissolved in water and shaking of the sample tube led to easy release of the acrylamide from the sample. Centrifuging the sample extraction at a low temperature (0 °C) led to solidifying of the oil layer, which made it easily to remove before the aqueous was collected and filtered.

The Maillard reaction is related to series reaction which occurs in many products. These products from the food matrix can also produce interference in the background which makes it difficult to analyse acrylamide using HPLC (Wang *et al.*, 2008). Clean-up of the acrylamide sample extraction was done using two SPE cartridges (Oasis HLB and MCX). The effect of the clean-up of the acrylamide sample extraction was tested using three clean-up methods, which differed in terms of the solvents and solvent amount used for activation of the SPE cartridges. These changes in the clean-up methods were used to improve the acrylamide peak separation by remove co-extraction components (Wang *et al.*, 2008). Sample extraction centrifuge and clean-up greatly reduced the interference effect, which was tested by LC-MS, as will be shown in the next chapter.

One of the aims of this work was to investigate the optimum method for determinate of acrylamide in foods using HPLC-UV detection. The use of HPLC-UV detection

in this study was selected because earlier studies have shown that acrylamide has high detection sensitivity (Wang *et al.*, 2008, Wang *et al.*, 2013), as shown in Table 2-2.

Methanol with water and acetonitrile with water mixture were used as mobile phases for acrylamide analysis owing to the fact that methanol and acetonitrile have a similar elution effect on acrylamide. However, the baseline separation of acrylamide and co-extraction of food samples was not satisfactory with methanol. After use of an acetonitrile mixture with water 4 and 100% (v/v) as a mobile phase, when the B solvent increased to 80% after the first second at a flow rate of 0.5 or 0.4 mL/min, the acrylamide peak appeared at retention times 4.6 and 10 min with the X-Bridge and C18 columns, which satisfactorily separated the acrylamide peak for the acrylamide standard but not the food sample, as shown in Figures 2-7. The acetonitrile was chosen as an optimal solvent due to it producing minimum co-extraction peaks with a shorter UV wavelength (210 nm) deduction, reducing the column pressure and acrylamide separating from the food co-extraction (Wang *et al.*, 2008).

To select the appropriate wavelength for acrylamide detection in the HPLC-UV method, acrylamide standards were obtained at 210 and 245 nm. The highest absorption of acrylamide was observed at 210 nm. A small absorption was observed at 254 nm. This means that the detection sensitivity of acrylamide was higher at 210 nm wavelength, as shown in Figure 2-10. This finding is in agreement with earlier studies (Wang *et al.*, 2008, Wang *et al.*, 2013). Consequently, from this range of data, a wavelength of 210 nm was selected for the acrylamide detection. The separation of acrylamide at this wavelength was good, with stable retention time (from both columns). Also, at this wavelength, acrylamide standard had good baseline resolution (Figure 2-10). Acrylamide HPLC analysis showed a low UV detection sensitivity with variation in the peak areas when the same concentration was analysed at different times under the same conditions.

After all optimisations in extraction and the HPLC method, , the results show that the acrylamide peak cannot be resolved when analysing food samples which means that in this case, the HPLC is not a suitable method to analyse acrylamide in foods.

According to Wang *et al.* (2008), the HPLC method is not as accurate as an LC-MS/MS technique for acrylamide analysis. LC-MS, as will be explained in the next chapter, can be applied to screen food samples for acrylamide and once high contents of acrylamide are found in a sample, LC-MS can be used to determine the concentration accurately.

The Institute for Reference Materials and Measurements (IRMM) of the European Commission's Joint Research Centre (JRC) in 2007, requested expertise test on acrylamide determination in potato products in 42 laboratories from 16 EU Member States. The 42 laboratories analysed acrylamide by HPLC-MS/MS and GC-MS methods. The results were different from laboratories to another and from instrument to another. The study recommended the importance of participation in proficiency testing systems on acrylamide determination to achieve comparable result. The LOD and LOQ in this study were in the lower range comparing with the values shown in Table 2-9 (3 and 11 µg/kg respectively).The LOD and LOQ of these instruments shown in Table 2-9 (Wenzl and Anklam, 2007).

Table 2-9: The LOD and LOQ with different type of instruments in 42 different laboratories from 16 EU Member States.

Information	**Equipment**				
	GC-MS (derivatis)	GC-MS (non-derivatis)	GC-MS/MS	LC-MS	LC-MS/MS
No. of lab.	5	4	3	2	16
LOD (µg/kg)	2-10	10-30	5-11	12	<5-30
LOQ (µg/kg)	1.7-40	30-100	10-40	45	2.4-60

2.6 *Conclusion*

To determine acrylamide in foods, several HPLC-UV analysis methods and extractions were used and modified. The binary gradient methods separated the acrylamide standard after modification. However, the HPLC-UV analysis and extraction modification methods could not resolve the acrylamide peak in the food samples. The acrylamide content were overestimated because the peaks could not be

resolved. For this reason, LC-MS was used to determine acrylamide in the same food sample as described in the next chapter.

3 Chapter 3: Acrylamide quantification by LC-MS

3.1 *Introduction*

3.1.1 Liquid chromatography mass spectrometry (LC-MS) for acrylamide determination

LC-MS can be used for quantitation and conformation of the components. It is recommended for analysing low molecular weight compounds such as acrylamide because it minimizes the interference ions which increase the background signals from sample and mobile phases (European Food Safety Authority, 2013). For the determination of acrylamide in food, the FDA has recommended using LC-MS/MS and acrylamide $^{13}C_3$ as an internal standard (Nemoto *et al.*, 2002).

Acrylamide can be identified in a chromatograph from the retention time, which is similar in the standard and samples. A change in retention time can occur because of column contamination and so the internal standard was used to identify acrylamide (European Food Safety Authority, 2013). The main ions observed for acrylamide are m/z 72 (protonated molecular ion), 55 (loss of amino) and 27 (later loss of CHO) (Riediker and Stadler, 2003). However, in LC-MS single quadruple the detection ion is m/z 72. Acrylamide is analysed in positive ion mode because it is the most sensitive mode (Rosen and Hellenas, 2002). In acrylamide analysis some contaminations which are similar to acrylamide's molecular weight can interfere with sample quantification when acrylamide content is low (Govaert *et al.*, 2006, European Food Safety Authority, 2013).

It is difficult to choose an appropriate mobile phase for acrylamide analysis because of high polarity, which affects retention time (Zhang *et al.*, 2005b). In the LC-MS method the acidity of a solvent increases acrylamide ionisation. Due to this, the mobile phase can contain organic acid (formic or acetic) and organic modifiers (acetonitrile or methanol) to improve ionisation and reproducibility. Also, acid concentration should be optimised to avoid a decrease in the signal to noise ratio due to matrix effects (European Food Safety Authority, 2013). The most common solvent to extract acrylamide from foods is water which is compatible with LC using

an aqueous mobile phase with a small amount of organic modifier (Zhang *et al.*, 2005b). As demonstrated in Chapter 1, the Maillard reactions have many products may also elute and appear in chromatography. Melanoidins is one of the Maillard reaction products and it has 30 different fragments. The m/z for some fragments were 519, 147, 135 and 69 (O'Brien, 1960). HMF (hydroxymethylfurfural) is another MRP which is known as an indicator of quality worsening in foods. HMF ions monitored are m/z 109 and m/z 127 (Agilent Technologies, 2006).

3.1.2 Internal standard

An internal standard (IS) is a suitable compound added to a sample in an early stage and is designed to improve accuracy (Sumner *et al.*, 1999). It should be similar to acrylamide in terms of chemical and physical properties but with a different molecular weight and it cannot be present naturally in the sample. An internal standard was used for acrylamide quantification, location, verification of non-detection and confirmation of identity. The most common used as an internal standard in published methods are $^{13}C_3$ 2-bromo propenamide, $^{13}C_1$ acrylamide, D_3-acrylamide $^{2}H_3$, N,N- dimethylacrylamide, methacrylamide and propionamide, as used by Zhang *et al.* (2005a).

The loss of acrylamide and IS should be the same and the appearance of an internal standard and absence of acrylamide means there is no acrylamide in the sample. If the internal standard does not appear during analysis that means there are problems which need to be corrected before analysis can take place. Whereas if it is compared with an external standard response, the data will be read as non-detected or a low result (Roach *et al.*, 2003). The retention time of the internal standard is the same as acrylamide. If the acrylamide retention time is changed when the samples run over night because of contamination, the acrylamide peak can by identified in the chromatography due to the use an internal standard (European Food Safety Authority, 2013).

C_{13}-labelling is recommended due to protons in the α-position to carbonyl-functions which may exchange with protons from the sample. The labelling should increase the molecular weight of the standard by at least two units to minimums interferences with masses originating from the analyte (Vikstrom *et al.*, 2010). The acrylamide

C_{13} molecular weight is 74.03 which higher than acrylamide with 3 units. Figure 3-1 shows acrylamide C_{13} structure (C_3H_5NO).

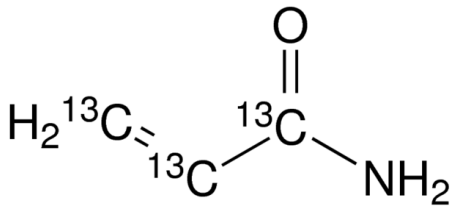


Figure 3-1: Acrylamide C_{13} structure

3.1.3 Aims

The specific aims of this chapter are to:

- develop methods for acrylamide quantification in extracted starchy food samples using LC-MS,
- determine the acrylamide level of starchy foods consumed by Saudi infants,
- determine the effect of high temperature on acrylamide level in infant cereal and bread loaf.

3.1.4 Hypotheses

- The acrylamide level in baked and fried foods consumed by Saudi infants is high.
- There is no acrylamide in infant cereals consumed by Saudi infants.

3.2 *Methods*

3.2.1 Materials used for acrylamide analysis

3.2.1.1 Reagents and consumables

- Most of the reagents and consumables were mentioned in section 2.2.1,
- Acrylamide (C_{13}), isotopic purity 99% (Sigma, UK, cat. No. 586617-0),

- Acetonitrile for HPLC and LC-MS grade (VWR; UK, cat. No.83640-320),
- Formic acid 99% (v/v) (AR Cat No 33015-54930R),
- Methanol LC-MS grade, 95% (v/v) (VWR, UK, cat. No.83638-320).

3.2.1.2 Equipment

- Liquid chromatography mass spectrum (LC-MS) (Shimadzu, UK limited), which consisted HPLC components with:
    - Mass spectrum (MS) (LCMS 2020), which is an analytical technique that measures the mass-to-charge ratio of charged particles,
    - Rotary pump E2M28,
    - Gas control system, nitrogen is the gas used in LCMS2020, and it used as three types of gas; nebulizer gas, drying gas and standard sample feed gas,
    - LabSolution program version 5.53, Shimadzu Corporation.
- Incubator (Sanyo: MIR-262, Incubator limited),
- Microwave (SKU:123464155 Sainsbury's supermarket),
- Pushdown toaster (Argos NO. LW-619).

3.2.1.3 Sampling

All sample information has been mentioned in Chapter 2, section 2.2.4.

3.2.2 Acrylamide determination: analytical methods

3.2.2.1 Acrylamide C_{13} standard preparation

1 mg of acrylamide C_{13} was weighed in a Falcon tube then 1 mL of water was added to make the stock solution (mg/mL). A series of dilutions were done to enrich the final concentration of 1 µg/mL. The stock solution and the dilution solutions were kept at 4 °C in a Falcon tube for 2 months.

3.2.2.2 Acrylamide standard preparation

The standard was prepared as mentioned in section 2.3.1. Preparation of acrylamide calibration standards (0.01, 0.05, 0.1, 0.5 and 1 µg/ mL) was as mentioned in the table below (3-1). All standards were stored in Falcon tubes at 4 °C for further use. The maximum storage time for the working standard was two months.

Table 3-1: Series of different acrylamide concentrations (µg/l) prepared from the stock solution (10000µg/l) by being diluted in water

Acrylamide concentration (µg/mL)	Acrylamide 10 µg/mL (mL)	Acrylamide C_{13} 1 µg/mL solution (mL)	Millipore water (mL)
0.0	0.0	2.0	8.0
0.01	0.01	2.0	7.99
0.05	0.05	2.0	7.95
0.1	0.1	2.0	7.9
0.5	0.5	2.0	7.5
1.0	1.0	2.0	7.0

3.2.2.3 Sample preparation and extraction

The samples were prepared as mentioned in section 2.3.2. However, there was a difference in that 2 mL of acrylamide C_{13} (1 µg/mL) was added instead of 2 mL of water. So the sample was prepared by adding two grams of solid sample into separate 50 mL Falcon centrifuge tubes, and then 18 mL of water and 2 mL of

acrylamide C_{13} (1 µg/mL) were added as shown in Figure 3-2. For recovery purposes, the sample was prepared similarly with 2 mL of 1 µg/mL acrylamide standard with 16 mL of water and 2 mL of C_{13} (1 µg/mL) as shown in Figure 3-3.

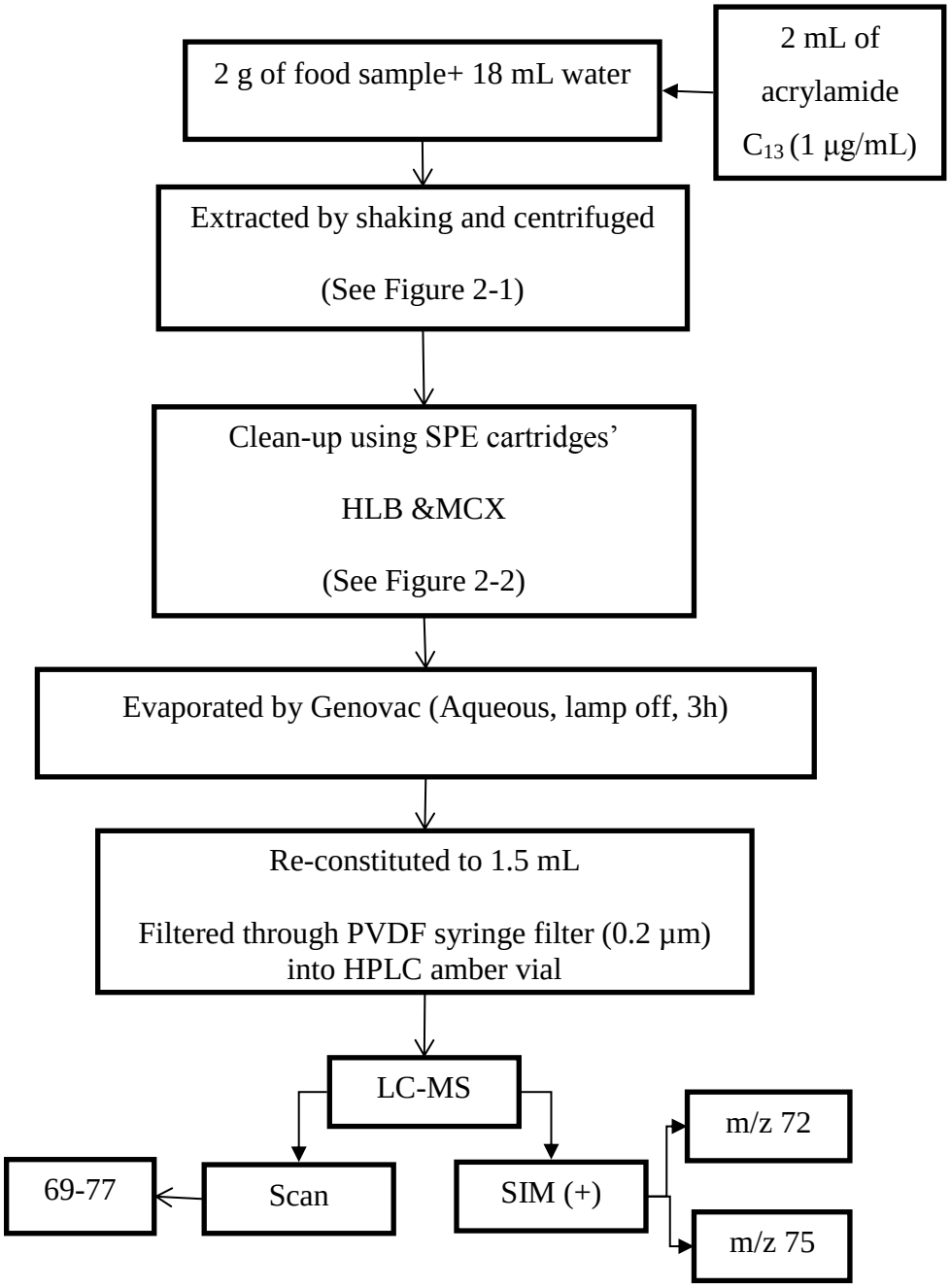


Figure 3-2: Schematic representation acrylamide treatment methods in food sample spiked with 2 mL of acrylamide C_{13} (1 µg/mL) before LC-MS analysis.

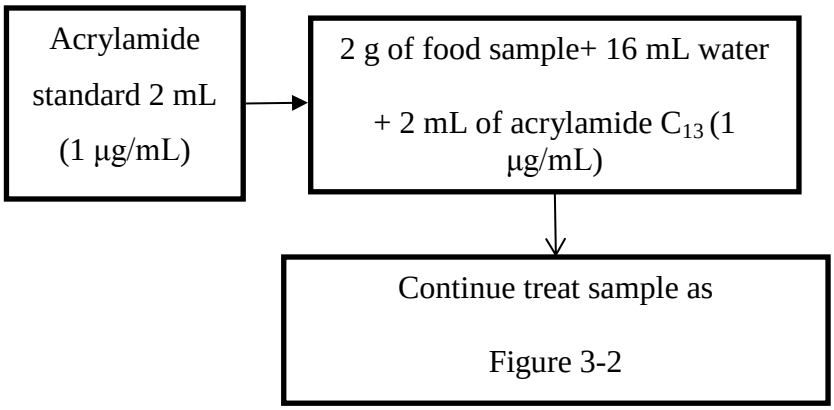


Figure 3-3: Schematic representation food sample spiked with 2 mL of acrylamide standard (1 µg/mL) for measure acrylamide recovery by using LC-MS analysis.

3.2.2.4 Acrylamide determination using liquid chromatography mass spectrometry LC–MS

The calibration standards and sample extracts were injected via a 20 µL sample loop, and detected in MS at positive mass m/z 72 and 75 for acrylamide and acrylamide C_{13} respectively. The LC-MS gradient elution program used 4% acetonitrile with 0.1% formic acid and Millipore water (v/v) as solvent A, and 95% acetonitrile LC-MS guard with Millipore water (v/v) as solvent B, at a flow rate of 0.4 mL/min using a C18 column. The elution program applied was 0% B, then at 0.1 min it was increased to reach 80% B at 22 min, then kept for 5 min and decreased to 0% B from 25 to 30 min. It was then kept at 0% B for 10 min. The total run time for the LC-MS gradient was 39 min. Before starting the run of the standard and the samples, Millipore water was injected several times to check for the absence of any contaminations with masses similar to acrylamide mass. Also, Millipore water was injected before the run of different standards or sample extractions to avoid any acrylamide contamination from the previous sample or standard.

The MS was setup for two events: a positive scan and selected-ion monitoring (SIM). In the scan event, the scan range was set up between 69 and 77 to reduce the

noise. In the SIM event, channels were chosen to be (positive ion) m/z 71, m/z 72 and m/z 73 for acrylamide and m/z 75 for acrylamide C_{13}. As mentioned in Chapter 1 acrylamide molecular weight (MW) is 71 and acrylamide C_{13} MW is 74, for that the channels were decreased to be m/z 72 and m/z 75 for acrylamide and acrylamide C_{13}.

The LC part was prepared as mentioned for HPLC in section 2.3.4.3. The MS source was wiped carefully after each run using a Mira-cloth with 50% isopropanol. Methanol (50%) and Millipore water were run before and after running the samples. Water vial was put between the samples to avoid contamination and water chromatography was used to check this. A mobile phase equilibration was needed for an hour before analysis of the standard and sample and due to the short equilibration period may have resulted in shifting of acrylamide retention time. The standards set used in these methods were high concentration 0.5 or 1.0 µg/mL.

3.2.2.5 Optimisation LC-MS methods

Method 1

A method from adapted Mojska *et al.* (2012) was used with an X-Bridge column.

Method 2

A method adapted from Hoenicke *et al.* (2004) was used with an X-Bridge column.

Method 3

A method adapted from Rosen and Hellenas (2002) was used with an X-Bridge column.

Method 4

A method adapted from Govaert *et al.* (2006) was used with an X-Bridge column.

Method 5 (optimal method)

A method adapted from Wang *et al.* (2008) was used which consisted of elution of the sample with 4% acetonitrile and 0.1% formic acid in Millipore water (v/v) as solvent A, and 95% acetonitrile as solvent B, at a flow rate of 0.5 mL/min, through

an X-Bridge and a C18 column and at a temperature of 25 °C. The method was run for 40 min with 0% B and this was increased until it reached 80% after 20 min, remaining as 80% for 5 min (20 to 25), after which it was decreased gradually to 0% B (at 25 min) and kept at 0 for 10 min (Figure 3-4). The injection volume was 20 µL and m/z was chosen to be 72 and 75 for acrylamide and acrylamide C_{13} respectively. This method was used as the initial method in this study.

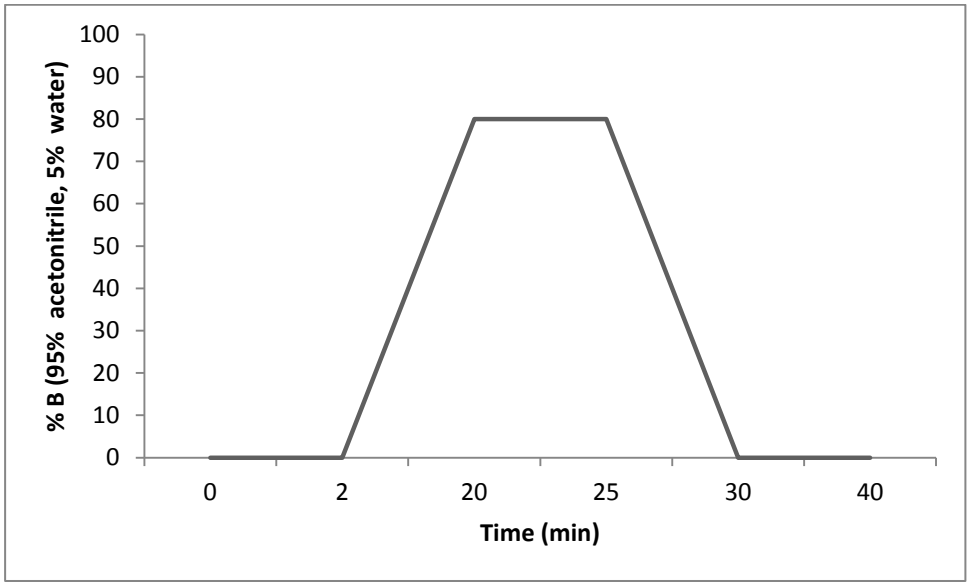


Figure 3-4: LC-MS optimal method curve (method 5) for acrylamide determination in starchy foods. The SIM set in m/z 72 for acrylamide and m/z 75 for acrylamide C_{13}.

3.2.2.6 High temperature effects

Some samples were treated before extraction to test the effect of temperature on acrylamide formation, as described below.

3.2.2.6.1 Infant cereal

Infant cereal samples were treated with two different treatments, storage and heating.

Storage

Five infant cereals (Cerelac wheat, wheat with honey, wheat with dates, rice and oats) were stored in incubation in special conditions similar to storage room conditions in Saudi Arabia with 37 °C and 25% moisture. The samples were left in these conditions for a month, then stored in a freezer at -18 °C.

Heating

Three infant cereals, Cerelac wheat, wheat with honey, and wheat with dates, were prepared by adding 2 g of each cereal to 4 mL of hot water then mixing. After that, the sample was heated in a microwave for 20 sec at high power (700 W). Then the sample was analysed as usual.

3.2.2.6.2 Loaf bread

Two slices of bread were toasted in a pushdown toaster (Argos NO. LW-619) for 1 and 2 min to give a light and medium colour respectively (visually as shown in Figure 3-5). After that, the bread was removed and analysed using LC-MS.

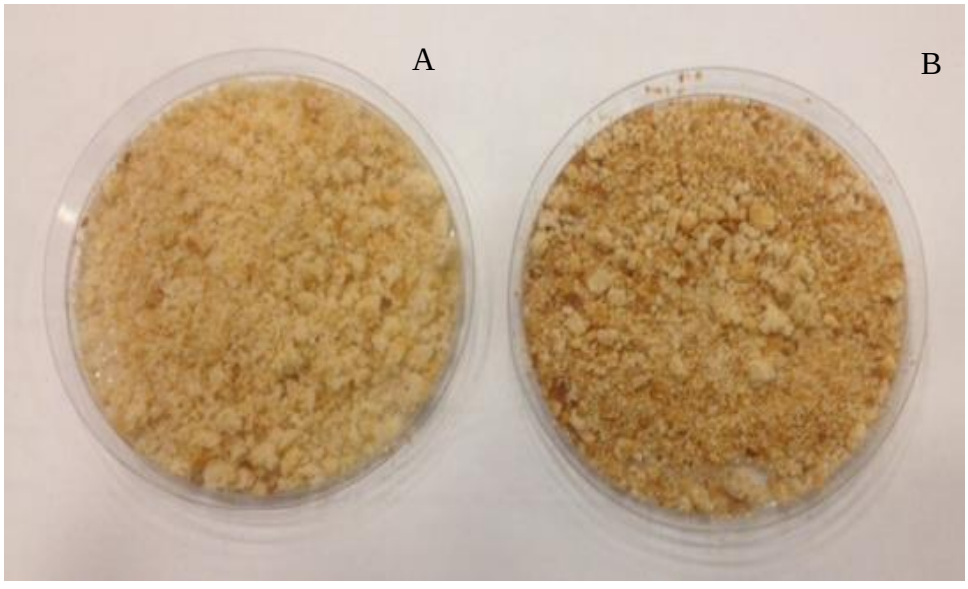


Figure 3-5: Toasted white loaf. (L'usine) A) light toasted colour and B) medium toasted colour.

3.2.3 Calculation of acrylamide

3.2.3.1 Calculation of acrylamide content in food

The acrylamide content in food was calculated as mentioned in section 2.3.5.1.

3.2.3.2 LOD and LOQ

The LOD and LOQ of using LC-MS for acrylamide detection were described in section 2.3.4.2.

3.2.3.3 Recovery of acrylamide

The extraction efficiency and sample loss during the preparation can be determined by adding 2 mL of acrylamide standards concentration 1 µg/ml in the sample before extraction instead of 2 mL of Millipore water. Once acrylamide peak areas for the spiked and un-spiked sample are known, acrylamide concentration can be calculated to determine the recovery factor. Also, recovery test can use to determine the extraction efficiency and acrylamide loss during preparation. The percentage of recovery was calculated as in Equation 3-1 after the total acrylamide in the spiked sample extraction was subtracted from the un-spiked sample extraction.

$$\textbf{Recovery Calculate (RC \%)} = \frac{\textbf{Measured } \boldsymbol{concentration}}{\textbf{known concentration}} \times \mathbf{100}$$

Equation 3-1

3.2.4 Statistical analysis

Microsoft Excel 2007 was used to generate a calibration curve and the coefficient of variation (CV) (sections 3.3.1.1 and 3.3.3). The acrylamide sample extraction was analysed triplicate from two extraction and the results were expressed as mean ±standard deviation.

3.3 *Results*

3.3.1 Optimisation LC-MS methods

For acrylamide determination several LC-MS methods were used, differing in terms of the solvents, run times and columns. When the five LC-MS methods were used to

choose the LC-MS optimal method for acrylamide analysis, the fifth method was chosen (the same method used in HPLC). In methods 1 and 3 the acrylamide peak shape was not good. Whereas the acrylamide peak was satisfactory in methods 2 and 4 for acrylamide standard but not sample (Figure 3-7 A, B, C and D). Therefore optimal method (5) was used due to good separation for acrylamide in standard and most samples (Figure 3-7 and 3-8). The use of C18 column and acetonitrile solvent was shown to be ideal in the HPLC chapter.

3.3.1.1 Initial calibration curve of acrylamide

The acrylamide standard calibration curve was obtained by plotting acrylamide (m/z 72) peak area against acrylamide concentration. Acrylamide solutions were prepared at 0.01, 0.05, 0.1, 0.5 and 1.0 µg/mL with a known amount of acrylamide C_{13}, which was 2 mL of 1 µg/mL (2 µg), as shown in Table 3-1. The linearity of the acrylamide standard was tested in the range of 0.01 to 1 µg/mL, with a correlation coefficient equal to 995 (R^2 = 0.995), as shown in Figure 3-8. The error bars point to the precision of triplicate injections. Also, Figure 3-7 shows the LC-MS chromatograph of 72 and 75 ions mode for acrylamide standard (0.05 µg/mL) and the TIC for both ions 72 and 75.

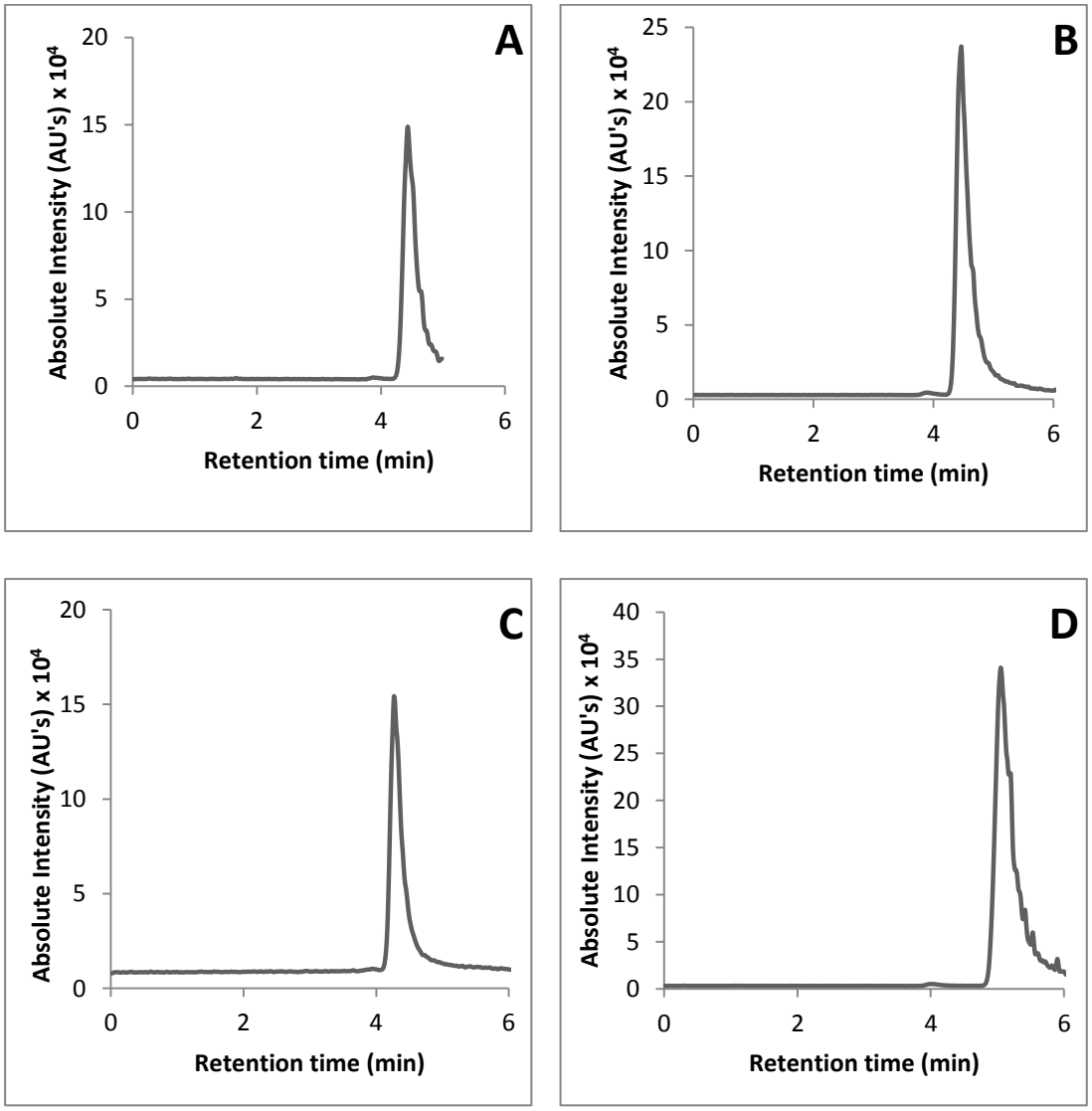


Figure 3-6: Acrylamide standard (1.0 and 2.0 µg/mL) chromatograph SIM m/z 72 (peak area). Analysed using LC-MS separation methods A) 1, B) 2, C) 3 (1.0 µg/mL) and D) 4 (2.0 µg/mL) were used with the C18column.

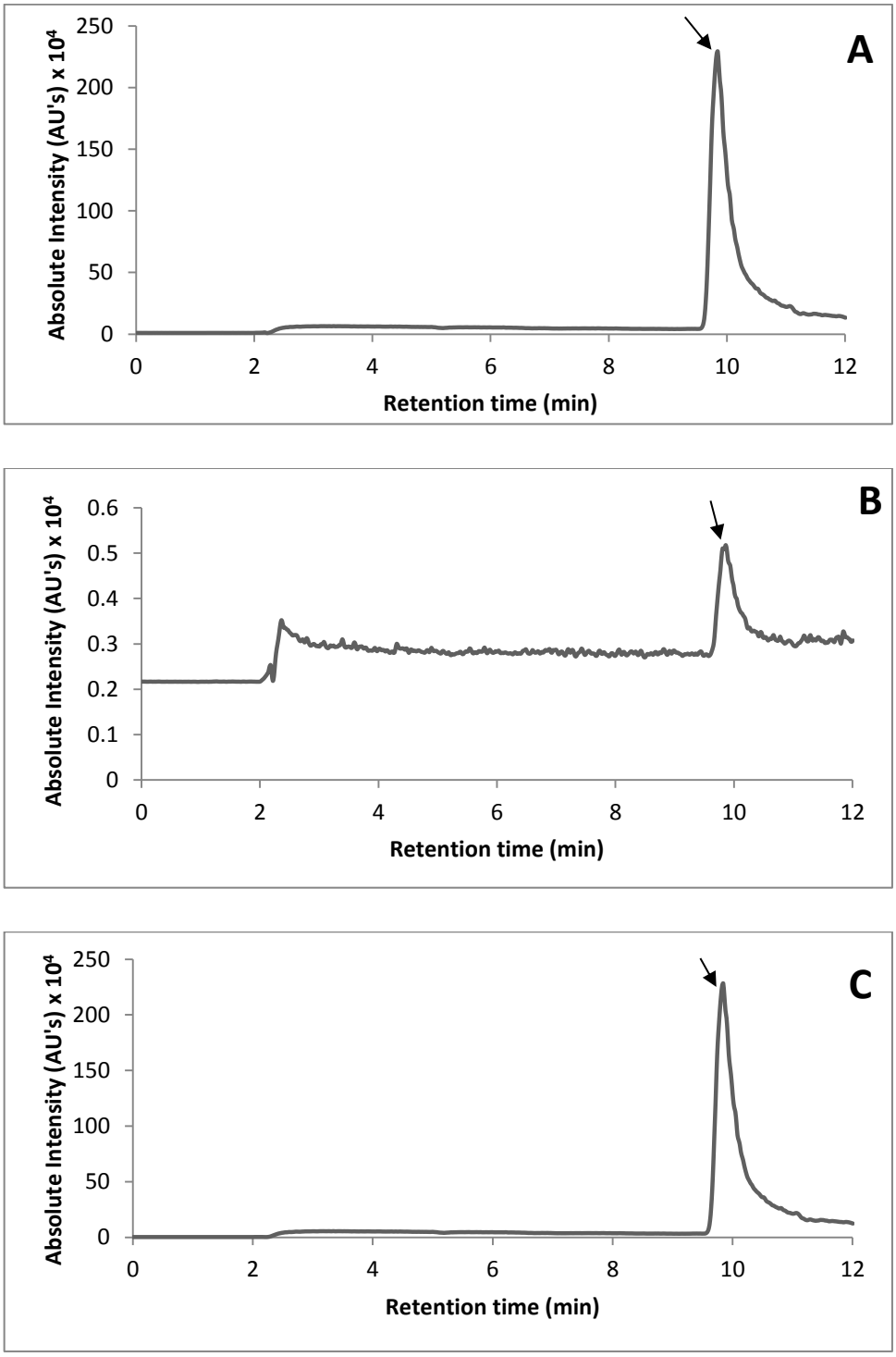


Figure 3-7: Acrylamide standard (0.05 µg/mL) chromatograph SIM with detection mode A) TIC, B) m/z 72 and C) m/z 75 (peak area), analysed using LC-MS separation method 5 with the C18 column (arrow indicates the acrylamide peak).

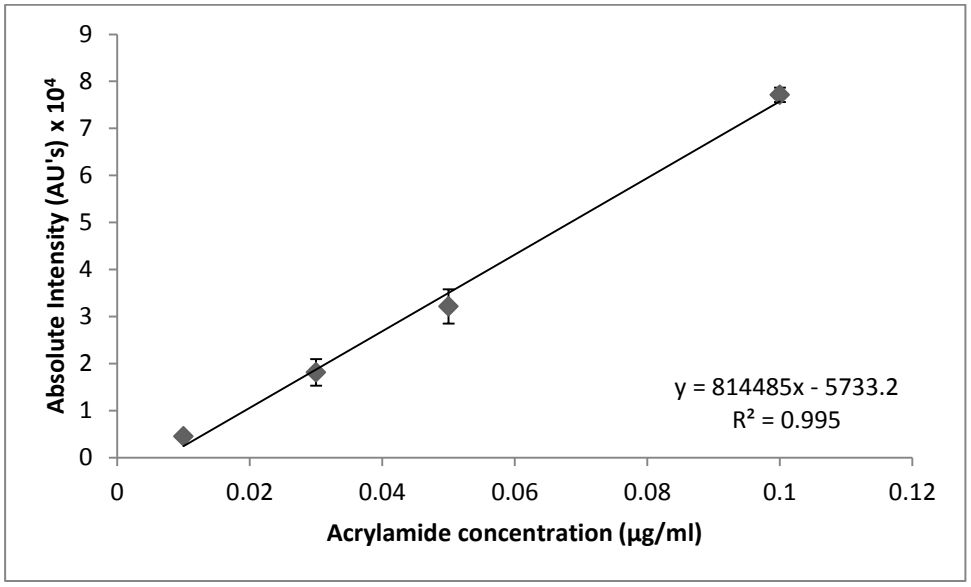


Figure 3-8: Acrylamide standards curve. Standard concentrations range from 0.01 to 0.1 µg/mL analysed using LC-MS SIM m/z 72 (LOD = 0.017 µg/mL).

3.3.1.2 Limits of detection and quantification

The LOD and LOQ were evaluated for a signal to noise ratio of 3.3 and 10 at a concentration of 0.017 to 0.052 µg/mL respectively. In another word the LOD and LOQ are 17.2 to 52.2 µg/L or µg/kg. This means the LC-MS LOD in this study was higher than the LOD in previous studies as shown in Table 2-2, ranging from 1 to 15 µg/kg.

3.3.2 Mass spectrum

Acrylamide and acrylamide C_{13} molecular weight are 71.08 and 74 g/mol respectively (as mentioned in sections 1.3.2 and 3.1.2). The mass spectrum in LC-MS was setup for SIM positive events, as mentioned in section 3.2.2.4. From the result the SIM channels were chosen to be m/z 72. The main ions observed for acrylamide are m/z 72 (protonated molecular ion) and m/z 75 (protonated molecular ion) for acrylamide C13 (section 3.2.2.1), due to the acrylamide and acrylamide C13 were found in channel 72 and 75 as shown in Figure 3-9.

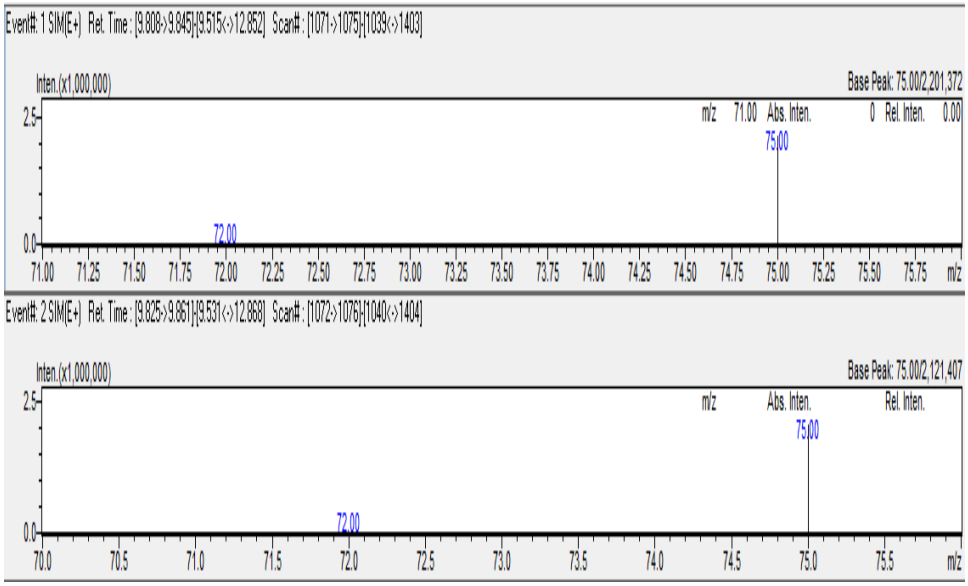


Figure 3-9: Mass spectra SIM of acrylamide and acrylamide C_{13} m/z 72 and m/z 75 (positive mode).

3.3.3 Calculation of acrylamide content in food sample

The standard curve slope equation was used to find the acrylamide level in food with an addition calculation to take account of dilution, as mentioned in section 2.4.7. For conformation of acrylamide peak in the sample a criterion was used: acrylamide standard and sample must occur at the same retention time, the signal of acrylamide at m/z 72 and the internal standard C_{13} m/z 75 (Zeng *et al.*, 2011). In the acrylamide calculation the TIC could not be used because it gave an overestimated value. For this reason the m/z 72 was used for determining acrylamide area.

The CV obtained for 6 analyses was 11.3% for the intraday precision of the method. The CV was similar to that found by Bent *et al.* (2012), who found that the CV was equal to 10%.

The acrylamide (m/z 72) peak area was calculated by using standard slope equation. For example, cornflakes (Nestle country) samples area reading was 5733.2 (AU's/min). This reading was used as the (y) value in the acrylamide standard (µg/mL) curve equation [x=(y- 5733.2)/814485] to find the value of (x) which was equal to 0.016 µg/mL. After that, the x value was multiplied by the dilution factor ×20 (2 g of the sample dissolved in 20 ml of water), so 0.016 µg/mL ×20 mL = 0.3

µg. The result was multiplied by 500 (2 g was used) to find the amount of acrylamide per kilogram of food, which was equal to 0.4 µg × 500 = 157.2 µg/kg.

3.3.4 Ratio between acrylamide (72) and acrylamide C_{13} (75)

The mass spectra of acrylamide and acrylamide C_{13} were m/z 72 and m/z 75. The LC-MS chromatograph set to show acrylamide, acrylamide C_{13} and total ion current (TIC) modes. The ratio between acrylamide and acrylamide C_{13} m/z 72:75 ranged between 0.36 and 0.44. In Figure 3-10 the acrylamide peaks in the popcorn (Butterkist) sample are shown in three different moods ions 72, ions 75 and TIC, to show the difference in peak size in each.

3.3.5 Recovery of acrylamide

The acrylamide recovery determined from the food samples may reflect loss during sample preparation and extraction, as well as clean-up efficiency (Wang *et al.*, 2008). The acrylamide recovery in different food samples determine by adding 2 mL of acrylamide standard (1 µg/mL) to food samples and the percentage of recovery was calculated as equation 3-1.

The recovery in this study ranged from 84.3% to 106.1%, which means that the sample preparation and LC-MS analysis was acceptable (Table 3-2). The variation in the result may be due to the differences in the food matrix between categories. The water was used as blank; also, no detectible samples were used as blank in most groups. The Figure 3-11 shows chromatograph for biscuits (Bahlsen) sample non spiked and spiked with 2 mL acrylamide standard (1 µg/mL).

The recovery percentages in Senyuva and Gokmen (2006) study were 96.8% and 94.0% for biscuits and breakfast cereal respectively, which is slightly higher than the acrylamide recovery reported in this study. Kim *et al.* (2007) estimated acrylamide recovery in steamed rice, bread and French fries by adding acrylamide standard; the recovery rates were 100%, 99.3% and 102.2% respectively, which for bread is higher compared with this study.

Table 3-2: Acrylamide recovery percentage in different food groups.

Food group	Number of samples	Acrylamide standard spiked µg/mL	Acrylamide recovery µg/mL	Acrylamide recovery (%)
Blank (water)	6	1	1.0	99.6
Bread	5	1	0.8	84.3
Biscuits and cake	3	1	0.9	93.8
Cereal	3	1	0.9	91.8
Potato Crisps & popcorn	3	1	1.0	99.9
Noodles, macaroni, oats and rice	4	1	1.1	106.1

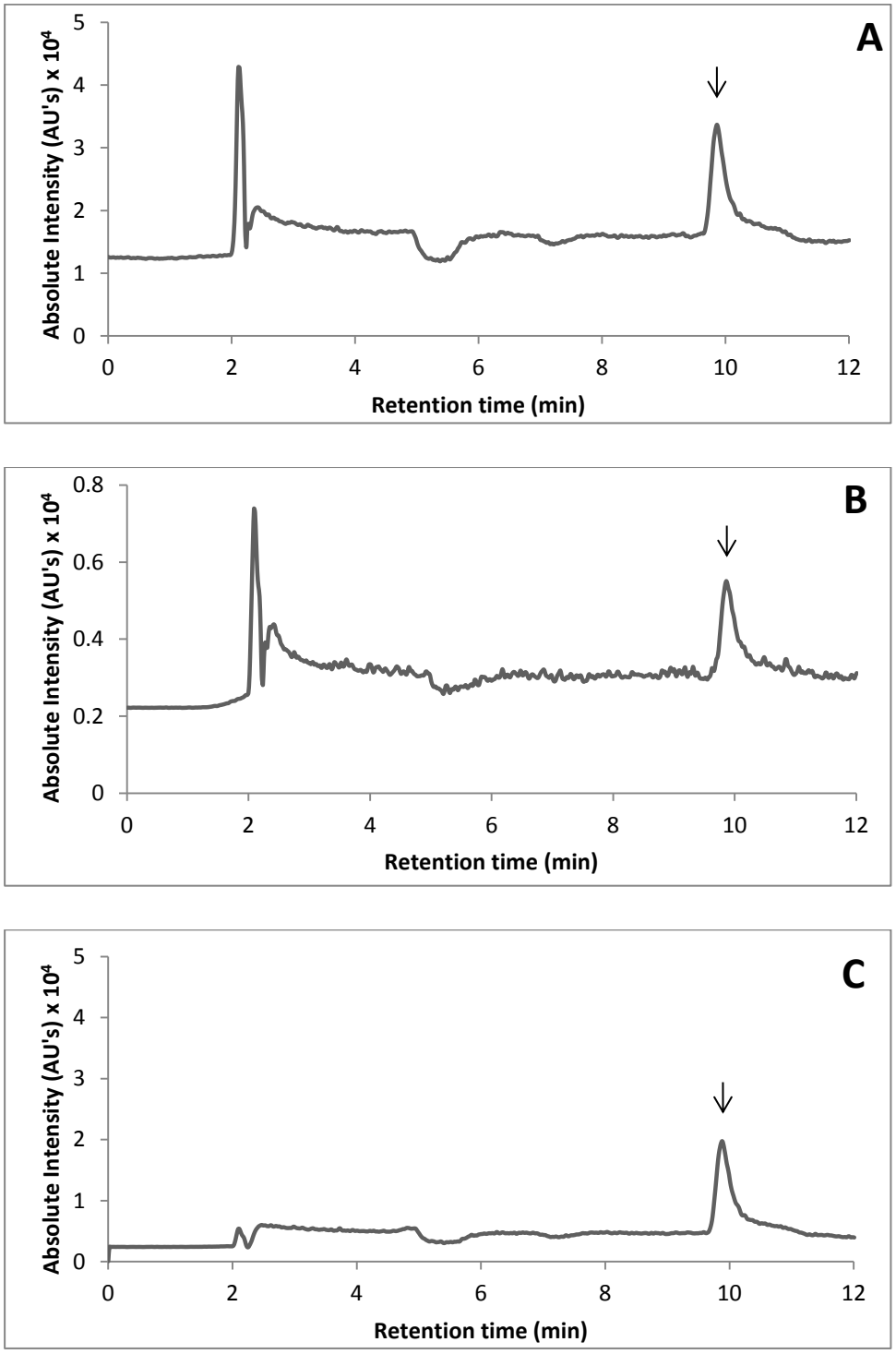


Figure 3-10: Acrylamide chromatographs SIM, A) acrylamide peak TIC mode (m/z 72) B) acrylamide peak (m/z 72) and C) acrylamide C_{13} peak (m/z 75) (peak area), in Popcorn (Butterkist) sample, analysed using LC-MS separation method 5 with the C18 column (arrow indicates the acrylamide peak).

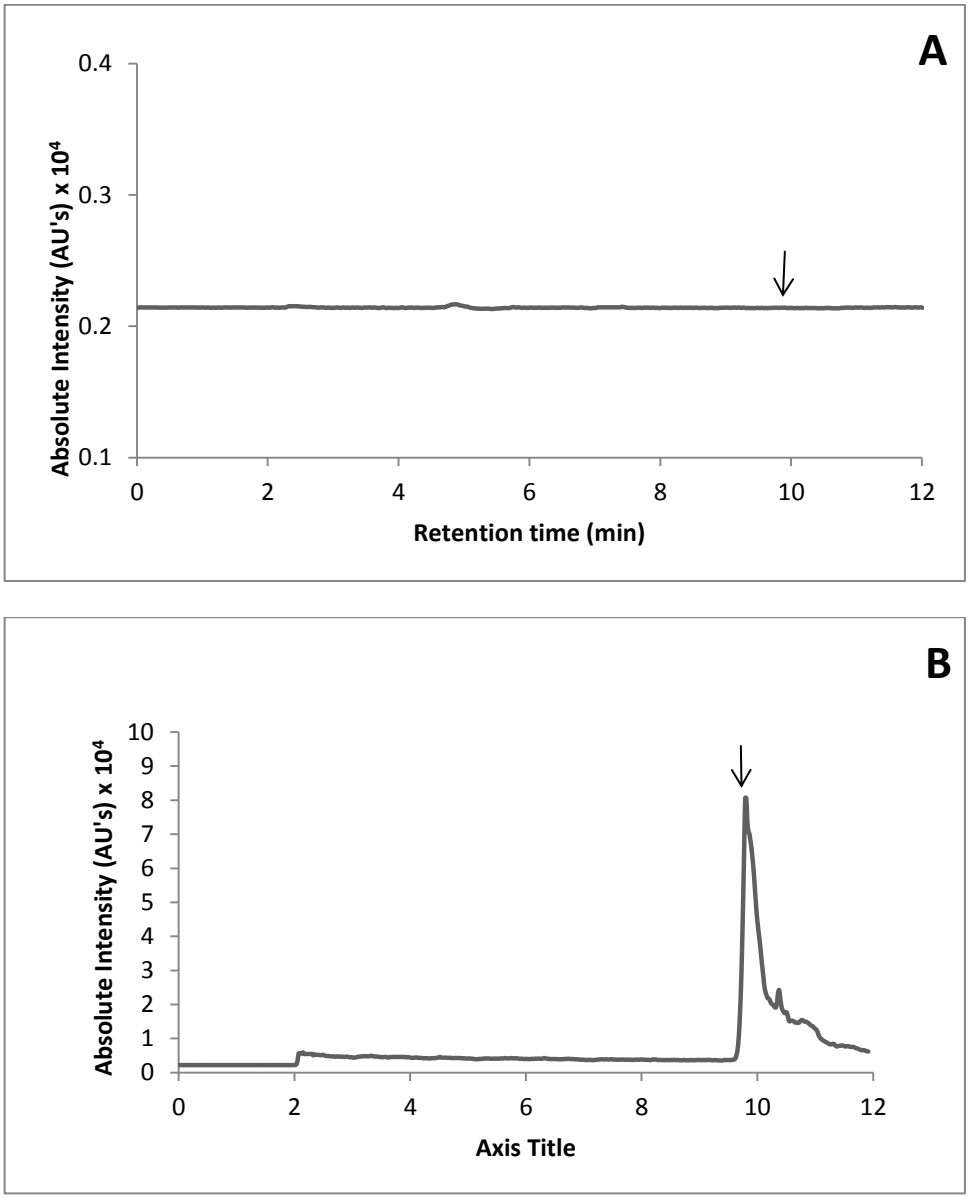


Figure 3-11: Acrylamide chromatograph SIM m/z 72 in Biscuits (Bahlsen) sample A) non spiked B) spiked with 2 mL acrylamide standard (1 µg/mL), analysed using LC-MS separation method 5 with the C18 column (arrow indicates the acrylamide peak).

3.3.6 Acrylamide determination by using LC-MS optimal method

It was difficult to analyse acrylamide in different food matrices. The results of analysing the acrylamide content are shown in Tables 3-3, 3-4, 3-5, 3-6 and 3-7 and Figures 3-8, 3-11, 3-12, 3-13, 3-14 and 3-15. The acrylamide levels in starchy foods

consumed by Saudi infants vary from not detected (ND) to 755.5 µg/kg. Of the 16 types of starchy foods analysed by LC-MS, potato crisps of Albatal and Lay's have the highest acrylamide content at 755.5 and 743.2 µg/kg respectively, followed by Butterkist popcorn at 427.8 µg/kg and Members Bambini biscuit at 208.2 µg/kg. According to Boroushaki *et al.* (2010), potato contains more asparagine and reducing sugar than cereal, for that it contains more acrylamide.

Most Saudi infants foods consumed, such as croissant, white loaf, white Arabic bread, infant cereal, oats and rice, were below the limit of detection, as shown in Tables 3-3, 3-5 and 3-7. The level of acrylamide was different between studies for the same types of food and groups. The acrylamide levels determined in this study using LC-MS showed lower contents compared with the findings of other studies, as shown in Tables 3-3, 3-4, 3-5, 3-6 and 3-7. These studies are a Dutch study by Boon *et al.* (2005), a Turkish study by Senyuva and Gokmen (2005), a Food Standards Agency (2010b) study, a Saudi study El-Ziney *et al.* (2009), a Swedish study by Svensson *et al.* (2003), and UK study by Food Standards Agency (2010b). From these studies the minimum and maximum of each food type were chosen to setup a range of acrylamide. Cereal, bread and potato crisps are the most stable food in Saudi infants' diets. Both infant cereal and white bread did not have acrylamide, whereas potato crisps were found to contain the highest acrylamide level in this study. The high consumption of potato crisps and other foods containing acrylamide may cause health implications, especially for infants.

Acrylamide stability in food sample extraction is dependent on pH, exposure to light, reactive co-extractives and microorganisms. The acrylamide was stable in the food extracted due to it having similar results when the same sample extraction vial was analysed by LC-MS. The samples were analysed on the same day of preparation and after weeks. This finding was in agreement with the study of Roach *et al.* (2003) when they reanalysed some samples to study the repeatability of the study, although, the study did not specify the length of the acrylamide stability in the samples. Croft *et al.* (2004) concluded that extracted samples can be stored at 4 ºC for short periods of time to prevent decreased acrylamide level.

In contrast, Andrzejewski *et al.* (2004) found acrylamide levels are not stable at room temperature, when they analysed acrylamide extraction in ground coffee stored at room temperature (25 ºC). The acrylamide level was decreased from 40% to 65% after 6 months of storing a sample at room temperature. Hoenicke *et al.* (2004) suggest that a reduction in acrylamide level in potato chip extractions stored at room temperature was due to acrylamide degradation.

3.3.6.1 Bread

The acrylamide mean (min-max) in the white and wholemeal (brown) Arabic bread in the Food Standard Agency (2010b), were detected as 12 (4-30) and 31 (9-51) µg/kg respectively, whereas the levels of acrylamide found in this study for the same foods were ND (ND-ND) and 91.6 (78.2-103.9) µg/kg respectively. The Food Standards Agency (2002) reported bread average portion size is around 85-95 g for Samoli (hamburger bun) and brown Arabic bread (pitta bread) respectively which means the acrylamide levels are around 7.0 and 8.7 µg per portion of Samoli and brown Arabic bread respectively.

In the study by El-Ziney *et al.* (2009) the acrylamide level in traditional Saudi food, was determined and they found the level of acrylamide in white and brown Arabic bread were 90 and 40 µg/kg respectively, which is in contrast with this study's results as shown in Table 3-3. The Samoli bread is similar to roll bread. However, the colour of the Samoli bread analysed varied from light gold to dark brown and the results of analysing the Samoli varied from ND to 88.4 µg/kg. The variation in result may be linked to the colour of Samoli (Danube) bread which was light gold, whereas, L'usine and Aljazei colour were light brown. The acrylamide level cannot be detected in different croissant samples as shown in Table 3-3. Figure 3-12 illustrates the croissant (Tasbeera) chromatograph.

Table 3-3: Acrylamide levels (µg/kg) in bread samples consumed by Saudi infants and published range for food groups from the literature: a (Boon *et al.*, 2005) b (Food Standards Agency, 2010b) and c (El-Ziney *et al.*, 2009).

Sample name (n=6)	Acrylamide level (µg/kg)	
	Result (±SD)	Published range
White loaf (Al-rashed food company)	ND	10-2883 [a]
White loaf (L'usine)	ND	
White loaf (Aljazei)	ND	
Samoli (Danube)	ND	17-446 [b]
Samoli (L'usine)	77.1±3.2	
Samoli (Aljazei)	88.4±2.6	
White Arabic bread (Danube)	ND	20-49 [b]
White Arabic bread (Al-raya)	ND	
White Arabic bread (Othaim)	ND	
Brown Arabic bread (Othaim)	78.2±2.2	90-180 [c]
Brown Arabic bread (Danube)	92.7±4.5	
Brown Arabic bread (Al-raya)	103.9±1.2	
Croissant (Tasbeera)	ND	<30- 70 [a & b]
Croissant (Al-raya bakery, fresh)	ND	
Croissant (Danube bakery, fresh)	ND	

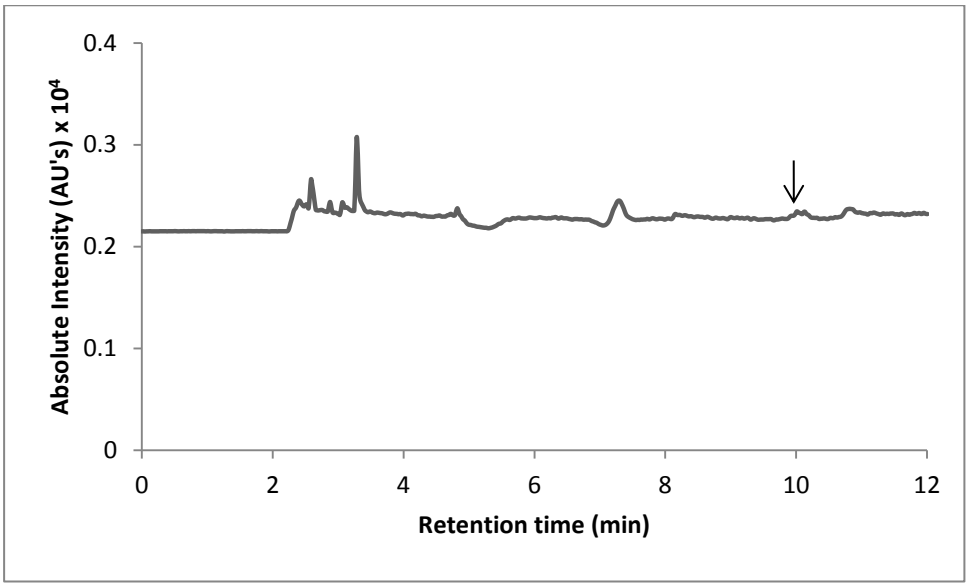


Figure 3-12: Acrylamide chromatograph SIM m/z 72 in Croissant (Tasbeera) sample analysed using LC-MS separation method 5 with the C18 column (arrow indicate the acrylamide peak).

3.3.6.2 Biscuits and cakes

In four different biscuits samples analysed by LC-MS the acrylamide level was not detectable for three of them, whereas the fourth one (Members Bambini) contained 208.2 µg/kg of acrylamide. In Mojska *et al.* (2012), 15 different infant biscuits were analysed by LC-MS/MS, and the mean was 219 µg/kg.

Three different cake samples analysed showed a similar result, ranging between 82.1 and 87.4 µg/kg as shown in Table 3-4. The colour of these types was different, ranging from gold to brown, but the acrylamide level was the same. This may be as a result of the food additives which reduce acrylamide levels as mentioned in section 1.3.7. Figure 3-13 shows the chromatography of biscuits (Members Bambini). According to the Food Standards Agency (2002) the acrylamide level is around 5.3 µg in a slice of cake (60 g). The biscuit (Members Bambini) average portion size is 20 g, so one piece would contain 12.5 µg acrylamide.

Regarding the sample colour and acrylamide content, this study found 208.2 µg/kg of acrylamide in biscuit (Members Bambini), the colour of this biscuit was dark gold compared with the other biscuit types analysed in the study which were light golden in colour.

Table 3-4: Acrylamide levels (µg/kg) in biscuits and cakes samples consumed by Saudi infants and published range for food groups from the literature: a (Senyuva and Gokmen, 2005) and b (Food Standards Agency, 2010b).

Sample name (n=6)	Acrylamide level (µg/kg)	
	Result (±SD)	Published range
Infant biscuits (Hero baby biscuits)	ND	<15-1326 [a]
Infant biscuits (Farley's Rusks)	ND	
Biscuits (Bahlsen)	ND	29-1600 [b]
Biscuits (Members Bambini)	208.2±21.0	
Marble cake (Danube, fresh)	82.1±1.5	<15-734 [a]
Marble cake (Americana)	87.4±1.9	
Marble cake (Sarita)	84.8±0.4	

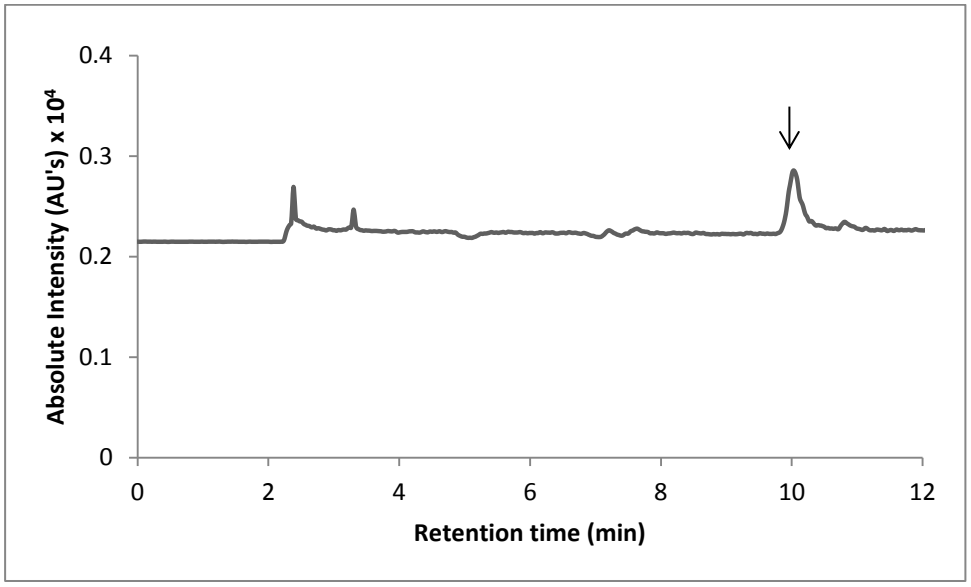


Figure 3-13: Acrylamide chromatograph SIM m/z 72 in biscuits (Members Bambini) sample analysed using LC-MS separation method 5 with the C18 column (arrow indicate the acrylamide peak).

3.3.6.3 Cereals

For infant cereal, acrylamide was not detected in this study, while the level of acrylamide mean (min-max), in this type of food analysed by the Food Standard Agency (2010b) was 30 (5-162) µg/kg. In Mojska *et al.* (2012), acrylamide content in infant cereal powder (19 samples) analysed by LC-MS/MS ranged between 65-296 µg/kg (Table 3-5 and Figure 3-14). Roach *et al.* (2003), on the other hand, reported that infant cereal did not contain acrylamide. The Food Standards Agency (2002) reported that medium portion size of cereal is equal to 30 g, from that the amount of acrylamide ranged from 2.4 to 4.9 µg. El-Ziney *et al.* (2009) determined the acrylamide level in four infant cereal samples consumed by Saudi infants and they found acrylamide levels ranging from 7 to 30 µg/kg, which is below the LOQ. Also, Roach *et al.* (2003) showed that there was no acrylamide level or small amount in the infant cereals and biscuits analysed.

The acrylamide level in breakfast cereal according to the Food Standards Agency (2010b) was 161 (12-403) µg/kg, whereas, in this study result showed acrylamide

levels in breakfast cereals to be 110.1 (79.2-163) µg/kg as illustrated in Table 3-5. Figure 3-11 shows the chromatography of cornflakes (Nestle country).

Table 3-5: Acrylamide levels (µg/kg) in cereal samples consumed by Saudi infants and published range for food groups from the literature: a (El-Ziney *et al.*, 2009), b (Senyuva and Gokmen, 2005)and c (Boon *et al.*, 2005).

Sample name (n=6)	Acrylamide level (µg/kg)	
	Result (±SD)	Published range
Cerelac wheat	ND	<15-293 [a & b]
Cerelac honey & wheat	ND	
Cerelac dates & Wheat	ND	
Cerelac rice	ND	
Oats (Heinz)	ND	
Cornflakes (Nestle country)	163.0±6.6	10-163[c]
Cornflakes (Kellogg's)	88.0±2.7	
Cornflakes (Briiggen)	79.2±0.9	

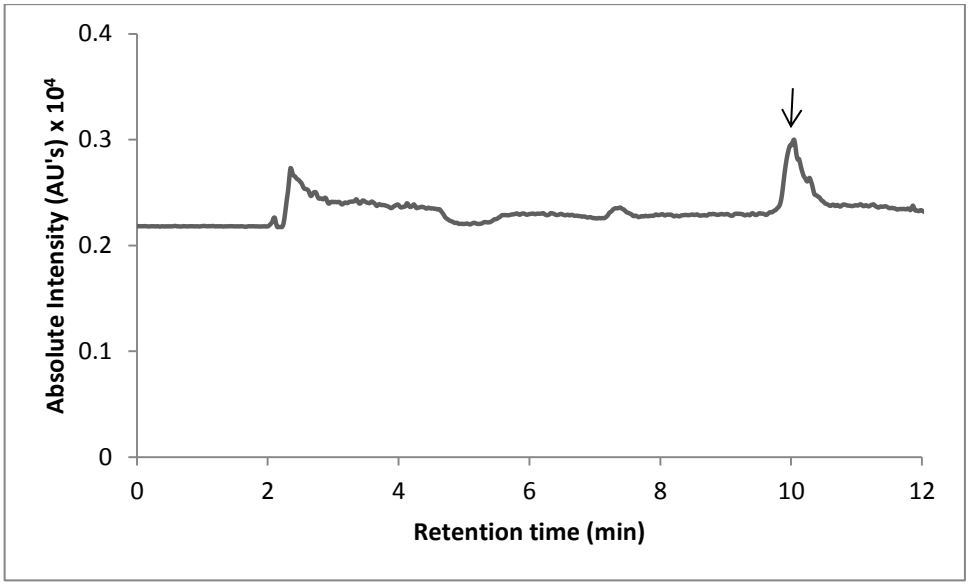


Figure 3-14: Acrylamide chromatograph SIM m/z 72 in cornflakes (Nestle country) samples analysed using LC-MS separation method 5 with the C18 column (arrow indicate the acrylamide peak).

3.3.6.4 Potato crisps and popcorn

According to the Food Standards Agency (2010b), the mean and range of acrylamide content in potato crisps was 1066 (144-4686) µg/kg, which is higher than the finding of this study, which was 499.6 (ND-755.5) µg/kg. However, the mean (min-max) in potato crisps was different between seasons: for March and November it was 1591 (305-4686) and 553 (144-1844) µg/kg respectively for the same number of samples (24). This shows that acrylamide varies between seasons. The result for potato crisps (Pringles, original) was below the detection level due to the ingredients and processing (they are not fried like other crisps). Also, these crisps are light golden in colour. According to the Food Standards Agency (2002), the popcorn and potato crisps (medium) portion sizes are around 75 g. The acrylamide levels in popcorn and potato crisps (medium) portions size are equal to 13.0 and 37.5 µg/portion respectively. The result was shown in Table 3-6, and Figure 3-10 (in section 3.3.1) shows the popcorn (Butterkist) result.

Popcorn content of acrylamide ranges from ND to 427.8 µg/kg in this study. However, in Boon *et al.* (2005) the acrylamide content in popcorn ranged from 60 to 715 µg/kg. According to Food Drink Europe (2011), the acrylamide content in pre-prepared food can be low if the customer follows the written instructions. However, in microwaved popcorn the level of acrylamide was found to be 427.8 µg/kg, despite following the package instructions. Yuan *et al.* (2007) studied the formation of acrylamide in model system (asparagine with fructose and asparagine with glucose) for 30 min at different microwaved power. The result showed increase in acrylamide level linked with increase microwave power from 300 to 600 W with levels ranging from 106 to 455 µg/kg, which agrees with the result from this study.

Table 3-6: Acrylamide levels (µg/kg) in potato crisps and popcorn samples consumed by Saudi infants and published range for food groups from the literature: a (van Klaveren, 2007) and b (Boon *et al.*, 2005).

Sample name (n=6)	Acrylamide level (µg/kg)	
	Result (±SD)	Published range
Potato Crisps (Lay's, salt)	743.2±41.3	10-4215 [a]
Potato Crisps (Albatal, salt)	755.5±38.5	
Potato Crisps (Pringles, original)	ND	
Popcorn (Al-batal)	91.9±3.2	60-715 [b]
Popcorn (Al-alali)	ND	
Popcorn (Butterkist)	427.8±47.3	

3.3.6.5 Noodles, macaroni, oats and rice

Boiled food such as rice and oats did not contain acrylamide which may be due to a high level of water content (moisture), which is similar to Svensson *et al.* (2003) results. Most macaroni and noodle types contained levels of acrylamide ranges between 85.3 to 112.0 µg/kg for macaroni and from ND to122.2 µg/kg for noodle, this may be as a result of the cooking process because these foods were all cooked in a similar manner. However, these levels were higher than Food Standards Agency (2010b) and Svensson *et al.* (2003) results, which showed the acrylamide levels were below the LOD in both studies (Table 3-7). Figure 3-15 shows noodles (Andomie) chromatography. According to Food Standards Agency (2002) the average portion size of noodles, macaroni (medium) and rice (medium) are; 280, 230 and 180 g. The acrylamide level in noodles, macaroni (medium) and rice (medium) is around 18.7, 21.9 and ND µg/portion of food respectively.

Table 3-7: Acrylamide levels (µg/kg) in noodles, macaroni, oats and rice samples consumed by Saudi infants and published range for food groups from the literature: a (Food Standards Agency, 2010b)and b (Svensson et al., 2003).

Sample name (n=6)	Acrylamide level (µg/kg)	
	Result (±SD)	Published range
Noodles (Andomie)	122.2±7.0	ND [a]
Noodles (Toya)	77.9±3.1	
Noodles (Mamee)	ND	
Macaroni (Goody)	85.3±2.9	<30 [b]
Macaroni (Alalali)	87.8±1.6	
Macaroni (Perfetto)	112.0±2.3	
White oats (Quaker)	ND	0.9- <30 [b]
White oats (Alalali)	ND	
White oats (Captin oats)	ND	
Rice (white swan)	ND	1.5- <30 [b]
Rice (Alwalimah)	ND	
Rice (Two girls)	ND	

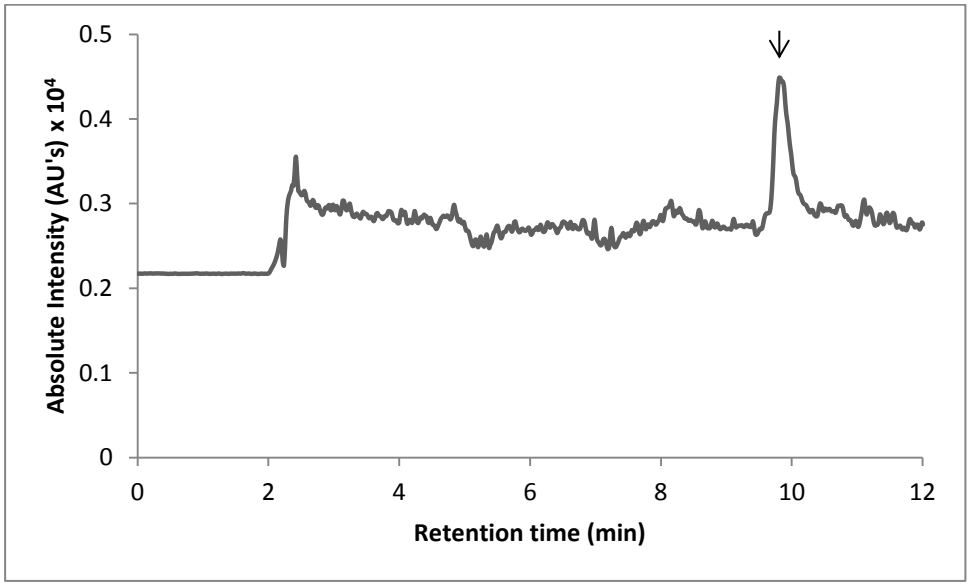


Figure 3-15: Acrylamide chromatograph SIM m/z 72 in noodles (Andomie) sample analysed using LC-MS separation method 5 with the C18 column (arrow indicate the acrylamide peak).

3.3.6.6 Food groups

In Tables 3-8 and 3-9 all the food samples are grouped into 5 different groups based on the type of food. These groups are bread, biscuits and cake, cereal, potato crisps and popcorn and noodles, macaroni, oats and rice. It should be noticed that in most groups there are products with low and high acrylamide levels.

The mean of acrylamide in these food groups varies from ND to 755.5 µg/kg and all groups in the range between minimum and maximum (Table 3-8). In the cereal group, acrylamide content in breakfast cereal was found to vary from 79.2 to 163.0 µg/kg, whereas acrylamide in infant cereal could not be detected. The table also shows a high variation in acrylamide levels between foods in each group, represented as minimum and maximum for this study's results and published range from other studies. The maximum of most food groups was at least three times less compared with the maximum published range. Whereas the minimum in all groups was below the LOD (17.2 µg/kg) which is close to the published range. However, the maximum for the noodles, macaroni, oats and rice group was three times higher than the reference level. This difference comes from the noodles and macaroni analysis result.

Table 3-8: Acrylamide minimum and maximum content in different food groups (6 triplicates for each sample).

Food group	Number of samples	Acrylamide Min –Max (µg/kg)	
		Result	Published range
Bread	15	ND-103.9	10-2883
Biscuits and cake	7	ND-208.2	<15-1600
Cereal	8	ND-163.0	10-846
Potato Crisps & popcorn	6	ND-755.5	10- 4215
Noodles, macaroni, oats and rice	12	ND-112.0	0.9- <30

LOD= 17.2 µg/kg

The acrylamide means of the food groups were calculated in three different ways based on the assumption of the ND value. The ND value was assumed to be zero, half the LOD (8.6 µg/kg) and equal to the LOD (17.2 µg/kg), as shown in Table 3-9.

In each group the ND was replaced with a value (zero, 8.6 or 17.2) to calculate the new mean. For example the biscuits and cake group had 7 different samples: three samples were non-detectable. To calculate the mean, first each ND value (n=3) was replaced with zero µg/kg (0.0, 0.0, 0.0, 208.0, 87.4, 84.8 and 82.1 µg/kg) and the mean was 66.1 µg/kg. Second, the three ND values were replaced by half the LOD (17.2) with 8.6 µg/kg (8.6, 8.6, 8.6, 208.0, 87.4, 84.8 and 82.1 µg/kg). The result show the mean value was equal to 69.8 µg/kg. Third, the ND values were changed to be equal to the LOD 17.2 µg/kg and the mean value of this change was (17.2, 17.2, 17.2, 208.0, 87.4, 84.8 and 82.1 µg/kg) 73.4 µg/kg.

According to the World Health Organization (2009) and European Food Safety Authority (2011b), the acrylamide value below the LOD was set to; 1) the LOD value, 2) half of the LOD and 3) zero as upper, middle or lower bound scenarios to report descriptive statistics for the characteristics of the data distribution.

The mean acrylamide level varied when the ND value was set to zero, half of LOD or the LOD value, which may have affected the acrylamide consumption estimation. The number of samples and the variation between samples were small and did not show large differences in term of mean acrylamide in the three assumptions as illustrated in Table 3-9.

Table 3-9: Acrylamide content in different food groups calculated according to different ND value, ND= 0, ½ LOD or LOD (6 triplicates for each sample).

Food group	Number of samples	Number of ND samples	Mean acrylamide level (µg/kg)		
			ND= 0	ND= ½ LOD	ND= LOD
Bread	15	10	29.4	35.1	40.8
Biscuits and cake	7	3	66.1	69.8	73.4
Cereal	8	5	41.3	52.0	64.7
Potato Crisps & popcorn	6	2	336.4	339.3	342.1
Noodles, macaroni, oats and rice	12	7	40.0	45.5	50.5

LOD= 17.2 µg/kg, ½ LOD= 8.6 µg/kg

3.3.6.7 High temperature effects

3.3.6.7.1 Infant cereal storage

The average temperature in Jeddah, KSA, is 37 ºC most of the year. Storage temperatures may be 37 ºC or more, which may affect acrylamide levels in stored food. For that, the infants' cereal was stored in two different conditions: the first condition was -18 °C and the second condition was storage for a month in an incubator at 37 °C with humidity caused by putting water in a tray at the bottom of the incubator. However, all samples with different storage conditions analysed by LC-MS acrylamide were not detectible, as shown in Table 3-10.

Table 3-10: Acrylamide level in infant cereals at different storage temperatures.

Sample name (n=6)	Brand	Acrylamide level (µg/kg)	
		-18º C	37º C
Wheat	Cerelac	ND	ND
Wheat & honey	Cerelac	ND	ND
Wheat & dates	Cerelac	ND	ND
Rice	Cerelac	ND	ND
Oats	Heinz	ND	ND

3.3.6.7.2 Infant cereal heating

Heating infant cereal in a microwave after adding water is one way a mother may make it warm for her infant. Heated and unheated infant cereal samples were determined using LC-MS and acrylamide levels were found to be below the limit of detection, as shown in Table 3-11.

Table 3-11: Acrylamide level in infant cereals before and after microwaved heating.

Sample name (n=6)	Brand	Acrylamide level (µg/kg)	
		No heating	Microwave
Wheat	Cerelac	ND	ND
Wheat & honey	Cerelac	ND	ND
Wheat & dates	Cerelac	ND	ND
Rice	Cerelac	ND	ND
Oats	Heinz	ND	ND

3.3.6.7.3 Loaf bread toasting

No acrylamide was detected in untoasted white loaf bread. However, toasting bread was found to increase acrylamide levels as shown in Table 3-12 and Figure 3-16 and 3-18. In Granby *et al.* (2008) study the acrylamide level of untoasted bread was found to be below the limit of detection, which is similar to the finding of this study. However, the levels of acrylamide found in light and dark toasted bread in this study were higher than the level reported in Granby *et al.* (2008), at 11 and 15 µg/kg respectively. However, the analysis result for both light and medium toasted was 10 times lower than the maximum in the reference level (2883 µg/kg). The toasted loaf (white) average portion size is 24 g (Food Standards Agency, 2002), containing 2.0 and 3.1 µg acrylamide in light and medium toasted levels respectively.

Table 3-12: Acrylamide levels in bread toasted at two different times.

Sample name	Acrylamide level (µg/kg)		
	Untoasted	Light toasted	Medium toasted
White loaf (Al-rashed food company)	ND	84.6±1.4	90.9±1.3
White loaf (L'usine)	ND	81.3±2.8	219.0±9.6
White loaf (Aljazei)	ND	78.5±1.6	83.3±1.3

Figure 3-166: Acrylamide level in three different white loaf bread with three different toasted levels (ND assumed to be 8.6 µg/kg).

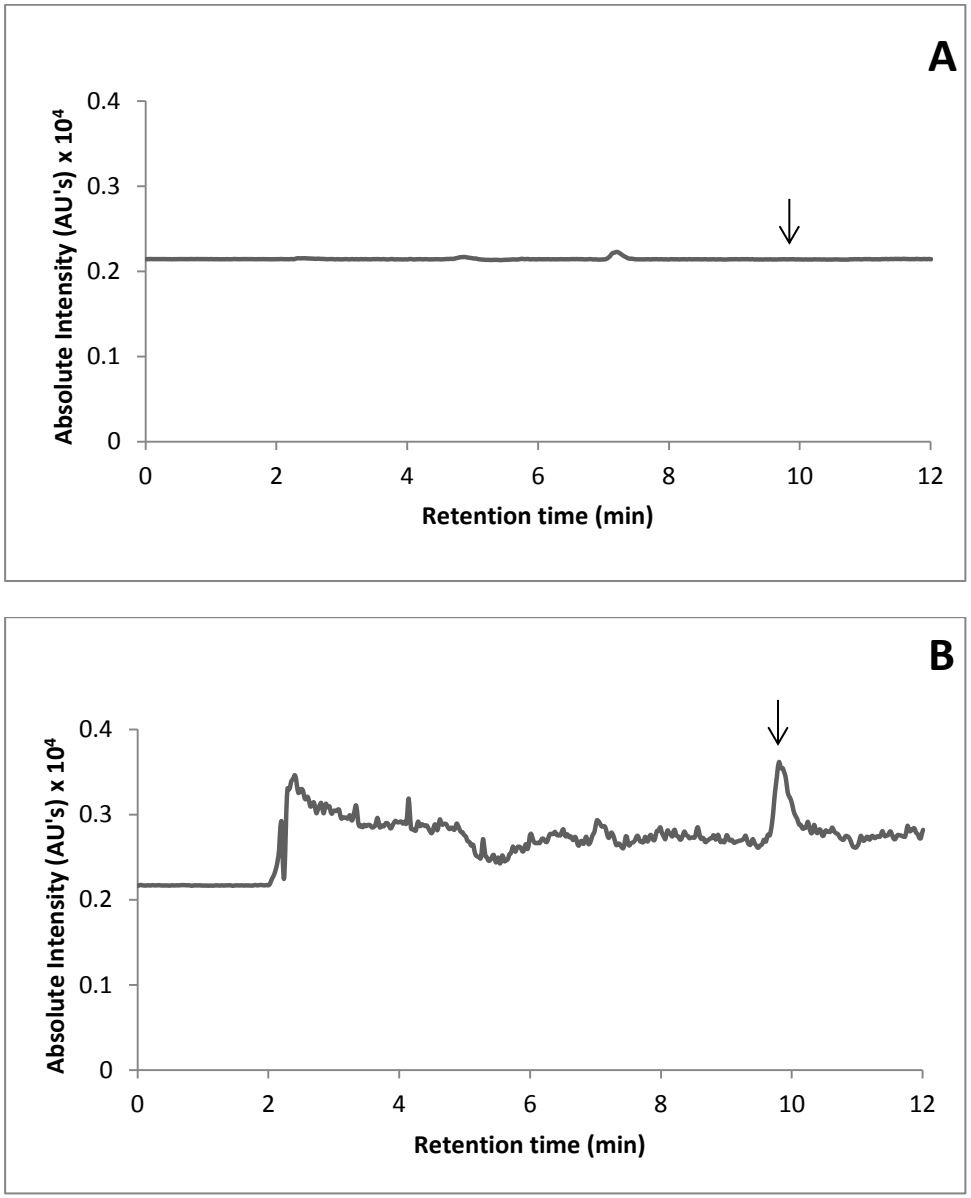


Figure 3-17: Acrylamide chromatograph MIS m/z 72 in white loaf (L'usine) sample A) untoasted B) medium toasted bread, analysed using LC-MS separation method 5 with the C18 column (arrow indicate the acrylamide peak).

3.4 *Discussion*

The optimisation methods in this study differed in terms of solvent, run time and column. These changes were similar to the changes in HPLC methods; the only difference was adding the acid to LC-MS solvents (1% formic acid in 4% acetonitrile). The LC-MS solvent should contain a small concentration of organic acid such as formic acid and acetic acid to improve component ionization (Fluka analytical, 1996). Longhua *et al.* (2012) reported that acetonitrile solution makes acrylamide easily adsorbed. The acetonitrile (4%) and formic acid (0.1%) with Millipore water was used in this study as LC-MS solvent (A) and acetonitrile (95%) as solvent (B).

In this study the chromatograph for analysis of acrylamide in infant food extracts there were strange peaks presenting at different retention times. The appearance of such peaks means there are unknown components in the food extraction which cannot be eliminated by clean-up method. These peaks are unknown and are possibly from food matrix and may be one or more of the Maillard reaction products or amino acid. These results were in agreement with Jiao *et al.* (2005) results, also, Govaert *et al.* (2006) pointed out the difficulty of determining acrylamide in food with lower content.

In this study the acrylamide C_{13} was used in the acrylamide analysis and it helped to indicate the acrylamide peak amongst the appearance of other unknown components. Regarding the study of Roach *et al.* (2003) and as mentioned in section 3.1.2 an internal standard can be added to a food matrix before acrylamide analysis for four reasons, confirmation of acrylamide location, non-detection, quantitation, and identification. Based on these reasons, the appearance of an internal standard (acrylamide C_{13}) and absence of acrylamide in the LC-MS result means the acrylamide level in the food sample is not detectable or there is no acrylamide in the sample. This can be applied to this study's results due to the response of acrylamide C_{13} in all food samples with non-detectable acrylamide level. In this study acrylamide C_{13} was used as an internal standard and it helped with the identification of acrylamide in the chromatograph, especially when there was no acrylamide peak shown.

This study was eluting the SPE cartridges with water in the sample extraction clean-up to wash from the cartridges. Also, the recovery percentage results evidenced that the sample extraction, clean-up and instrument performance were satisfactory. These results are in agreement with Roach *et al.* (2003).

Food processing affects acrylamide content in starchy foods. This study found there is no acrylamide in boiled rice and oats which were in agreement with Bent *et al.* (2012) results. However, the acrylamide level in boiled macaroni and noodles may come from the processing steps.

In this study the acrylamide levels found low in most of the bread sample comparing with the published range. However, the acrylamide level increased when the loaf bread was toasted from ND to 219 µg/kg. These results are agreed with other studies. Capuano *et al.* (2009) found that no acrylamide was detected in untoasted bread slices. Granby *et al.* (2008) pointed out that toasting wheat bread reduces the asparagine level and increases the acrylamide level and that a darker toasted bread colour represents a higher amount of acrylamide. In Surdyk *et al.* (2004) study it was found that 99% of acrylamide was present in the bread crust, whereas the bread crumb did not contain acrylamide due to the inner temperature not exceeding 100 ºC. Capuano *et al.* (2009) also reported that bread toasted at 180 ºC for 22 min decreased in moisture content rapidly in the first 15 min and then slower after that (reduced the moisture level led to formed acrylamide).

In this study heating the food using a microwave has been found to lead to the formation of acrylamide. Microwaving the popcorn as the instruction produced acrylamide (427 µg/kg), whereas microwaving the infant cereals did not (ND). Yuan *et al.* (2007) compared the acrylamide content in French fries with a model system treated via microwave, boiling or frying. The results showed that microwave treatment (750 W) increased the acrylamide level in both French fries and the model system and the level was higher than in the case of traditional frying treatment at 897 and 35 µg/kg respectively, which is agree with this study results.

In this study the main ingredients in all food sample groups (bread, biscuits and cake, cereal, noodles, macaroni, oats and rice and potato crisps and popcorn) were, yeast, milk, vegetable oil and salt. The quantity of reducing sugar and asparagine

content in the food sample ingredients (wheat flour, oats, rice, potato or popcorn, sugar and milk) are responsible for acrylamide level formation when cooked at a high temperature, as shown in Chapter 1. Whole wheat foods contain higher levels of acrylamide as shown in chapter 3, the Arabic bread made from whole wheat (brown) was contain acrylamide content higher than bread made from white flours. Capuano *et al.* (2009) found that the acrylamide content of whole wheat flour was higher than in white wheat flour, which may as a result of high asparagine level. Also, Bent *et al.* (2012) found that products made with whole wheat or wheat bran contained over twice the amount of acrylamide compared with products made with white flour. This finding was the result of high asparagine and reducing sugar in whole wheat and wheat bran compared with white wheat. A similar finding was made by this study relation to white and brown Arabic bread. They have similar ingredients and the same baking methods, but they were found to differ in term of acrylamide level, which may be as result of fibre content in brown bread. Also, the study of Rufian-Henares *et al.* (2006) shows the effect of fibre on acrylamide level. They found significant difference in acrylamide level between breakfast cereal with and without dietary fibre at 401 and 273 µg/kg respectively.

In this study brownish colour of heated food samples (toasted, baked or fried) is linked to higher acrylamide levels. The results showed the dark coulur of toasted bread was correlated with high level of acrylamide, for example, toasting a bread slice in two different colours (light and medium) causes differences in the level of acrylamide (81.3 and 219.0 µg/kg respectively). In other types of bread and biscuits, colour is associated with acrylamide level: the darker the colour the higher the level of acrylamide, which is in agreement with previous studies. These results were in agreement with other studies. Surdyk *et al.* (2004) pointed out the significant correlation between acrylamide level and bread crust colour from white to dark brown, in the same bread recipe. However, Wang *et al.* (2013) reported that the correlation between surface colour and acrylamide content in different foods is not linear. This study's results show that. Thinner slices of food usually contain a higher amount of acrylamide due to the brown colour being spread over a large surface (Wang *et al.*, 2008, Capuano *et al.*, 2009).

This study shows, in white bread, croissant and biscuits the colour may be gold but the acrylamide level is not detectable. This may be due to moisture level, fermentation and food additives in these foods which is agree with Surdyk *et al.* (2004). Fredriksson *et al.* (2004) fermented bread dough and this led to a reduction in the asparagine content during the fermentation time, which can be linked to the reduction of acrylamide level when baking bread. The acrylamide level in baked bread was reported to be 180 and 24 µg/kg for short and long fermenting times respectively (15 and 180 min).

Regarding the portion size and the acrylamide content of carbohydrate rich foods, this study results show a small amount of acrylamide in a single average portion size. However, consuming large amount of foods containing a small quantity of acrylamide increases the acrylamide exposure level of the individual, as will be discussed later in Chapter 5.

In this study some samples and standards were analysed within two months and the results were similar which prove that acrylamide was stable in the sample at that time when stored it at -4 ºC (no shown data). In case of infant cereal stored in conditions similar to storage in Jeddah, KSA, acrylamide could not be detected. The storage of samples with a high level of acrylamide may be required. These results are agree with Hoenicke *et al.* (2004) result. They suggested that storage time (over 100 days) affects acrylamide levels due to acrylamide instability.

In this study analysing acrylamide in the same food products showed a wide variability of acrylamide contents (higher than 10%), even in the same batch. This is in agreements with Hoenicke *et al.* (2004). They showed that analysis of acrylamide using HPLC-MS/MS or GC-MS/MS in over 2000 packages of potato crisps from one batch found that it ranged between ±10% and 50% from the mean. They assumed the reason for the variation was sample inhomogeneity or acrylamide stability. Roach *et al.* (2003) reported the variation in acrylamide level from batch to batch of potato crisps (23 bags) and bag to bag in the same batch, ranging in one bag, for example, from 425 to 550 µg/kg and between bags from 250 to 550 µg/kg. Regarding Mojska *et al.* (2012), within the same group, the acrylamide content varied from low to high level. This variation may be due to factors such as, the

amount of reducing sugar and asparagine in raw material, or temperature and moisture in the heating process, as explain in section 1.3.7. Also, the variation in acrylamide level may be due to variation in the sample precursor and preparation (Bent *et al.*, 2012).

In this study the ND values were set as zero, half of the LOD and the LOD value. These values results were similar. For this reason the middle scenario was chosen for use when estimating acrylamide consumption in Saudi infants, as will be illustrated in Chapter 5. This study's finding, where similar to the World Health Organization (2009) and European Food Safety Authority (2011b) finding. They are used three different scenarios to represent the value of non-detectable acrylamide. However, they found similarities between the results which led to use of the middle scenario (half of LOD value) only as a value for the ND level of acrylamide.

3.5 *Conclusion*

The acrylamide level was difficult to detect by using the HPLC. However, the LC-MS detected acrylamide using positive ion m/z 72 and the internal standard acrylamide C_{13} (m/z 75) was used to identify the acrylamide peak. Starchy Saudi infant foods were found to have levels of acrylamide ranging from ND to 755.5 µg/kg, which is in general lower than that found in other studies. The acrylamide recovery from food samples was high. Infant cereals were found not to contain acrylamide, even when stored at 37 ºC or microwaved. Toasted bread showed increased levels of acrylamide. The acrylamide recovery in food groups ranged between 84.3% and 106.1%. This chapter hypotheses are "the acrylamide level in baked and fried foods consumed by Saudi infants is high" and "there is no acrylamide in infant cereals consumed by Saudi infants" have been confirmed.

3.6 *Limitation*

The limitations of the acrylamide analysis included the fact that the LC-MS used was heavily used by other students and commonly broken, which led to there being only a short time to analyse the samples. This limitation affected the results in many aspects; the chromatograph for certain sample results could not be improved, the effect of storage extraction on the acrylamide level could not be tested, and most

samples and extraction could not be analysed in the same conditions. However, using LC-MS single quadrupole, which is less sensitive, was the only available option due to the breakdown of the GC-MS and the overestimating of HPLC results.

In some chromatographs there were many peaks, which may be as a result of poor extraction or clean-up. However, many changes were made at the beginning which did not lead to any differences in the chromatograph. The limitation on time after analysis of all samples was the main reason for not trying to improve the extraction method. Also, the centrifuge speed was not as high as recommended in previous studies, which may have been the cause of interference in the chromatographs.

Different factors affecting acrylamide levels in starchy food as explained in Chapter 1, such as reducing sugar and asparagine could also affect results. These factors will be determined in the next chapter (soluble sugar and total protein).

4 Chapter 4: Determination of soluble sugar and protein in selected infant cereals

4.1 *Introduction*

Infant cereals are popular products in both Saudi Arabia and the UK, particularly during the early weaning stages. Commonly consumed infant cereals in the UK are based on wheat, rice or mixed cereals (Lunn and Buttriss, 2007) .

Cereal grains are made of a starchy endosperm, a fibrous aleurone layer (seed coat) and a protein rich embryo. Most processed cereal products are refined into flours containing mainly endosperm fractions, with a high proportion of digestible carbohydrate (60-70% dry weight basis), of which the majority is starch (40-50%) and the rest is composed of soluble sugars (Figure 4-1). (Griffith, 1993). Sucrose in most abundant sugar in infant cereals is high, because it is added as ingredient to cereal products. Glucose and maltose content come from hydrolysis of starch, while fructose may have added or derived from enzymatic breakdown of sucrose (Fernandez-Artigas *et al.*, 2001).

Based on the composition of cereal flours, they appear to be a good medium for the formation of acrylamide. Amongst the soluble sugars, fructose is more efficient in the formation of acrylamide (Dybing *et al.*, 2005, Zhang *et al.*, 2005b).

Cereal flours also contain around 20% (dry weight basis) protein. The amino acid asparagine is the main source for the formation of acrylamide in food. The three carbon group atoms and the nitrogen atoms of acrylamide are derived from asparagine (Figure 1-2). Other amino acids that form low levels of acrylamide include, alanine, aspartic acid and cysteine (Ezeji, 2003). Cereal proteins are known to contain asparagine, alanine, cysteine, and aspartic acid (Stadler *et al.*, 2002).

The conventional process for making infant cereals include the stages of mixing cereal flour with water, and then heating the mixture to temperatures equal or over 100 ºC for 15-60 minutes to gelatinize starch. After cooling, the hydrolytic enzymes using α or β-amylase and glucoamylase (5% by weight) are added. The enzyme is inactivated by boiling it to over 100 ºC. More chemical reagents are added to adjust

the mixture's pH and by the addition of vitamins, minerals, fats, milk, fruits or vegetables. Finally the cereals are dried by roller, drum or drying to form flakes, pellets or powder (Gil *et al.*, 1997).

4.1.1 Acrylamide formation in cereal products

The high temperatures (above 100 ºC) involved in the drying process produce compounds by the Maillard reaction, including acrylamide, and reduce the microbiological activity. Cereal products including pastries, biscuits, breads and processed cereal products have been shown to contain acrylamide (Dybing *et al.*, 2005, Zhang *et al.*, 2005b). The level of acrylamide in infant's cereals and biscuits were ranged from <15 to 293 and 1326 µg/kg respectively as shown in Chapter 3, Section 3.3.6 (reverence value).

Some mechanisms have been reported for acrylamide formation in potato, bread and other cereal foods involving asparagine and reducing sugar during high temperature processing (Mottram *et al.*, 2002a, Amrein *et al.*, 2003, Surdyk *et al.*, 2004).

Surdyk *et al.* (2004) reported that increasing asparagine content by spiking dough before baking leads to a strong increase in the level of acrylamide from about 80 to 600 µg/kg and above. The study revealed that during baking, reducing sugar was formed due to heat, indicating that reducing sugar may not be limiting acrylamide formation in wheat bread. The increase in acrylamide was concomitant with a decrease in reducing sugar, but there was no correlation with asparagine content. Acrylamide formed from fructose more than glucose and sucrose. Acrylamide formation in crust wheat bread was found to increase with an increase in temperature and time of baking (Becalski *et al.*, 2004). According to Fredriksson *et al.* (2004), free asparagine content was 0.5 g/kg in wheat. They found a decrease in the asparagine level by around 40% after an hour of yeast and sourdough fermentation. These results affected the acrylamide formation; they found the acrylamide level decreased the longer the fermentation time.

El-Ghorab *et al.* (2006) correlated acrylamide and asparagine at different heating temperatures. At 150 ºC the amount of acrylamide was 318 µg/g asparagine, whereas, at 170 ºC the amount of acrylamide was 3329 µg/g asparagine.

Elmore *et al.* (2005) found that when cake (made from wheat, barley or rye) was cooked at 140 ºC or less for 180 min, there was a reduction in sugar (glucose, fructose and maltose) and free amino acids in the cake samples, which is associated with acrylamide formation. Pollien *et al.* (2003) studies show that in model systems, asparagine with fructose can generate more acrylamide than asparagine with glucose at 120 ºC. Robert *et al.* (2005) suggest the acrylamide formation temperature is similar to sugar melting temperature. For example glucose and asparagine react at 120 ºC after 60 min and 160 ºC after 5 min.

There is no published information on the effect of composition or processing on the acrylamide content of infant cereal.

The aim of this chapter is to determine the content of soluble sugar and protein in infant cereal, to evaluate the potential for acrylamide formation during high temperature processing. The levels of sugar and protein in infants' cereal cannot be correlated with acrylamide content due the non-detectable levels reported in Chapter 3.

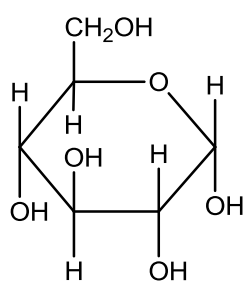
4.1.2 Aims

The specific aims of this chapter are to:

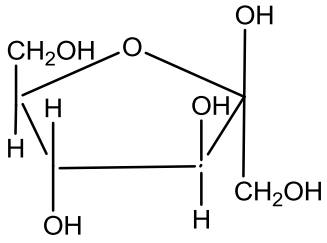
- determine soluble sugar level in certain infant cereal,
- analyse soluble protein in certain infant cereal.

4.1.3 Hypothesis

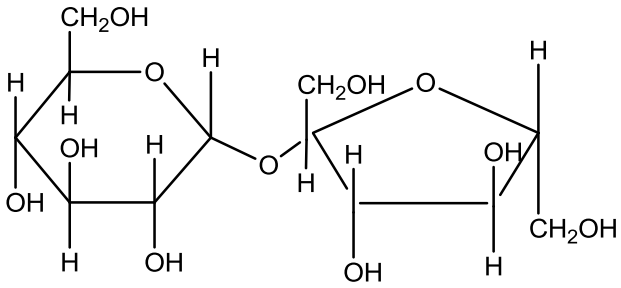
Infant cereals contain a sufficient amount of reducing sugar and protein for acrylamide formation during storage or microwaving.



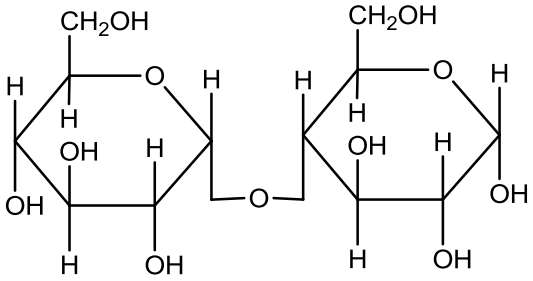
Glucose



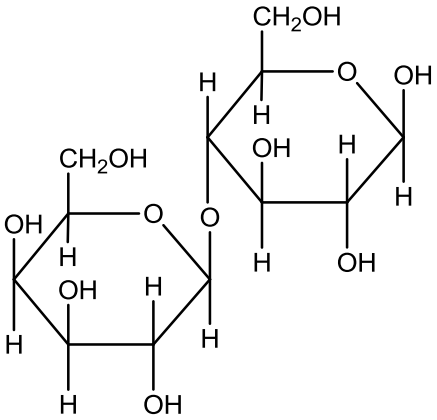
Fructose



Sucrose



Maltose



Lactose

Figure 4-1: The structure of some simple carbohydrates found in foods; glucose, fructose, sucrose, maltose and lactose

4.2 *Methods*

4.2.1 Materials used for carbohydrate and protein analysis

4.2.1.1 Reagents

- Ethanol, 95 and 78% (v/v),
- MES-TRIS buffer solution (0.5 M pH 8.0 at 23 ºC): 19.52 g of 2 (N-morpholino) ethanomethane acids (MES) (Sigma UK, cat. no.M-8250) and 14.2 g tris (hydroxymethyl) aminomethane (TRIS) (Sigma cat. no.T-1503) are dissolved in 1.7 L deionized water. Then the pH is adjusted to 8.0 with 6.0 M NaOH (Merck, Darmstadt, Germany). Dilute to 2 L with water. NOTE: the pH of the buffer must be adjusted at approximately 23 ºC. The buffer is stable at 4 ºC,
- Sulfuric acid concentration (Fisher Scientific; UK; code S/9240/PB17, batch 0944541),
- Phenol 80% (Acros Organic; USA; code 149340500),
- D-glucose, Glucose (Fisher Scientific, UK, cat. No.10005167),
- 200 mM sodium hydroxide was prepared by dissolving 32 mL of NaOH into 2 L water (Fluka, UK, cat. No.BCBK7141V),
- Bradford reagent (Sigma, UK, cat. No.B-6916),
- Bovine serum albumin BSA (Fisher Scientific UK, code 1605-100),
- Alkaline 3,5-dinitrosalicyclic acid (DNS) reagent 98%. In 250 mL of water, 5 g of 3,5-dinitrosalicyclic acid was dissolve at 80 ºC and 2 N NaOH was added. Finally, 159 g of potassium sodium tararate-4-hydrate was added and the volume was completed to 150 mL with water. (Sigma UK, cat. No.128848),
- A Millipore Elix water purifying system (Milli-Q Advantage A10, Millipore UK Ltd, UK) was used to provide ultrapure, nuclease free water (≥ 18.2 MΩ).

4.2.1.2 Consumables

- Syringe filters 0.45 µm Nylon (13mm) (Millex -HN, UK, cat No 2013-04),
- Syringe with needle (21 g x 38 mm, 2 mL), (UK, cat No 613-2001, 17193L),
- Micro test tube EZ, with cap, 2 mL (Bio-Rad UK, cat No 223-9430),
- Falcon tubes, polypropylene 15 and 50 mL (VWR; cat No 188271, 18010L and 227261, 18040L),

- Vial 30511P-1232 snap ring autosampler with crimp top polypropylene clear 0.500 mL (Thermo Scientific, cat No 12376999),
- Plastic cuvettes (1 cm light path UV),
- The CarboPac PA-20 (Dionex, 3×150 mm) column with guard column (3×30 mm) will be used for mono and disaccharides,
- Microtiter plates (96-well, MaxiSorp) (Nunc, Rosklide, Denmark),
- Whatman No. 1 Filter paper (GE Healthcare UK Ltd., UK)
- Mira-cloth (Merck Chemicals Ltd, cat No. 475855-1R),
- Pipette tips (10 µl-5 mL) (Starlab Ltd, Milton Keynes, UK).

4.2.1.3 Equipment

- Centrifuge (Eppendorf Centrifuge 5810R),
- Spectrophotometer (Talbot Scientific LTD, UK),
- Shaker incubator (Orbet incubate 100),
- A high resolution (0.001 Abs) photodetector Multiskan FC® Plate Reader (Thermo Scientific, Vantaa, Finland) was used to determine the ELISA microplate well optical densities,
- Dionex DX500 is the HPAEC-PAD system, consisting of:
  - A GP40 gradient pump, PAD system ED40 electrochemical detectors including gold working, silver and titanium electrode,
  - LC20 Chromatography Enclosure column oven was used for detection of soluble sugars,
  - The autosampler AS500. The analytical column used was CarboPac PA-20 (Dionex, 3×150 mm) with guard (3×30 mm). For extending the analytical column life, the guard column was used in front of the analytical column. The anion exchange capacity for Carbo Pac PA-20 is 65 µeq/column and the maximum operating pressure is 3500 psi. The carbohydrates undergo ionisation at high (12.8) pH, followed by positive charge on the column.

Regeneration of the column with the same conditions after the run should take place to remove strongly bonded contaminations such as peptides and amino acids by using 200 mM sodium hydroxide solution for 10 minutes at a flow rate of 1 mL/min

to minimize the contamination. Before the run, the column should re-equilibrate with 16 mM sodium hydroxide solution at a flow rate of 1 mL/min for 10 minutes.

Solvents

Solvents such as sodium hydroxide and water should have little dissolved carbonate to avoid reducing column selectivity, resolution and efficiency. Also, the solvent should be degassed by sparging with helium for 15 min.

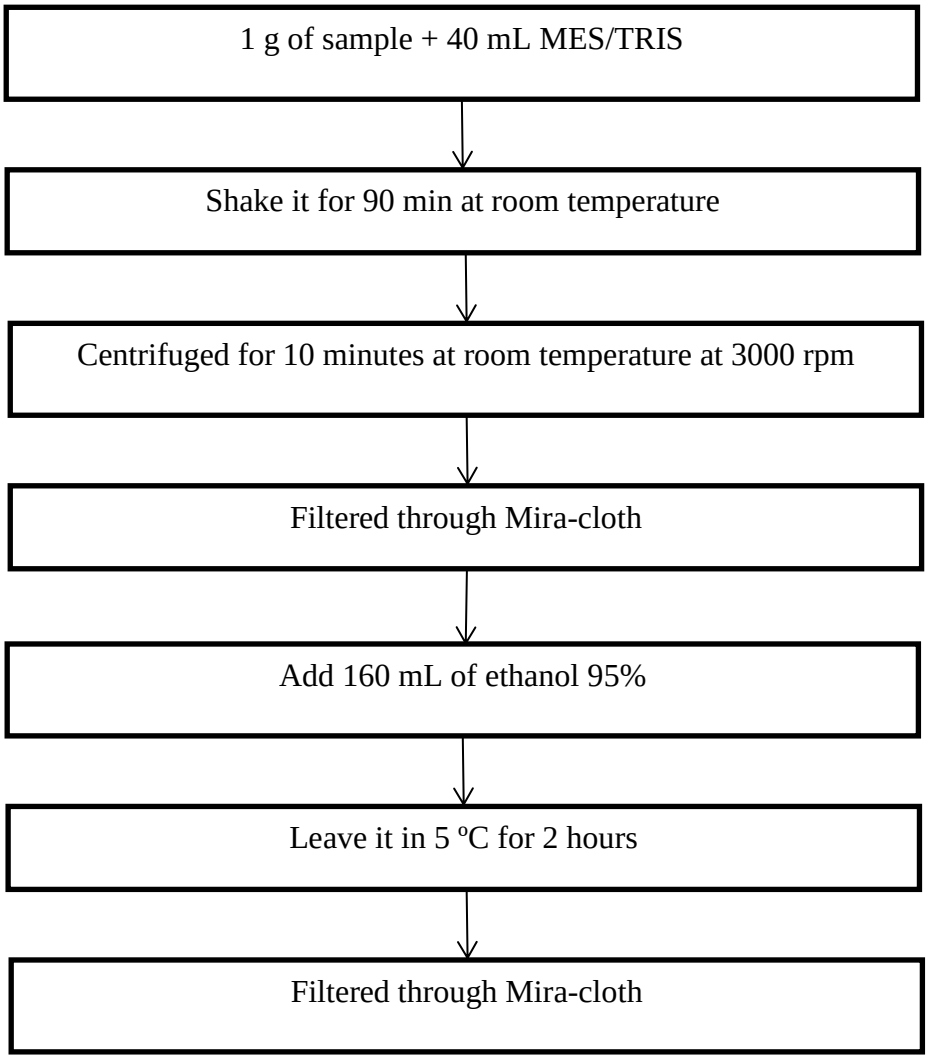


Figure 4-2: Analytical scheme for extraction of free sugar and protein from infant cereal samples.

4.2.1.4 Sampling

All sample information has been mentioned in Chapter 2, section 2.2.4. The samples comprised five types of infant cereal consumed by Saudi infants'.

4.2.2 Soluble sugar and protein determination analytical methods

4.2.2.1 Sample preparation

One gram of dry sample was weighted into a 50 mL Falcon centrifuge tube to which 40 mL 0.05 M MES/TRIS (pH 8.2) was then added. The centrifuge tube was shaken horizontally for 90 min in an incubator at room temperature, then centrifuged for 10 min at room temperature at 3000 rpm. The supernatants were filtered through Mira-cloth filter, the 4 volumes of ethanol 95% (160 mL) was added and kept in the fridge (5 ºC) for more than 2 hours, then filtered through Mira-cloth filter. The supernatants for each sample were stored in –18 ºC until used. Analytical scheme for extraction of free sugar and protein from infant cereal samples is shown in Figure 4-2.

The soluble sugar was determined by using three different methods, phenol sulfuric, DNS and HPAEC-PAD, as shown in sections 4.2.2.2, 4.2.2.3 and 4.2.2.4. Also, soluble protein was determined by using Bradford assay section 4.2.2.5. The soluble sugar was determined to investigate the possibility of acrylamide forming during storage or heating from the amount of soluble sugar available. Figure 4-3 shows the analytical scheme for soluble sugar determination methods in infant cereal samples.

4.2.2.2 Phenol sulfuric acid assay for soluble sugar determination

The method for free sugar assay was taken from DuBois *et al.* (1956) but with some modifications. In the presence of sulfuric acid, sugar reacts with the phenol and produces a stable orange-yellow colour. From the diluted sample solution (as mentioned in Section 4.2.2.1), 400 µL was put in a tube, then 10 µL of phenol 80% (w/w) and 1 mL sulfuric acid concentrated were added and the tube was shaken by vortex. After that, the absorbance of each standard sugar concentration and samples were read in plastic cuvettes using a spectrophotometer at wavelength 495 nm (Figure 4-3).

4.2.2.2.1 Standard preparation

The calibration standard was glucose. For the purpose of standard preparation, the stock solution of D-glucose anhydrous 0.1 mg/mL (1mg in 10 mL water) was prepared followed by dilution with Millipore water (1:10) to make a new stock solution (0.01 µg/mL). Then a series of standard sugar solutions with known concentrations were made, as shown in Table 4-1.

Table 4-1: Series of different glucose concentrations (standard) (µg/L) prepared from the stock solution (0.01µg/L) by being diluted in water.

Glucose solution µL	Water µL	Concentration µg/mL
0	400	0
100	300	1
200	200	2
300	100	3
400	0	4

4.2.2.2.2 Sample preparation

The samples were prepared as mentioned in section 4.2.2.1. The sample solution was diluted to (1:40), prepared to determine the sugar level using phenol-sulfuric assay. The extraction was stored at -18 ºC for future use.

4.2.2.2.3 Calculation of free sugar

The sugar amount was calculated with reference to the glucose standard curve slope and multiplied by the dilution factor (400). Finally, the sugar amount was expressed in grams of soluble sugar per hundred grams of dried cereal (g/100 g). All solutions were prepared in triplicate.

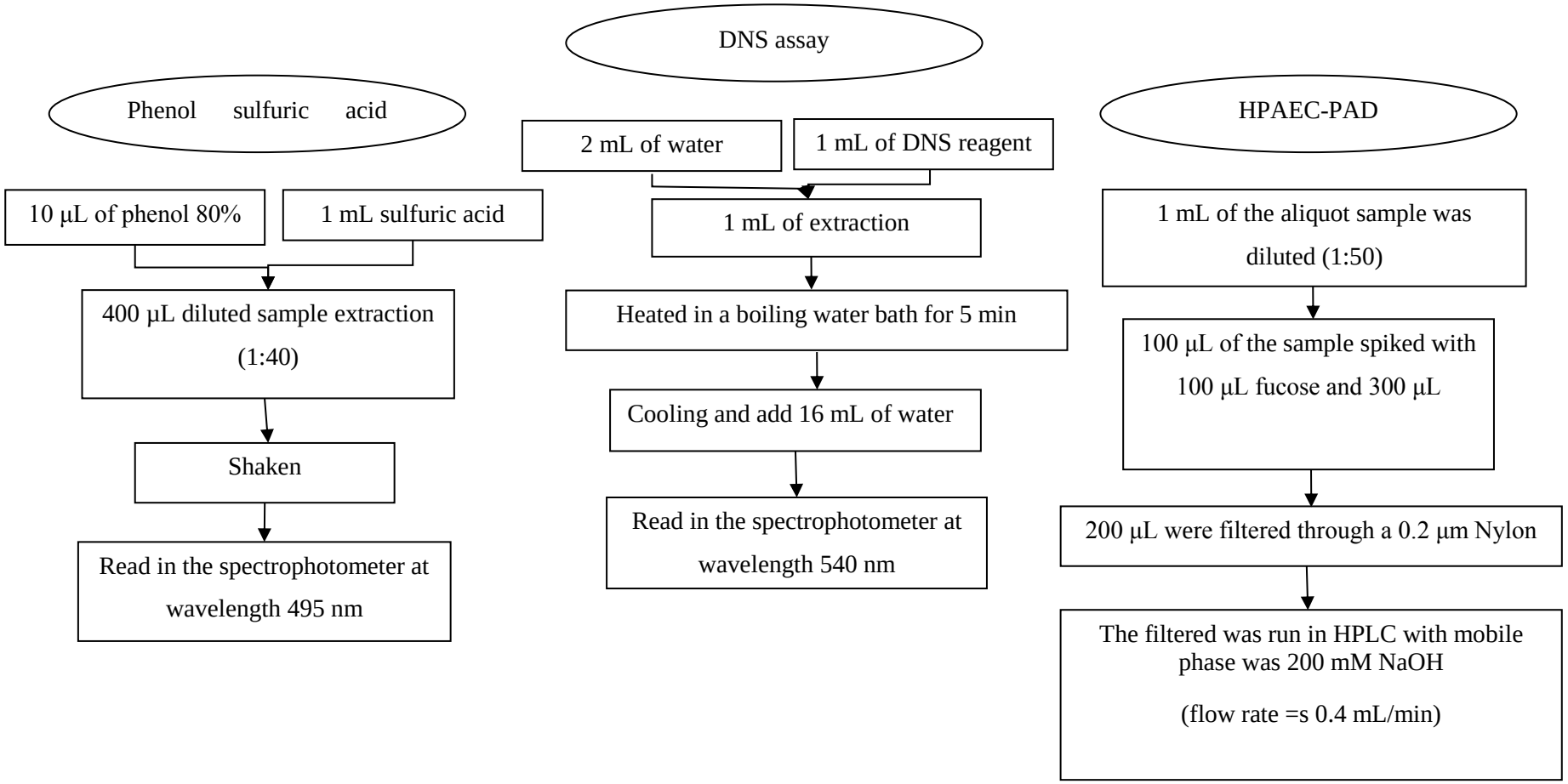


Figure 4-3: Analytical scheme for soluble sugar determination methods in infant cereal samples.

4.2.2.3 DNS assay for soluble sugar determination

This procedure was modified from Miller (1959). Reducing sugars will react with alkaline 3,5-dinitrosalicyclic acid (DNS) and form a red-brown reduction product, 3-amino-5-nitrosalicylic acid. The colour absorbance at 540 nm allows measurement of reducing sugars present in food (glucose, fructose).

In a Falcon tube (50 mL), 1 mL of standard or sample, 1 mL of DNS reagent and 2 mL of distilled water (total volume= 4.0 mL) were added. The tubes were heated in a boiling water bath for 5 min for the glucose to react with the DNS. After cooling, 16 mL of water was pipetted into each tube and mixed to stop the reaction. The absorbance reading was taken for each solution at 540 nm using a spectrometer (Figure 4-3).

4.2.2.3.1 Standard preparation for soluble sugar quantification

Glucose was used as standard (2 mg/mL). A series of standard concentrations were prepared (0.0, 0.5, 1 and 2 mg/mL).

Table 4-2: Series of glucose concentrations (mL) prepared from the (2 mg/L)) by being diluted in water and DNS reagent.

Concentration (mg/mL)	DNS reagent (mL)	Water (mL)	Glucose (mL)
0.0	1	3.0	0.0
0.5	1	2.5	0.5
1	1	2	1
2	1	1	2

4.2.2.3.2 Sample preparation for soluble sugar quantification

A sample was extracted with ethanol, as mentioned in section 4.2. 2.1.

4.2.2.3.3 Calculation for soluble sugar quantification

The amount of glucose (mg) present in each sample was calculated using the glucose standard curve slope. This was multiplied by the dilution factor (200) and this is referred to g of reducing sugars present in 100 g of food.

4.2.2.4 High-performance anion-exchange chromatography amperometric detection (HPAEC-PAD) for soluble sugar determination

High performance anion-exchange chromatography amperometric detection (HPAEC-PAD) was used to separate carbohydrates. HAPEC-PAD detects carbohydrates by oxidation of the functional group by voltage. The reducing sugar ionisation becomes greater when the hydroxide is increased.

The mobile phase was 200 mM NaOH and the flow rate was 0.4 mL/min. The injection volume was 10 μL and the column temperature was 30 ºC. The elution after injection was 30% of 200 mM NaOH for 8 min, followed by an increase in the mobile phase concentration to 70% for 17 min. After that the concentration was dropped to 30% for 6 min to wash the column. Therefore the run time was 30 min for soluble sugar separation (Figure 4-3).

Oxidation of the carbohydrate at the surface of the gold electrode caused the electrical current which was measured for mono and disaccharide detection. The electrode potential was first +0.05 V for 0.00 to 0.40 sec, then it was raised to +0.75 V from 0.41 to 0.60 sec, and finally decreased to -0.15 V from 0.61 to 1.00 sec. Consequently, pulsed amperometric detection caused a repeating sequence of three potentials. The carbohydrate oxidation current was measured during the integration period (between 0.20 and 0.40 sec) and the detector response was measured in columns (Hanko and Rohrer, 2000). For the purpose of washing the columns, the concentration of 60 mM of NaOH was used for 10 min followed by an increase in the concentration by 100 mM for 5 min. Then the concentration was reduced backed down to 60 mM and washing was carried out for the next 10 min.

4.2.2.4.1 Standard preparation for soluble sugar quantification

For determination of mono and disaccharides in the samples, the standard stock solutions of each glucose, fructose, sucrose, maltose and lactose were made individual by adding 1 mg of sugar to 1 mL of water to have a stock solution with 1 mg/mL, diluted to 0.1 mg/mL. The serial of mixed standard was prepared in different concentrations using the sugar solution concentration 0.1 mg/mL and spiked with internal standard fucose (100 µg/L), as shown in Table 4.2. Each sugar standard was run individually to find each separation time.

Table 4-3: Series of different of mix sugar concentration (glucose, fructose, sucrose, maltose and lactose) prepared from the stock solution (0.1µg/L) as HPAEC-PAD standards and IS.

Sugar solution (µL)						**Water (µL)**	**Concentration µg/mL**
Glucose	Fructose	Sucrose	Lactose	Maltose	Fucose		
20	20	20	20	20	40	860	2
40	40	40	40	40	40	760	4
60	60	60	60	60	40	660	6
80	80	80	80	80	40	560	8
100	100	100	100	100	40	460	10

4.2.2.4.2 Sample preparation for soluble sugar quantification

The sample was extracted as mentioned in section 4.2.2.1. Then, 1 mL of the aliquot sample was diluted in Milipore water (1:50) and 100 µL of the sample was spiked with 100 µL fucose (IS) and 300 µL of water was added to have 500 µL in total. Then, 200 µL were filtered through a 0.2 µm Nylon filter into vial.

4.2.2.4.3 Calculation for soluble sugar quantification

A standard curve was prepared with known standard sugar concentrations (plotting peak area vs. concentration of standard sugar). For the analysis of soluble sugar in the samples, the retention times and the peak area of each standard sugar were compared with those of the unknown sugars present in the samples. The dilution factor was of concern and the final result was represented as grams per hundred grams (g/100 g).

4.2.2.5 Bradford assay for soluble protein determination

In order to give an indication about the soluble protein content in infant cereal the Bradford assay was used for determining the total soluble protein, but not free amino acid. Depending on the protein content, standard or micro assay can be used or depending on the volume of the protein sample, the assay can be performed in test tubes or micro-plates.

The Bradford reagent solution was gently mixed by inverting the bottle a few times before each use and it was equilibrated to room temperature. Then 250 µL of the Bradford reagent was added to 5 µL standard or sample to be assayed into individual micro-plate wells in duplicate. After that, the micro-plate was mixed for 30 sec and incubated at room temperature for 5 to 45 min using the plate reader. The plate reader was set to 595 nm to measure the absorbance of standards and sample solutions (Bradford, 1976) (Figure 4-4).

This work was carried out prior to analysing the acrylamide level in infant cereal with the intention of then analysing the asparagine levels. However, due to the acrylamide level in infant cereal being ND (as shown in chapter 3) the analysis of asparagine was then not carried out.

4.2.2.5.1 Standard preparation soluble protein quantification

The standard assay in micro-plates was used to determine the amino acid. The bovine serum albumin (BSA) superior linear range is between 50 and 1000 µg/mL as shown in Table (4-3).

Table 4-4: Series of BSA concentrations (µg/L) prepared from the stock solution (µg/L) by being diluted in water as Bradford assay standard.

BSA solution µL	Water µL	Concentration µg
0	1000	0.0
50	950	0.5
250	750	2.5
500	500	5.0
750	250	7.5
1000	0	10.0

4.2.2.5.2 Sample preparation

The sample was extracted with methanol, as mentioned in section 2.3.2, and 5 µL of the extraction was used.

4.2.2.5.3 Calculation of soluble protein

A standard curve was created by plotting the absorbance at 595 nm vs. protein concentration of each protein standard (mg/mL). The protein content of unknown samples was determined by comparing their absorbance values against the standard curve.

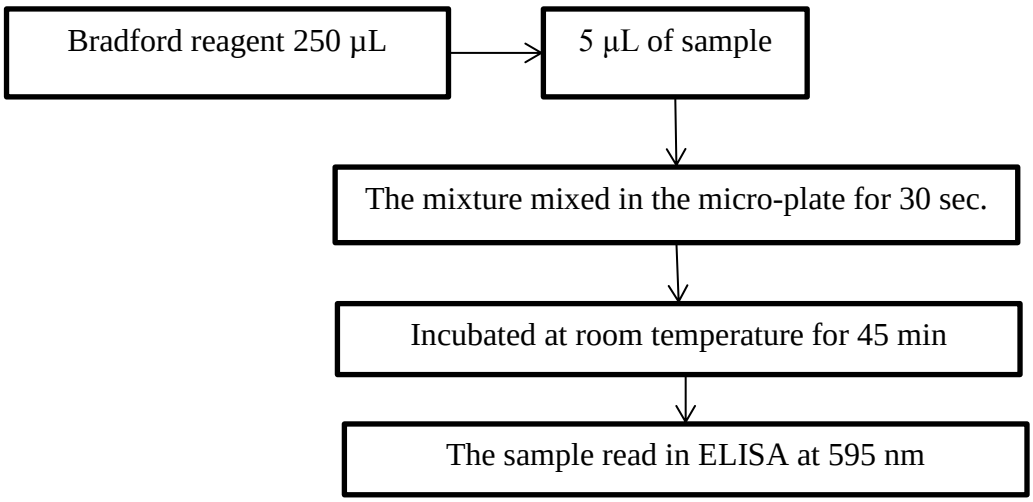


Figure 4-4: Analytical scheme for protein determination method in infant's cereal samples.

4.2.3 Statistical analysis

Microsoft Excel 2007 was used to generate calibration curve, which was used to quantify the level of sugar within food samples. One way between methods analysis of variance (ANOVA) was used to evaluate the three methods used to determine soluble sugars in the infant cereal samples phenol sulphuric, DNS and HPAEC-PAD. The p-value expected to be less than 0.05. The Tukey HSD test was used to indicate the significant difference between the methods. The eta squared was used to calculate the effect size.

4.3 *Results*

4.3.1 Soluble sugar determination

Three different methods were used to determine free sugar in the infant cereal samples (n=5) using phenol sulfuric, DNS assays and HPAEC-PAD. The standard curve of sugar was plotted vs. absorbance for phenol sulfuric and DNS or peak area for HPAEC-PAD. The amount of sugar was calculated with reference to the glucose for phenol sulfuric and DNS detection assays for the standard curve, as shown in Figure 4-5. In these colorimetric methods the colour intensity depended on the reaction between sugar and sulfuric acid in the phenol sulfuric assay and DNS reagent in the DNS assay method with wavelengths 495 and 540 nm in order.

For the HPAEC-PAD method, however, the calculation of the sugar amount was done by injecting different concentrations of mixed standards of glucose, fructose, sucrose, lactose and maltose with 100 µL fucose as the internal standard. The peak area was related to the concentration of the sugar amount as illustrated in Figure 4-6. The elution order of each sugar is shown in Figure 4-9 with fucose at 2.7 min, glucose at 5.1 min, fructose at 5.7 min, sucrose at 8.3 min, lactose at 9.4 min and maltose at 17.8 min. The reducing sugar was expressed in grams of soluble sugar per 100 g of dried cereal.

4.3.1.1 Phenol sulphuric assay

In the infant cereals, the amount of soluble sugars detected ranged between 19.1 and 47.4 g/100 g, as shown in Table 4-4. The soluble sugar levels in wheat cereal based samples (3) ranged between 27 and 32 g/100 g food, whereas the level of soluble sugars in rice and oats cereal based samples (1) was 19.1 and 33.8 respectively. The mean soluble sugar in infant cereal was 29.8 ±4.2 g/100 g. However, McCance and Widdowson (2002) found the amounts of total carbohydrate in wheat and rice cereal based infant cereals were 70.3 and 79.6 g/100 g (Table 4-5) respectively, which is approximately double and triple the soluble sugars analysed here in wheat and rice cereal based infant cereals respectively. The results are illustrated in Table 4-4.

4.3.1.2 DNS assay

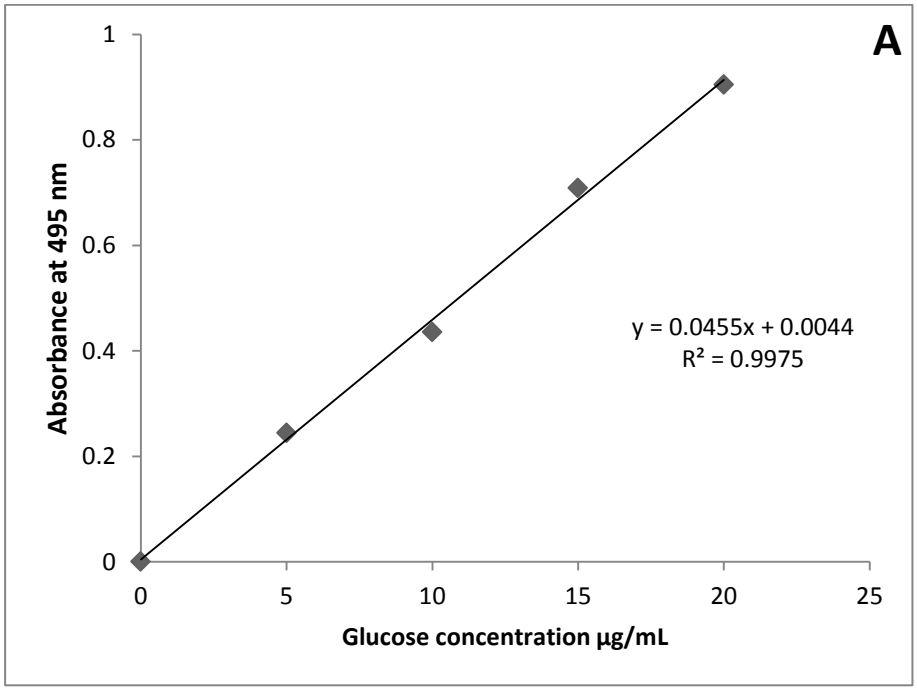
With DNS assay the soluble sugar amount detected was lower than phenol sulphuric assay by 50-30%. The mean soluble sugar in wheat based cereal was 11 g/100 g, whereas for rice and oats cereal based infant cereals the soluble sugar amount was 8.3 and 10.5 g/100 g respectively, as shown in Table 4-4. These soluble sugar results were around 23% and 6.6 % of the total content of carbohydrate in the wheat and rice based cereals respectively according to the study by McCance and Widdowson (2002) of the food composition of infant cereals. The results are shown in Table 4-4.

4.3.1.3 High-performance anion-exchange chromatography amperometric detection (HPAEC-PAD)

The reducing sugars determined by HPAEC-PAD were glucose, fructose, maltose, lactose and sucrose. The amount of most sugar determined using HPAEC-PAD was higher than the findings of McCance and Widdowson (2002). The amount of sugar recovered from HPAEC-PAD in infant cereal made from wheat and rice was 68.0% and 27.1 % lower than the amount of sugar found by McCance and Widdowson (2002). The results are shown in Table 4-5 and the sample and standard chromatograph is shown in Figure 4-7.

Glucose, for example, was 8 times higher for the wheat based infant cereals, whereas for the rice based cereal there was no determination of glucose using HPAEC-PAD. The fructose found in wheat cereal with dates and honey only was 10 times higher than that found by McCance and Widdowson (2002) (Tables 4-4 and 4-5). Sucrose and lactose contents were found to be high in most infant cereals, as shown in Table 4-4. The reason for the high sucrose content may be due to it being added during the process, as shown in.Fernandez-Artigas *et al.* (2001) study, whereas, the high level of lactose may be the result of milk being added as an ingredient in most infant cereals, as shown in Table 2-5 (Chapter 2).

In general, from phenol sulphuric and DNS assays the reducing sugar was found to be high in cereals that were made from oats, wheat and rice cereal, in order. However, regarding HPAEC-PAD, the reducing sugar was found to be high in cereals that were made from wheat, oats and then rice cereal, in order.



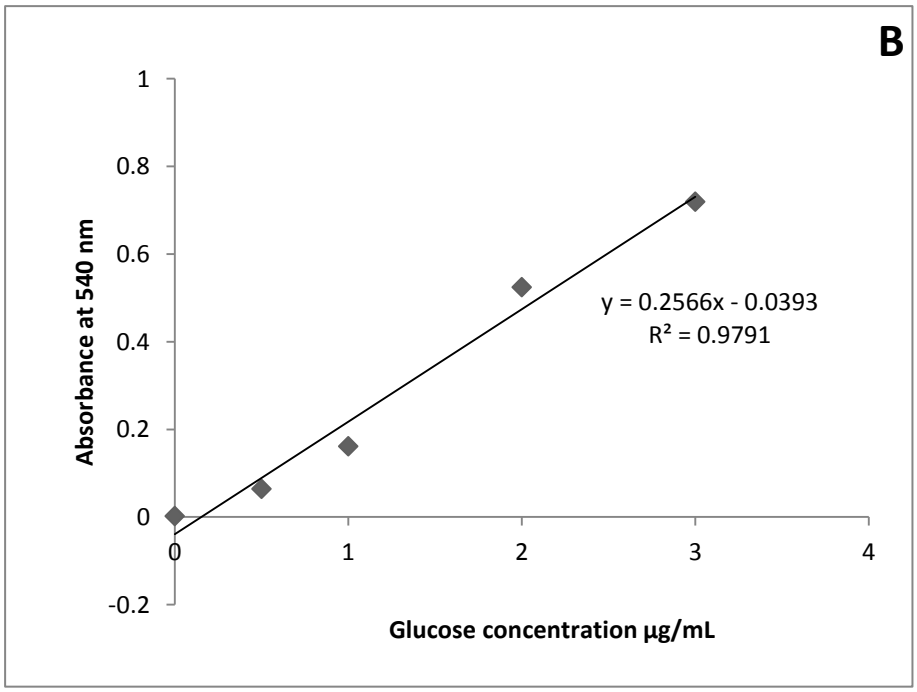


Figure 4-5: Standard curve for measuring reducing sugar content in food by using glucose solution as standard; A) glucose detected by phenol-sulphuric assay method the absorbance was at 495 nm (n=6), B) glucose detected by DNS assay method the absorbance was at 540 nm (n=6).

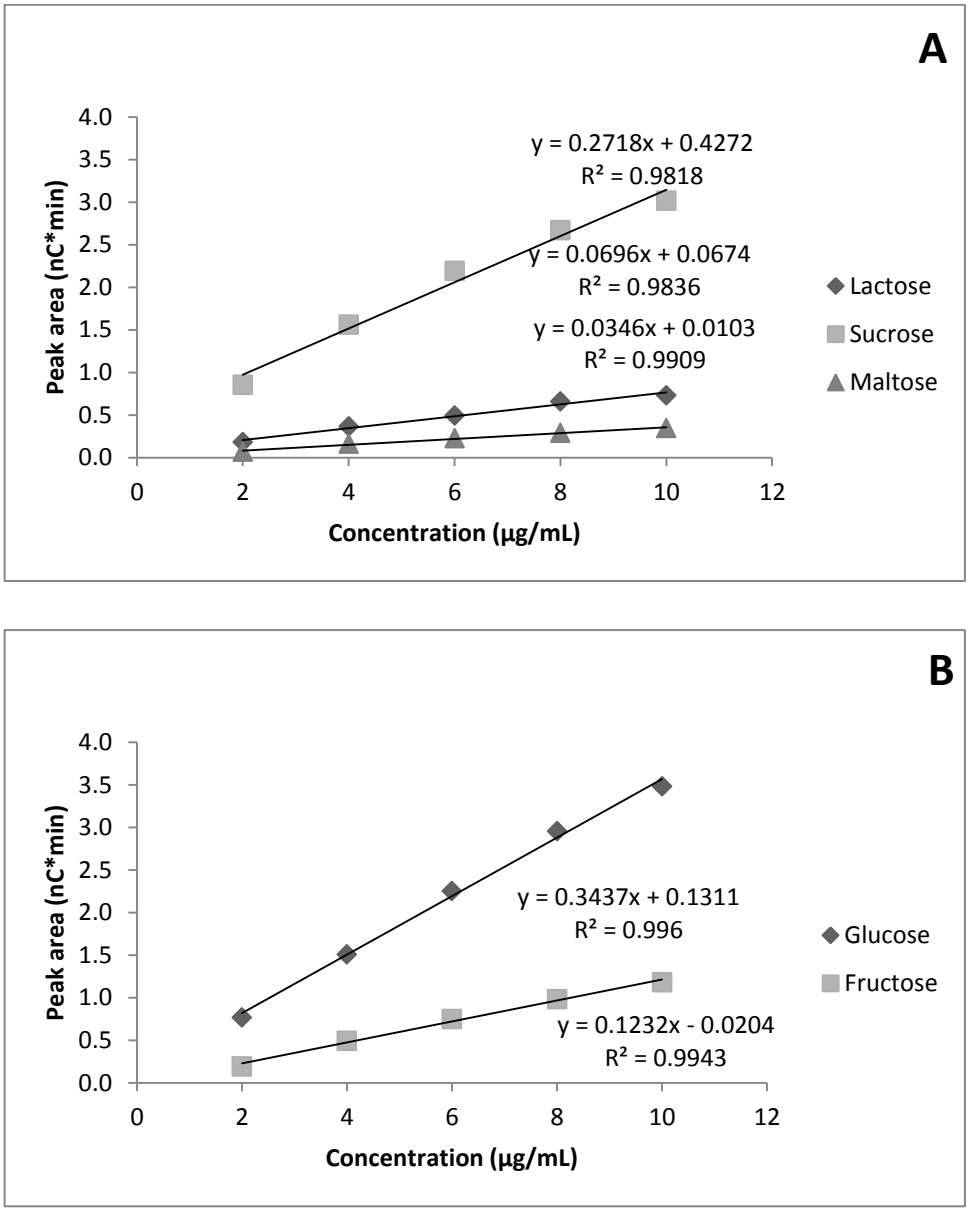


Figure 4-6: Five standard curves for measuring reducing sugar content in food by detection using HPAEC-PAD detector (peak area) (n=6); A) maltose, sucrose and lactose, B) fructose and glucose.

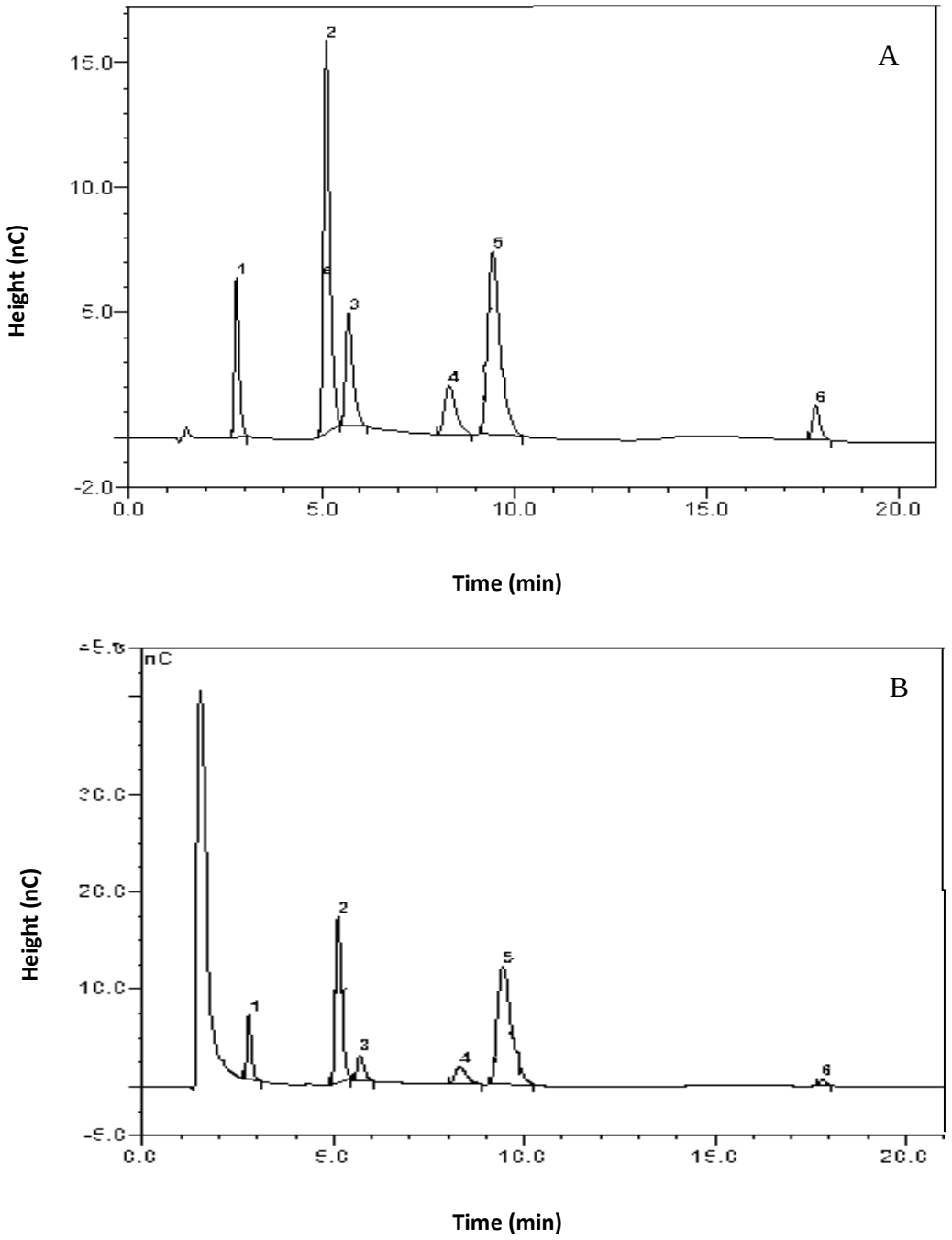


Figure 4-7: HPAEC-PAD chromatograph; A) mixed standards of free sugars (glucose, fructose, sucrose, lactose and maltose), B) of Cerelac wheat and dates for content of free sugars glucose, fructose, sucrose, lactose and maltose; 1) fucose (IS), 2) glucose, 3) fructose, 4) sucrose, 5) lactose, and 6) maltose.

Table 4-5: Soluble sugar content of some types of infant cereals (g/100g dry matter), analysed by three different methods (Phenol sulphuric, DNS and HPAEC-PAD; (n=3).

Sample name	Phenol sulfuric	DNS	HPAEC-PAD					
	Total soluble sugar (±SD)	Total reducing sugar (±SD)	Total specific sugar (±SD)	Glucose (±SD)	Fructose (±SD)	Sucrose (±SD)	Lactose (±SD)	Maltose (±SD)
Cerelac rice	19.1±1.0	8.3±0.0	21.6±0.1	0.0±0.0	0.0±0.0	9.5±0.1	12.1±0.1	0.0±0.0
Heinz Oats	33.8±3.8	10.5±0.0	31.3±0.2	2.4±0.0	0.0±0.0	11.0±0.1	11.2±0.2	6.7±0.4
Cerelac wheat & dates	32.2±4.2	10.2±0.0	57.9±0.5	2.5±0.0	27.2±1.1	7.7±0.4	17.4±0.5	3.1±0.4
Cerelac wheat & honey	27.0±5.3	12.8±0.0	49.3±0.4	2.4±0.0	12.8±0.3	12.0±0.7	18.0±0.4	4.1±0.6
Cerelac wheat	30.1±3.0	10.0±0.0	36.3±0.0	2.8±0.3	0.0±0.0	12.9±0.4	17.3±0.4	3.3±0.8

Table 4-6: The composition of infant cereal per 100 g in terms of soluble sugar.

Food Name	Glucose (g)	Fructose (g)	Sucrose (g)	Maltose (g)	Lactose (g)
Baby cereals, wheat-based	0.3	0.2	2.9	2.1	4.2
Baby cereals, rice-based	0.5	0.1	3.3	0.8	1.6
Baby cereals, various cereal-based	3.2	1.8	7.6	1.8	9.1

(McCance and Widdowson, 2002).

4.3.1.4 Statistical analysis

A one-way between methods analysis of variance (ANOVA) was conducts to explore the impact of method on reducing sugar analysis. The methods used in the analysis were three methods phenol sulphuric, DNS and HPAEC-PAD. There was a statistically significant difference at the $p<0.05$ level in soluble sugar analysis for phenol sulphuric and HPAEC-PAD and reducing sugar analysis for DNS analysing methods $F(2, 15) = 4.74$, $p<0.05$. Despite reaching statistical significance, the actual difference in mean scores between methods was large. The effect size, calculated using eta squared, was 0.38. Post-hoc comparisons using the Tukey HSD test indicated that the mean score for DNS method ($M=14.1$, $SD = 9.3$) was significantly different from phenol sulphuric method ($M = 31.6$, $SD =9.3$) and HPAEC-PAD method ($M = 34.6$, $SD =17.1$). There was no statistically significant difference in mean scores between phenol sulphuric method and HPAEC-PAD method. That due to different measurement for soluble sugar between methods as mentioned in sections 4.3.1.1, 4.3.1.2 and 4.3.1.3.

4.3.2 Soluble protein determination

The Bradford assay was used to determine soluble protein in the infant cereals. For calculation of soluble protein amount the BSA standard was used in different concentrations to draw the standard curve (see Figure 4-8).

The amount of soluble protein was low in all infant cereals, ranging between 0.2 and 0.8 g/100 g, as shown in Table 4-6. These results were below the amount of total protein in the infant cereals studied by McCance and Widdowson (2002), at 96.6%, 95.4% and 94.7% for infant cereals that were wheat, rice and oats cereal based respectively.

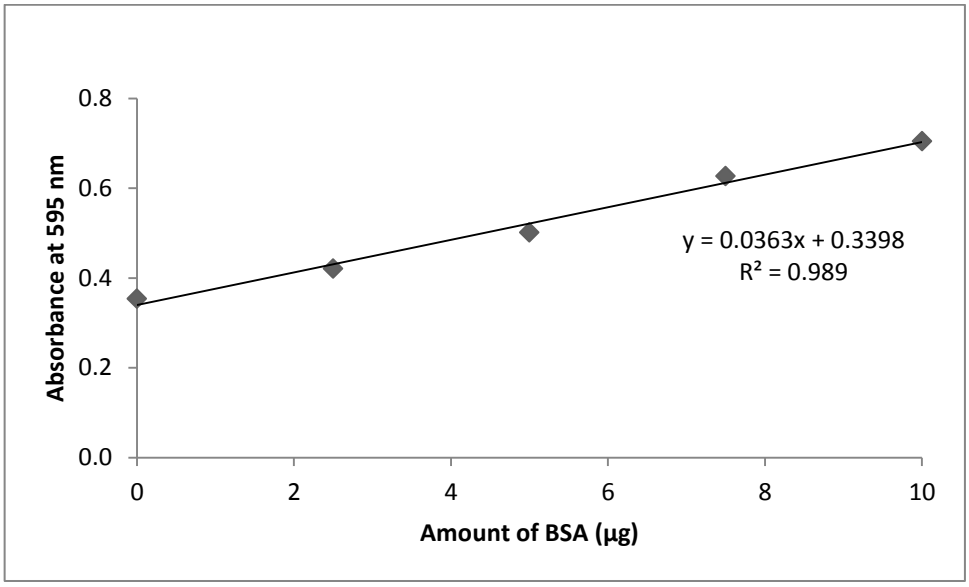


Figure 4-8: BSA standard curves for measuring soluble protein content in food by Bradford method the absorbance was at 595 nm (n=6); error bar represents the standard error of the mean.

Table 4-7: Soluble protein content of infant cereal samples (g/100g) analysed by Bradford method (n=3).

Sample name	Total soluble protein g/100 g (±SD)
Cerelac Rice	0.5±0.0
Heinz Oats	0.8±0.0
Cerelac wheat	0.2±0.0
Cerelac wheat & dates	0.4±0.0
Cerelac wheat & honey	0.7±0.0

4.4 *Discussion*

In these conditions, if the heating is maintained for a long time acrylamide may be formed, unless there are any additives that reduce the acrylamide formation. In cereal processing, when using a roller drying process, all sugars were found to decrease or be lost and browning reaction was observed. However, the browning in oats cereal was found to be lower than in rice cereal. This was due to the presence of reducing sugar (Fernandez-Artigas *et al.*, 2001). During the processing and storage

of cereals, sugar can react with amino acids and so it is likely that Maillard reaction products will be formed (Fernandez-Artigas *et al.*, 2001).

The soluble sugar in cereal is present in small quantities. Hydrolysis of infant cereal during processes increased the sugar profile, such as glucose, fructose and maltose. The increase in glucose came from hydrolysis starch, whereas the increase in fructose was derived from sucrose, and sucrose was increased by adding additional sucrose in the hydrolysis step during the cereal process. Sucrose in most infant cereals is high because it is added as an ingredient to cereal products (Southgate *et al.*, 1978, Fernandez-Artigas *et al.*, 2001). Fernandez-Artigas *et al.* (2001) found that hydrolysis of rice starch increases the content of glucose and maltose. The rice contains more starch which is easy to hydrolyse.

The high level of reducing sugar content in commercial wheat, rice-corn-soy and 7-cereal with fruit was 23.7, 3.7, 1.3 and 0.3 g/100 g for sucrose, maltose, glucose and fructose respectively. The total reducing sugar in breakfast cereal without added sugar was found to be as low as flours, whereas with added sugar it was over half of the food weight (Southgate *et al.*, 1978, Fernandez-Artigas *et al.*, 2001). The content of reducing sugar in Spanish infant cereal was studied by Fernandez-Artigas *et al.* (2001). They found that the reducing sugar was high in wheat and oats than rice. Also, in the processing of cereal-based foods more sugar is added, such as glucose, sucrose and fructose. The sample which contains soy and honey had the highest content of fructose and glucose as expected (Fernandez-Artigas *et al.*, 2001).

The study of Fernandez-Artigas *et al.* (2001) on Spanish infant cereal showed that the rice cereal sugar profile was similar to normal rice products (fructose, glucose and sucrose). Also, Fernandez-Artigas *et al.* (2001) was found that oats cereal contained the same sugar profile as wheat. The sample which contained honey had the highest content of fructose and glucose, as expected. Fernandez-Artigas *et al.* (2001) found that the highest sugar concentration was in ethanol extraction with shaking.

The phenol sulphuric and DNS assays were used for soluble sugar analysis. The phenol sulphuric assay is an inexpensive assay with available material. In contrast, all the chemicals used in the phenol sulphuric assay are dangerous (DuBois *et al.*,

1956). The DNS assay is simple, sensitive and reproducible. However, the DNS assay works only with reducing sugar such as glucose and fructose, but not with non-reducing sugar such as sucrose (Miller, 1959). HPAEC-PAD can identify and quantify each sugar individually, which makes it appear to be suitable for soluble sugar determination (Southgate *et al.*, 1978). Southgate *et al.* (1978) suggest that ion-exchange chromatography is suitable for the analysis of low sugar contents in food. Rivers *et al.* (1984) illustrate a lack of agreement in sugar analysis between DNS and HPLC methods. This is because DNS analyses only reducing sugar in a sample, whereas HPLC analyses specific sugars, and because of that the agreement should not be accepted.

Asparagine, which is a soluble amino acid, was recognised as the response amino acid for acrylamide formation by (Stadler *et al.*, 2002, Tareke *et al.*, 2000). The Bradford assay was used to determine the soluble protein (not free amino acid) in infant cereal. This method is widely used because of its simplicity of performance, rapidity and sensitivity (Bradford, 1976).
In this study only five samples from one patch were tested, whereas the results in the food composition table produced by McCance and Widdowson (2002) were established by analysing different batches and varieties, which may be the reason for the differences between McCance and Widdowson's results and this study's results. McCance is total protein which is done by the Kjeldhal process.

Analyses of infant cereals have shown that there may be enough reducing sugar for acrylamide formation. However, the storage and heat treatment of these cereals (Section 3.3.6.1) means that the acrylamide levels were below the limit of detection or the conditions of storage and heating may not be good for acrylamide formation. Another possible reason for the absence of acrylamide in infant cereals is that the producer may add some substrate to inhibit acrylamide formation during the various processes may be vitamins such as ascorbic acid.

4.5 *Conclusion*

There are significant differences between soluble sugar determination methods. The HPAEC-PAD results show that reducing sugar is high in infant cereals of wheat, oats and rice cereal based in that order. Whereas determination of soluble sugar

using phenol sulphuric and DNS assays showed that reducing sugar is high in oats, wheat and rice cereals based. However, these substrates did not lead to acrylamide formation. This hypothesis “infant cereals contain a sufficient amount of reducing sugar and protein for acrylamide formation during storage or microwaving” was not confirmed from the results in Chapters 3 and 4.

4.6 *Limitation*

In the analysis of soluble carbohydrate, the small sample size was one weakness. There are other limitations, such as those connected to time and the tools used for detecting the asparagine in these products.

After determination of acrylamide content in Saudi infant food as shown in Chapter 3, the estimation of acrylamide exposure by Saudi infants and UK infants, children and whole population will be estimated in the next chapter.

5 Chapter 5: Acrylamide exposure

5.1 *Introduction*

Children and infant populations are seen as a sensitive sub-group as they have a reduced capacity for detoxification reactions putting them at a higher risk of the toxic effects of acrylamide. (Bongers *et al.*, 2012). They also have a high consumption to low body weight ratio, therefore higher exposure to acrylamide per kilogram of body weight, especially in the case of French fries, potato crisps, bread and biscuits (Stopper *et al.*, 2005, European Food Safety Authority, 2011a). Acrylamide is toxic, as explained in Chapter 1, section; 1.3.8. Acrylamide dietary assessment methods, acrylamide dietary composition and acrylamide dietary composition were all explained in Chapter 1, sections; 1.4.1, 1.4.2 and 1.4.3 respectively.

An exposure estimate can be determined using several different techniques; in the following section, methods for assessing dietary consumption, comparison of point estimation and probabilistic exposure estimates are described, relying basically upon the quantity and quality of the available data with which to construct the estimate. Essentially, the total individual exposure in a given day will be the product of the quantity of food consumed which contains acrylamide and the content levels of acrylamide within the food. For example if 1 kg of affected food is consumed then we require the content level within that food to determine the total exposure. So, we require the food items which are likely to contain acrylamide, the quantities consumed of these foods and the content levels within these foods. This clearly will necessitate information on the dietary consumption of the target population (e.g. UK children), sampled levels of acrylamide within selected foods (e.g. bread) and also groupings of associated foods (e.g. breads or cereals). This latter point will facilitate the assessment but will also permit evaluation of consumption to exposure correlations, determination of main exposure contributions and it will enable the delivery of appropriate risk advice.

According to a study by Pedreschi *et al.* (2014) approximately 40% of foods contain acrylamide with a wide variation of levels. Friedman (2003) found the sources of

acrylamide consumed in USA were potato crisps, French fries, breads, cereal and biscuits. In European countries, bread, rusks, coffee and potatoes contribute more than half of the acrylamide exposure. However, in developing countries there have been few acrylamide exposure assessments made (European Food Safety Authority, 2011a).

In Saudi Arabia, Ahmed and Mousa (2013) estimated the consumption of staple foods, such as bread and rice, and found that wheat consumption amounted to around 241 g/day/person. This consumption is in the form of bread, such as Arabic bread (pita), roll bread (Samoli), and western style bread such as French bread and pizza. Rice is the staple food in Saudi Arabia and the consumption mean is around 117 g/day/person. The traditional dish made from rice is named Kabsah (rice with meat or chicken). Acrylamide exposure is primarily associated with the consumption of bread, cake and biscuits (made from wheat) and potato crisps.

For this study, a sample of Saudi infants' (4-24 months) was taken from Thaiban (2006). Also, a sample of infant, children and the whole population will be taken from National Dietary Nutrition Survey (NDNS) SN3481 (1.5-4.5 years) and SN6533 (1-2, 1-4 and 1-92 years) (the details of which will be explained in Table 5-2), using an approach used previously for other toxicants. The results of this study will allow an assessment of the risk to Saudi Arabian infants and the UK population with regards to acrylamide exposure.

Table 5-1 shows acrylamide content data (µg/kg) found in different food groups from different countries. Similar food items in each study were grouped into one group, for example, all biscuit types in the biscuit group. However, some studies contain a special food which is not included in the table due to it does not contain acrylamide or it is traditional foods.

5.1.1 Aims

The specific aims of this chapter are to:

- estimate the acrylamide exposure of Saudi infants; and compare it with UK infant and children,

- compare different age groups' acrylamide exposure found in the NDNS SN6533 (2008/2009 – 2011/12) and SN3481 (1992-93).

5.1.2 Hypotheses

- Saudi and UK infants' exposures to acrylamide are high.
- The UK children population's exposure to acrylamide has decreased from 1992 to 2011

Table 5-1: Acrylamide content data (µg/kg) found in food groups consumed by populations in different studies and countries.

Country	Food group [mean, SD (µg/kg)] (number of samples)*										Reference
	Bread	French fries	Potatoes	Cereal	Biscuits	Coffee	Pastry	Popcorn	Infant food	Composite (food)	
Sweden	[50,-] (21)	[540,-] (7)	[1360,-] (11)	[220,-] (14)	[300,-] (11)	[25,-] (2)	-	[500,-] (3)	-	-	(Svensson *et al.*, 2003)
Saudi Arabia	[50,-] (-)	[206,-] (-)	-	-	[220,-] (-)	-	-	[820,-] (-)	-	-	(El-Ziney *et al.*, 2009)
Austria	[153,-] (38)	-	[627,-] (44)	[95,-] (28)	[275,-] (30)	[204,-] (7)	-	[106,-] (15)	-	-	(Murkovic, 2004)
Germany	[25,-] (67)	-	[330,-] (330)	[104,-] (178)	[269,-] (545)	[12,-] (102)	-	[75,-] (4)	-	-	(Boon *et al.*, 2005)
Germany	[916,-] (-)	[152,-] (-)	[567,-] (-)	[36,-] (-)	-	-	[410,-] (-)	-	[ND] (-)	-	(Hilbig *et al.*, 2004)
Belgium	[30,-] (6)	[254,-] (33)	[676,-] (29)	[135,-] (20)	[143,-] (6)	[114,-] (11)	-	[160,-] (5)	-	-	(Matthys *et al.*, 2005)
France	[34, 17] (14)	-	-	[16, 10] (6)	[954, 240] (16)	[80, 40] (9)	-	-	-	[14, 11] (12)	(Sirot *et al.*, 2012)
Canada	[32,-] (28)	[1053,-] (18)	[524,-] (12)	[102,-] (16)	[120,-] (16)	-	-	[329,-] (4)	-	-	(Normandin *et al.*, 2013)
Brazil	-	[264,-] (7)	[591,-] (12)	[30,-] (8)	[116,-] (3)	[174,-] (3)	-	-	-	-	(Arisseto *et al.*, 2009)

(-) Indicates number not supplied

5.2 *Methods*

Acute acrylamide exposure was estimated for Saudi infants' by using data from Al-Theeban (2006) study data. Also, exposure was estimated UK infant, children and whole population by utilizing the NDNS SN3481 and SN6533.

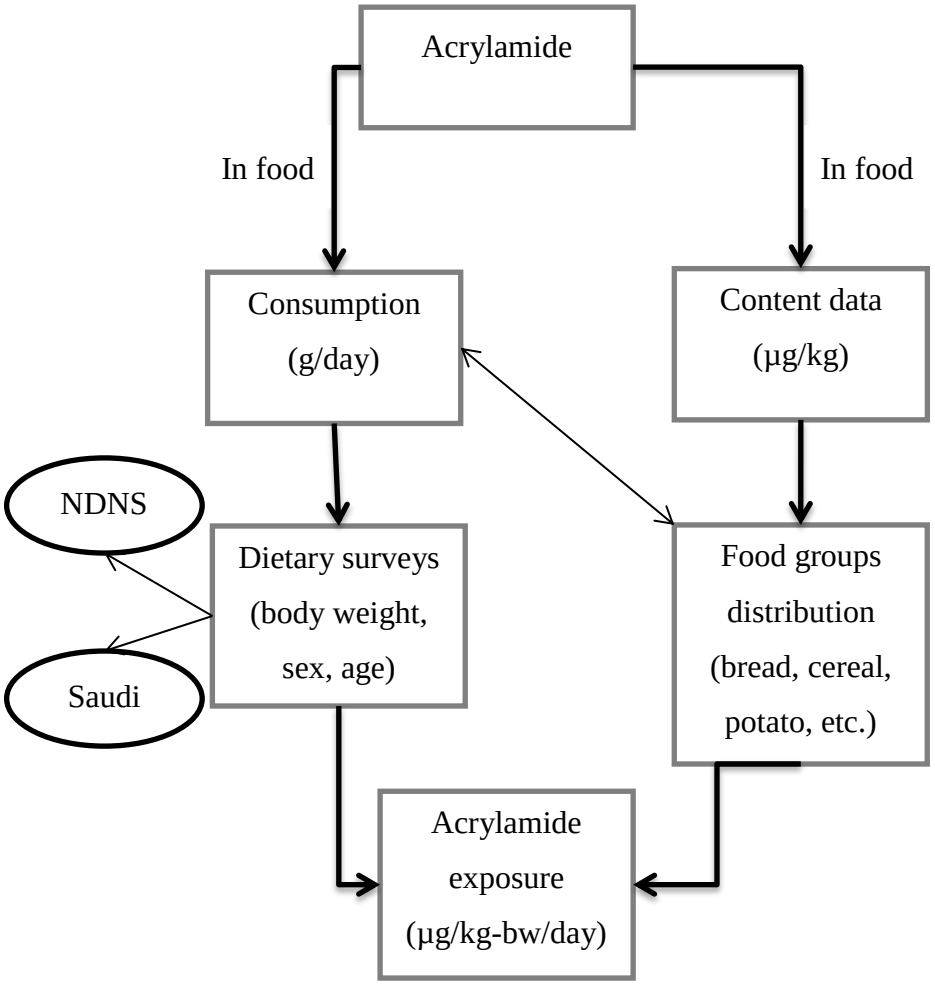


Figure 5-1: Flow chart summarising the methods used to estimate the exposure of acrylamide from NDNS and Saudi surveys data.

5.2.1 Dietary surveys

Food consumption data can be collected using dietary methods such as FFQs, 24-hour recalls and food records (as mentioned in section 1.4.1). In addition, factors may be considered such as age, sex, ethnicity, socioeconomic group, body weight and region. After collecting consumption data they should be formatted to match content data (using the same unit) in preparation for calculation of acute dietary exposure estimation (Intrenational Programme on Chemical Safety, 2009).

Wilson *et al.* (2009) used both a FFQ method and acrylamide haemoglobin adducts to assess the dietary exposure of the Swedish population. These methods led to people reporting their consumption data with wide variation and inaccuracies. Epidemiological studies carried out in different populations can lead to different results because of the difference in acrylamide exposure due to dietary and non-dietary factors (Olesen *et al.*, 2008).

The FFQ method used in acrylamide consumption estimation studies has limitations, including 1) it is not designed to assess exposure to food toxicants, 2) it does not take into account the way foods are cooked at home, 3) it does not include all possible dietary sources of a food toxicant and 4) it could be subject to misclassification (Wilson *et al.*, 2009).

5.2.2 Consumption data

5.2.2.1 NDNS consumption data

The NDNS participants were drawn randomly from a postcode file for the UK. Information about the survey's purpose was sent to all selected addresses by post. This was followed by face-to-face interview to complete the food diary (weighing and recording everything participants consumed over a four-day or seven-day period). Their age, ethnicity, religion, socio-economic status and anthropometric measurements (weight and height) were taken. Length, height and weight were measured by a nurse at the first interview using a stadiometer and weighing scales. BMI (weight (kg)/ height (m^2)) was calculated using the interviewer's laptop. Growth charts for the UK (World Health Organization (WHO)) from birth to four years were used. For clinical purposes, the charts define overweight as above the

91st but on or below the 98th percentile for BMI and obesity as above the 98th percentile. The interviewer also collected information about food shopping and food preparation, cooking skills and facilities in the household (National Diet and Nutrition Survey, 2008).

Consumption diaries were compiled using a four or seven day estimated diary, the assessed days in each case included two weekend days because there are differences in consumption of some foods associated with certain days of the week. Also, for some foods there is different consumption base upon season (National Diet and Nutrition Survey, 2008).

The previous NDNS (SN3481) covered children aged 1.5 years to 4.5 years in 1992-1993 with 1717 participants completing four day dietary record (Gregoryd *et al.*, 1995), children aged 4 to 18 years in 1997 (SN4243) with 1701 participants completing seven consecutive day dietary record (Gregory *et al.*, 2000), adults aged 19 to 64 years in 2000-2001 (SN5140) with 1724 participants completing seven-day weighed intake record (Henderson *et al.*, 2002), and senior people aged 65 years and over in 1994-1996 (SN4036) with 1275 people not living in institutions (free-living group) and 412 people in institutions completing, four day record of weighed dietary intake (Smithers *et al.*, 1998). The NDNS 2008-2012 survey (SN6533), was a rolling programme for people aged 1 year and over. The NDNS's used in this study are shown in Table 5-2. A total of 3073 individuals aged a years and older completed four day dietary record (Bates *et al.*, 2011).

As shown in Table 5-2, the NDNS SN6533 survey (combined 2008/2009 – 2011/12) divided participants into infants aged 1 to 2 years old (158 participants), children aged 1 to 4 years (335 participants) and whole population aged 1 to 92 years old (2844 participants). The number of participants used for the estimation of exposure to acrylamide was less than the number of NDNS participants due to the exclusion of some participants because of missing information such as age and body weight (Whitton *et al.*, 2011). A screenshot from the NDNS individual foods consumed and recorded in dietary intake shown in Appendix A.

In the NDNS, foods were grouped into hierarchical groups based on nutrients and similarities (Whitton *et al.*, 2011). There have been number of studies of risk

assessment in different countries that have grouped foods into similar groupings as this study (see Table 5-1), but they do not mention any reason for the food groupings. These studies include Matthys *et al.* (2005) study of Flemish adolescents, Normandin *et al.* (2013) study of Canadian adolescents, Hirvonen *et al.* (2011) study of Finnish adults and children, Mojska *et al.* (2010) study of the Polish population, Cengiz and Gündüz (2013) study of Turkish toddlers and Sirot *et al.* (2012) study of French population.

Table 5-2: NDNS survey numbers used in this study, with participant numbers, their ages and group code numbers.

NDNS number (year)	Participants numbers	Age group (years)	Group code
NDNS SN3481 (1992-93)	1717	Children (1.5-4.5)	SN3481 children
NDNS SN6533: (combined) of the rolling programmed (2008/2009 – 2011/12)	158	Infant (1-2)	SN6533 infant
	335	Children (1-4)	SN6533 children
	2844	Whole population (1-92)	SN6533 whole population

Mills *et al.* (2008) studied risk assessment in the United Kingdom and Ireland among adults and they used a Codex classification system with some modifications related to acrylamide occurrence in food groupings. Foods in their study were grouped into 10 groups and each group contained subgroups. The groups were; bakery (516), beverages (314), biscuits (1238), cereal and cereal products (245), confectionery (70), fruits, vegetables and nuts (141), potato and potato products (1130), infant food (215), snack products (52) and sugar syrup (3). Boon *et al.* (2005) studied risk assessment in the Dutch population. Foods were grouped into 14 groups relating to Dutch food classifications, food properties, cooking methods and acrylamide levels.

Overall, 10,784 individual food items have been researched across all surveys. All items were grouped into 10 groups base on food similarity. These groups were, 1)

grain based products, 2) potato products, 3) beverages, 4) meats, poultry and fish, 5) legumes, nuts and nut butters, 6) dairy, 7) confectionary, 8) composite (food), 9) soups and sauces and 10) fruit and vegetables. Overall, 1699 food items were collected, grouped and associated into eight food groups with some groups disregarded. Food groups were established which combined foods of similar raw ingredients e.g. potato, cereal, coffee. This enabled a reduction of the 1699 items into smaller food sets making them more amenable to subsequent analysis.

The final groups are; 1) bread, 2) pastry, 3) biscuits and cake, 4) cereal, 5) French fries, 6) potato products, 7) composite (food) and 8) coffee. For each group there was subgroup containing different foods, as shown in Appendix B. For each group, the foods from the same group and with similar levels of acrylamide were put into one group to make estimation of acrylamide consumption and identification of the main acrylamide sources easier and more accurate (Office of Environmental Health Hazard Assessment, 2005). In this study foods were grouped based on food processing and acrylamide content as in Boon *et al.* (2005) study. The groupings are similar to those of other studies, as shown in Table 5-2.

The foods were divided into eight groups as described previously. The bread group contained all bread types eaten by participants such as white and whole bread (sliced) milk loaf, pitta bread and rolled bread. The pastry group included all types of pastries, pies and shortbread, whereas, the biscuits and cake group included all types of biscuits and cakes. The cereal products group contained breakfast cereal and infant cereal. The French fries group contained French fries, whereas potato products were grouped with popcorn and potato crispy snacks. The coffee group contained all coffee types. The composite (food) group contained mixed foods, such as vegetables with rice casserole and lamb casserole as shown in Appendix B. Also, Excel sheet shows food groups, code and description for food consumed by the NDNS population (Appendix C). FDA content data had a LOD of 5 µg/kg (Food and Drug Administration, 2005). Dairy, fruit and vegetables, meat, poultry and fish, soups and sauces and confectionary were disregarded from the assessment due to them having low acrylamide levels (<LOD) (Can and Arli, 2014). The grain-based products group was redefined into smaller groups allowing trends to be analysed more accurately.

The individual exposure days allowed a distribution for the population exposure estimate. In constructing individual exposure days, variation in consumption behaviour relative to body weight could be quantified. Also, there is variation in the number and quantity of food items consumed by individuals from day to day, which leads to variations in acrylamide levels. (National Diet and Nutrition Survey, 2008, World Health Organization, 2009, Elbashir *et al.*, 2014).

Food consumption data for the NDNS SN3481 children were collected over a four day period. The data provided 6868 (1717 x 4) individual exposure days to estimate. Whereas, in the NDNS SN6533, dietary information for a four day period were recorded, providing 632, 1340 and 11,376 individual exposure days to estimate a distribution for infants, children and the whole population respectively.

5.2.2.2 Saudi consumption data

For the Saudi infant population, dietary information was collected from 150 infants (≤ 2 years) over a three day period and recorded. The Saudi data were taken from a study of Thaiban (2006) in Jeddah, Saudi Arabia.

The sample comprised 150 healthy infants aged up to two years old (4 to 24 months). Data were collected randomly from the well-baby clinic of three of the most popular infant hospitals in Jeddah. These hospitals are located in the south east, north west and the central areas which are the King Abdul-Aziz University Hospital (King Abdul-Aziz University, Ministry of Higher Education), the King Fahad Armed Forces Hospital (Ministry of the Army) and the Maternity and Children's Hospital (Ministry of Health) respectively. The interviewer interviewed the infants' mothers, in waiting areas for approximately 1 hour in order to collect data about their infant's health and family's socio-economic background. Also, the mothers were taught about household measurements using food models, household measurement and pictures to identify food portions (Thaiban, 2006).

In the interview, the mother was asked about the diet history of her infant and she was asked to complete a 24-hour record for three days (two weekdays and one day at the weekend). At the end of the interview, mothers were supplied with pictures to use to determine their infants' food intake and to complete the 24-hour recall form.

The anthropometric data and details of the infant's health were taken from the infant's file by a nurse assistant (Thaiban, 2006). Table 5-3 summaries the methods used to collect the dietary intakes, location of meetings the participants' ages and number of participants.

After estimation of Saudi infant exposure of acrylamide, the results were compared with UK infant, children and whole age range population. This comparison will allow a relative assessment picture of the differences between infants and children or whole population. The best dietary data currently available in the UK is the NDNS SN6533 (2008/2009 – 2011/12) and SN3481 (1992-93) daily consumption surveys which were utilized to estimate and compare the differences in acrylamide exposure between the groups indicated.This comparison will allow a relative assessment between Saudi infants and all UK age groups and potentially elucidate the main routes of acrylamide exposure in the dietary context.

Table 5-3: Samples groups and dietary food methods used in all NDNS surveys and the Saudi survey

Group (date)	Sample		Dietary method	Location
	Age (years)	Size (individual)		
NDNS (1992-2001)	1.5-4.5	1717	4-day dietary record	Houses
NDNS (2008-2012)	1 -92	2,844	4-day dietary record	Houses
Saudi Arabia (2006)	≤ 2	150	3-day 24-hour recall and diet history	Hospitals

In the Saudi infants' data, 50 types of foods were recorded as consumed including milk and milk products (breast milk, bottle milk, milk, yogurt, and cheese), bread, cereals (infant cereal and breakfast cereal), biscuits and cake, potato crisps and popcorn, eggs, vegetables and fruit and composite (food) (any type of meat with macaroni, oats, noodles and rice). Infant foods were grouped into five groups similar to the NDNS groups (pastry, French fries and coffee did not consume by Saudi infants). The foods analysed amounted to 16 types (47 samples), each food was analysed in three different brands, as explained in Chapter 2. Also, the content of each group has been described in section 2.2.4. The main limitations of the data are

that there is no information about the food brand name, the recipe, the preparation or the packaging. The data were collected for nutritional assessment which concentrating on nutrient intake.

The similarity and simplicity of infant food and the fact that there is no difference in food ingredients or cooking type made the number of groups and content of each group smaller. Traditional Saudi composite (food) such as Kabssa (boiled rice, with meat or chicken), macaroni (boiled macaroni, with meat or chicken), oats soup (boiled oats, with meat or chicken) and other composite (food) are basically boiled foods that contain meat.

In the Saudi infant study the dietary information for 150 infants over three days provided 450 individual exposure days. Infants' exposure may not vary from day to day due to the limitation of infants' consumption of Saudi dataset food.

5.2.3 Content data

Data for acrylamide content in food consumed by the NDNS SN3481 and SN6533 surveys were obtained from several primary sources including the UK Food Standards Agency (FSA), the US Food and Drug Administration (FDA), as well as smaller studies including a Dutch study (Konings *et al.*, 2003) and Swedish study (Svensson *et al.*, 2003). Different sources from different countries were used to gain a wide, representative sample of foods that are consumed. Acrylamide analysis results differ between laboratories and different brands and packaging from the same food batch and other factors can affect the analysis results. Regarding the difference in results for the same food item, a sampling method should include a wide range of sources. Infant foods were not included in the NDNS studies as it was not relevant to the age group under consideration. Both general and branded food products were assessed (Food and Drug Administration, 2005). Appendix D contains a list of acrylamide content (µg/kg) in the NDNS food group

Acrylamide data from the FSA were part of a three year study running from 2007 to 2010. Samples were collected twice a year to allow seasonal variation (European Food Safety Authority, 2011a). The location of purchase, production method,

storage method and sample time were recorded for each product. (Food Standards Agency, 2008, Food Standards Agency, 2009, Food Standards Agency, 2010a) Data from the FDA were part of a study published in March 2005 that measured content levels in a wide range of food items. Food products, groups, acrylamide contents (µg/kg), number of samples and references used for the NDNS acrylamide exposure estimation was shown in Appendix E.

All datasets were analysed statically for how good they fit to distributions by using Q-Q plot and Shapiro-Wilk test with p-value (<0.05). All food groups are transformed to be normally distributed by taking natural logarithms (log normal distribution) and had a statistically significant fit. Consequently, data was log transformed, resulting in data being modelled by normal distributions. In addition, with the final food groups selected, many ND content values were reported. In the numerical simulations these were modelled using uniform distributions in the range LOD to the ND values quoted in the content data survey (5 µg/kg). Foods that were part of a combination such as meals as identified in NDNS were placed in the composite (food) group, for example, steak pies. These foods, essentially recipes, only partially contribute towards exposure. For example, if no data are available for steak pies but data are available for pastry, an estimate may be made as to the exposure contribution. If we assume, in this case, that between 10% – 60% (most likely 30%) of the food mass is pastry, we multiply the food weight by an extrapolation factor. This will reduce the exposure contribution based upon the contributory mass estimate. To further the study, recipe assumptions needed to be identified as they may have a significant effect on potential exposure.

Acrylamide food content data regarding that consumed by Saudi infants' were taken from starchy foods analysed in this study (as shown in Chapter 3) and the results were used to estimate Saudi infants' consumption of acrylamide. The foods were assessed once by using individual food values and food group values. As explained in Chapter 3, half the LOD value was used as the ND value.

The Saudi infants foods were analysed in the laboratory using LC-MS as mentioned in Chapter 3. The contributes to acrylamide levels in Saudi infants' was calculated twice; the first time using mean concentrate level values to calculate the food

contribution and the second time the mean food group value. These two values were used to study whether there were any differences in acrylamide exposure between the two values.

5.2.4 Exposure assessment

There are several considerations when estimating dietary exposure, such as the duration of exposure and age sub-groups and requires a calculation incorporating the quantity of ingested material and the content in the consumed material. Exposure estimation basically includes a number of subjects with food consumption data (descriptions and quantities), and may include the information for example preparation recipes and whether the food is homemade or ready-to-eat. Exposure assessment may be conducted to address particular target groups, for example to assess the whole populations and especially sensitive groups such as infants and children (Intrenational Programme on Chemical Safety, 2009). The exposure can be assessed by using deterministic or probabilistic methods. To compare exposure estimation between groups, there are several points which should be considered such as the type of estimation, the population age range and food groups and sub-groups (Mills *et al.*, 2008).

The minimum components which are needed to make individual exposure estimation are 1) individual food consumption this could be estimated or known from diary, 2) individual personal data such as age, body weight and sex, 3) acrylamide content in food, 4) grouping of food and 5) estimation methods.

5.2.5 Exposure estimation

5.2.5.1 Deterministic estimation

Deterministic (point estimate) is a single value which describes consumer exposure and may provide mean exposure of a population or upper percentile of population, requires limited information such as content data (Intrenational Programme on Chemical Safety, 2009).

Point estimate may be estimated as a worst case scenario and therefore not a true representation of a population’s or an individual’s exposure. Point estimation

assumes that parameters are at fixed values and that consumption and body weight are also fixed for example, 1 kg of food of interest consumed and a 70 kg adult. To provide a relative measure between individuals, exposure results need to be quantified in a kilogram per body weight basis. This measure will reveal, that despite consuming more, a heavier individual may in fact have lower exposure than a lower bodyweight individual who consumes less in absolute terms (Andritz 2014). The point estimates also do not account for intra-individual variation (individual consumption vary from day to day) (Mills *et al.*, 2008) and do not express the number of individuals who are at risk, how often and how much they may exceed risk levels (Wang *et al.*, 2008) .

In Saudi and NDNS data for each individual's exposure level; the sum of the contents in each of the food items they consume is calculated giving an exposure per day (Equation5-1) (van Klaveren *et al.*, 2006, Obón-Santacana *et al.*, 2014).

$$\textbf{Exposure to acrylamide from specific food item (µg/kg-bw/day)} = \frac{\textbf{Acrylamide content in food (µg/kg)} \times \textbf{Food consumption (kg)}}{\textbf{Body weight (kg)}}$$

Equation 5-1

The sum of all food items consumed by the individual in that day is taken to estimate the total daily acrylamide exposure (Equation5-2).

$$\textbf{Total exposure} = \textbf{Exposure item 1} + \textbf{Exposure item 2} + \textbf{Exposure item 3} \ldots$$

Equation 5-2

Implementing dietary surveys enables estimates to be calculated for each individual; each day the reflect intra variation can be estimated. Also, assuming we have a representative sample, we can estimate a population's exposure profile and so assess intra-individual variation.

Using individual body weight to rescale into µg/kg-bw/day enables relative exposure assessments to be made across the population and increase the precision for 15-20% (Dybing *et al*., 2005).

Deterministic estimate= 1 kg of bread (food amount) ×0.16 µg/kg (acrylamide content) divided by 30 kg (body weight).

5.2.5.2 Probabilistic estimation

Probabilistic is an exposure assessment method which uses distributional data to evaluate and describe consumer exposure. Probabilistic estimates provide a range of exposure dependent upon the variability in consumption of foods across consumers. In contrast, deterministic methods can only supply single point estimates. Exposure varied due to different consumption; 1) from day to day, due to preference, 2) in each age group as a result of different body weight, 3) food types and 4) in terms of sex, region and ethnicity etc. Both of these models use the same equation to estimate the dietary exposure combined from food consumption with food content. Probabilistic models may account for exposure in one food (simple) or more foods consumed by the same individual in a single meal or day (complex).

Probabilistic risk assessment limitations are; 1) inaccurate in representative input data, 2) requires significant data such as dietary data, contamination data and distribution data and 3) in random samples it may generate extremely high exposures which are never observed. For that 95% CI are used to minimize the error (Chatterjee, 2006).

The Monte Carlo assessment is an example of a probabilistic assessment where the model samples from distributions generated from input data (Intrenational Programme on Chemical Safety, 2009). The Monte Carlo simulation uses randomly sampled values from the input distributions. It can be concluded that with appropriate data and when the simulation is conducted with a sufficiently large number of iterations, the results will provide a representative estimate of the actual exposure. This simulation may be inaccurate at the extreme upper and lower ends of a distribution, which is particularly true when using parametric distribution rather

than nonparametric distribution data (Intrenational Programme on Chemical Safety, 2009, Andritz 2014).

The Monte Carlo analysis starts by selecting an individual from the selected population. Individual exposure was simulated for each food consumed, then the intakes are combined to provide total individual intake for a single day. The content in each food will vary so random samples taken from representative distributions must be selected. Distribution for specific foods for example, bread or food groups must be defined or alternatively, samples from appropriate dataset chosen. Calculating multiple days is the next step as individual intake varies from day to day. Simulation for each individual in the population is repeated and the results are presented as a frequency distribution of daily doses or average doses for the population. The simulation is repeated many times until a reproducible estimate is obtained (Petersen, 2000).

The Monte Carlo model may address variation in many factors such as chemical contents, food consumption patterns and individual personal characteristics. In the Monte Carlo model the most desirable data are measured directly, whereas unavailable data can be defined by extrapolated from alternate data (Petersen, 2000).

In the Monte Carlo ideally, data from large sample of individuals (for example 2000-10000) and food dataset are required. In the NDNS in each group, individual has four days and in each day a food can be consumed several times in different amounts. Consequently, many hundreds of thousands of samples can result.

5.2.5.2.1 Modelling of exposure

A two-dimensional Monte Carlo simulation in MATLAB (Mojska *et al.*, 2010) was used to estimate exposure by modelling the uncertainty in the content data and using the precise consumption quantities provided in the NDNS surveys. Consumption quantities in the surveys indicate the variability within and between consumers.

A single iteration of the model provides the variation in exposure due to differences in consumption and for a specific selection of concentration values within the individual foods consumed. If another iteration of the model is executed, different content values are sampled for the same foods and consumption quantities. Many

iterations of the model therefore permit estimates or confidence intervals of the exposures due to the uncertainty in the content datasets.

Essentially, for each iteration of the simulation the algorithm checks the diary entries of each individual identifying the consumer identify (ID), consumer weight, age, gender and the food items consumed. For the foods consumed the food ID code, associated food group, and quantity consumed are recorded. When the associated food group has been identified, a randomly sampled content value is obtained to provide a representative acrylamide level within the food. Multiplication of the food consumption quantity with the content value gives the individual food exposure contribution.

The program accumulates all contributions of the exposure for the whole day and gives an exposure-day estimate for the individual. This may be presented as (µg/person/day) or more conveniently as (µg/kg-bw/day) to facilitate easier comparison. The algorithm retains the source of each exposure so that the relative contribution from each food group can be provided and be represented as the percentage contribution to exposure from each group.

Numerically, we may write an iteration of the algorithm as in Equation 5-3. The exposure estimate Ejk (µg/kg-bw/day) for individual j consuming up to n(k) contributory items on day k (eg. k=1, 2, 3) may be calculated by using Equation 5-3.

$$E_{\mathrm{jk}} = \frac{1}{W_j} \sum_{l=1}^{n(k)} w_{jkl} c_{jkl}$$

Equation 5-3

Here, j is individual, Wj is the weight of individual j and c_{jkl} is the content of acrylamide (µg/kg) in the food commodity l, w_{jkl} denotes the weight of the consumed item l as identified in the NDNS diary. Included items are only those which have been identified as being potentially contributory toward the acrylamide exposure. It is important to recognise the content c_{jkl} is sampled from the appropriate food group distribution and that actual distribution. So for example, if 100 g of bread

are consumed, a random sample is taken from the content distribution group to which bread belong. Similarly, if French fires are consumed, a random sample from the French fries content distribution will be sampled to provide reasonable acrylamide content in that food type (see Figure 5-2 for the process). As previously stated, the (log transformed) content distributions are described using the log normal distribution parameters of mean and standard deviation which have been calculated from the actual content values measured in actual foods within their respective groups (Holmes et al., 2008). Actual measured values have been selected from a variety of independent literature sources.

For each individual, an exposure value for each affected food consumed in the whole day is estimated and the sum of which provide and exposure day. This is repeated across all survey days and all individuals within the survey to provide a total exposure-day sample of size determined by the product of number of consumers and days in the survey period e.g. 4 or 7 days. These exposure-day estimates are then ranked from smallest to largest to give a cumulative distribution which permit estimation of proportion of consumers which exceed or be lower than specific daily intake thresholds which may have been established by regulatory bodies e.g. TDI values. Several different formulas may be used for symmetrical plotting positions.

Such formulas have the form with the value of *a* in the range from 0 to 0.5:

$$(\boldsymbol{j}-\boldsymbol{a})/(\boldsymbol{n}+\mathbf{1}-\mathbf{2}\boldsymbol{a})$$

Equation 5-4

The Hazen plotting position, has a=0.5. For large sample size, n, there is little difference between the various expressions and j is the individual number (Holmes *et al.*, 2008).

If the process is repeated and another iteration of the model is conducted, different concentration values are then re-sampled providing an alternative yet representative exposure estimate in the population. We define the outer iteration loop of the model by the index *i* which effectively explores the uncertainty in the concentration data values in the log normally distributed content data. Clearly if sufficient outer loop

iterations are completed the confidence intervals of the estimates stabilise to repeatable ranges due to the fact that log normal distributions describe the content data. It was found that by conducting 1000 outer loop iterations repeatable estimates were achieved. In the following chapter stable estimates are reported. The inner loop captures the variability between all individuals through their dietary choices and consumption levels. The complete algorithm may define as in equation 5-5.

$$E_{ijk} = \frac{1}{W_j} \sum_{l=1}^{n(k)} w_{jikl} c_{jikl}$$

Equation 5-5

Where *i* denotes the iteration of the model (Holmes *et al.*, 2008).

Figure 5-2 shows Monte Carlo simulation model and equation.

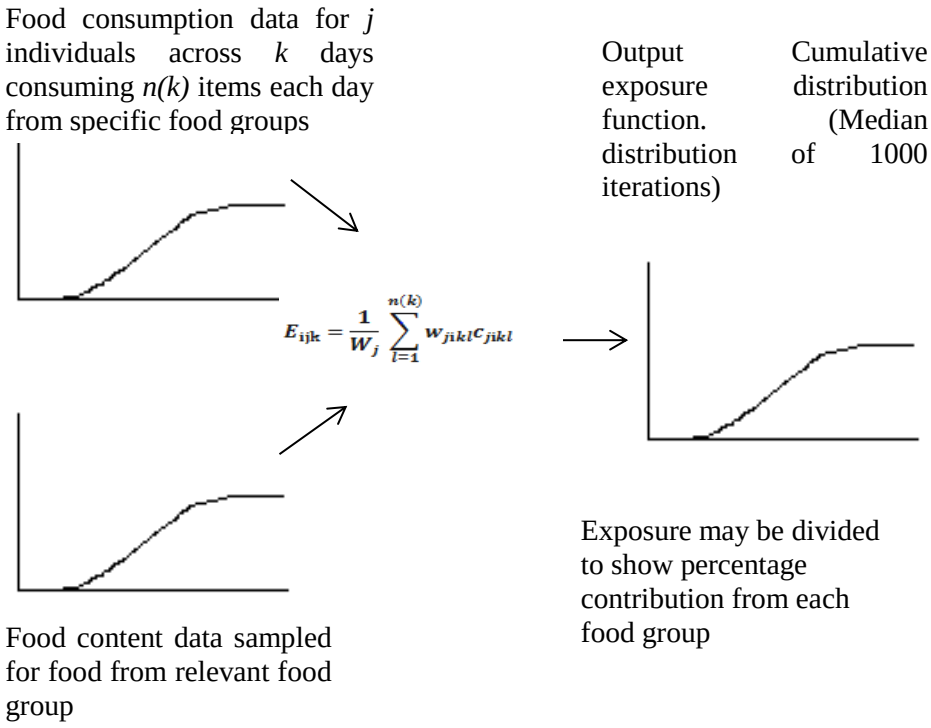


Figure 5-2: Monte Carlo simulation model and equation.

In order to verify the stability of the exposure estimates, Monte Carlo simulations were conducted with increasing numbers of iterations ranging between 1000 and 5000 outer-loop iterations as shown in Figure 5-10. The resulting 95 % confidence intervals of the exposure estimates at both 1000 and 5000 iterations were then compared at each percentile to ensure repeatable estimates were obtained. The iteration numbers may affect the result accuracy with a too few iterations leading to inaccurate outputs and too many iterations taking long simulation time(EPIX analytics, 2010). Accordingly, for efficiency in computational time, 1000 iterations were used in the Monte Carlo simulations. Furthermore, in this study, the distributions used to describe the concentration data for particular food groups e.g. bread, have fixed parameter values (i.e. mean and standard deviation), therefore, the uncertainty arising from sampled concentration values used to represent levels of acrylamide in consumed foods tend toward the average values for large numbers of samples providing further justification for this decision.

The exposure estimate was simulated with 1000 iterations which was shown to be sufficient for stable estimated assessing each individual exposure day, shown in Section 1.2.4. For example, 960 individuals over four days in NDNS SN3481 create 3840 exposure days. Individual exposure days are ranked in increasing order of exposure with contributor from each food group being additionally quantified. Monte Carlo simulation in Matlab was used to estimate individual exposure. Selected food consumed by participants are identified in each day, and associated with the appropriate food groups which contain acrylamide (Mojska *et al.*, 2010). Acrylamide concentration with consumption foods are sampled from the log normal distributions which model each food group as fitted from concentration data. So, for each individual within the survey, samples from these food group distributions are taken whenever a food from that group is consumed. In the next iteration of the model new parameter values are then re-sampled (Holmes *et al.*, 2005). All estimated acrylamide daily exposure were divided by individual body weight in kilograms to achieve exposure (µg/kg bw/ day).

Different assumptions may be appropriate when modelling acute and chronic dietary exposures, since the contents of the substances will not always be high (Intrenational Programme on Chemical Safety, 2009).

The actual exposure has contributions from the 8 food groups as defined earlier. To assess the contribution of exposure from each group we calculate the associated percentage contribution for each exposure day event. So to find the food group percentage contribution we can divide the actual individual exposure of each group into the total exposure from groups (Equation 5-6).

$$\%\boldsymbol{Group\ contribution}_{\boldsymbol{food}} = \frac{\boldsymbol{Food\ group\ exposure}}{\boldsymbol{Total\ exposure}}$$

Equation 5-6

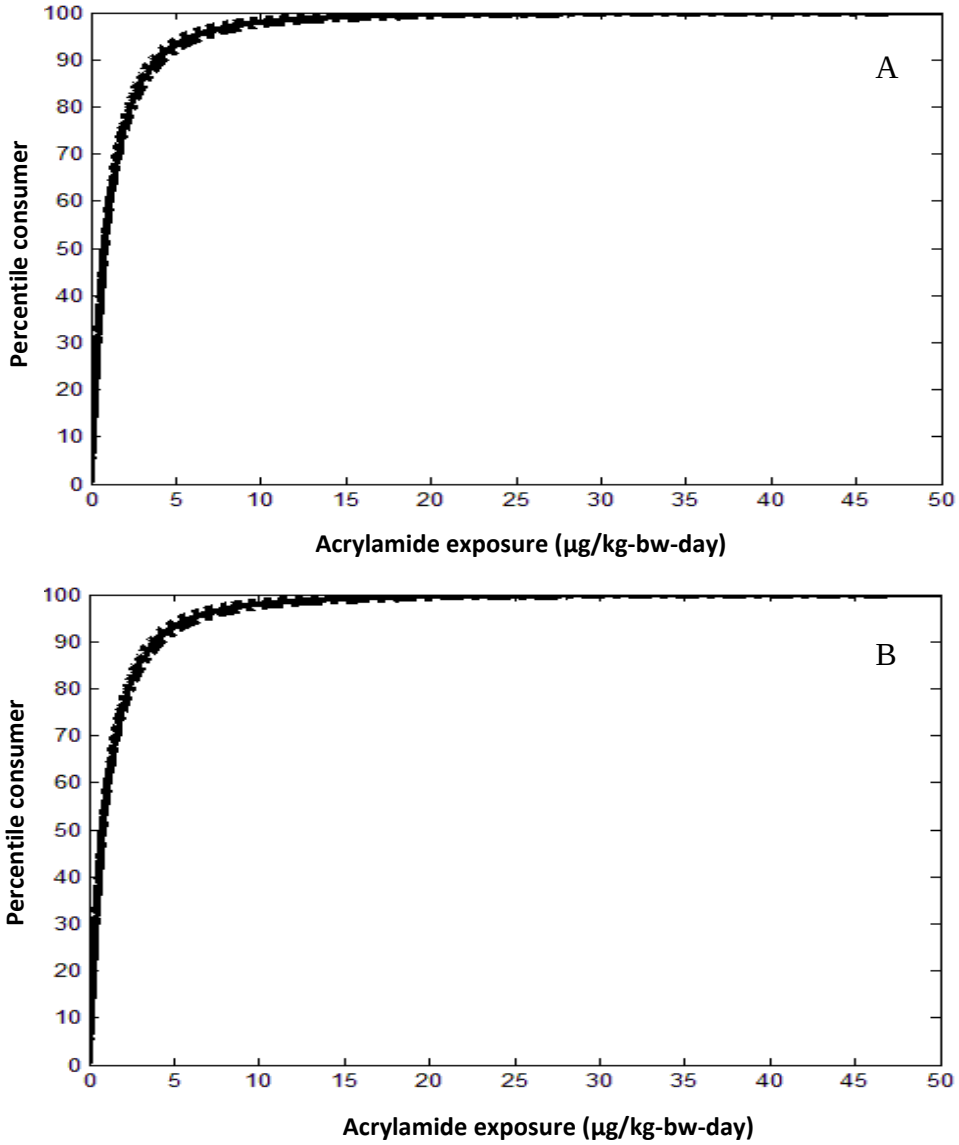


Figure 5-3: Daily consumer acrylamide exposure (µg/kg-bw/day) for NDNS SN3481 children (aged 1-4 years) with A) 1000 iterations and B) 5000 iterations.

5.2.5.2.2 Modelling parameter uncertainty

Although when the content datasets comprise of $n>1$ samples, sampling parameter uncertainty may be modelled based on a log normally distributed dataset and expresses the true mean (μ) and standard deviation (σ), qi-square (χ^2), distributed as follows,

$$\sigma \sim \sqrt{\frac{(n-1)s^2}{\chi^2_{n-1}}}$$

Equation 5-7

$$log\ \mu |\ log\ \sigma \sim normal\left(\overline{log\ x}, \frac{\sigma}{\sqrt{n}}\right)$$

Equation 5-8

Here $\bar{x}$ relates to the data mean and *s* the associated data standard deviation of the relevant food group dataset. If the parameters, mean and standard deviation of each concentration distribution are uncertain they will change across each iteration of the outer loop and be distributed as per equations 5-7 and 5-8. The variation in the exposure will be wider than in the previous fixed parameter case but will account for the uncertainty in the content parameters due to sample size. This assumes that the samples are normally distributed which was established in the data analysis. This study confine the estimates to fixed parameter values to investigate exposure estimates which are obtained from variability in consumption and not the additional potential exposures which may be estimated form parameter uncertainty. This approach is adopted since the comparable data from the Saudi data is of significantly lower quantitative level.

5.2.5.2.3 Uncertainty and variability

Uncertainties in acrylamide risk assessment occurs in all assessment aspects; for example, acrylamide levels are different in various dietary foods (there are differences between foods and brands of the same foods), as explained in Chapters 2 and 3. Also, the factors affecting acrylamide levels in food, which cause the variation, were discussed in Chapter 1. There are many possible reasons for explaining the uncertainty in acrylamide exposure levels, such as 1) matrix effects on dietary acrylamide, 2) population (regions, countries and age groups) (Dybing and Sanner, 2003), 3) analysis techniques (LC-MS and GC-MS), 4) sample preparation, 5) analysing different brands and different regions, 6) different cooking habits (Xu *et al.*, 2014), 7) food recipe items and 8) underestimation or overestimation of food consumption (Holmes *et al.*, 2008). This has the potential to

affect the overall exposure estimate significantly. Xu *et al.* (2014) that are should not generalize estimates from developed countries to developing countries. However, creating international data may reduce the level of uncertainty.

Variability occurs between individuals within a population, across populations and within individuals over time due to differences in consumption (Tucker and Ferson, 2003).

Uncertainty may be reduced by obtaining more data or re-analysing samples, for example, acrylamide content data in bread samples. In contrast, variability cannot be reduced since it is inherent in populations, for example due to different body weights and consumption behavior (Wang *et al.*, 2008). It is important to factor in uncertainty and variability so a more precise exposure assessment can be made and to identify the assumptions implemented in the model so that critical reviews may be made.

Risk assessment models can be very complex when quantitative techniques are used to identify acrylamide content or food consumption data. Analyses that evaluate inputs identified as the most important sources of uncertainty may be expected to be the most useful. Sensitivity analysis may also be used to evaluate the effects of factors on the exposure output estimate (Wang *et al.*, 2008).

5.2.6 Formatting data

NDNS data was converted from SPSS files to Excel files (Microsoft Excel 2007). These files included; participants' personal information and dietary intake for 4 days. The files were compared to be sure the conversion was done correctly and data was cleaned to exclude any individual with missing data such as body weight and age or non-consumer. Also, the acrylamide content in foods was recorded in an Excel file with a sheet containing the acrylamide levels of all food groups. This formatting was required for calculation of the acrylamide exposure by the Matlab program (The MathWorks© 2013) (Works©, 2011).

The files were saved and run using Matlab coding in preparation for using the program to estimate acrylamide exposure in the infant, children and whole population for NDNS surveys.

Dietary exposure (µg/kg-bw/day) for acrylamide was generated in a probabilistic way for the whole NDNS population. The distributions of food consumption and the acrylamide levels in actual food consumed were sampled repeatedly. During this sampling, the individual intake linked to acrylamide data generated individual exposure to acrylamide.

Saudi infants' data was formatting similar to NDNS data formatting. The acrylamide exposure in Saudi infants was estimate using two different ways, depending on the mean values: 1) using individual food levels and 2) using food groups' levels. The acrylamide exposure rates from each food group were added and divided by the body weight of each individual to provide the distribution of daily acrylamide exposure.

5.2.7 Statistical analysis

Normality of concentration data distributions were tested with a Shapiro-Wilk when sufficient samples were available. The content data were transformed by taking the logarithm of sample values which in most cases the transformed data were normally distributed. Descriptive statistics for acrylamide levels in different food items included the mean and 95% confidence intervals (CI), min, max and percentiles.

The acrylamide exposure was presented as median estimated with a 95% CI ranging from 2.5^{th} to 97.5^{th} percentile range. Exposure was expresses as µg/kg-bw/day.

5.2.8 Ethics

Before using the Saudi study data (2006), the Food Science and Nutrition Department at the King Abdul-Aziz University gave permission and the Ethics Committee of the Faculty of Mathematics and Physical Science at the University of Leeds stated that the published anonymous data did not need an application for ethical review from the University of Leeds (Appendix F contains a copy of the King Abdul-Aziz University letter and the University of Leeds email).

5.3 *Results*

5.3.1 Participant characterization

5.3.1.1 NDNS

Details of participants in certain age related groups are shown in Table 5-4 and 5-5. The NDNS SN6533 (2008-2012) sample was 52.4% and 47.6% female and male respectively. Age of the NDNS survey ranged between 1 and 92 years old. There were a total of 2844 participants and their ages were 1-2, 1-4 and 1-92 years old with percentages of 5.6%, 11.8% and 100% respectively. For, previous NDNS SN3481 the sample size was 1717 with 49.3% and 50.7% female and male respectively.

5.3.1.2 Saudi infants

The study population was characterized by age and gender. The Saudi infants had a female to male split of 42.7 % and 57.3% respectively. The total sample size amounted to 150 participants aged from 4 to 24 months (Thaiban, 2006).

The Saudi infant data (n=150) showed that the number of infants who consumed food which detectable levels of acrylamide amounted to 124 infants, whereas, 25 infants had consumed only milk (breast milk or formula milk). One infant from the sample had consumed only milk, vegetables and fruits.

5.3.2 Food group exposure contribution

This section identifies the percentage of contributions to exposure for each of the food groups. As mentioned in section 5.2.3, the foods containing acrylamide were grouped into eight groups, which were bread, pastry, biscuits and cake, cereal, French fries, potato products, composite (food) and coffee.

Among the eight food groups, in the NDNS surveys and for the Saudi infant data, the potato products food group contained the highest acrylamide mean levels, at 486.8 and 339.3 µg/kg for the NDNS and Saudi infant data respectively. The food group with the lowest acrylamide level was the composite (food) group at 28.8 µg/kg for the NDNS and bread group at 35.1 µg/kg for the Saudi infant data.

Table 5-4: Mean acrylamide contents in the eight NDNS food groups (number of samples (No.), mean content (µg/kg), log mean content (µg/kg), standard deviation and p-value analysed by Shapiro-Wilk test.

Food group	No.	Mean (µg/kg)	Log mean (µg/kg)	SD µg/kg	p-value (<0.05)
Breads	69	53.6	1.4	0.5	0.000
Biscuits and cakes	28	50.4	1.5	0.4	0.072
Pastry	151	193.1	1.9	0.6	0.027
Cereal products	99	155.1	2.0	0.5	0.611
French fries	309	343.5	2.3	0.4	0.000
Potato products	308	486.8	2.4	0.6	0.017
Composite (food)	37	34.3	1.4	0.3	0.000
Coffee	80	201.3	2.0	0.7	0.000

Table 5-4 summarises the NDNS datasets and provides the mean parameter values of the normal distributions implemented. These data are transformed to be normally distributed by taking natural logarithms. Goodness of fit (Q-Q plot and Shapiro-Wilk test with p-value (<0.05)) were tested against normality showed normal fit was reasonable distributions (breads, French fries, composite (food) and coffee) except in the case of biscuits and cakes, potato products, pastry and cereal which showed p>0.05 due to variation in acrylamide values. The Shapiro Wilk test can produce misleading results and so a standard Q-Q plots were used to assess normality of content data within food groups and which showed an acceptable fit of data {See scatter plots Figure 5-4). The Q-Q plot shows the normality of biscuits and cakes, potato products and cereal groups, but not pastry.

The potato products had the highest levels of acrylamide, giving a log mean of 2.4 µg/kg. Whereas the lowest acrylamide content was in the bread group, with a log mean of 1.4 µg/kg. Appendix G shows the food groups, acrylamide content distributions (µg/kg) in the NDNS. The groups are breads (169), pastry (28), biscuit and cake (151), cereal (99), French fries (309), potato products (308), composite (food) (37) and coffee (80).

The acrylamide content in foods ranged from ND to 4686 µg/kg, depending on the food type. The highest mean value of acrylamide was in potato products (487.0

µg/kg) and this group is the highest consumption in UK infant, children and whole UK population.

Table 5-5: Mean acrylamide content in the eighth Saudi infants' food groups (number of samples (No.), mean content (µg/kg), log mean content (µg/kg), standard deviation.

Food group	No.	Mean (µg/kg)	Log mean (µg/kg)	SD µg/kg
Breads	15	35.1	1.9	0.1
Biscuits and cakes	7	69.8	2.0	0.2
Pastry and Products	0	-	-	-
Cereal products	9	52.0	2.0	0.2
French fries	0	-	-	-
Potato products	6	339.3	2.6	0.4
Composite (food),	12	45.5	2.0	0.1
Coffee	0	-	-	-

Saudi foods were grouped into eight groups comparable to the established in the NDNS food groups. Table 5-5 summarises the Saudi infants' datasets and provides the mean parameter values. The highest mean value was potato products with 339.3 µg/kg.

Tables 5-6 and 5-7, estimated that potato products provide the largest percentage contribution towards acrylamide exposure, with estimated contribution percentages of 40.9%, 42.8%, 43.3% and 49.8% for the SN3481 children, SN6533 infants, SN6533 children and Saudi infants respectively. The potato products group also provided the largest percentage contribution towards acrylamide exposure in the whole NDNS SN6533 population, with an estimated mean contribution of 38.9%. The second highest percentage contribution was from French fries for SN3481 children and biscuits and cake group for SN6533 infants, SN6533 children and Saudi infants, with estimated mean percentage contributions of 23.0%, 19.0% 19.0% and 21.6% respectively. However, NDNS SN6533 whole population the second highest percentage contribution was coffee with 20.1%.

Potato products and biscuits and cake are a staple of the UK which are frequently consumed by the UK child population (van Klaveren *et al.*, 2006). The staple foods in Saudi Arabia are rice, bread and other starchy foods (Shommo *et al.*, 2014).

Although the coffee group has the third highest log mean acrylamide content (2.0 µg/kg, Table 5-5), coffee has a very low percentage contribution to acrylamide exposure among UK children and Saudi infants. Coffee is not consumed frequently by UK children or Saudi infants, thus explaining the low percentage contribution, as shown in Tables 5-6 and 5-7. Due to high levels of acrylamide in coffee group, represents the second highest percentage contribution to exposure due to the higher consumption of coffee among the whole population (Tables 5-6 and 5-7). The mean exposure of acrylamide for coffee was 20.1 µg/kg-bw/day, as shown in Table 5-7.

The top-ten most frequently consumed foods sorted by the amount consumed as opposed to the frequency of consumption in the NDNS (Appendix H) show for all age groups that the bread group was the most consumed food. This indicates that although bread contains a smaller amount of acrylamide compared with potato products, when consumed in high quantities it may contribute more acrylamide.

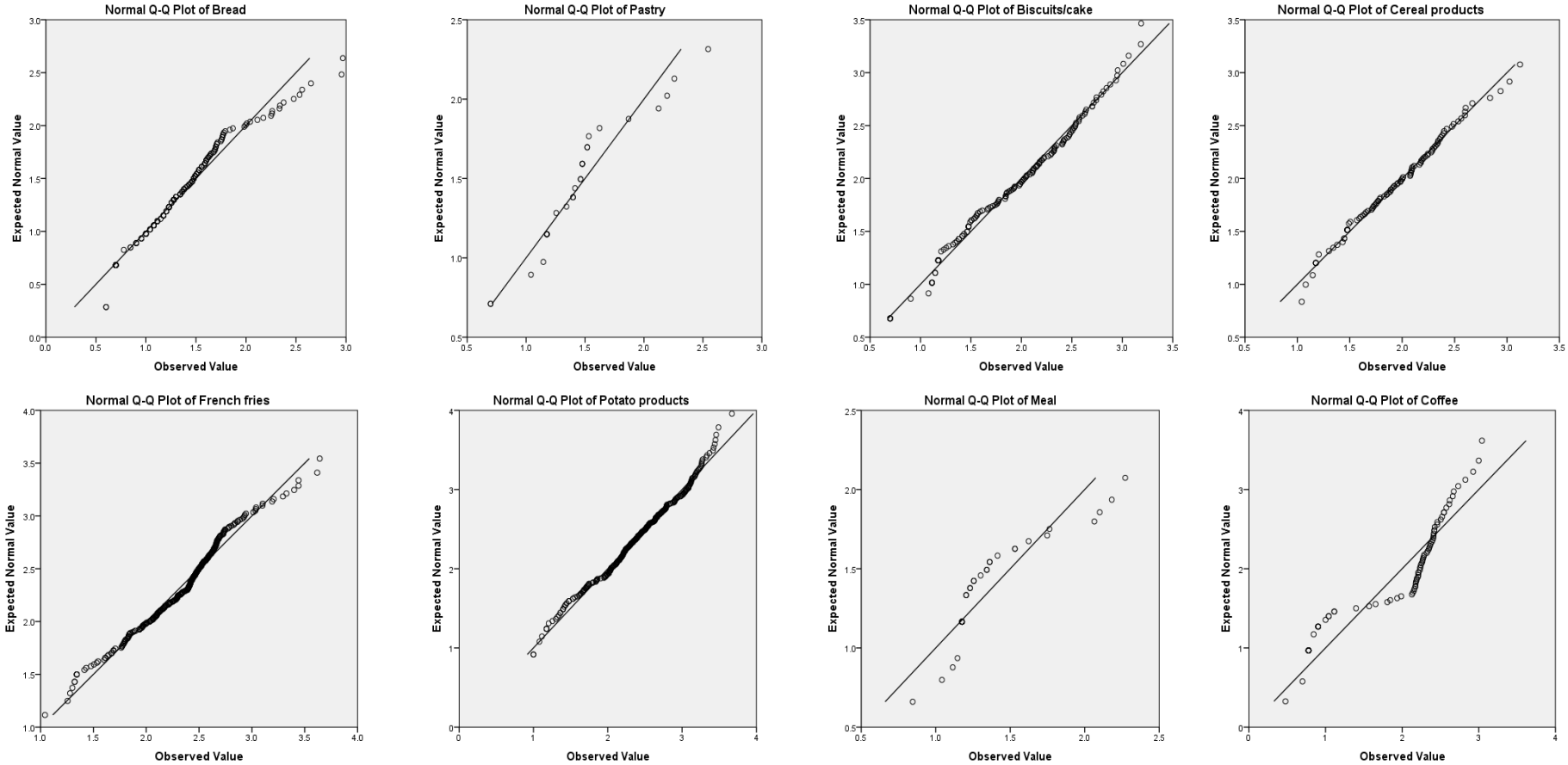


Figure 5-4: Q-Q plot for food groups content distributions (µg/kg) breads (169), pastry (28), biscuit and cake (151), cereal (99), French fries (309), potato products (308), composite (food), (37) and coffee (80) (acrylamide content taken from FSA, FDA and other studies).

Table 5-6: Acrylamide exposure percentage contributor (mean, SD, minimum and maximum) from food groups in Saudi infants and NDNS SN3481 children, SN6533 infants and children.

Food groups	Saudi infants'		SN6533 infant		SN3481 children		SN6533 children	
	Mean ±SD (%)	Min-Max (%)	Mean ±SD (%)	Min-Max (%)	Mean ±SD (%)	Min-Max (%)	Mean ±SD (%)	Min-Max (%)
Bread	8.2±1.0	1.7-34.6	8.5±0.6	6.7-10.1	6.4±0.2	6.0-6.9	8.3±0.4	7.2-9.4
Pastry	0.0-0.0	0.0-0.0	1.3±0.2	0.9-1.7	1.0±0.1	0.8-1.1	1.5±0.2	1.2-2.0
Biscuits/cake	21.6±3.1	5.2-38.7	19.0±2.1	15.5-26.0	19.0±0.7	17.3-20.9	19.1±1.3	16.6-23.2
Cereals	10.3±1.1	0.3-38.7	11.8±1.0	9.0-14.2	8.8±0.3	8.2-9.4	10.3±0.7	8.4-12.6
French fries	0.0-0.0	0.0-0.0	15.8±1.8	11.3-20.2	23.0±0.8	21.2-24.7	16.7±1.2	13.7-19.5
Potato products	49.8±1.8	46.9-83.7	42.8±3.5	32.0-50.8	40.9±1.1	38.5-43.5	43.3±2.1	37.4-50.3
Composite (food)	10.2±56.8	3.4-11.5	0.7±0.2	0.3-1.3	0.6±0.2	0.2-1.0	0.6±0.2	0.2-1.1
Coffee	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.4±0.3	0.2-2.2	0.1±0.2	0.0-1.5

Table 5-7: Acrylamide exposure percentage contributor (mean, SD, minimum and maximum) from food groups in NDNS SN6533 whole population.

Food groups	Mean ±SD (%)	Min-Max (%)
Bread	7.2±0.3	6.5-8.0
Pastry	1.6±0.1	1.2-2.0
Biscuits/cake	12.2±0.8	10.3-14.2
Cereals	4.2±0.2	3.7-4.6
French fries	15.3±0.9	13.3-17.7
Potato products	38.9±1.8	33.7-43.7
Composite (food)	0.5±0.2	0.2-0.9
Coffee	20.1±2.0	15.8-27.6

5.3.3 Exposure to acrylamide

Current estimates of dietary exposure to acrylamide, made by FAO/WHO, range from 0.4 to 3.0 and from 0.2 to 1.0 µg/kg-bw/day for children, adolescents and adults in the whole population (Food and Agricultural Organization and World Health Organization, 2002a). These estimates have been made using point estimation. Using actual dietary records in the form of the NDNS and the Saudi data, to be able to better estimate UK and Saudi infant exposure to acrylamide as well as quantify actual exposure estimates. The variability distribution can be described by referring to representative members of the population.

5.3.3.1 NDNS

For example individual exposure is calculated on a daily basis by summing exposures from consumed food items. Content within specific foods are randomly sampled from the appropriate concentration distribution, e.g. if bread is consumed then the content of acrylamide in the bread is assigned by sampling from the bread distribution, this content value is then multiplied by the consumed quantity providing the exposure.

All individual exposures were ranked in increasing order, producing a cumulative distribution function curve (CDF). From the CDF confidence intervals can be provided at each percentile consumer (95% CI). This approach addresses extremely high exposures which are in artifact of probabilistic approach and of debate able.

Tables 5-8 and 5-9 and Figure 5-5 show the results of all individual exposure after ranking them from smallest to largest. Then from these results the 2.5th, 50th and 97.5th percentiles were selected to show the variation and uncertainty in exposure estimates. From the CDF the median exposure (50th percentile) of each group can be determined. Also, the 2.5th and 97.5th percentiles for low and high consumers give an exposure estimate for each group. The median (50th percentile) estimates with 95% CI for acrylamide exposure among NDNS SN3481 children, SN6533 infants, children and the whole population are 0.128 (0.118-0.135), 0.78 (0.72-0.86), 0.430 (0.419-0.440) and 0.43 (0.42-0.44) µg/kg-bw/day respectively. The lower (2.5th percentile) estimates with CI 95% for acrylamide exposure among NDNS SN3481 children, SN6533 infants, children and the whole population are 0.014 (0.010-0.017), 0.334 (0.304-0.370), 0.015 (0.014-0.016) and 0.015 (0.014-0.016) µg/kg-bw/day respectively. Whereas the higher (97.5th percentile) estimate with CI 95% for acrylamide exposure among NDNS SN3481 children, SN6533 infants, children and the whole population are 9.847 (9.117-10.575), 9.134 (6.718-11.401), 0.430 (0.419-0.440) and 5.689 (5.286-6.087) µg/kg-bw/day respectively.

This analysis has identified little changes in acrylamide exposure when comparing children's data from 2008-12 with children's data from 1991-92, at 0.84 and 1.07 µg/kg-bw/day respectively.

Table 5-8: Acrylamide exposure cumulative distribution function of NDNS SN3481 and SN6533 children's population with fixed parameters (2.5th 50th and 97.5th percentile).

percentile	SN3481 children			SN6533 children		
	2.5th	50th	97.5th	2.5th	50th	97.5th
2.5th	0.010	0.014	0.017	0.014	0.015	0.016
50th	0.118	0.128	0.135	0.419	0.430	0.440
97.5th	9.117	9.847	10.575	5.347	5.620	5.922

Table 5-9: Acrylamide exposure cumulative distribution function of NDNS SN6533 infant's and whole population with fixed parameters (2.5^{th} 50^{th} and 97.5^{th} percentile).

percentile	SN6533 infant			SN6533 whole population		
	2.5^{th}	50^{th}	97.5^{th}	2.5^{th}	50^{th}	97.5^{th}
2.5^{th}	0.304	0.334	0.370	0.014	0.015	0.016
50^{th}	0.722	0.780	0.858	0.423	0.429	0.442
97.5^{th}	6.718	9.134	11.401	5.286	5.689	6.087

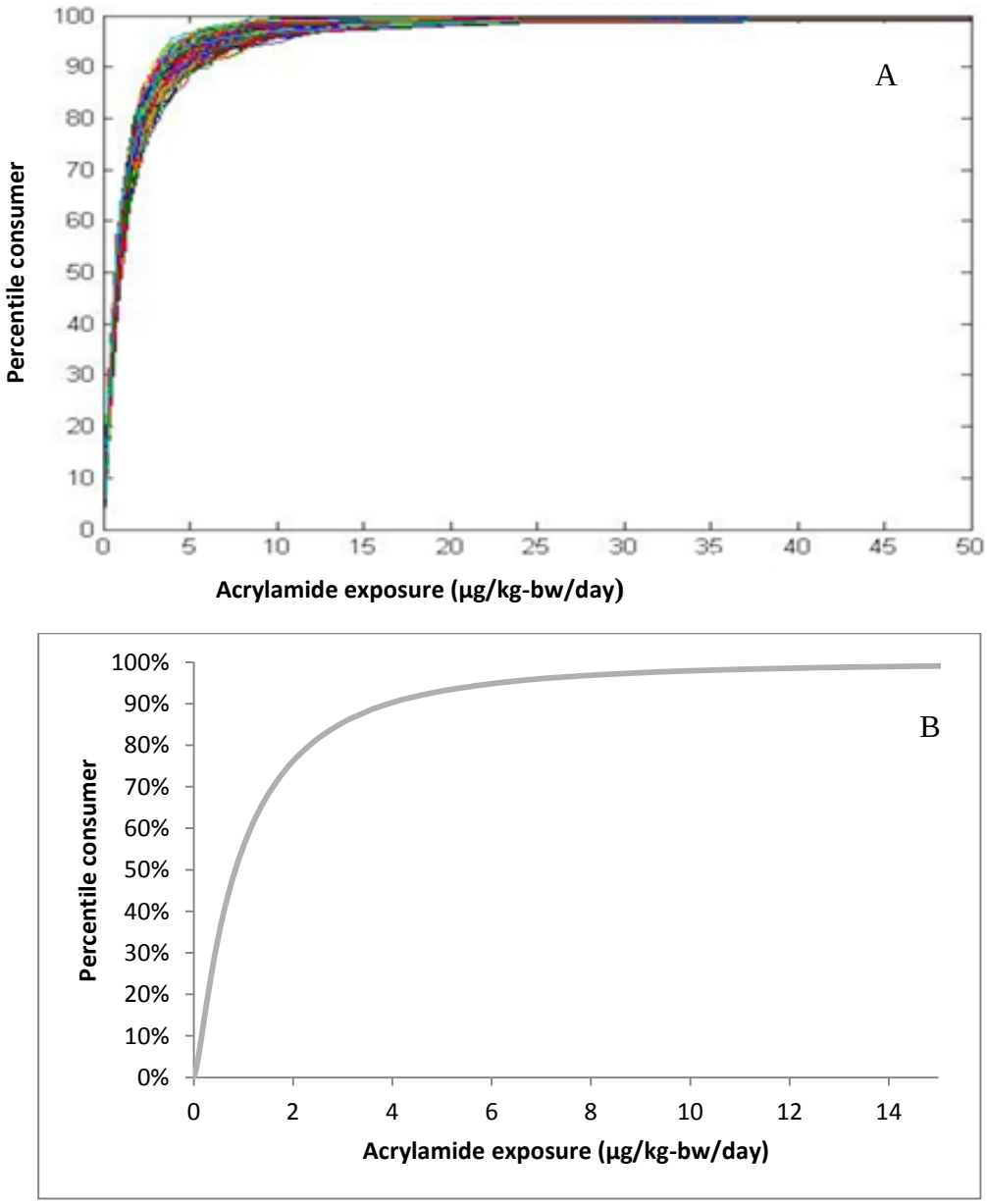


Figure 5-5: Exposure to acrylamide showing the 95th confidence interval for each consumer percentile in NDNS SN3481 children, A) the model output and B) zoom in with 50th percentile.

5.3.3.2 Saudi infants'

The percentile consumer of Saudi infants' exposure to acrylamide for the 150 infants was calculated using the acrylamide content value from each individual food and food group mean values. The results show the values are different below the 70th percentile, then they become similar. The median exposures for Saudi infants, including non-consumers were 0.06 and 0.22 µg/kg-bw/day for individual foods and food groups respectively. Whereas when the non-consumers were excluded the median exposures were 0.17 and 0.22 µg/kg-bw/day for individual foods and food groups respectively. The Saudi infants' median (50th percentile) exposure to acrylamide is 0.17 µg/kg-bw/day if the estimate is based on the use of individual foods or 0.22 µg/kg-bw/day if the estimate is based on the use of food groups (non-consumers were excluded) as shown in Figure 5-6.

When comparing the Saudi infants' median (50th percentile) exposure to acrylamide result from the food groups for consumers only (0.22 µg/kg-bw/day) with the result for NDNS SN6533 infants (0.78 µg/kg-bw/day), the result shows Saudi infants have less acrylamide exposure than UK infants of the same age. However, the difference in results due to the use of different acrylamide content references, less food variety for Saudi infants or the Saudi infants consumer participants being less in number than the UK participants for the same age group (≤ 2 years) so potentially less variation in consumption.

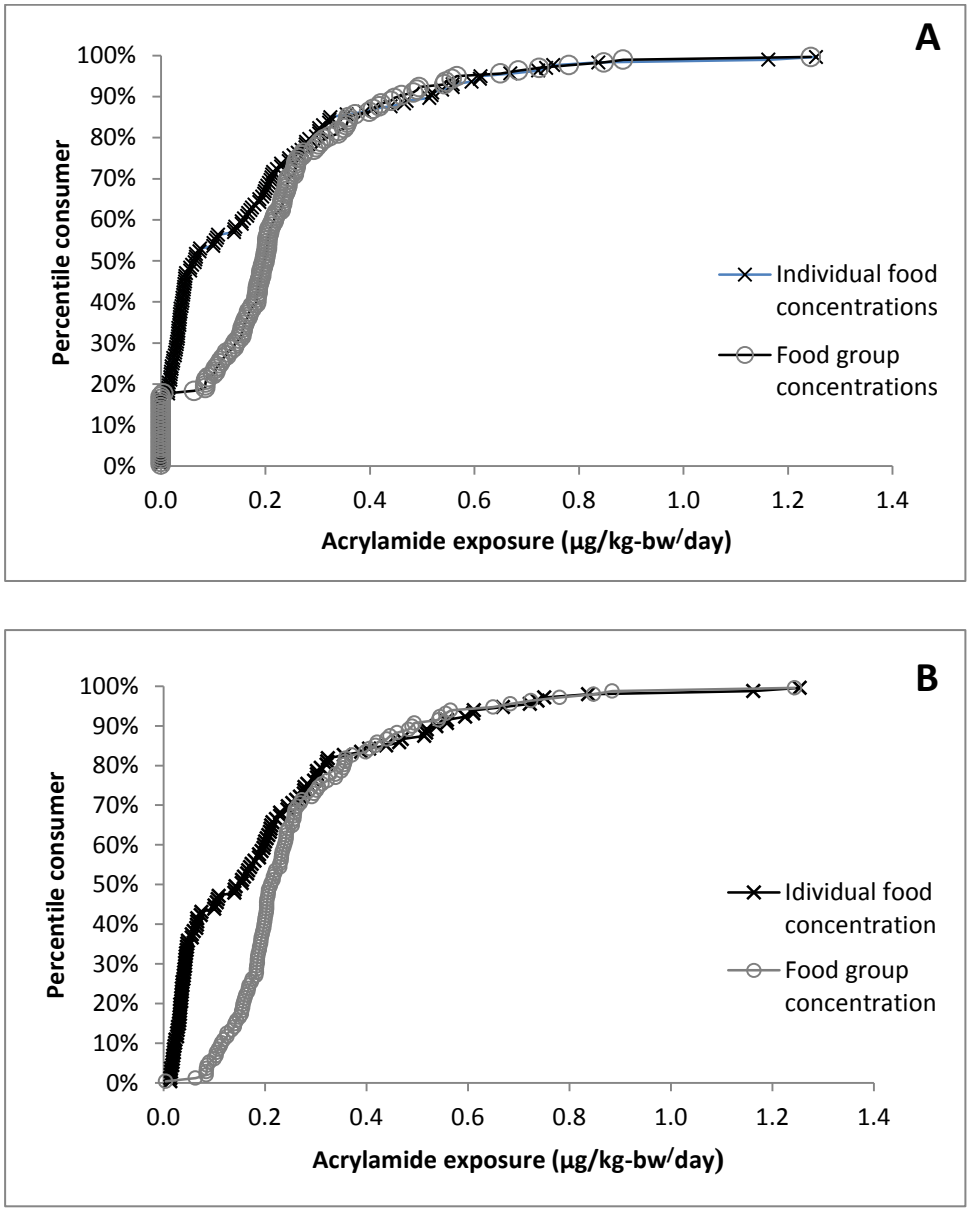


Figure 5-6: Percentile consumers of Saudi infants' exposure of acrylamide in Saudi survey A) consumer and B) non-consumer ((×) shows the median exposure of individual food content and (o) shows the median exposure of food group content).

5.3.3.3 Statistical analysis

A one-way between age groups analysis of variance (ANOVA) was conducted to explore the impact of age on acrylamide exposure. Participants were divided into five groups according to their age and survey (NDNS SN3481 children, SN6533 infants, children and the whole population and Saudi infants). There was a statistically significant difference at the $p<0.001$ level in acrylamide exposures for five age groups (except between SN6533 children and infant) $F(4, 10) = 201.01$, $p<0.001$. Despite reaching statistical significance, the actual difference in mean acrylamide exposure between age groups was large. The effect size, calculated using eta squared, was 0.99. Post-hoc comparisons using the Tukey HSD test indicated that the mean score for NDNS SN6533 infants (M=0.79 µg/kg-bw/day, SD=0.07) was significantly different from Saudi infants (M=0.22 µg/kg-bw/day, SD=0.00) and NDNS SN6533 whole population (M=0.43 µg/kg-bw/day, SD=0.01). Also, that for NDNS SN6533 children (M=0.84 µg/kg-bw/day, SD=0.06) was significantly different from that for NDNS SN3481 children (M=1.07 µg/kg-bw/day, SD=0.03) and the NDNS SN6533 whole population (M=0.43 µg/kg-bw/day, SD=0.01). There was no statistically significant difference in mean scores between NDNS SN6533 infants and children. This was due to the fact that the infant group (1-2 years) was included in the children's group (1-4 years).

5.3.4 Comparison with TDI and other reference values

As shown in Chapter 1, section 1.3.8, the lower value of LD_{50} is 1.7×10^3 µg/kg-bw (European Commission, 2002), which is 1000 times higher than the median exposure to acrylamide for all groups. Also, the NOAEL is higher (0.5×10^3 µg/kg-bw/day) than acrylamide exposure in all groups (Food and Agricultural Organization and World Health Organization, 2002a). In terms of dietary exposure to a chemical, if the exposure level does not exceed its relevant toxicological reference value, then the level should be acceptable. This applies to both acute and chronic exposure assessments (Food and Agricultural Organization and World Health Organization, 2005).

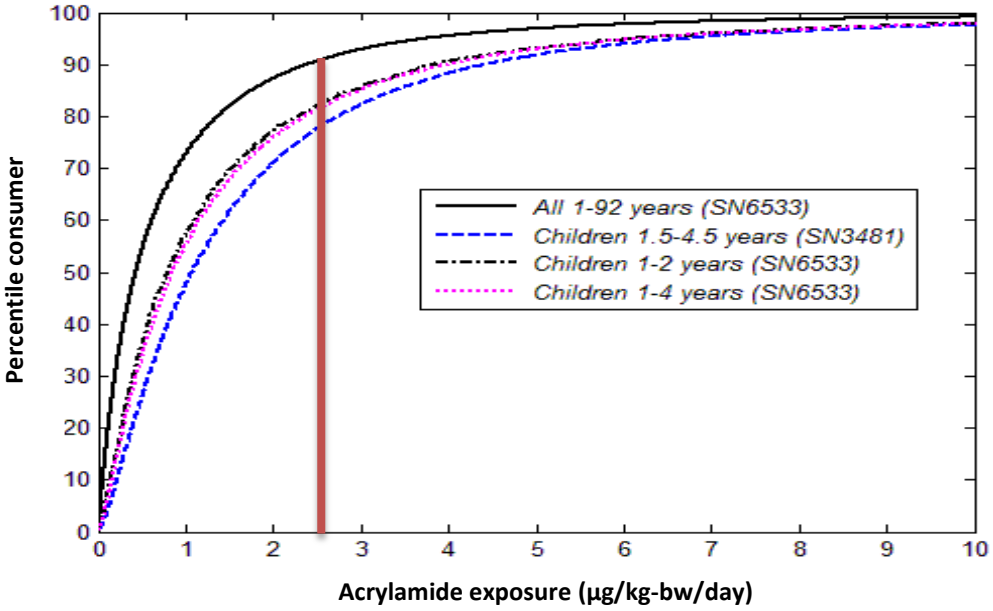


Figure 5-7: Acrylamide exposure (µg/kg-bw/day) for NDNS children and the whole population and TDI cancer (2.6 µg/kg-bw/day) (the red colour line indicates the TDI value).

Table 5-10: Assessment of acrylamide median exposure (µg/kg-bw/day) in the Saudi infants' and the NDNS population, and the percentage with cancer TDI (2.6 µg/kg bw/day).

Age group	**Median** (µg/kg-bw/day)	**TDI with median** (%)
Saudi infants'	0.2	8.1
NDNS SN6533 infant	0.8	30.0
NDNS SN6533 children	0.8	32.3
NDNS SN3481 children	1.1	41.2
NDNS SN6533 whole population	0.4	16.5

(Statistical difference within age groups $p<0.001$)

Figure (5-7) shows median acrylamide exposure (µg/kg-bw/day) for UK consumers, NDNS SN3481 children, SN6533 infants, children and the whole population and the TDI cancer value (2.6 µg/kg-bw/day). This figure (5-7) allows estimation of the proportions of individuals who may be at potential risk from exposure exceeding the TDI cancer value (2.6 µg/kg-bw/day). It is also possible to identify the frequency of exposure days where risk occurs and by how much individuals may exceed the TDI. Table 5-10 shows the median exposure value and the TDI cancer value percentage in Saudi infants, NDNS SN3481 children, SN6533 infants, children and the whole population. In general, the results show the acrylamide exposure for all groups is less than the TDI for at least more than 50%. When comparing between UK children (≤ 5 years) the results show a decrease in the potential risk from acrylamide exposure at 41.2% and 32.3% for children of SN3481 and SN6533 respectively as shown in Table 5-10. Also, the results shows the lowest risk percentage was for Saudi infants at 8.1%, being three times less than the NDNS SN6533 for the same age group (≤ 2 years) and one time less than the SN6533 whole population.

According to the Office of National Statistics (2013), the total UK population is 64.1 million person, who have an average life expectancy of around 82 years. Using the assumption those age groups are equally proportioned, this is quick estimate for the number of people of each age, the whole population is divided by the life expectancy, which results in 781,707 for each year of age. So for children aged from 1 to 4 years old the estimate is 2,345,122 persons. Twenty five percent of those children are exposed to acrylamide levels higher than the cancer TDI (2.6 µg/kg-bw/day) (Tardiff *et al.*, 2010), which means 781,707 children may be at risk of exceeding TDI for acrylamide, as illustrated in Figure 5-7.

The Central Department of Statistics and Information (2010) has shown that the total Saudi population amount to 29,195,895 people and their life expectancy is on average (2006-2010) 72.8 years. The total number of infants aged ≤ 2 years is estimated to be 802,085 persons. These infants are not exposed to high levels of acrylamide due to the highest exposure percentage being less than the cancer TDI (2.6 µg/kg-bw/day).

5.4 *Discussion*

According to WHO (2000), nutrition is important throughout life, from fetal development to the elderly stage. Childhood nutrition affects an individual's health in future (Baker-Henningham *et al.*, 2004). The importance of estimating acrylamide allows the levels to which the UK and Saudi Arabia infants and children are exposed due to the consumption of staple foods. As illustrated in Chapter 1, acrylamide occurs in most starchy foods and these foods are staples in many countries.

In this study the acrylamide exposure estimated for Saudi infant and NDNS infant, children and whole population using 24-hours recall (3-day intake) and dietary record (4-day intake) can be compared. According to Whitton *et al.* (2011) there is no difference in the intake patterns of individuals between three and four day dietary data, since same food groups (different foods) are consumed.

In this study, not all foods consumed by Saudi infants were analysed due to primary knowledge that they did not contain acrylamide or that the level of acrylamide was below the LOD, for example, all type of milk and milk products, and raw vegetable and fruit. This was similar to method employed by Matthys *et al.* (2005) whom estimated acrylamide exposure among Flemish adolescents by analysing 150 food items using LC-MS. They also anticipated some foods did not contain acrylamide and were not analysed.

There is much debate about how food items should be grouped, as this can create uncertainty in the results due to variation in acrylamide level between foods in a group. In this study, eight food groups were chosen for placement of relevant food items. The importance of food groups is displayed when estimates are produced; assuming parameters are calculated for the distributions for all foods within the same group. Using distributional content data will overcome some of the problems caused by food groupings. To improve reliability, food items need to be studied further to obtain information about levels of acrylamide. Boon *et al.* (2005) differentiated between spiced biscuits and normal biscuits to study if the separated gave significantly different results. In this study we differentiate between potato products and French fries. Since French fries are routinely consumed in isolation and in large quantified ranged from 23.0% to 19.0% for NDNS age groups. Also,

Table 5-1 shows that most similar studies have divided these two groups and justified this separation similarly.

This study was used NDNS and Saudi data. The NDNS data is real data which can respects the actual correlations between consumed foods and it provides realistic consumption quantities and biometric data. Additionally, NDNS data collected from around UK and Ireland. Saudi infant data is also real data collected from Saudi Arabia.

In this study, in each food group, the variation in acrylamide levels was high and log-normally distributed (see Appendixes E and G) for both Saudi and NDNS food which is similar to all other studies. Among the eight food groups created in the NDNS surveys, the food group of potato products contained the highest acrylamide mean level, at 486.8 µg/kg, followed by the French fries group with a mean value of 343.5 µg/kg. In the Saudi infants' food groups the highest acrylamide mean level was in the potato products group (potato crisps and popcorn), at 339.3 µg/kg, followed by the biscuits and cake group at 69.8 µg/kg (as shown in section 3.3.6.6). These results are in agreement with those of Mojska *et al.* (2010) whom estimated the acrylamide exposure of Polish population using 225 foods taken randomly from all over Poland, the level of acrylamide was found between 11 and 3647 µg/kg with acrylamide mean values ranging between 100 and 300 µg/kg.

This study results showed the acrylamide level Arabic white bread and infant cereal being found as ND, as shown in Chapter 3, and the bread group contribution to acrylamide being low. In contrasts, El-Ziney *et al.* (2009) found that Arabic white bread and infant cereal were the highest contributors to acrylamide exposure among Saudi adults and infants, at 11.3 and 3.45 µg/day respectively.

In this study the potato products group and the biscuits and cake group were the major contributors in all NDNS and Saudi groups, with percentage contribution of 40.9%, 42.8%, 43.3% and 49.8% for SN3481 children, SN6533 infants, SN6533 children and Saudi infants respectively. The French fries group was one of the major contributors in NDNS children and infants. These results are in agreement with the vast majority of published results, such as EFSA (2011a) which found that potato products are the most important contributor to acrylamide exposure in children due

to the high consumption of these products by children and their high acrylamide level. The second largest contributors to acrylamide are bread and biscuits. This was in agreement with Boon *et al.* (2005), who studied Dutch children aged 1 to 6 years and the whole population, found that French fries (28% and 31%) and potato products (18% and 15%) were the major acrylamide contributors, followed by biscuits in children, at 17%, and coffee in the whole population, at 13%. Also, Saleh and El-Okazy (2007) found that the potato products group contributed most in Alexandria children (≥6 years), at 57.2%. The French fries group and the bread group also contributed to the total acrylamide content, at 14.6% and 11.1% respectively.Croft *et al.* (2004) found that the potato products group was the largest contributor in Australian children (≤ 2-6 years), at 15%. Konings *et al.* (2003) found that the potato products group was the largest contributor in Dutch children (1-6 years) and the whole population (1-97 years), at 40% and 31% respectively. This was followed by French fries, at 18% and 21% respectively, and then Dutch spiced honey cake, at 20% and 16% for both groups respectively.

In this study, the bread group in NDNS infants and children was the sixth largest acrylamide contributor (less than 10% from total acrylamide), because the bread group contains a low amounts of acrylamide but is the most consumed food among children (1-5 years), as Cengiz and Gündüz (2013) showed in Turkish children and Matthys *et al.* (2005) showed in Flemish adolescents. These findings are in agreement with the finding in present study. However, it is in contrast with Hirvonen *et al.* (2011) conclude that the mean sources of acrylamide were due to foods low or moderate in acrylamide levels and due to consumption of large amounts of these foods and the high frequency their consumption. The food contribution to acrylamide exposure depends on food consumption habits and these vary between countries (Cengiz and Gündüz, 2013).

The food items consumed in large quantity tends to be mean items such as bread, so although acrylamide exposure from those will be less proportional contributor rather than the full item consumed. The bread group which has the most consumers (mean) contains levels of acrylamide 9 times lower than potato products group which is the largest contributor by acrylamide. The findings of Mojska *et al.* (2010) are in contrast with this study's results. They found that the bread group was the highest

consumed and that which gave the highest acrylamide exposure in Polish people (1-96 years) and children (1-6 years), at 45% and 31% respectively.

In this study, coffee was found to be one of the major contributors in the NDNS over whole population group, which is in agreement with Hirvonen *et al.* (2011) who found the most important source of acrylamide in Finnish adults from six groups (190 foods) was coffee at 39%, potato crisps at 16%, and French fries and bread at 11% for each group.

In this study, the contribution of coffee was 19% for the whole population and most coffee was consumed by adult. The potato products group and French fries group contributed towards acrylamide level 16% and 26% respectively. Mills *et al.* (2008) estimated the acrylamide exposure in UK adults by using NDNS data; they found that the main acrylamide contributor was white bread due to the high consumption in the UK.

In this study, when calculating the dietary exposure of a population, it is vital that the most representative exposure estimate is selected. In this study deterministic point estimations and probabilistic methods were used to determine the exposure of UK children and the whole population to acrylamide, whereas in Saudi data only deterministic method was used due to sample size. Probabilistic methods tend to provide a more realistic estimate as actual dietary information and consumer information such as body weight and acrylamide content data are used. Unlike deterministic exposure estimates, using probabilistic assessment accounts for variations within a population consumption, body weight, consumption behavior and food grouping. Hirvonen *et al.* (2011) and Mojska *et al.* (2010) used means of a probabilistic Monte Carlo simulation techniques to calculate acrylamide exposure in the Finnish and Polish populations respectively in adults and children. Whereas in the Dutch population acrylamide exposure was estimated by a deterministic approach by Boon *et al.* (2005).

Uncertainty in content data can affect acrylamide estimation when using a probabilistic method, but this does reflect the stat of knowledge of the problem and so may identify where additional measurement of samples are required. In the

present study the effect of uncertainty of parameters estimated is not fully explored the objective being of consumption of mean estimates of UK and Saudi populations.

In this study the ND value was set as half of LOD, as explained in section 3.3.6.6. Also, as shown in Figure 5-6, a comparison was made between acrylamide exposure in consumers and in all infants using either individual food or food group contents. In the case of Boon *et al.* (2005) the median did not change significantly when they assumed the LOD result was taken as means zero value.

In this study the results for individual foods show lower acrylamide exposure in non-consumers than consumers (0.06 and 0.17 µg/kg-bw/day respectively), whereas for the food group acrylamide exposure in non-consumers was similar to that of consumers (0.22 µg/kg-bw/day). That as result of with food group acrylamide mean is fixed. This result is close to non-consumer individual foods. Most studies have used food groups for acrylamide estimation, although some of them have used a specific food (highly consumed food in the country) as a group and some studies have used individual foods, such as Cengiz and Gündüz (2013), Boon *et al.* (2005) and El-Ziney *et al.* (2009).

In this study the median acrylamide exposure of NDNS infants and Saudi infants was estimated to be 0.78 and 0.22 µg/kg-bw/day respectively. The Saudi infants finding was below level in other studies, whereas in the NDNS infant result was higher than that of who found acrylamide exposure in Australian infants (≤ 2 years) to be 0.45 µg/kg-bw/day. Also, both groups were below the median acrylamide exposure of Finnish children (3 years), which was at 1.01 µg/kg-bw/day (Hirvonen *et al.*, 2011). Turkish children's (1-3 years) median exposure to acrylamide was estimated to be 1.20 µg/kg-bw/day, which is higher than this study's finding for Saudi and UK infants' acrylamide exposure (Cengiz and Gündüz, 2013).

In this study, the median acrylamide exposure in NDNS SN3481 and SN6533 children was estimated to be 0.128 (0.118-0.135) and 0.430 (0.419-0.440) µg/kg-bw/day respectively, which is similar to the finding for Dutch children's (1-6 years) exposure, with the median and 97.5th percentile being 1.04 and 1.30 µg/kg-bw/day (Konings *et al.*, 2003). Also, Croft *et al.* (2004) found acrylamide exposure in Australian children (2-6 years) to be 1.20 µg/kg-bw/day, which is higher than the

NDNS exposure. However, NDNS children's median exposure was higher than Mojska *et al.* (2010) estimation for exposure in Polish children aged 1-6 years, which was 0.35 µg/kg-bw/day.

In this study, the NDNS whole population exposure was estimated to be 0.43 µg/kg-bw/day, which is in between the Polish population (1-96 years) median acrylamide exposure (0.27 µg/kg-bw/day) (Mojska *et al.*, 2010) and the Dutch whole population (1-97 years) median and 97.5th percentile, at 0.48 and 0.80 µg/kg-bw/day respectively. However, acrylamide exposure in the Alexandria population was found to be higher than the NDNS, at 1.75 µg/kg-bw/day (Saleh and El-Okazy, 2007). The mean acrylamide estimation and consumption for several age groups in different countries with the number of participants and assessment methods are shown in Table 1-6.

This study found that children aged 1-2 years old have lower exposure compared with children aged 1-4 years old (from the same survey). This is in contrast with Cengiz and Gündüz (2013) results, which found that Turkish children aged from 1-1.5 years had a higher acrylamide exposure level compared with children aged 2-3 years. The lower acrylamide exposure in children aged 1-2 years may be the result of their food being based on milk or boiled food, rather than baked or fried food. For the same reasons the lower level of acrylamide exposure in Saudi infant population. Variation in results including different subpopulations, applying different methodologies and including more food items (Matthys *et al.*, 2005). Boon *et al.* (2005) have shown that

Calculations from this study estimate a mean exposure for the NDNS SN3481 children, SN6533 infants, SN6533 children and Saudi infants to be 2.03, 1.76, 1.70 and 0.17 µg/kg-bw/day respectively. Whereas mean exposure estimated for the whole population is 1.04 µg/kg-bw/day. The NDNS infants' and children's results were higher than the FAO/WHO estimates, whereas for the Saudi infants they are in agreement. The 97.5th percentile of the NDNS SN3481 children, SN6533 infants, SN6533 children, Saudi infants and the whole population had exposures estimated at 9.847, 9.134, 5.620, 0.720 and 5.689 µg/kg-bw/day, suggesting that UK infants and children are exceeding levels stated by FAO/WHO estimation. Food and

Agricultural Organization and World Health Organization (2002a) estimated a mean exposure of 0.20-1.00 µg/kg-bw/day for the general adult population, with exposure estimated to be two to three times higher for children when calculated on a body weight basis.

The results from this study show that the UK infant and child populations have a median and upper exposure approximately two times higher than those of the whole UK population. The upper estimate finding exceeds the FAO/WHO estimates for children's exposure to acrylamide.

This study results illustrate, high end child consumers are potentially exposing themselves to increasing the risk of detrimental health effects, especially the risk of cancer (TDI <2.6 µg/kg/day). From the results it was found that the upper exposure in UK infants, children and the whole population exceeded the TDI value by approximately five times for UK infants and children, and three times for the whole population. However, all UK groups are not exceeding the TDI for neurotoxicity (40 µg/kg-bw/day). For Saudi infants the upper acrylamide exposure was (1.3 µg/kg-bw/day) lower than the TDI.

This study result conclude the importance of further explore the diets of Saudi and UK infants and children in order to understand why exposure levels may lead to these levels. It may be possible to compare estimates for children's populations between countries and the main diets of these countries to determine the main factors influencing exposure of Saudi and UK infants and children (van Klaveren *et al.*, 2006).

5.5 *Conclusion*

Through the different exposure assessment methods used in this study, it can be concluded that probabilistic modelling provide a more realistic and informative approach for assessing dietary acrylamide exposure. Unlike deterministic methods, probabilistic assessments can be used to estimate how many children may be potentially at risk, by how much and how often they exceed critical limits. Estimates from this study can be used for future decisions and policies to reduce exposure

levels and lower potential health risks. From these estimates it will be possible to tell how exposure changes with age, which may help to estimate chronic risks.

The potato product group was the highest contributor in Saudi infants and all UK age groups. Saudi infants' exposure was lower than that for NDNS infants, children and the whole population. The upper percentile of acrylamide estimation was higher than the TDI cancer value for all UK groups.

Children are at higher risk acrylamide exposure in comparison with adults, due to lower body weight and higher consumption per kilogram body weight. However, this risk can be reduced by changing food habits and reducing the acrylamide content in frequently consumed foods. Consumers should avoid foods with high acrylamide levels and reduce the temperature when they fry or bake starchy food to reduce the formation of acrylamide. The hypotheses of this chapter "Saudi and UK infants' exposures to acrylamide are high" and "the UK children population's exposure to acrylamide has decreased from 1992 to 2011" have been conformation for UK infant and children, whereas, the Saudi infant exposure is high was rejected

5.6 *Limitation of the study*

Estimation of acrylamide exposure is a challenge due to the various sources of uncertainty and measurement error. Variance in acrylamide levels in some food groups was found to be large, although it can be minimized be analysing a higher number of samples from the same product and other products in the same group. Home cooked food can also vary because acrylamide formation is affected by many conditions, such as cooking time, temperature and other factors, as mentioned in Chapter 1, section 1.3.5.

Self-reported dietary recording is an important limitation. Also, participants did not record whether the food was toasted or baked to reach a brown colour. This point may increase the formation of acrylamide as mentioned in Chapter 3, section 3.3.6.7.1.

The number of Saudi participants (n=150) was small and this was the only data on food consumption available for use. Also, the limited number of food samples. In the UK, the NDNS provides the best representative consumption data.

Chapter 5: Acrylamide exposure

The problem is in the difficulty to reduce acrylamide in foods e.g. through processing without changing the food itself and not increase the level of another genotoxic compound, such as furans in coffee (Guenther *et al.*, 2010).

6 Chapter 6: General conclusion and future work

Acrylamide, which is one of the Maillard reaction products, is known as a probable carcinogen by the International Agency for Research on Cancer (IARC). It is found in food, food packaging, cosmetic additives and other useable things. It was first discovered in food in 2002 by the Swedish National Food Administration (SNFA) and the University of Stockholm, who published a list of certain foods that are processed at high temperature. Asparagine and reducing sugars are the main causes of acrylamide formation in certain conditions, such as low food moisture, low food pH and high cooking temperature. These factors are found in the heating process for most starchy foods, examples being potato crisps, French fries, bread and biscuits.

Most studies into acrylamide have focused on either acrylamide analysis or acrylamide exposure estimation or effect of acute doses on animal or cell behaviour. This thesis, for the first time, has explored the analytical aspect of acrylamide in Saudi infant foods and the estimation of Saudi infants' exposure to acrylamide. Also, estimates of UK infants' and children's exposure to acrylamide have been undertaken through analysis of National Diet and Nutrition Survey (NDNS) data. Chapters 2 and 3 dealt with acrylamide analysis of Saudi infant food using high-performance liquid chromatography (HPLC) (Chapter 2) and liquid chromatography mass spectrometry (LC-MS) (Chapter 3) analytical methods. Carbohydrates (free sugar) and amino acid (total amino acid) have been determined as the main factors leading to acrylamide formation (Chapter 4). The findings presented in Chapter 5 were obtained from an estimate of acrylamide exposure using Saudi infants' diets and NDNS data. To estimate acrylamide exposure among UK infants, children and the whole population, NDNS data were used and compared with data for Saudi infants. The aim of this chapter is to summarise the main findings and suggest future work.

6.1 *Acrylamide determination in food*

6.1.1 Methods

There is no AOAC method for acrylamide determination and extraction with different food matrices. Published studies have used several extraction, clean-up and

analysing methods. In this study, acrylamide was extracted from Saudi infants' starchy foods by using water due the solubility and stability of acrylamide in water and the samples were shaken to make acrylamide extraction easer. This was followed by centrifugation and then the samples were cleaned-up with two SPE cartridges (Oasis HLB and MCX). This method is similar to the methods used in other studies. Rosen and Hellenas (2002) state that acrylamide occurs in heated starchy foods (fried and baked). Acrylamide was extracted from starchy foods (potato crisps, crisp bread, breakfast cereal and biscuits) by using water and then centrifuged and cleaned-up with SPE cartridges. After that the extraction was analysed using LC-MS/MS. They found the contents of acrylamide in food samples ranged from 300 to 1600 µg/kg. GC-MS was used to analyse the same samples and the results were in agreement with LC-MS/MS (no figure was provided in the article). Following this, Mottram *et al.* (2002a) and Mottram *et al.* (2002b) studied acrylamide formation in heated starchy food; they found that asparagine and reducing sugar are the main factors leading to acrylamide formation.

In this study acrylamide in the extracted food samples were detected using HPLC-UV or LC-MS methods. Several changes were made to sample extraction to reduce the interference of components by centrifuging and cleaning-up (SPE cartridges), and the gradient elution program, flow rate and solvent mixture were modified, although there was no good resolution for the acrylamide peak using HPLC-UV methods. The acrylamide peak was eluted with other peaks (a lot of interference) so the acrylamide could not be resolved, even when the peak was spiked with standard acrylamide. Therefore, this study concludes based on published and experimental data that HPLC-UV is not a good method for acrylamide determination. This was in agreement with Paleologos and Kontominas (2005), Wang *et al.* (2008), Longhua *et al.* (2012) and Wang *et al.* (2013) all used HPLC for acrylamide determination in foods. However, Wang *et al.* (2008) determined acrylamide in deep fried Chinese foods. The results show there was acrylamide content in foods ranging between 27 and 198 µg/kg. The chromatograms from the Chinese study appear to show multiple peaks and interfering compounds and when they spiked with acrylamide standard other peaks were increased as well, making it difficult to say whether the estimation was accurate. Also, the results for the food samples were at the lower end of the

standard curve, possibly lowering the precision of the measurement. They improved the methods a few years later but the sensitivity and resolution of acrylamide did not improve. Wang *et al.* (2013) established another HPLC method for acrylamide determination in deep fried Chinese foods using the same standard contents. The difference was they used solvents (Carrez 1 and 2) to precipitate protein in sample extraction and they changed the gradient elution program, flow rate and solvent mixture. The results showed acrylamide content in foods ranged between 86 and 151 µg/kg. Both studies used a standard range 10 times higher than the food content. Therefore, LC-MS was used to determine acrylamide.

Acrylamide molecular weight is small, thus difficult to detect. For that, the internal standard was used to improve acrylamide detection using LC-MS due to identifying, quantifying and verifying of non-detection. Also, acids such as formic acid were used to improve acrylamide ionisation. In this study acrylamide in the extracted food samples was detected using a modified LC-MS method with acetonitrile (4%) and formic acid (0.1%) to give a good chromatogram. In some chromatographs extra peaks were found with different retention times. These peaks were from unknown components in the food extraction. Thus, acrylamide C_{13} was used as an internal standard in the analysis to indicate acrylamide availability with the appearance of other unknown components, acrylamide quantitation and identification of non-detection acrylamide.

There have been three studies based in Saudi Arabia analysing acrylamide in food items. Chapter 3 shows how the results of this study have some similarities and differences with other Saudi studies. In the El-Assouli (2009) study, high acrylamide levels were found in Pringle's crisps, Samoli bread, sliced bread and white Arabic bread, whereas in this study acrylamide was not detectable in those samples. It is possible that the processing or formulation of those products changed from 2009, or differences may due to analytical methods. GC-MS was not carried out in the present study. El-Assouli (2009) studied acrylamide anaylsed 23 food items commonly consumed in Saudi Arabia (including French fries, potato crisps, corn crisps, cereal, bread and coffee) using GC-MS. Wistar rats were fed these foods at a final acrylamide content of 0.4 µg/g in food for 30 days.

This study results were in contrast with El-Ziney *et al.* (2009) study results, as acrylamide could not be detected in white Arabic bread whereas in brown bread the mean was 90 µg/kg, explained by a high level of asparagine in brown bread. Whereas El-Ziney *et al.* (2009) study were analyse acrylamide levels in a selection of traditional Saudi foods (26), infant milk powder (6) and infant cereal (4). They Found the acrylamide level in Arabic bread, white (90 µg/kg) and brown (40 µg/kg).

In this study the same sample vial did not give good results (acrylamide peak was dissolve with another peak) with HPLC-UV, but with LC-MS a resolved peak for acrylamide was obtained which suggest that HPLC-UV not appropriate method for acrylamide detection. Gokmen *et al.* (2005) compared HPLC and LC-MS methods for analysing acrylamide in French fries and potato crisps. They concluded that the HPLC method is not a sufficient method for acrylamide detection in foods, especially in complex matrices due to acrylamide's high polarity and poor retention, as well as the appearance of interference components. For this reason MS is preferred for acrylamide analysis.

In this study the acrylamide was quantified in the food consumed by Saudi infants for the first time. Results of acrylamide quantification using the modified LC-MS method showed that the level of acrylamide in Saudi infant food varied between food samples in the same food group. The acrylamide levels in Saudi infant food varied from non-detectible to 755.5 µg/kg. The LOD and LOQ were 17.2 and 52.2 µg/kg respectively. Acrylamide in food was found to be low in content, but an increase in the concentration was associated with higher interference and lower recovery. Roach *et al.* (2003) describes in detail the use of LC-MS/MS for acrylamide quantification. The method used for acrylamide extraction was similar to this study's extraction method. Jiao *et al.* (2005) developed an LC-MS method for acrylamide determination in infant powdered milk and infant foods in jars. The extraction, clean-up methods and internal standards were similar to this study's. The results showed non-identified acrylamide peak and unknown interference components as this study.

This study found the levels of acrylamide in infant cereal and biscuits were not detectible (Chapter 3). Whereas, Zhang *et al.* (2005a) LC-MS/MS analysed results showed a high acrylamide level in one sample of infant biscuits (1568.9 µg/kg).

In this study results show variation between samples in food groups is great, which reflects the differences in food ingredients, heating methods, temperature and time of heating, as well as other treatments (fermentation or adding of acid). In this study a high level of acrylamide in Saudi infant food was found in potato crisps, with a range varying from ND to 755.5 µg/kg; biscuits and cakes ranged from ND to 208.2 µg/kg. The acrylamide recovery in food groups ranged between 84.3% and 106.1%. To use this data for estimate the exposure of acrylamide in Saudi infant, the ND value was assumed to be zero, half the LOD or the LOD value. However, half LOD value was used for estimation as a result of small differences with other values as shown in the World Health Organization (2009) and European Food Safety Authority (2011b) reports.

In conclusion, LC-MS method is an appropriate method for acrylamide determination. Using acetonitrile and formic acid for analysing make it easier.

6.1.2 Factors

In 2009, Food Drink Europe released a toolbox aimed at reducing acrylamide formation in the potato, cereal, coffee and cereal based food industries. The regulations recommended for industry were: 1) choose a raw material with less reducing sugar and/or asparagine; 2) control storage conditions to prevent an increase in the reducing sugar level; 3) reduce the food surface by using steam frying; and 4) cook to a golden colour (175 ºC). They have also made general recommendations for preparing food at home (Food Drink Europe, 2011).

Differences between potato products and cereal products in terms of the level of asparagine and reducing sugar, processing methods and other factors are the main cause of acrylamide variation in foods. Additionally, some foods, such as infant cereal, tend to have reduced acrylamide levels due to the food industry choosing raw materials that contain less reducing sugar and asparagine, or new processing techniques. It is expected that the industry may reduce acrylamide by lowering

temperatures or adding certain treatments to prevent acrylamide formation during heat treatment. In this study, infant cereals were found to have high levels of soluble sugar, but not found to have acrylamide, even when stored or heated. This could indicate the lack of asparagine or the presence of inhibitors of the Maillard reaction in these products. Ascorbic acid is a good candidate as the infant cereals are fortified with vitamins and minerals. In this study, different methods of sugar analysis were used, giving different results. Using a standardised method for sugar determination is important to investigate the role of sugar as a factor in acrylamide formation. Asparagine was not measured in this study due to acrylamide not detected in infant cereal. HPLC or GC-MS can be used to analyse asparagine content.

In this study, soluble sugars and total protein were analysed in five types of infant cereal commonly used in Saudi Arabia. The results of three different methods showed the presence of reducing sugar and protein in the infant cereals analysed. This result means there is the possibility of formation of acrylamide in certain conditions.

This study results showed toasted bread increased acrylamide level. This increasing correlated with dark colour. The acrylamide content in untoasted bread was not-detectable, whereas, toasted bread contained different levels of acrylamide depending on the toasting level and bread colour. For example, acrylamide contents in L'usine (white loaf) were 81.3 and 219.0 µg/kg for light and medium toasted (colour). Also, the acrylamide content varied between the three types of white loaf bread when toasted similarly with 83.3, 90.9 and 219.0 µg/kg for Aljazei, Al-rashed food company and L'usine respectively. This result was in agreement with Ahn *et al.* (2002) who showed that sliced bread toasted for longer had increased acrylamide levels. This study illustrated that the acrylamide contents were different in food made from white wheat with the range between ND to 208.2 µg/kg, for biscuit, cake, breakfast cereal, noodles and macaroni. Furthermore, the acrylamide contents were different between potato crisps, popcorn and were non-detectible for oats and rice.

In this study different bread type such as Samoli, white Arabic bread, brown Arabic bread and croissant have different acrylamide contents, even between the same types

or from type to type with acrylamide content varying from ND to 103.9 µg/kg. These differences may be as a result of different content of reducing sugar and asparagine in the raw material and after fermentation as shown in Fredriksson *et al.* (2004). Fredriksson *et al.* (2004) aimed to reduce acrylamide formation in fermented whole grain wheat flour using yeast to reduce asparagine content. Asparagine level was reduced by 40% and 90% after 1 and 2 hours of fermentation respectively. Acrylamide level formation dropped from 180 to 24 µg/kg for shorter and longer fermentation respectively.

This study results show different acrylamide level between white and brown Arabic bread between ND to 103.9 µg/kg. These differences are likely to be of high asparagine level in brown bread (whole wheat). This was also observed by Capuano *et al.* (2009). They studied the effect of flour type (wheat, whole wheat and rye) in acrylamide formation in crisp bread by analysing different factors, such as asparagine and reducing sugar. They found higher levels of asparagine in whole wheat and rye, at 0.45 and 0.55 g/kg respectively. Reducing sugar was 18.0, 21.2 and 15.4 g/kg for whole wheat and rye respectively. They found high acrylamide levels in whole wheat and rye as a result of asparagine level.

In conclusion, there are many different factors affecting acrylamide content. For that to have reliable acrylamide data, samples should take from many different brand, different session, different patch, repeated many times and repeated all the time away.

6.2 *Acrylamide exposure*

It is important to estimate acrylamide exposure due to most staple foods in the Saudi Arabia and UK containing acrylamide, such as bread and potato crisps. This study did not analyse foods where it is well known that an acrylamide level cannot be detected. To estimate acrylamide exposure needs individual information (age, bodyweight and sex) and food consumed by the individual and food content. The food grouping may create uncertainty in the results and there is no specific grouping agreement found across acrylamide studies. This study's groupings numbered eight, based on similar food items being in one group. The highest mean acrylamide

content was found in potato products, at 486.8 and 339.3 µg/kg respectively for the food used to estimate acrylamide exposure in NDNS and Saudi infants'.

Mills *et al.* (2008) estimated UK adults' acrylamide exposure using NDNS data (2000) and compared it with Irish adults' data using the North/South Ireland Food Consumption Survey (1997-1999) and the European Union's acrylamide database and Dublin Public Analyst's Laboratory of acrylamide levels in food. They grouped the food items together to combine consumption data with the acrylamide database using Codex classification (based on nutrients). Acrylamide levels had shown included the mean, minimum and maximum. For the UK data the FSA semi-probabilistic in house intake model (statistical program) was used for estimating chronic acrylamide exposure in both populations, whereas for Irish data the probabilistic was used with another program. Monte Carlo was used for acrylamide dietary exposure disruption. The results showed that the mean UK adults' exposure to acrylamide was 0.6 and 1.3 µg/kg-bw/day and the 97.5th percentile respectively, and for the Irish adults it was 0.6 and 14.8 µg/kg-bw/day and the 97.5th percentile respectively. They did not use all food sources of acrylamide in the Irish population and the number of groups was different between the UK and Ireland, at 10 and 6 groups respectively.

Boon et al. (2005) estimated acrylamide exposure among the Dutch population using the Dutch National Food Consumption Survey (DNFCS) (1997-98), with 6250 participants from the whole population (1-97 years), 530 participants being young children (1-6 years). The participants recorded weighted food over two consecutive days. Acrylamide levels were used from the IRMM database (Institute for Reference Materials and Measurements) and samples with a ND value were set as half LOD. Foods containing acrylamide were grouped into 14 different groups based on a Dutch food composition table. However, they separated spiced biscuits from the biscuit group due to differences in acrylamide level, and they differentiated between French fries (French fries group) and potato crisps and baked potato (potato products). A probabilistic model was used to estimate chronic and acute acrylamide exposure. One hundred thousand iterations were run by the Monte Carlo program to multiply consumption data and acrylamide level (non-consumers were excluded). The results showed that the usual median acrylamide exposure and 97.5th percentile

was 0.5 and 1.2 µg/kg-bw/day for the whole population and 1.1 and 2.0 µg/kg-bw/day for young children. They found the median acute intake was higher than the usual intake. The French fries group was the main contributor in both groups, at 31% and 28% for the whole population and young children. The study had a good methodology. However, in the discussion they compared between the whole population's exposure and that of Swedes (18-74 years) and Belgians (adolescents), whereas they did not compare children's exposure. They gave a good explanation of the differences between the results of the various groups.

6.2.1 Children's exposure

This study used Saudi infant and UK infant, children and whole population data. Saudi infant data was taken from the study of Thaiban (2006) which collected data using 24-hour recall (3 days) from 150 infants (≤ 2 years), and the acrylamide content in starchy foods were analysed in this study (as mentioned in section 6.1). However, the UK data was taken from NDNS surveys SN3481 and SN6533 which collected using 4-day dietary intake from 158, 335and 1717 for SN6533 infant (≤ 2 years), children (≤ 4 years), and SN3481children (≤ 4 years) respectively. The acrylamide contents in food were taken from FSA, FDA and smaller studies. The approaches used in this study were probabilistic and deterministic. Individual exposure was represented as 2.5^{th}, 50^{th} and 97.5^{th} percentile and the food group contributing to acrylamide was representing as percentage.

The mean daily exposure to acrylamide in this study for all age groups (Saudi and UK) was lower than the WHO/FAO estimation which range 0.4 to 3.0 µg/kg-bw/day. However, The 97.5^{th} percentile median acrylamide exposure was higher than the WHO estimation; for NDNS NS3481 children, NS6533 infants and NS6533 children exposure was estimated at 9.847, 9.134 and 9.20 µg/kg-bw/day, suggesting UK infants and children are exceeding levels stated by WHO/FAO predictions, whereas Saudi infants, at 0.72 µg/kg-bw/day in 97.5^{th} percentile acrylamide exposure, are in the WHO estimation range.

In this study the upper percentile median acrylamide exposure (97.5^{th}) was higher than the tolerable daily intake (TDI) for cancer risk (TDI <2.6 µg/kg/day) among UK infants, and children. Whereas the Saudi infants' upper percentile median

acrylamide exposure for all groups was lower than the TDI. The mean and median acrylamide exposure were lower than the TDI value. Potato products group was found to be the main contributor in this study for most age groups ranged between 40.9% to 49.8%, followed by biscuit and cake group ranged between 21.6% to 19.0%.

In this study acrylamide exposure was estimated from foods consumed by 150 infants over three days (24-hour recall) and acrylamide exposure in the age bracket less than two years was 0.22 µg/kg-bw/day, based on each infant's bodyweight. They also reported that exposure to acrylamide was high as a result of a high consumption of infant cereal, whereas levels in infant cereal ranged between 10-30 µg/kg. However, El-Ziney *et al.* (2009) found that infants' exposure to acrylamide from these two types of foods was 0.5 µg/kg-bw/day in the age range 7 to 12 months, based on a mean bodyweight of 5.5 kg. In general, the differences between this study and El-Ziney *et al*. (2009) study are the number and types of food analysed and number of participants. They analysed only infant milk powder and infant cereal, then the estimated acrylamide exposure for 50 infants from only these two types of food. Whereas, this study analysed 16 different types of food consumed by 150 infants (24-hour recall was used to recall infants' intake). After analysing the food acrylamide content, exposure in infants was estimated based on infant food consumption record for 3 days and infant body weight. From these differences this study was more realistic in estimating Saudi infant exposure to acrylamide.

Cengiz and Gündüz (2013) also studied acrylamide exposure by analysing selected cereal based infant food consumed by Turkish toddlers (1-3 years). A 24-hour recall was used for a day to collect toddlers' (302 participants) consumption data. The samples were analysed using GC-MS and exposure estimates were made using a deterministic approach. The foods were grouped into 7 groups (infant biscuits, bread, infant rusks, crackers, biscuits, breakfast cereal, and powdered infant foods), with ND set as zero. Results showed that mean and 95th percentile acrylamide intake was 1.4 and 3.76 µg/kg-bw/day. Also, they did not find a significant difference between boys and girls. The bread group was the main contributor of acrylamide, at 41%.

In conclusion, children in risk of high acrylamide exposure due to consumption potato products, French fries and cereal foods. Parents who are concerned about their children's health should decrease their acrylamide exposure by minimizing the amount of potato crisps, French fries and toasted bread they consume. Also, general instructions for preparing food at home should be followed.

6.2.2 Whole population exposure

In this study UK data was taken from NDNS survey SN6533 which was collected using 4-day dietary intake from 2844 individual aged 1 to 92 years (whole population). The acrylamide contents in food were taken from FSA, FDA and smaller studies. The approaches used were probabilistic and deterministic.

The upper percentile median acrylamide exposure (97.5^{th}) was higher than the tolerable daily intake (TDI) for cancer risk (TDI <2.6 µg/kg/day) among UK whole population. Also, potato products group was found to be the main contributor flowed by coffee group with 38.9% and 20.1% respectively. Whereas Mojska *et al.* (2010) estimated acrylamide exposure among the Polish population by analysing acrylamide in food samples using GC-MS. The consumption data were taken from the Household Food Consumption and Anthropometric Survey (2000), which covered 4131 participants aged 1-96 years using a 24-hour recall (number of days were not provide). For estimation a probabilistic approach was used, namely a Monte Carlo simulation technique (100,000 iterations). They grouped foods into 12 groups (no reason for the groupings was mentioned). They calculated exposure based on the person's bodyweight. The estimation method was similar to that used in this study. The results showed the mean, median and 97.5^{th} percentile acrylamide exposure were 0.8, 0.4 and 4.5 µg/kg-bw/day for children (1-6 years), and 0.4, 0.3 and 2.1 µg/kg-bw/day for the whole population (1-96 years). The bread group was the main contributor, at 40% and 45% for children and the whole population respectively.

Svensson *et al.* (2003), Sirot *et al.* (2012) and Delgado-Andrade *et al.* (2012) estimated acrylamide exposure in Sweden, the whole French population and Spanish boys (11-14 years) respectively. They all analysed acrylamide in food samples using LC-MS. However, they did not provide enough information about the methods used.

Chapter 6: General conclusion and future work

In general, some studies' results cannot be compared with those of other studies due to differences in 1) the type of estimate (population or consumer), 2) the age range, 3) the calculation method, 4) food groupings, 5) the use of median or mean exposure and 6) the use of different percentiles for upper intake. Differences in exposure may be a result of previous differences acrylamide levels in food and ND values.

There is no information on acrylamide exposure among Saudi infants. There were two studies on acrylamide exposure which analysed specific foods. However, the issue of generic food consumed by infants has never been analysed before. Indeed, there have been only a few studies regarding acrylamide consumption among infants around the world.

This study results raise concerns about the positive health risk for the general population in the case of consumption of a large amount of acrylamide. Eating habits and food types should be modified to reduce acrylamide exposure level. More damage can come from the home rather than industry due to acrylamide levels not being under control in home cooking. Also, acrylamide from food, coffee (even decaffeinated), cigarettes and cosmetics increases the bad effect, especially among sensitive age groups and those who are pregnant.

Although most people in this study are under the TDI level there are a number of people who exceed the legislation or advised level. There are other MRPs, for example HMF and Furan, which may increase health risk, especially cancer risk, for consumers (Heat-generated Food Toxicants, 2007). Additionally, other components may form during acrylamide reduction. Thus, due to other toxic components, acrylamide level should be reduced.

As stated above, people make their own food and this may have more acrylamide present than that from the food industry. Consumers need to be advised, therefore, to reduce acrylamide formation. Recommendations and advice should include the following: 1) acrylamide formation increases when food is fried at a temperature higher than 175 ºC (Matthäus *et al.*, 2004); 2) food should not be overcooked due to an increase in acrylamide at that time (Ahn *et al.*, 2002); 3) the food surface should be reduced, such as thinner potato slices (0.07-0.1 inches) as there is lower acrylamide formation compared with thicker slices (Food Drink Europe, 2011); 4)

increased peel removal, which contains high amounts of reducing sugar, may lead to a decrease in acrylamide formation (Food and Drug Administration, 2013); 5) following package instructions when cooking pre-prepared food is advised (Food Drink Europe, 2011); and 6) cook food (roast or fry) at 170-175 ºC until a light golden colour is obtained (Van Boekel *et al.*, 2010). This advice will not affect the food's taste or colour.

6.3 *Recommendations for future work*

6.3.1 Laboratory

- Results should be confirmed through a larger number of food samples and replicates. To reduce the variation in foods from each food type, different brands should be taken and from each brand samples should be taken from different batches. Also, to reduce variation in acrylamide analysis, the food extraction should be analysed using LC-MS/MS or GC-MS. The same extraction should be analysed using the same method in different laboratories.
- More studies are needed to measure the free asparagine and soluble sugars in starchy foods. The amount of acrylamide formed after heat treatment should be determined and the change in free asparagine and soluble sugars should be measured to study the correlation strength. For example, the amount of free asparagine and soluble sugars in bread dough, then after fermentation, after baking and after toasting at different temperatures and times.
- The effect of storage of starchy food in storage conditions similar to those found in hot areas, such as high temperatures and humidity, should be studied to determine the effect on acrylamide formation. This can be achieved by storing foods with known acrylamide levels.
- The effect of cooking methods should be studied by comparing between baking and frying. Also, the differences between old and new frying and baking methods based on acrylamide formation should be included.
- Study the correlation between acrylamide and colour levels in cereal products to confirm if there is any correlation.

- Each country should have a database of acrylamide content in foods that are traditional or the most used recipes in their country. The database should contain the foods consumed in all seasons and on all occasions.

6.3.2 Assessment

- The results should be confirmed through a large sample of participants, using more than one dietary method for assessment so as to reduce underestimation of food consumption. These methods should contain questions about ingredients, type of packaging and recipes or the name of the restaurant or industry.
- It would be interesting to use biomarkers with dietary assessment methods to better estimate actual acrylamide exposure and a wider cohort could be used to allow stratification based on smoking and genetic factors. Also, well designed methods should be used when estimating the correlation between acrylamide and diseases.
- Another potential future work would be to have a national dietary survey in the Kingdom of Saudi Arabia similar to the NDNS survey covering all age groups to estimate acrylamide intake based on Saudi food data.
- The correlation between the effect of social-economic level and acrylamide exposure should be studied.
- There is a need for validation of the dietary assessment methods used for measuring acrylamide exposure in different age groups.
- There is a need to study and estimate acrylamide exposure in the most sensitive age groups and groups, such as pregnant women, infants and children as they have not been the concern of most researchers.

6.3.3 Other

- Based on the acrylamide risk assessment results, various strategies and methods could be developed to reduce acrylamide levels in food and thus reduce the human intake of acrylamide.
- Education programs for parents and food service workers are needed to teach them how to reduce acrylamide during household cooking. Also, they should learn how to modify dietary habits and consumption patterns among sensitive groups, especially infants and children.

- In summary, it is useful to combine laboratory analysis with assessment studies in order to measure acrylamide content in foods participants are exposed to. However, this type of study would have a high cost and be rather time consuming.

6.4 *Conclusions*

In this thesis, for the first time, Saudi infants' exposure to acrylamide was estimated by analysing 16 types of starchy foods consumed by Saudi infants (≤ 2 years) using the LC-MS and HPLC-UV. Therefore this study recommends using LC-MS for acrylamide estimation. The results have shown that acrylamide levels in Saudi infant starchy foods are similar to those found in other studies. Due to variation in acrylamide content it is suggested to continue monitor acrylamide contents in food. Estimation of acrylamide exposure in Saudi infant and UK infant, children and whole population was made using probabilistic and deterministic approaches. The data was taken from a Saudi study and the NDNS survey. Acrylamide exposure in Saudi infant was find to be low and this is due to lower consumption of food contain acrylamide, this might increase with age which suggested to extend Saudi study to include all children. Whereas, an upper percentile exposure of acrylamide in the UK infant, children and whole population have exceeds the tolerable daily intake (TDI) cancer value. The study highlights the importance of reducing the consumption of foods high in acrylamide by education people or reducing the levels of acrylamide in the most commonly consumed foods in the country by optimaising formulation processing.

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Appendix

Appendix A: The NDNS individual foods consumed and recorded in dietary intake (4 day).

	A	B	C	D	E	F
1	case No	DayNo	FoodNumber	FoodName	Total_Grams	
2	10,101,032	1	10,040	FAT SPREAD (62-72% FAT) NOT POLYUNSAT	18.00	
3	10,101,032	1	608	MILK SEMI-SKIMMED PASTEURISED SUMME	106.80	
4	10,101,032	1	126	BREAD WHITE TOASTED	65.88	
5	10,101,032	1	10,072	PRAWN COCKTAIL SNACKS, MAIZE / RICE FI	17.00	
6	10,101,032	1	2,251	BOILED SWEETS BARLEY SUGAR BUTTERS	42.00	
7	10,101,032	1	9,508	HAM UNSPECIFIED NOT SMOKED NOT CANN	22.00	
8	10,101,032	1	10,040	FAT SPREAD (62-72% FAT) NOT POLYUNSAT	14.00	
9	10,101,032	1	5,000	WATER NOT AS A DILUENT	250.00	
10	10,101,032	1	120	BREAD, WHITE SLICED, NOT FORTIFIED	175.68	
11	10,101,032	1	1,952	APPLES EATING RAW FLESH & SKIN ONLY	100.00	
12	10,101,032	1	2,358	MIXED FRUIT JUICE DRINK RTD NOT LOW CA	208.00	
13	10,101,032	1	2,251	BOILED SWEETS BARLEY SUGAR BUTTERS	35.00	
14	10,101,032	1	2,253	CHEWING GUM NOT SUGAR FREE	2.00	
15	10,101,032	1	9,508	HAM UNSPECIFIED NOT SMOKED NOT CANN	5.50	
16	10,101,032	1	10,072	PRAWN COCKTAIL SNACKS, MAIZE / RICE FI	17.00	
17	10,101,032	1	10,040	FAT SPREAD (62-72% FAT) NOT POLYUNSAT	7.00	
18	10,101,032	1	120	BREAD, WHITE SLICED, NOT FORTIFIED	43.92	
19	10,101,032	1	1,853	CHIPS OLD POTATOES FRESH FRIED IN BLE	105.00	
20	10,101,032	1	1,115	COATED CHICKEN PIECES TAKEAWAY	96.00	
21	10,101,032	1	2,425	GRAVY THICKENED NO FAT	90.00	
22	10,101,032	1	7,784	SAUSAGES ECONOMY FRIED	45.00	
23	10,101,032	1	2,262	ICE LOLLIES, JUICE BASED, NOT FORTIFIED	70.35	
24	10,101,032	1	2,269	POPCORN SWEET	100.00	
25	10,101,032	1	2,259	FRUIT GUMS WINEGUMS	15.60	
26	10,101,032	2	8,159	KELLOGGS COCOPOPS COCOROCKS	32.00	
27	10,101,032	2	608	MILK SEMI-SKIMMED PASTEURISED SUMME	50.00	
28	10,101,032	2	970	BEEF TOPSIDE ROAST LEAN ONLY	37.00	
29	10,101,032	2	1,146	TURKEY ROAST LIGHT AND DARK MEAT AND	35.00	
30	10,101,032	2	1,841	POTATOES OLD ROAST IN BLENDED VEGET	150.00	
31	10,101,032	2	721	ICE CREAM, DAIRY, VANILLA, SOFT SCOOP	120.00	
32	10,101,032	2	2,425	GRAVY THICKENED NO FAT	73.70	
33	10,101,032	2	9,249	POTS BOILED/MASHED OLD WITH LOW OR F	45.00	
34	10,101,032	2	5,215	YORKSHIRE PUDDING MADE WITH S SKIM M	40.00	
35	10,101,032	2	9,451	PORK LOIN JOINT ROASTED LEAN ONLY	40.00	
36	10,101,032	2	8,464	FRUIT DRINK CONC BLACKCURRANT LOW CA	62.50	
37	10,101,032	2	5,101	WATER FOR CONCENTRATED SOFT DRINKS	187.50	
38	10,101,032	2	1,711	CARROTS, OLD, FRESH, BOILED	20.00	
39	10,101,032	2	2,617	CABBAGE WHITE BOILED	30.00	
40	10,101,032	2	1,713	CARROTS, YOUNG, FRESH, BOILED	40.00	
41	10,101,032	2	871	BLENDED VEGETABLE OIL	1.70	
42	10,101,032	2	1,921	SWEDE BOILED	20.00	
43	10,101,032	2	2,259	FRUIT GUMS WINEGUMS	20.80	
44	10,101,032	2	9,382	HAM NO ADDED WATER NOT SMOKED	38.00	
45	10,101,032	2	255	CREAM CRACKERS	32.00	

Appendix

Appendix B: Food groups and subgroups used for the NDNS acrylamide exposure estimation.

No.	Group	Subgroup	Description
1	Breads	1	White bread sliced, unsliced, toast, fried; includes French stick, milk loaf, slimmers, pitta bread, rolls, chappatis, and soda bread.
		2	Wholemeal bread sliced, unsliced, toast, fried; includes chappatis, pitta bread, rolls, hi-bran bread, and wholemeal soda bread.
		3	Softgrain bread sliced, unsliced, toast, fried, rolls, fortified and not fortified.
		4	Other breads sliced, unsliced, toast, fried; includes brown, granary, high fibre white, rye bread, gluten free, garlic bread, continental breads e.g. ciabatta, oatmeal bread, Vitbe, Hovis, crumpets, English muffins (white & wholemeal), pikelets, brown and granary rolls, bagels, brioche, naan and paratha.
		5	Pizza all types - thin & crispy, deep pan, French bread, pie and shortbread
2	Pastry	1	pastries includes Danish pastries, bakewell tarts, jam tarts
		2	Fruit pies all types, one and two crusts; includes apple strudel and individual fruit pies from takeaways.
3	Biscuits and cake	1	Biscuits all types, sweet and savoury; includes cream crackers, flapjacks, breadsticks, crispbread, cereal crunchy bars and ice cream cornet.
		2	Buns, cakes and, currant bun, doughnuts, eccles cakes, scones (sweet and savoury), sponge cakes, fruit cakes, eclairs, currant bread, malt loaf, gateaux, pastry, mince pies, sponge fingers, scotch pancakes, croissants, custard tart and lemon meringue pie.
		3	Cereal-based puddings
4	Cereal	1	Wholegrain breakfast cereals, e.g. All Bran, muesli, Shredded Wheat. Includes porridge and Ready Brek.
		2	Other breakfast cereals, e.g. cornflakes, Coco Pops and Sugar Puffs. Includes Pop Tarts.

Appendix

Continued:

No.	Group	Subgroup	Description
5	French fries & products	1	French fries fresh and frozen, including oven and microwave, French fries.
		2	Fried potatoes fried sliced potato with or without and fried potato products batter, fried waffles, croquettes, crunchies, alphabites, fritters and hash browns.
6	Potato products	1	Crisps and savoury snacks include all potato and cereal based savoury snacks, popcorn (not sweet) and twiglets.
		2	Potato products not fried croquettes, waffles, fritters, hash browns, alphabites, grilled or oven baked.
		3	Other potatoes, potato includes baked or roast (with or without salads and dishes fat), cheese and potato pie.
7	Meal	1	Any foods contain type of cereal with meat and vegetables (exclude potato).
8	Coffee	1	Coffee (made up) includes instant and leaf bean, decaffeinated, vending machine with whitener and coffee essence.

Appendix

Appendix C: The sheet shows food groups, code and description for food consumed by the NDNS population.

	A	B	C	D	E	F	G
1	Food Group	Food Code	Food Description				
2	1	4182	4180 BREAD TOASTED				
3	1	101	ALL-BRAN LOAF				
4	1	3356	APPLE CHARLOTTE WITH W/M BREAD				
5	1	3903	APPLE PUDDING WITH BREAD +SUET				
6	1	80188	BAGEL				
7	1	9373	BAGELS PLAIN ONLY				
8	1	10,771	BAGELS PLAIN TOASTED				
9	1	9909	BANANA AND RAISIN BREAD				
10	1	2795	Banana bread				
11	1	103	BR. BREAD FRIED BLEND OIL				
12	1	4167	BRAN LOAF				
13	1	4180	BREAD				
14	1	9520	BREAD & BUTTER PUD MADE W REDCD FAT SPR PUFA H/M				
15	1	9919	BREAD & BUTTER PUDDING FORTIFIED BREAD + PUFA				
16	1	9621	BREAD & BUTTER PUDDING MADE WITH WHOLEMEAL BREAD				
17	1	9887	BREAD & BUTTER PUDDING WITH ARTIFICIAL SWEETENER				
18	1	3245	Bread & butter pudding with cream				
19	1	4175	BREAD +BUTTER PUDDING				
20	1	3337	BREAD +BUTTER PUDDING MADE WITH KRONA				
21	1	3612	BREAD +BUTTER PUDDING NO FAT				
22	1	3242	BREAD +BUTTER PUDDING WITH SKIMMED MILK +PUFA MARG				
23	1	507	BREAD AND BUTTER PUDDING				
24	1	3122	Bread and butter pudding made with brioche and cream				
25	1	6474	Bread and butter pudding made with flora light, no dried fruit				
26	1	6592	Bread and butter pudding made with semi-skimmed milk, wholemeal bread and Aldi beatifully butterfully				
27	1	9625	BREAD AND BUTTER PUDDING S/SKIM MILK & LOW FAT SPR				
28	1	3534	Bread and Butter Pudding With Apricots and Mixed Berries				
29	1	6939	Bread and butter pudding with chocolate and whipping cream				
30	1	3562	Bread and butter pudding with purchased fruit loaf				
31	1	6676	Bread and butter pudding with utterly butterly, sultanas, dates and walnuts				
32	1	9623	BREAD AND BUTTER PUDDING W'MEAL BREAD + ARTI SWEET				
33	1	102	BREAD BROWN				
34	1	107	BREAD BROWN TOASTED				
35	1	108	BREAD CURRANT				
36	1	109	BREAD CURRANT TOASTED				
37	1	3323	BREAD FRIED KRONA AND PUFA				
38	1	112	BREAD GRANARY				
39	1	113	BREAD GRANARY TOASTED				
40	1	7611	BREAD HIGH FIBRE FRIED IN DRIPPING				
41	1	7612	BREAD HIGH FIBRE FRIED IN LARD				
42	1	7613	BREAD HIGH FIBRE FRIED IN PUFA				
43	1	7609	BREAD HIGH FIBRE WHITE				
44	1	7610	BREAD HIGH FIBRE WHITE TOASTED				
45	1	111	BREAD HOVIS TOASTED				
46	1	7618	BREAD OATMEAL TOASTED				
47	1	116	BREAD PITTA WHITE				
48	1	117	BREAD PITTA WHOLEMEAL				
49	1	2746	Bread pudding (wholemeal bread)				
50	1	6214	Bread pudding made with olivio, rum and olive oil. No milk				
51	1	6186	Bread pudding made with water and pufa spread				
52	1	3697	BREAD PUDDING NO FAT				
53	1	115	BREAD RYE TOASTED				
54	1	114	BREAD RYE/PUMPERNICKEL				
55	1	5037	BREAD SAUCE MADE SKIM				

Appendix

Appendix D: Food products, groups, acrylamide contents, number of samples and references used for the NDNS acrylamide exposure estimation

	A	B	C	D	E	F
1	Food Product	Group	Concentration µg/kg	# of samples	Comments	Reference
2	Chips - Khangs Fish & Chips	Potato Products	136	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
3	Chips - Marco's Fish Bar	Potato Products	136	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
4	Side Chips - The Scott Arms	Potato Products	425	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
5	Chips - Newton Fish Bar	Potato Products	43	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
6	Extra Chips - The Railway Inn	Potato Products	390	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
7	Chips - The Hot Spot	Potato Products	363	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
8	Large Fries - McDonalds	Potato Products	273	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
9	Chips - Stavros Fish Bar	Potato Products	68	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
10	Chips - Zero Plus Fish Bar	Potato Products	66	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
11	Chips - Tuck in Cafe	Potato Products	411	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
12	Chips - Cafe Fresco	Potato Products	96	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
13	Chips - Hightown Fish & Chip Shop	Potato Products	684	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
14	Chips - Moto The Kitchen	Potato Products	668	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
15	Regular Fries - KFC	Potato Products	148	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
16	Regular Fries - Burger King	Potato Products	481	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
17	Fries - Perfect fried Chicken	Potato Products	79	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
18	Chips - Loch Fyne	Potato Products	1089	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
19	Fries - Chicken Cottage	Potato Products	460	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
20	Chips - City Restaurant	Potato Products	69	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
21	Chips - Windmill Fish Bar	Potato Products	163	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
22	Chips - Clifton Fish Bar	Potato Products	41	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
23	Chips - The Abbey	Potato Products	210	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
24	Chips - The Rosery	Potato Products	434	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
25	Chips - Relish	Potato Products	128	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
26	Chips - Silver Lounge Cafe	Potato Products	130	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
27	Chips - Apsley's	Potato Products	168	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
28	Chips - Arcade Chip Shop	Potato Products	252	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
29	Chips - The Trinity	Potato Products	556	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
30	Chips - The Upper Crust	Potato Products	264	1		Mar-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
31	Side Chips - Wimpy	Potato Products	265	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
32	Chips - Sainsbury's Cafe	Potato Products	184	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
33	Chips - Khangs Fish & Chips	Potato Products	123	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
34	Chips - Central Fish Bar	Potato Products	93	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
35	Chips - The Rosery	Potato Products	280	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
36	Chips - Silver Lounge Cafe	Potato Products	275	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
37	Chips - The Upper Crust	Potato Products	109	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
38	Chips - Apsley's	Potato Products	108	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
39	Chips - Relish	Potato Products	100	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
40	Chips - Arcade Chip Shop	Potato Products	93	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
41	Chips - Marco's Fish Bar	Potato Products	27	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
42	Chips - Bar-B-Que	Potato Products	47	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
43	Chips - The Abbey	Potato Products	63	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
44	Chips - City Restaurant	Potato Products	35	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201
45	Chips - Newton Fish Bar	Potato Products	345	1		Nov-09 FSA. 2010. Survey of Process Contaminants in Retail Foods 2009 [online]. [Accessed 2nd November 201

Appendix

Appendix E: Acrylamide content (µg/kg) in NDNS food group

Bread	Pastry	Biscuits/ cake	Cereal	French fries	Potato crisps	Meals	Coffee
4	5	5	11	11	10	7	3
4	5	5	12	18	10	11	5
5	11	5	14	19	12	13	6
5	14	5	15	20	13	14	6
5	15	8	15	21	15	15	6
5	15	12	15	21	15	15	6
5	15	13	16	22	15	15	6
5	15	13	20	22	16	15	6
5	15	13	22	22	18	15	6
5	18	13	24	26	20	15	7
5	22	14	27	27	21	15	8
5	25	14	28	30	22	15	8
5	25	15	28	32	23	15	8
5	26	15	30	34	23	15	8
5	29	15	30	35	25	15	10
5	29	15	30	40	25	15	11
6	30	15	30	41	25	16	11
7	30	15	30	41	26	16	13
7	30	15	30	43	27	17	13
8	33	15	31	44	27	17	25
8	33	16	32	47	28	18	37
8	34	17	37	47	30	18	45
8	42	18	39	49	30	20	64
9	74	19	40	49	30	22	70
9	133	21	42	49	34	22	86
9	157	22	44	51	34	23	97
10	181	23	45	58	36	23	134
10	350	24	47	59	39	26	138
10		24	51	59	41	34	142
10		26	52	60	42	34	144
10		26	53	61	43	42	149
11		27	54	61	44	56	149
11		29	56	62	46	58	150
11		29	57	63	46	116	151
12		30	59	63	47	126	154
12		30	59	63	48	152	155
12		30	61	64	48	187	161
12		30	62	66	50		162
12		30	67	67	51		163
13		30	71	68	51		163
13		31	71	68	52		170
13		32	74	68	53		172
13		32	77	68	54		172
14		34	78	69	55		175
15		35	78	69	56		179

Appendix

Continued:

Bread	Pastry	Biscuits/ cake	Cereal	French fries	Potato crisps	Meals	Coffee
15		36	81	70	56		184
15		36	84	71	62		185
15		37	84	74	64		188
15		37	89	76	69		191
15		39	91	79	70		194
15		41	96	79	70		209
16		46	97	86	71		215
16		47	100	86	72		222
16		49	100	87	73		226
17		52	102	90	80		231
17		55	119	90	89		235
17		57	120	92	90		244
17		58	121	92	93		250
17		59	121	93	93		255
17		59	123	93	94		257
17		60	123	95	97		258
17		69	124	96	97		263
18		70	125	99	98		263
18		70	130	100	103		266
18		70	146	100	103		286
19		70	150	102	104		288
19		71	150	107	104		319
19		72	152	108	105		332
19		75	156	109	106		351
19		78	160	111	108		351
20		81	166	114	108		377
20		84	174	116	109		411
20		85	176	116	110		417
22		86	189	117	110		458
22		95	193	117	111		471
22		97	194	117	111		539
22		97	201	121	111		668
23		100	207	122	114		845
23		100	214	123	117		998
23		103	219	125	119		1100
24		105	220	125	119		
24		107	228	126	120		
24		110	233	126	124		
24		112	241	127	128		
25		113	249	128	128		
26		121	251	128	130		
26		128	266	130	131		
27		128	299	130	133		
27		130	309	133	133		
27		131	343	134	133		

Appendix

Continued:

Bread	Pastry	Biscuits/ cake	Cereal	French fries	Potato Crisps	Meals	Coffee
28		133	366	136	135		
29		133	397	136	135		
29		140	398	136	138		
29		143	403	139	141		
29		144	467	143	143		
30		150	689	143	144		
30		151	868	143	146		
30		152	1057	146	146		
30		159	1332	148	147		
31		159		148	149		
31		166		150	150		
31		168		150	152		
32		184		151	153		
32		194		151	155		
33		199		155	157		
33		199		157	157		
34		208		162	158		
34		211		163	160		
34		212		165	160		
34		213		166	161		
34		215		168	162		
36		223		168	162		
36		254		169	163		
36		255		177	164		
38		261		177	164		
39		262		180	164		
39		268		183	169		
39		283		184	169		
39		294		188	171		
40		300		190	172		
40		300		192	173		
40		309		193	173		
41		319		194	173		
42		324		196	175		
42		334		196	178		
43		342		197	181		
44		344		198	182		
44		348		198	186		
45		371		199	186		
47		373		199	190		
56		620		223	209		
57		647		230	215		
57		697		230	219		
58		760		235	219		

Appendix

Continued:

Bread	Pastry	Biscuits/ cake	Cereal	French fries	Potato Crisps	Meals	Coffee
58		865		236	220		
59		890		245	222		
59		900		245	225		
60		1024		246	228		
62		1156		247	230		
69		1526		249	235		
74		1538		250	237		
96				250	240		
99				252	240		
102				252	241		
110				253	248		
130				254	249		
149				254	251		
178				257	253		
182				257	254		
183				258	254		
216				259	255		
218				260	260		
238				260	266		
300				261	267		
343				262	270		
364				263	274		
				291	343		
				291	345		
				292	351		
				292	352		
				294	352		
				295	355		
				296	356		
				299	364		
				301	364		
				301	366		
				302	374		
				303	387		
				308	399		
				310	408		
				310	419		
				312	420		
				313	426		
				315	429		
				318	433		
				319	434		
				323	438		
				326	438		
				327	440		

Appendix

Continued:

Bread	Pastry	Biscuits/ cake	Cereal	French fries	Potato Crisps	Meals	Coffee
				328	441		
				333	443		
				334	461		
				389	604		
				390	606		
				396	610		
				398	613		
				400	616		
				407	618		
				411	619		
				415	624		
				416	627		
				419	659		
				425	693		
				425	693		
				432	752		
				432	782		
				433	786		
				434	797		
				440	798		
				441	802		
				443	807		
				450	823		
				452	832		
				453	847		
				454	870		
				459	879		
				460	955		
				462	958		
				558	1323		
				590	1325		
				605	1343		
				610	1360		
				638	1362		
				668	1431		
				684	1485		
				692	1492		
				728	1515		
				736	1538		
				767	1572		
				810	1602		
				833	1661		
				847	1712		
				856	1770		
				879	1820		

Appendix

Continued:

Bread	Pastry	Biscuits/ cake	Cereal	French fries	Potato Crisps	Meals	Coffee
				1030	1822		
				1077	1844		
				1089	1853		
				1096	1895		
				1258	2097		
				1265	2167		
				1556	2336		
				1605	2605		
				1965	2655		
				2123	2748		

Appendix

Appendix F: The Research ethics administrator in Leeds university response for unneeded the ethical review and King Abdulaziz University permission for using the data

1) Research ethics administrator in Leeds university response for unneeded the ethical review

RE: Ethical Approval

Jennifer Blaikie on behalf of ResearchEthics

You forwarded this message on 17/09/2014 09:29.

Sent: 17 September 2014 09:09
To: Maha Thaiban

Dear Maya

Thank you for your application. If the data is anonymous and publicly available, then you do not need to apply for ethical review from the University of Leeds.

Best wishes
Jennifer

~~~~~~
Jennifer Blaikie

Senior Research Ethics Administrator
Performance, Governance and Operations
Research & Innovation Service
University of Leeds
Leeds LS2 9LJ

Tel: 0113 34 34873
Email: j.m.blaikie@leeds.ac.uk
www.leeds.ac.uk/ethics
Follow @UoLResEthics

-----Original Message-----
From: Maha Thaiban [mailto:ml08mat@leeds.ac.uk]
Sent: 15 September 2014 14:28
To: ResearchEthics
Subject: Ethical Approval

Dear Ethical Committee,

Please find attached the ethical review form for this part of my PhD research project which will use anonymised data collected in 2005/6. The data is owned by Kind Abdul Aziz University (Saudi Arabia) which has given permission for its use.

I look forward to hearing from you.
Best regards,

Maha Thaiban
~~~~~~

Appendix

2) King Abdulaziz University permission for using the data

Kingdom of Saudi Arabia
Ministry of Higher Education
King Abdulaziz University
Faculty of Home Economics

قسم الغذاء والتغذية
Department of Food and Nutrition

المملكة العربية السعودية
وزارة التعليم العالي
جامعة الملك عبد العزيز
كلية الاقتصاد المنزلي

King Abdul-Aziz University
P.O. Box 80200
Jeddah, 21589
Kingdom of Saudi Arabia

1 September 2014

To whom it may concern,

Maha Thaiban was involved in a study carried out at the King Abdul-Aziz University in Jeddah, which studied nutrient intake and food consumption by healthy Saudi infants. In this study, data was obtained from mothers of 150 Saudi infants from public hospitals in Jeddah. Participants were recruited voluntarily and were fully briefed on the aims and objectives of collecting the data and on the methods of research required to obtain the data. Participants were informed that the data obtained would be used for research into nutrient intake and food consumption by healthy Saudi infants. Participants were interviewed face-to-face in the hospital and were asked questions concerning the parent's level of education and their socio-economic status. Participants were then asked to measure and record the food intake for their child by a 24 hour recall, over three days. This research data was approved by King Abdul-Aziz University, King Abdul-Aziz University Hospital and King Fahd Armed Forces Hospital, Jeddah. The original data obtained was pooled and stored anonymously before it was published in 2006. The blind data is stored in the Food Science and Nutrition Department at the King Abdul-Aziz University where it is available for future research into nutrient intake and food consumption by Saudi infants.

This letter confirms that we the Food Science and Nutrition Department at the King Abdul-Aziz University give permission for Maha Thaiban to use this blind data, which has not been published, for her research into nutrient intake and food consumption by Saudi infants.

Yours sincerely,

Hanan A. Jambi
Food and Nutrition Department Chair
King Abdulaziz Universtiy

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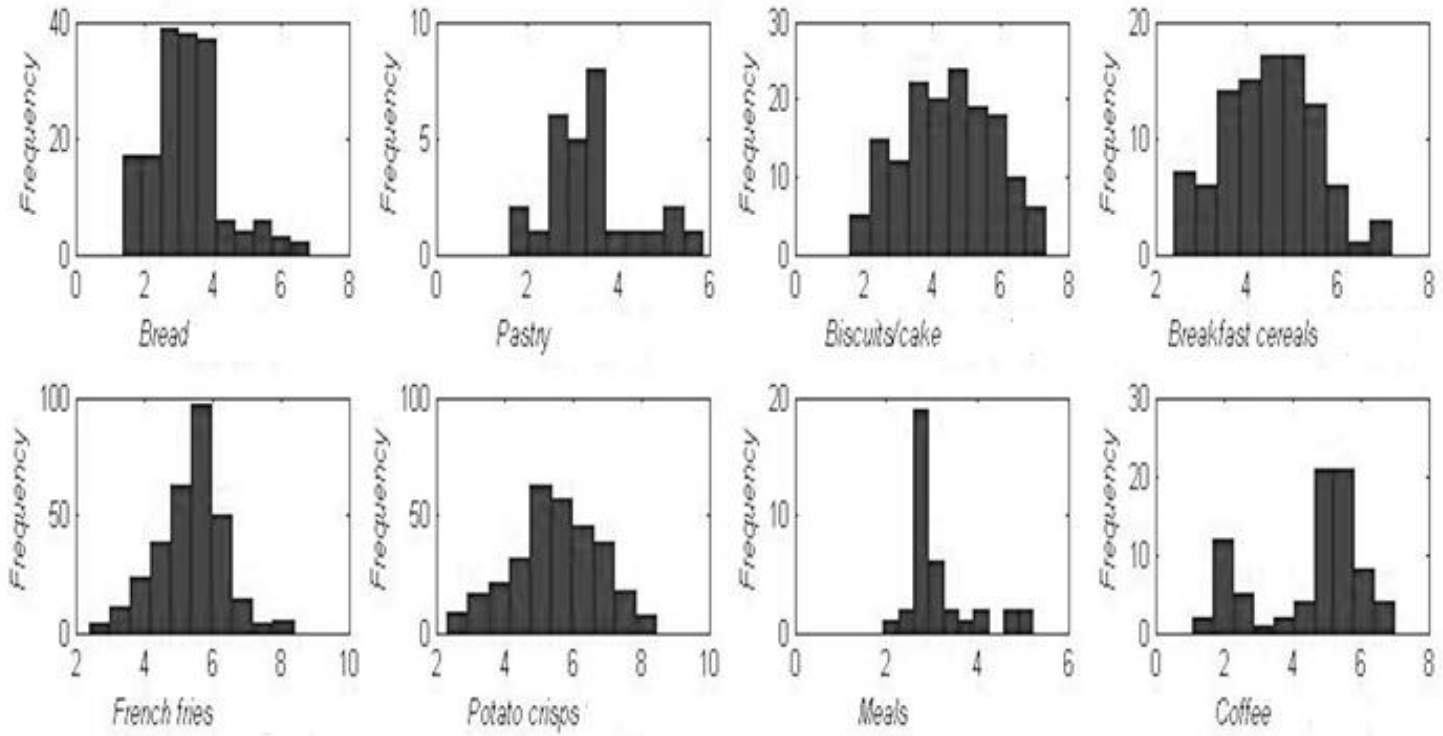
Tel: (012)6952000 Fax: (012)6952000 فاكس: (٠١٢)٦٩٥٢٠٠٠ هاتف: (٠١٢)٦٩٥٢٠٠٠
P.O.Box 42807 Jeddah: 21551 ص.ب ٤٢٨٠٧ جدة: ٢١٥٥١
HOM-023-FOO1 الإصدار: الأول

Appendix

Appendix G: Food groups content distributions (µg/kg) breads (169), pastry (28), biscuit and cake (151), cereal (99), French fries (309), potato products (308), meal (37) and coffee (80) (acrylamide content taken from FSA, FDA and other studies).



Appendix

Appendix H: The top-ten most frequently consumed foods of the UK.

H-1: Infant population NDNS SN6533 (1-2 years) that contribute to acrylamide exposure.

Food description	No.	Amount (g)	Consumers	Mean (g)	Max (g)
Bread white sliced	317	18590	148	59	288
White chapati	305	308	42	1	2
Bread white toasted	279	10338	139	37	131.76
Weetabix	217	6036	91	28	80
Bread wholemeal	135	5805	73	43	144
Potato crisps in sunseed oil	132	3302	76	25	75
Bread wholemeal toasted	130	4453	67	34	75.64
Rolls white soft	90	4931	63	55	220
Rice krispies kelloggs only	80	1571	47	20	51.4
Hovis, white bread with added wheatgerm, toasted	79	3056	31	39	84

H-2: Children population NDNS SN6533 (1-4 years) that contribute to acrylamide exposure.

Food description	No.	Amount (g)	Consumers	Mean (g)	Max (g)
Bread white sliced	506	506	28718	243	57
Bread white toasted	486	486	17362	230	36
Weetabix	323	323	9239	143	29
White chapati	313	313	314	44	1
Bread wholemeal	215	215	9801	116	46
Potato crisps in sunseed oil	211	211	5161	125	24
Bread wholemeal toasted	170	170	5659	94	33
Rolls white soft	135	135	6841	97	51
Fish fingers grilled	127	127	7995	115	63
White and wholemeal bread with added wheatgerm	119	119	6844	58	58
Bread white sliced	506	506	28718	243	57

Appendix

H-3: Children population NDNS SN3481 (1.5-4.5 years) that contribute to acrylamide exposure.

Food description	No.	Amount (g)	Consumers	Mean (g)	Max (g)
Bread white sliced	2778	282430	60007	30	94
Bread white toasted	1931	173670	14016	26	151
Weetabix	1406	124721	5009	20	69
Potato crisps	1331	85724	42020	22	125
Potatoes old boiled	875	202933	15020	58	219
Cream sandwich biscuits	845	55800	90010	17	56
Bread wholemeal	792	86467	93009	27	85
Cornflakes fortified	747	50420	75018	20	92
Corn snacks (monster munch wotsits)	624	40765	91002	18	35
Short sweet biscuits	590	29230	13022	13	55

H-4: Whole population NDNS SN6533 (1-92 years) that contribute to acrylamide exposure.

Food description	No.	Amount (g)	Consumers	Mean (g)	Max (g)
Coffee instant powder or granules	5268	5886	805	1	20
Bread white sliced	2866	198259	1378	69	288
Bread white toasted	2366	107747	1132	46	162
Potato crisps in sunseed oil	1474	41405	850	28	175
Bread wholemeal	1223	77653	587	63	216
Rolls white soft	1187	74153	750	62	220
Weetabix	981	35144	473	36	120
Bread wholemeal toasted	884	41622	451	47	186
Coffee instant decaffeinated powder	729	819	126	1	3
White and wholemeal bread with added wheatgerm	630	44900	307	71	300