Sudan University of Science and Technology

College of Graduate Studies

Determination of Acrylamide in Sudanese Homemade Biscuits and Wheat Flakes (Rugag) by Liquid Chromatography Tandem Mass Spectrometry

تحديد الأكريلاميد في البسكويت المصنع في المنزل السوداني والرقاق باستخدام الكروماتوغرافي السائل المقترن بالكتلة المطيافية

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By:

Rayan Adil Elhig Widaa

B.Sc. (Honours) in Food Science and Technology,

Sudan University of Science and Technology (2014)

Supervised by

Prof. Dr. Yousif Mohamed Ahmed Idris

Department of Food Science and Technology,

College of Agricultural Studies

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الآية

قال تعالى:

(فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ أَنْ يُقْضَى إِلَيْكَ وَحْيُهُ
وَقُلْ رَبِّ زِدْنِي عِلْمًا)

صدق الله العظيم

سورة طه الآية (114)

Dedication

To

My fabulous parents

Nuha and Adil

To

My sisters, brothers and all members in my family

To

All my teachers and friends with great regard and respect.

Acknowledgements

All thanks are due to Almighty Allah (SWT) who gave me health, strength and helped me tremendously to produce this work.

I would like to express my deepest gratitude to my supervisor **Prof. Dr.Yousif Mohamed Ahmed** for his guidance, patience, interest, continuous participation throughout this study and providing me with valuable advice to finalize this research.

Thanks are also extended to all those who gave me hand and helped me in producing this work.

Last, but not least I am greatly indebted to my parents for supporting and encouraging me to believe in myself, and to my family who stood beside me.

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Abstract

This study was carried out to estimate acrylamide content in some Sudanese foods including Rugag (thin sheets of baked wheat flour), homemade biscuit with ammonium bicarbonate and homemade biscuit without ammonium bicarbonate.

Rugag was prepared according to the traditional method employed in Sudanese house hold. Prepared from 72% extraction wheat flour, sugar, milk powder, vanilla, corn flour, salt, and baking powder, baked in thin sheet on hot steel plate at 170 ˚C for 40 seconds. Two types of homemade biscuits were prepared according to the traditional method employed in Sudanese house hold, one type with added ammonium bicarbonate, and the other prepared similarly but without ammonium bicarbonate. Biscuits were prepared from wheat flour 72% extraction, ghee, sugar, vanilla, corn flour, salt, baking powder and one type with added ammonium bicarbonate, shaped in small pieces baked in oven at 240 ˚C for 15 minutes.

Proximate analysis was carried out for the samples. Acrylamide content of sample was determined using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methods by liquid chromatography tandem mass spectrometry (LC/MS). The results showed significant levels of acrylamide in Rugag and homemade biscuits with ammonium bicarbonate 1.391,0.904 ppm respectively, while no detection of acrylamide in homemade biscuits without ammonium bicarbonate. The estimated dietary intake of acrylamide in Rugag would be 103.12 and 312.97 µg/kg.bw/day for adult and child, respectively, while in homemade biscuit with ammonium bicarbonate would be 29.78 and 90.4µg/kg.bw/day for adult and child, respectively. Can be concluded that Rugag processing conditions lead to formation of acrylamide and addition of ammonium bicarbonate as leavening agent assisted formation of acrylamide in homemade biscuits.

ملخص البحث

أجريت هذه الدراسة بغرض تقدير نسبة الأكريلاميد في بعض الأغذية السودانية التي تشمل الرقاق، بسكويت مصنع بإضافة بيكربونات الأمونيا وبسيكويت من دون بيكربونات الأمونيا.

تم تصنيع الرقاق على حسب الطريقة التقليدية التي تستخدم في المنازل السودانية وتم تحضيره من الدقيق(نسبة الإستخلاص 72%)، سكر، لبن بودرة،دقيق الذرة، فانيليا، ملح وبيكنيج باودر. خبز الرقاق على شكل طبقة رقيقة على صاج في درجة حرارة 170 درجة مئوية لحوالي 40 ثانية.

وتم تصنيع نوعين من البسكويت على حسب الطريقة التقليدية التي تستخدم في المنازل السودانية إحدهما بإضافة بيكربونات الأمونيا والآخر من دون بيكربونات الأمونيا بإستخدام نفس مقادير المكونات ونفس ظروف التصنيع، وتم تحضيره من الدقيق (نسبة الإستخلاص 72%)، السمن، سكر،فانيليا، لبن، دقيق الذرة، ملح، بيكنيج باودر وبيكربونات الأمونيا تم تشكله إلى قطع صغيرة وخبزه في فرن على درجة حرارة 240 درجة مئوية لمدة 15 دقيقة.

تم إجرى تحليل تقريبي للعينات ومن ثم حضرت العينات لتقدير الأكريلاميد على حسب طريقة QuEChRS وقدرت كمية الأكريلاميد بإستخدام جهاز الكروماتوغرفي السائل مقترن مطيافية الكتلة. وأوضحت النتائج وجود نسبة من الاكريلاميد في الرقاق والبسكويت المضاف إليه بيكربونات الأمونيا 0.904,1.391 ppm على التوالي، بينما لم توجد نسبة من الاكريلاميد في البسكويت من دون بيكربونات الأمونيا.

وفي تقدير نسبة المستهلك يومياً من الأكريلاميد في الرقاق103.12, 312.79 ميكروجرام/ كيلوجرام /اليوم للبالغين والأطفال على التوالي ، أما في البسكويت المصنع قي المنزل فكانت النسبة 29.78 , 90.4ميكروجرام/كيلوجرام/اليوم للبالغين والأطفال على التوالي.

نستنتج أن عملية تصنيع الرقاق تؤدي لتكوين أكريلاميد كما أن إضافة بيكربونات الامونيا تؤدي لتكوين أكريلاميد في البسكويت.

CHAPTER ONE

INTRODUCTION

Acrylamide is considered as the most actively investigated compound among heat induced food contaminants (Krska *et al*., 2012; Wenzl *et al*., 2007). The first detection of acrylamide was in 2002 by Swedish researchers, further research determined that acrylamide forms in some foods during certain types of high-temperature cooking (Mottram, 2002).

It is a potential cause of a wide spectrum of toxic effects (Eriksson 2005; IARC 1994; European Union Risk Assessment Report 2002; Manson *et al*. 2005), including neurotoxic effects as has been observed in humans. Also, acrylamide has been found to be carcinogenic in animals, increasing incidences of a number of benign and malignant tumors identified in a variety of organs (for example thyroid, adrenals; FAO/WHO 2004). Several observations led to the hypothesis that heating of food could be an important source of acrylamide exposure to humans, if the heating/frying is done with a frying pan or in an oven, but no acrylamide has been detected in boiled food products (Törnqvist, 2005).

Acrylamide is naturally formed as a byproduct of cooking process in carbohydrate rich foods at high temperatures and low moist conditions (Franek, *et al*., 2014; Tareke *et al*., 2002). Maillard reaction of reducing sugars with asparagine at temperature higher than 120 ˚C is the most probable route to acrylamide formation during the browning process (Yaylayan and Stadler 2005; Özer *et al*., 2012).

In cereals such as wheat, asparagine is present in excess compared with reducing sugars. Therefore the concentration of asparagine, not reducing sugars, is the important factor driving acrylamide formation in cereal-based foods. Cereal grain type, grain variety, and growing conditions are some of the factors that affect asparagine levels.

Rugag is an analogue of flakey snacks prepared by Sudanese for their fasting month of Ramadan. Rugag is based on wheat flour with other ingredients that constitute ordinary meal having no dietetic claims.

Homemade biscuit is of wide use in Sudan, and some families use ammonium bicarbonate as an additive to improve texture of home-made biscuit. The practice is formed in a reaction between NH_3 and acrylic acid.

There is a few documented information published about the presence of acrylamide in Sudanese cereal based foods, in gorrasa and minnan, and the results have shown no detection of acrylamide (Ahmed *et al*., 2014).

Due to the high consumption of Rugag and home-made biscuits by Sudanese people in Ramadan and in special occasions, and the fact that they have high carbohydrates content and exposed to high heat treatment during processing which might lead to formation of acrylamide, this study is carried out to detect and estimate acrylamide in these Sudanese foods, since no information is available about their acrylamide content.

The objectives of this study are to analyze Rugag and home-made biscuit for:

1. Proximate composition.
2. Acrylamide content.

CHAPTER TWO

LITERATURE REVIEW

2.1 Acrylamide:

Acrylamide is an unsaturated amide that is produced mainly for use as an intermediate in the production of polyacrylamide. It is produced commercially by hydrolyzing acrylonitrile using nitrile hydrate and exists in two forms: monomer and polymer. It is used to produce polyacrylamide polymer, which find many uses as a coagulant in waste water treatment and clarifying drinking water. Grouting agents for the construction of dam foundations and tunnels and as electrophoresis gels (Eriksson, 2005).

2.1.1 Chemical and physical characteristics of acrylamide:

Acrylamide (CH_2=CH-CO-NH_2; 2-propenamide) is a white crystalline solid, and has low molecular weight, polar, low volatile, with a molecular weight of 71.08. It has a melting point of 84.5±0.3 °C, low vapor pressure (e.g. 0.007 mm Hg at 25 °C, 0.03 mm Hg at 40 °C, 0.07 mm Hg at 50 °C and 0.14 at 55 °C), a high boiling point (136 °C at 3.3 kPa/25 mmHg) (Norris, 1967; American Cyanamid, 1969; Habermann, 1991).

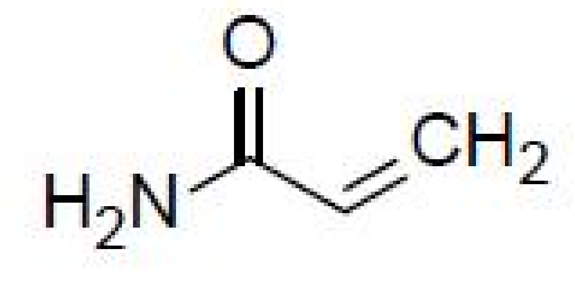
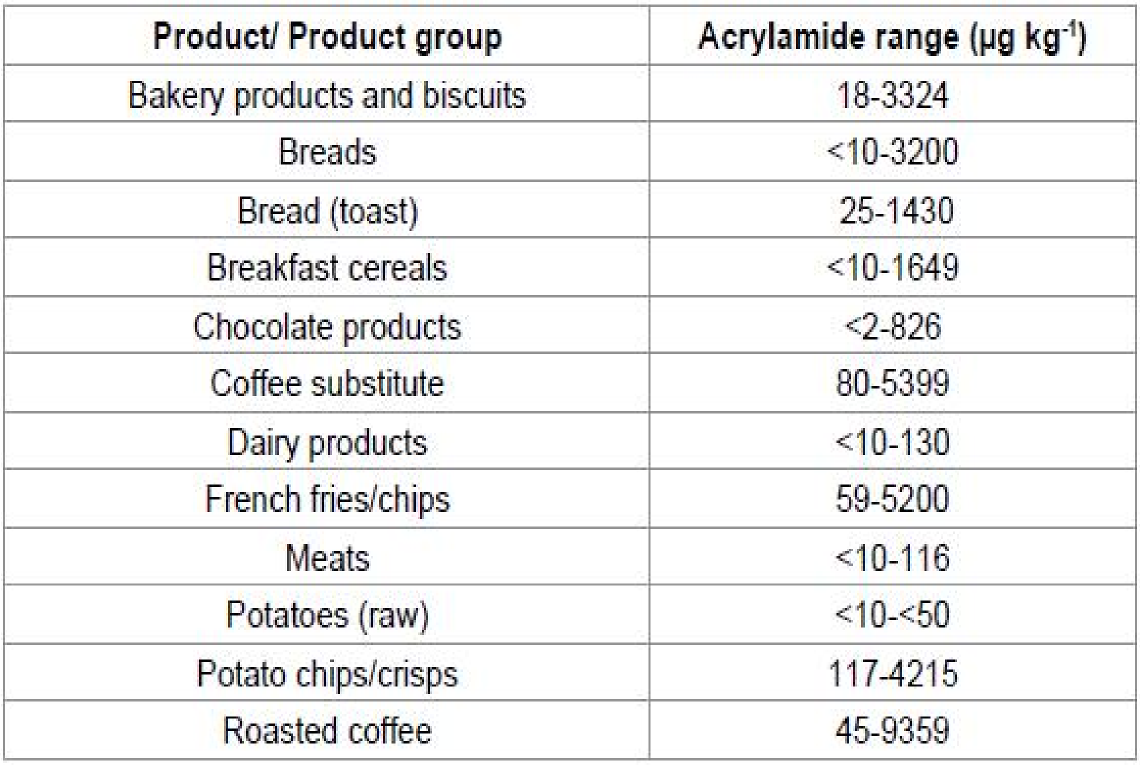


Figure 1: Chemical structure of Acrylamide

2.1.2 Acrylamide in foods:

Acrylamide was detected in wide range of foods, specially in high-carbohydrate foods when prepared at high temperatures during frying, baking and roasting (Tareke *et al*., 2002; Murkovic, 2004). Relatively small amounts can be found in microwaved (where temperatures can reach up to 260°C) foods, but not fresh ones. Even roasted tea leaves and roasted barley grains contain acrylamide in the concentration up to 570 and 320 ng/g respectively. In commercially processed foods as well as in home-cooked meals the acrylamide content tends to increase with cooking time and temperature. The surface color of the products correlates highly with acrylamide levels in food the darker surface, the more acrylamide it contains (Friedman and Mottram, 2005).

Table 1: Amount of acrylamide in some foods (Elder *et al*.,2004):



Product/ Product group	Acrylamide range (µg kg^{-1})
Bakery products and biscuits	18-3324
Breads	<10-3200
Bread (toast)	25-1430
Breakfast cereals	<10-1649
Chocolate products	<2-826
Coffee substitute	80-5399
Dairy products	<10-130
French fries/chips	59-5200
Meats	<10-116
Potatoes (raw)	<10-<50
Potato chips/crisps	117-4215
Roasted coffee	45-9359

2.1.2.1Acrylamide in potato and potato products:

Potato cultivar and temperature are important factors for the formation of acrylamide. Blanching before deep-frying did not affect the amount of acrylamide formed. However the content of reducing sugars determined the level of acrylamide after frying (Weisshaar, 2004; Wicklund, *et al.,* 2005). Others found an effect of blanching and reducing sugars in amount of formed acrylamide (Haase, *et al.*, 2004). Blanching in acidic solutions is an effective way of decreasing acrylamide levels in crisps (Kita, *et al*., 2004). Also linear correlation between acrylamide content of potato chips and their color, represented by redness, was found in the temperature range 120-180°C, and blanching was found to reduce the acrylamide formation (Pedreschi, *et al*., 2005). A physical tool to decrease the amount of acrylamide formed by up to 95% is to use low-temperature vacuum frying equipment for crisps (Granda, *et al*., 2004).

Fresh potatoes respond to cooling below about 10°C by increasing the content of reducing sugars, which results in high acrylamide concentration in fried, roasted or baked potato products. The potential for acrylamide formation increases approximately proportionally to the amount of reducing sugars (Noti, *et al*., 2003; Chuda, *et al*., 2003; Olsson *et al.*, 2004; De Wilde, *et al*., 2004, 2005). Extraction of surface asparagine and sugars with water, without washing out the starch, is a way to lower the acrylamide content in the final product. Since acrylamide formation increases exponentially towards the end of the frying process, the most important factor to keep acrylamide contents low is the determination of the proper end point of the frying process (Grob, *et al*., 2003). The acrylamide concentration depended on the above factors and on the surface to volume ratio of the French fries (Matthäus, *et al*., 2004; Taubert, *et al*., 2004).

Potato croquettes, which are coated with egg and bread crumbs, receive reduced formation of acrylamide, compared to without coating (Fiselier,*et al*., 2004).

2.1.2.2 Acrylamide in coffee:

The factor that affects acrylamide formation in coffee is roasting (Time and temperature), also acrylamide formation may depend on coffee species. Arabica and Robusta coffee beans differ in acrylamide amounts formed. When coffee was roasted in a laboratory roaster to common degrees for consumer, as well as in the thermostatic oven under standard roasting conditions, Robusta showed to have the highest amounts of acrylamide. It seems, that asparagine is a limiting factor for acrylamide formation in coffee, because Robusta coffees also contain higher amounts of asparagine than Arabicas, the highest acrylamide amounts in coffee are formed at the very beginning of the roasting process. After five minutes of roasting at temperatures higher than 220°C the amount of acrylamide is decreasing with increasing the roasting time. Furthermore, acrylamide forms in lower amounts at higher temperatures because of the faster elimination process (Kristina, 2007).

2.1.2.3 Acrylamide in cereal and cereal products:

The determinant factor for acrylamide in cereal products is free asparagine. The free asparagine content varies widely, in wheat it has been reported at 69 to 443 mg kg^{-1}. Revealed a significant impact of cultivar and fertilization on acrylamide levels in bakery products due to variations in the asparagine and crude protein contents of the raw material.The sulphate deficiency in soils causes wheat to accumulate asparagine. The effects of sulphate were greater than varietal differences (Muttucumaru, *et al*. 2006).

In gingerbread, ammonium hydrogen carbonate strongly enhanced acrylamide formation. Both acrylamide concentration and browning intensity are increased with baking time and correlated with each other. The use of sodium hydrogen carbonate as baking agent reduced the acrylamide formation.

Reduced levels of asparagine, replacing reducing sugars with sucrose or by adding organic acids, could also lower the acrylamide content (Amrein, *et al*., 2004). This also occurred to sweet bakeries, like biscuits (Graf, *et al*., 2005). Ammonium hydrogen carbonate also affects acrylamide content in other type of bread (Grothe, *et al*., 2005). Ammonium hydrogen carbonate is an effective amino source in the formation of acrylamide, i.e. acrylamide content increases when ammonium hydrogen carbonate is added, but decreases when sodium hydrogen carbonate is added, which only affect the pH of the product.

One way of reducing free asparagine is to ferment the dough with yeast. Sour dough inhibits the asparagine utilization of the yeast (Fredriksson, *et al*., 2004).

2.1.2.4 Acrylamide in almonds:

In almonds acrylamide increases with roasting time and temperature, but temperature have much higher impact on the formation than time. During the roasting procedure sugars are consumed faster and to a larger extent than free asparagine, suggesting that the content of reducing sugars is the critical factor for formation of acrylamide in roasted almonds. Acrylamide was found to decrease in roasted almonds during storage at room temperature (Amrein, *et al*., 2005).

2.1.3 Health implications of acrylamide in foods:

In 1994, the International Agency for Research on Cancer (IARC) classified acrylamide as a potential carcinogen to humans (Group 2A) based on its carcinogenicity in rodents. This classification was endorsed by the WHO Consultation in 2002, and took this fact as a matter of concern to human health. After rapid confirmation of this observation, the potential health risks of acrylamide in food have been evaluated by Scientific Committee on Food (SCF 2002) and British Food Standard Agency (FSA 2002). The major findings of these studies have additionally indicated that acrylamide is neurotoxic in animals and humans and it is a reproductive toxicant, is genotoxic and is a carcinogen in rodents. Consequently in order to accurately assess the amounts of acrylamide to which human are exposed, the content of acrylamide has been determined in a high number of processed foods.

The summary report of the 64th meeting of the Joint Food Expert Committee on food Additives (JECFA 2005) of Agriculture Organization of the United Nations/ WHO provided acrylamide occurrence data from 24 countries for different food items analyzed from 2002 to 2004. Analysis of the data showed moderate levels of acrylamide (<50 µg kg^{-1})in heated protein-rich foods such as meat, fish and seafood and higher contents (150-4000 µg kg^{-1}) in carbohydrate-rich foods such as cereal and cereal based product, beetroot and selected commercial . High acrylamide levels were also found in coffee extracts (up to 4948µg kg^{-1}) and substitutes (up to 7300 µg kg^{-1}) (WHO 2002).

2.1.3.1 Dietary acrylamide intake:

Estimation of dietary acrylamide intake has been made for several populations comprising different dietary records (Dybing *et al*., 2005; Fohgelberg *et al*., 2005; Hilbig *et al*., 2004; Hilbig and Kersting, 2006; Konings *et al*., 2003; Matthys *et al*., 2005; Sommerfeld and Dehne, 2006). The calculated average acrylamide intake ranges from 0.3 to 0.6 mg/kg body weight per day for adults, while children and adolescents tend to ingest more acrylamide on a per bodyweight basis (0.4–0.6 mg/kg). This can be ascribed to a combination of children higher caloric intake as well as their higher consumption of certain acrylamide rich foods, such as French fries and potato crisps (Dybing *et al*., 2005; Wilson *et al*., 2006). The foods with the highest contribution to acrylamide intake vary from country to country according to national dietary patterns and methods of food preparation.

2.1.3.2 Metabolism of acrylamide:

Owing to its polarity and low molecular mass, acrylamide is readily incorporated and distributed in animals and humans, as shown in isotope experiments (Sumner *et al.*, 1992). However, it is likely that interactions of acrylamide with the food matrix such as dietary proteins influence its uptake (Schabacker *et al.*, 2004). After ingestion, acrylamide is rapidly distributed through in the whole body via the bloodstream. It can be found in the thymus, liver, heart, brain, kidneys (Abramsson-Zetterberg *et al.*, 2005), and even in human breast milk (Sorgel *et al.*, 2002). The conjugation of acrylamide to glutathione, as well as its epoxidation to glycidamide in the liver via cytochrome P450, represents the major metabolic routes (Sumner *et al.*, 1992). Toxic kinetic studies revealed that the second pathway becomes increasingly important at lower acrylamide levels in the bloodstream (Doerge *et al.*, 2005). The formation of glycidamide is considered to be the critical step for the genotoxic effects of acrylamide and its metabolites. Acrylamide and glycidamide, the latter at a much higher rate, can react with macro-molecules such as hemoglobin and enzymes (Wilson *et al.*, 2006). Furthermore, glycidamide may react with DNA, leading to point mutations and cancer.

2.1.3.3 Carcinogenicity of acrylamide:

Earlier experiments showed two relevant effects of acrylamide on health carcinogenicity and neurotoxicity (Friedman, 2003). Since neurotoxic effects only occur at high acrylamide levels (NOAEL 0.5 mg/kg body weight per day), which cannot be reached via food, they can be disregarded when discussing health concerns from bakery products. In contrast, clear evidence of the carcinogenic and genotoxic effects of acrylamide and its metabolite glycidamide was found in various in vivo and in vitro studies (Wilson *et al.*, 2006). In cell culture studies, acrylamide induced chromosomal breaks and point mutations, while experiments with mice and human cell lines treated with acrylamide showed increased gene mutation rates, especially exchange of adenine by guanine and guanine by cytosine, respectively. In animal experiments with mice, tumors were observed in the thyroid gland, testes, mammary gland, lung, clitoral gland, and brain (Rice, 2005). Additionally, a lifetime oncogenicity study with Fischer rats receiving up to 2mg acrylamide per kg bodyweight revealed significant increases of tumors in the thyroid gland, testes, central nervous system, uterus, and other tissues (Johnson *et al*., 1986). These findings were only partly confirmed by Friedman *et al*. (1995). However, in all animal studies, high doses of acrylamide were used so several epidemiological studies were conducted (Wilson *et al*., 2006) to assess the cancer risk for humans. A study by Mucci *et al*. (2003), using data from an existing population-based case control study in Sweden, revealed no impact of dietary acrylamide on cancer of the bladder, bowel, and kidneys. Furthermore, no positive correlation was found between acrylamide and renal cell cancer, cancer of the pharynx, larynx, breast, or ovaries. Prospective studies, which are generally regarded to have a higher level of evidence than case control studies, could not establish a correlation between acrylamide ingestion and breast or colon cancer.

Nevertheless, acrylamide remains ‘‘potentially carcinogenic to humans’’ unless more detailed studies provide clear evidence of the opposite. Therefore, acrylamide levels in foodstuff should be as low as reasonably achievable (ALARA principle). For this purpose, the formation routes of acrylamide need to be elucidated so that suitable measures can be taken.

2.1.4 Mechanism of acrylamide formation in foods:

Acrylamide is not a substance that is added to food, but it is formed in food during heat processing. Research indicates that heating of food could be an important source of acrylamide formation (Becalski, *et al*., 2003). Acrylamide formed in a wide variety of foods, particularly carbohydrate (reducing sugars) rich foods cooked at above 120°C upon frying, baking and roasting (De Meulenear *et.al* 2008). However, acrylamide formation in potato fries taken place at below 120°C at low moisture content and prolonged heating conditions (Gökmen and Senyuva 2006).

Acrylamide formation follows different routes in conjunction with the Millard reactions system in food products, where the asparagine route is the major one for the formation of acrylamide (Eriksson, 2005).

2.1.4.1 Asparagine route of acrylamide formation:

One of the major pathways of acrylamide formation is the asparagine route (Gökmen and Palazoğlu 2008). Also different other routes have been suggested in conjunction with the Maillard reactions system in food products (Mottram *et al*. 2002; Stadler *et al*. 2002). Asparagine can principally be converted to acrylamide through thermal decarboxylation and deamination reactions, but the presence of reducing sugars is essential for these reactions to occur. Many carbonyl containing moieties can enhance a similar transformation (Stadler *et al*. 2004; Becalski *et al*. 2003; Yaylayan *et al*. 2003; Zyzak *et al*. 2003; Stadler *et al*. 2003). In model studies, it has been shown that α-hydroxy carbonyls are much more efficient than α-dicarbonyls in converting asparagine to acrylamide (Stadler *et al*. 2003).Furthermore, according to the results obtained from model systems, fructose increases the acrylamide content by about two times in comparison with other reducing sugars because it contains two α-hydroxylic groups rather one as is the case with other sugars (Eriksson, 2005).

The first step in the asparagine route is the formation of Schiff bases from asparagine and reducing sugars (intermediate I). The main part of this compound, i.e., carboxylate ion, enhances the decarboxylation to intermediate II. The resulting negatively charged α-carbon is directly transferred for removing the hydrogen from the α-hydroxy group. Furthermore, the resulting α-hydroxy anion (a strong base within the molecule) will now be able to remove the acidic α-hydrogen in a six-member ring formation when the last step of the degradation to acrylamide is initiated.

In addition, decarboxylated asparagine (3-aminopropionamide) can generate acrylamide in the absence of reducing sugars. Structural considerations dictated that asparagine alone might be converted thermally into acrylamide through decarboxylation and deamination reactions. However, the main product of the thermal decomposition of asparagine is maleimide, mainly due to the fast intermolecular cyclization reaction that prevents the formation of acrylamide. On the other hand, asparagine (in the presence of reducing sugars) is able to generate acrylamide in addition to maleimide (Yaylayan *et al*. 2003, Zyzak *et al*., 2003).

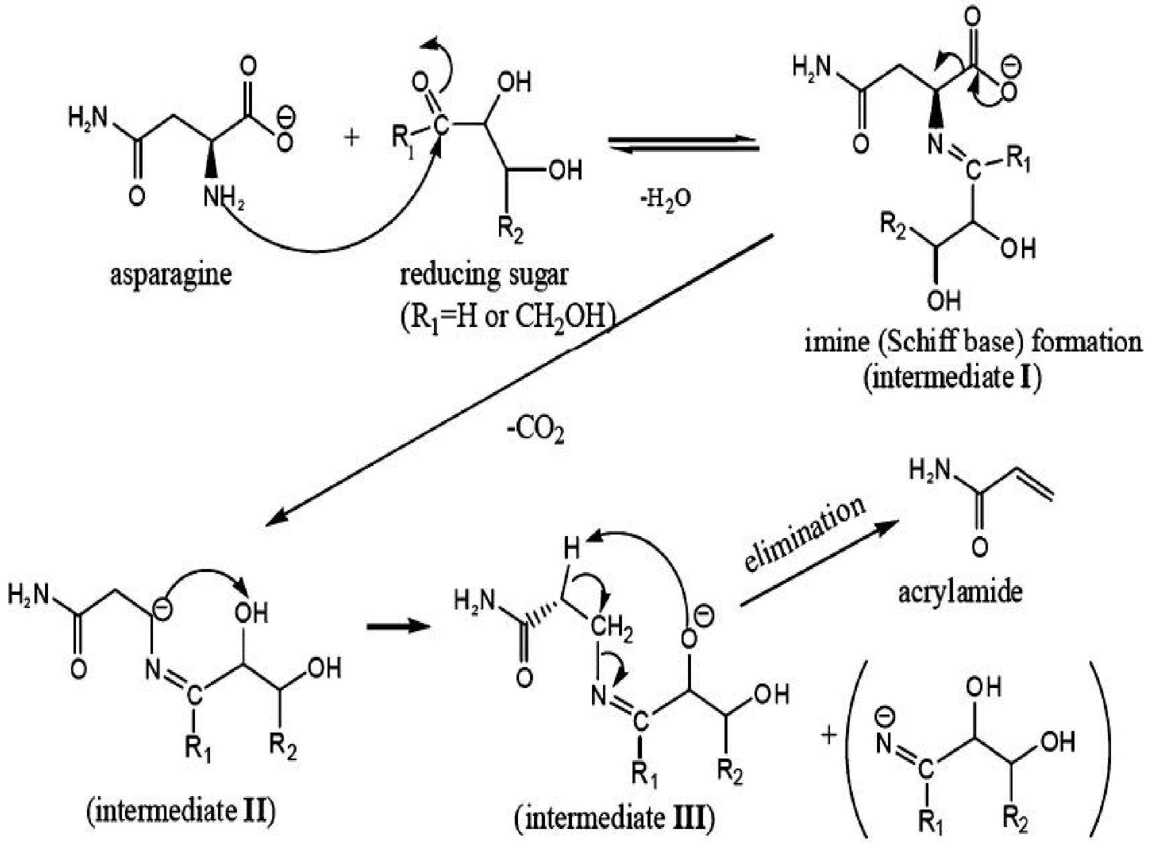


Figure 2:A proposed mechanism which follows the Maillard reaction, for formation of acrylamide from asparagine and reducing sugar (Eriksson,2005).

2.1.4.2 Alternative pathways of acrylamide formation:

Even though the asparagine route is the dominant route in the formation of acrylamide in foods, there are also alternative routes via the Maillard reaction system. The term Maillard reaction is a collective term to the reaction between amines and carbonyl compounds. The knowledge on the chemistry of color formation and toxic and mutagenic compounds is scant compared with flavor formation. The Strecker degradation is an important pathway via Maillard reaction, in which amino acids react with dicarbonyl degradation products which are suggested as a way of acrylamide formation. Another component produced from hexose degradation, as well as fructosyllysine dehydration, is hydroxyl methyl furfural (Kroh 1994; Ramírez-Jiménez *et al*. 2000).

Some pathways proposed as alternatives to the Maillard reactions include:

A: Aspartic acid, carnosine, and β-alanine can give rise to acrylamide formation through the formation of acrylic acid during their thermal decomposition in combination with available ammonia to convert acrylic acid to acrylamide (Yaylayan *et al*., 2004; Stadler *et al*., 2003; Yaylayan *et al*., 2005; Sohn and Ho, 1995).

B: Amino propionamide has been identified as an intermediate during acrylamide formation from asparagine. This compound is also formed in reactions between asparagine and pyruvic acid and is a very effective precursor to acrylamide formation (Stadler *et al*., 2004; Zyzak *et al*., 2003).

C: Pyruvic acid can be generated by dehydration and desulfidation of serin and cysteine, respectively. It can then be proposed as the reduction of pyruvic acid into lactic acid, with further dehydration into acrylic acid. Finally, acrylic acid is transformed to acrylamide (Yaylayan *et al*., 2005; Wnorowski and Yaylayan, 2003).

D: Benz aldehyde and styrene are formed as volatile compounds during pyrolysis of Amadori compounds. As the latter compound represents the decarboxylated
Amadori compound of phenylalanine, acrylamide may be formed from the decarboxylatedAmadori compound of asparagine (Stadler *et al*., 2004).

2.1.5 Factors affecting the kinetics of acrylamide formation and elimination:

The resulting acrylamide concentration in foods ultimately depends on both compositional and process variables. From a mechanistic point of view, acrylamide formation requires amino acid asparagine and a carbonyl compound as the Maillard reaction precursors (Mottram *et al*.2002; Stadler *et al*., 2004). The concentrations of acrylamide precursors and temperature mainly affect the rate of acrylamide formation (Mottram *et al*.2002; Tareke *et al*., 2002; Becalski *et al*.2003; Biedermann and Grob, 2003; Rydber *et al*.2003; Matthäus *et al*.2004; Pedreschi *et al*.2005; Gökmen *et al*., 2006).

2.1.5.1 Effect of raw materials:

The influence of variety, harvest year, fertilization and storage conditions on acrylamide formation have been studied in potato products (Truong *et al*., 2013) and also in cereal products .The composition of potatoes vary with variety (Torres, 2009; FIiselier and Groblk, 2005).Potato relatively contains high amounts of reducing sugars, which is the major limiting factor in potato products for acrylamide formation. While the major limiting factor in cereal products for acrylamide formation is asparagine. Therefore, controlling reducing sugars and asparagine may be a better option to reduce acrylamide in potato and cereal products respectively (David *et al*., 2012). Potato varieties with low concentrations of reducing sugars can be an effective way to reduce acrylamide concentration (Lindasy and Jang 2006; Sanny *et al*., 2012).

Climatic condition such as harvest year has a significant impact on asparagine and reducing sugars in potatoes. The asparagine content was significantly lower in all the samples from the 2004 harvest as compared to 2003(Park *et al.*, 2005). Fertilization is considered to be a key factor in crop production. A decrease in nitrogen fertilization enhanced reducing sugars concentrations, resulting in an increase of acrylamide formation in potato products (Mestdagh *et al.*, 2008), where inverse effects have been noticed for bakery products.

Generally, potato tubers are stored for several months in order to meet the supply throughout the year. Cold temperatures and senescent sweetening are the main causes of sugar accumulation in potatoes during storage (Blenkinsop *et al.*, 2002). Higher temperature storage (more than 8°C), which results senescent sweetening is also related to sprout formation in potatoes (Amrein and Schönbächler *et al.*, 2004). Storing potatoes at low temperature (below 8°C) was found to be an effective technique to inhibit sprouting, temperature below 4-6°C has a major effect on reducing sugar accumulation (Knutsen *et al.*, 2009;Viklund *et al.* , 2008).

2.1.5.2 Effect of temperature:

Temperature has an important role in the formation and elimination of acrylamide. It is well known that acrylamide is formed in foods that are cooked at high temperatures (>120°C) (Mottram *et al.*, 2002; Tareke *et al.*2002; Becalski *et al.*2003; BiedermannandGrob2003; Rydberg *et al.*, 2003). For shorter heating times, as in the frying operation of potato chips or strips, lowering the frying temperature may significantly reduce the amount of acrylamide formed. The same may not be true for longer heating periods as in the roasting of coffee beans where extending the operation may result in a decrease in the amount of acrylamide persisted in the final product (Şenyuva and Gökmen, 2005) There may be a critical temperature and time zone where acrylamide is formed at a greater rate than it is destroyed, and at temperatures outside of this zone little acrylamide is present.

The fact that acrylamide is not formed during boiling indicates that higher temperatures and/or low moisture conditions are needed for its formation. During heating under atmospheric conditions, higher temperatures can be reached only if simultaneous drying takes place, which is the case in frying, baking, and roasting. The loss of water as the food dries during heating extracts a large amount of the incoming energy, and hence, the bulk of the product is at a temperature very much lower than that of the heating medium. In this respect, temperature, time, and moisture are key drivers of acrylamide formation in foods during heating. The moisture content determines the physical state and mobility of chemical constituents in food matrix. In addition, water alone affects the chemical route and the mechanistic pathway for acrylamide formation.

Concerning reducing sugars as carbonyl source, fructose has been found more effective than glucose in forming acrylamide. Both the chemical reactivity of sugars and their physical state play an important role in acrylamide formation. The melting points of fructose and glucose are 126 and 157°C, respectively (Robert *et al*., 2004). This explains why fructose is more reactive than glucose on acrylamide formation during heating. Frying, baking, and roasting are simply characterized as open processes in which heat and mass transfer occur simultaneously. As the moisture reduces due to evaporation, sugars initially dissolved in water begin to form a saturated solution and then crystallize. After crystallization, melting is required to change their state to liquid, so as to make them chemically reactive. In this respect, reducing sugar having a lower melting point is expected to form acrylamide earlier during heating as the formation of acrylamide appears to be strongly linked to the Maillard reaction; factors affecting its rate also affect acrylamide formation. It is well known that the rate of Maillard reaction is strongly dependent on pH of the reaction environment. It has been reported that the addition of acids, by means of lowering pH, decreased the amount of acrylamide formed in foods during heating (Rydberg *et al*., 2003; Jung *et al*., 2003; Surdyk *et al*.,2004; Kita *et* *al*., 2004; Low *et al*., 2006). The protonation of the α-amino group of asparagines hinders the formation of the N-substituted glycosylamine, which may explain the reduced acrylamide content of acid-treated foods. However, the addition of citric acid into dough comprising sucrose has been shown to increase the susceptibility of acrylamide formation in cookies due to the excessive hydrolysis of sucrose, which increased the concentration of reactive sugars (Gökmen *et al*., 2007).

2.1.5.3 Effect of additives:

Asparaginase, an enzyme that converts precursor (asparagine) into ammonia and aspartic acid, can reduce acrylamide formation in foods (Kumarand Singh, 2004). It is commercially produced from *Aspergillus niger* or *Aspergillus oryzae* and found its most applications in potato and cereal products. Though it is a promising strategy for acrylamide reduction, it is rather expansive compared with other strategies.

The addition of amino acids or protein rich substances reduces the acrylamide content in foods (Rydberg *et al*., 2003; Vattemand Shetty, 2005). Amino acids such as glycine, cysteine, methionine, glutathione and lysine on acrylamide formation and its elimination kinetics was assessed in several studies (Brathen and Knutsen, 2005; Anese *et al*., 2009). Formation of acrylamide decreased by 50% when cysteine and methionine were added to cracker and potato dough (Elder, 2005). On the contrary (Flückiger and Salih,2006) studied the effect of cysteine on acrylamide formation in crisp bread and found that no such effects. Addition of antioxidants has been found to influence the Maillard reaction, which results in acrylamide formation (Zhang, 2005, Ou *et al*., 2010). Antioxidants present in the rosemary extracts, bamboo leaves and green tea extract (Zhang, 2008) could effectively reduce acrylamide presence in different heated foods. The exact mechanism on acrylamide formation is not yet understood, however it is proposed that it could interact with active aldehydes and block the oxidation of acrolein to a certain extent (Totlani and Peterson, 2006; Grangby *et al.*, 2008).

Mono and divalent cations (Na^+ and Ca^{2+} or Mg^{2+}) added to the dough showed a remarkable effect on acrylamide reduction (Elder *et al.*, 2008). In addition, polyvalent cations also capable to reduce acrylamide formation during heating. These ions could interact with asparagine so that prevent the Schiff base intermediate formation and thus acrylamide generation (Gökmen and Şenyuva, 2007; Tomoda *et al.*, 2004).

A significant effect of NaCl on acrylamide reduction via polymerization was reported by several studies. However, the acrylamide content increased at higher NaCl levels, which was attributed to an inhibition of yeast growth by the salt (Voelker, 2005). Acrylamide formation in potato strips was reduced by hydrocolloid coatings when alginic acid and pectin were used as coating agents (Zeng *et al.*, 2010) whereas carob gum, carrageenan, hydroxypropyldi starch phosphate and xanthan gum stimulated acrylamide formation.

2.1.5.4 Effect of processing conditions:

Most of the strategies proposed to reduce the acrylamide are focused on the processing stage. The important factors that influence the process of acrylamide formation are heating temperature and time, blanching and frying (Ciesarovà *et al.*, 2006). Several studies were conducted on the effects of baking temperature and time combination during baking and found strong correlation between baking temperature and time and acrylamide formation (Elmore *et al.*, 2005; HEATOX, 2007). However, the prolonged baking temperature and time combination (260°C, 20 min) decreased the acrylamide content in foods. Optimized conditions such as temperature and relative humidity profile resulted in a 50% reduction of acrylamide formation. Conduction and radiation heat transfer are more effective in acrylamide reduction than convection baking ovens. Moreover, the combination of conventional and dielectric (microwave) heating found to be suitable for reduction of acrylamide in bakery products. Baking at high relative humidity proved to be effective for reducing acrylamide in bakery products (Anese, Suman ,2010; Vleechouwer *et al*., 2007). This can be achieved not only by reducing the temperature, but also by using steam as heating medium during the final part of baking. Acrylamide formation mostly occurs in the outer crust than in the inner crust of bread, while only trace amount appeared in the crumb (Sadd and Hamlet, 2008).

Blanching is an important unit operation in the prediction of French fries production. This reduces acrylamide formation mainly by leaching the precursors (reducing sugars) prior to frying (Pedreschi *et al*., 2004; Samir *et al*., 2013). Blanching conditions (temperature and time) can be varied in order to maintain the final product specifications constant. Blanching at 70°C for 10 to 15 min, reduced acrylamide formation of 65% and 96% for French fries and potato chips, respectively. Frying is used in food processing both at industrial and home levels. Acrylamide formation is correlated to color development, which occurs in Millard reaction during heating process, mostly at the end of the frying process (Fiseiler *et al*., 2006; Amrein *et al*., 2007). Intensive frying conditions (temperature and time) lead to darker fries and higher acrylamide formation. Thus, two factors such as frying temperature (should not exceed 170-175ºC) and time to be considered as important for acrylamide reduction. Frying under low pressure conditions using vacuum fryer results in higher acrylamide reduction (Granda *et al*., 2004).

2.1.5.5 Effect of pH:

Maillard reaction has strong influence on pH; it is known that high pH affect nutrients in foods (Eriksson, 2005). Researchers showed that the reduced pH drastically reduces acrylamide content during frying and baking (Grob, 2007; Jung *et al*., 2003). Any acid treatment reduces the pH of foods and results in formation of Maillard associated substances. Addition of different acids decreased the amount of acrylamide in bakery products such as corn chips, semi-finished biscuits and cracker models (Graf *et al*., 2006;Jung*et al*., 2003). Reduced pH levels results in Millard reactions, accompanied by reduced acrylamide formation (Delatour *et al*., 2004).

2.1.5.6 Effect of water activity:

Water activity in food plays a major role in reducing acrylamide formation. Acrylamide forms in food when the water activity is below 0.8, whereas the acrylamide formation is high at water activity of 0.4 and below (Hoenicke and Gatermann, 2005). However, the removal of acrylamide from heated foods such as biscuits and potato chips increases with the increase of water activity.

2.1.5.7 Effect of fermentation:

Fermentation controls the rate of acrylamide formation in food by maintaining precursor composition and pH. Prolonged fermentation time (at least an hour) was found to be suitable for acrylamide reduction in bread and fried potato products (Fredriksson *et al*., 2004),combined lactic acid fermentation with blanching were found suitable for higher acrylamide reduction in potato products (Baardseth *et al*., 2006).

2.1.6 Methods of acrylamide analysis:

Many analytical methods have been developed for acrylamide determination in food. Extraction and cleanup of acrylamide from food matrices is a critical factor for analytic recovery (Elbasiher *et al*., 2014). Due to the high polarity of acrylamide water is a highly efficient extraction solvent for acrylamide from different food matrices. Because of low selectivity of water as extractant, tedious and time consuming cleanup procedures are needed to partially isolate the analytic from other matrix components. Mostly cleanup procedures consist of the combination of two different solid-phase extraction (Fernandes and Soares, 2007; Yusà *et al*., 2006).

QuEChERS (quick, easy, cheap, effective, rugged and safe) method was introduced by Mastovska and Lehotay in 2006 as high throughput determination method for acrylamide analysis in various food matrices using

LC-MS/MS or GC-MS. It involves miniaturized extraction with acetonitrile, liquid- liquid partition by salting out with sodium chloride and magnesium sulfate and cleanup the extract using dispersive solid- phase extraction(dSPE) step with loose sorbent such as primary secondary amine (PSA) (Oracz *et al*., 2011).

Analytical methods based on liquid or gas chromatography coupled to mass spectrometry (LC-MS/MS or LC-MS,GC-MS)have been used for the determination of acrylamide contents in food samples (Fernandes and Soares,2007;Hoenick and Gatermann,2005;Kaplan *et al*., 2009).For GC, a tedious procedure of derivatization is usually required, and the high injection temperature may result in acrylamide production from any co-extracted acrylamide precursors, and thus false results are obtained (Longhua *et al*., 2012).The main advantage of the LC-MS/MS based methods is that acrylamide can be analyzed directly without prior derivatization, which considerably simplifies and expedites the analysis (Krska *et al*., 2012;Riediker and Stadler, 2003).

To maintain sample throughput and cost effectiveness ratio, the development of generic liquid chromatography or ultra-high performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) screening methods is highly demanded (Gómez *et al*., 2012).The major advantages of UHPLC over classical HPlC utilizing columns packed with 5.0 µm particles include improved resolution within a shorter retention time and higher analytical sensitivity (Zhang *et al*., 2007).Due to low molecular mass of acrylamide and thus also its low mass fragment ions, confirmation of the analytic can be achieved with a two stage mass spectrometer(Riediker and Stadler, 2003).

2.1.6.1 Extraction and purification:

The high water-solubility of acrylamide means that extraction from foods using plain water is very effective, with no need for pH adjustment (Eriksson

2005). Water at room temperature has been used as an extractant in most LC methods (Becalski *et al*. 2003; Rosén and Hellenäs 2002; Tareke *et al*. 2000). Heating during the extraction should be avoided as this may generate the SPE columns used in further clean-up steps (US Food and Drug Administration 2003).

Purification of water extracts for acrylamide analysis is based on the following principles: purification with SPE columns and chemical purification (Eriksson 2005). However, the extraction solvent could be a mixture of water and organic solvents such as n-propanol or 2-butanone (Biedermann *et al*. 2002). Recovery rates of 68–75.4% have been reported when pure methanol was applied for the extraction of baked food products (Tateo and Bononi 2003). Also, the extracted amount of acrylamide, using Soxhlet extraction with methanol, far exceeded that by other extraction techniques (>90%). However, one drawback is the long extraction time of 10 days while no information is available on the potential acrylamide formation during the extraction (Wenzl *et al*. 2003). Although solubility of acrylamide in most organic solvents is lower than water, dichloromethane appears to be a promising extraction solvent, particularly addition of 2% ethanol as a modifier improves the results considerably. Re-extraction of the organic phase with water delivers an aqueous solution which is almost free of any interfering matrix components. For instance, fatty components remain in the organic phase; thus, defatting of the sample prior to extraction is not necessary any more. Applying this method, nearly all of the target foodstuffs could be analyzed in a similar fashion with satisfactory results (Brandl *et al*. 2002; Dionex, 2004).

Most purification procedures consist in combining several SPE, for instance combination of three different cartridges: mixed-mode anion exchange, mixed-mode cation exchange, and graphitized carbon have been used (Becalski *et al*. 2003). Takatsuki *et al*. (2003) also used a similar combination of SPE cartridges for the clean-up of samples, which were measured by LC-

MS with column switching. Also, a combination of SPE and filtration and/or ultracentrifugation has been used to avoid blockage of the chromatographic system (Wenzl *et al*. 2003). However, Höfler *et al*. (2002) reported that both SPE and liquid– liquid extraction did not lead to any significant improvement in the analysis. Therefore, filtration through a 0.22- µm nylon filter is the only sample treatment used as cleanup procedure after extraction and before applying to HPLC.

In contrast, other laboratories added acetonitrile to the aqueous extract and pipetted 0.5 ml Carrez I and Carrez II onto the sample in order to obtain a clear sample (Wenzl *et al*. 2003).One special aspect of the extraction procedure involves the swelling of the matrix in order to provide better access for the extraction solvents to potentially adsorbed or enclosed acrylamide. However, the side effect associated with swelling is that it provides some time for the development of matrix/internal standard interactions. For this reason, the homogenized sample is mixed with water and an internal standard solution and kept at a pre-specified temperature for 10–20 min. Depending on the matrix, swelling yielded an increase in analytic recovery of up to 100-fold (Biedermann *et al*. 2002).Although extraction at room temperature provides satisfactory results, hot water (60–80°C) can be used to enhance the extraction. Increased recovery has also been observed by treating the sample in an ultrasonic bath (30 min at 60°C; Schaller 2003). Problems with the high viscosity of the sample/water extraction mixture have been reported to be solved by the addition of small amounts of amylase to the mixture.

2.1.6.2 Derivatization:

In derivatization methods, acrylamide is converted to 2,3-dibromopropionamide which is volatile and can be detected on a GC with an electron capture or an alkali flame detector (Tekel *et al*. 1989; United States Environmental Protection Agency 1996). This bromination is mostly performed by ionic reaction (Hashimoto 1976; Arikawa and Shiga 1980). The analysis has been suggested to be performed on the more stable 2-bromopropenamide obtained after debromination of 2,3-dibromopropionamide (Andrawes *et al*. 1987; Martin *et al*. 1990).

Applications of SPE columns to obtain concentrated samples or utilizing a more sensitive derivatization technique may increase the possibility for determination of residual acrylamide in many types of food (Kawata *et al*. 2001; Pérez *et al*., 2003).

Bromination of acrylamide has the advantage that a more volatile compound is produced and the selectivity of determination is enhanced (Wenzl *et al*. 2003). However, some derivatization approaches are laborious and timeconsuming.

The procedure first reported by Hashimoto (1976) is carried out by adding a pre-prepared bromination solution containing potassium bromide, hydrogen bromide, and bromine to either the pre-treated or raw aqueous extracts (Tareke *et al*. 2002; Ahn *et al*. 2002; Castle 1993;Castle *et al*. 1991; Ono *et al*. 2003). In this method, the yield of 2,3-DBPA is constant and >80% when the reaction time is more than 1 h (United States Environmental Protection Agency 1996).Nemoto *et al*. (2002) improved the method by using different derivatization reagents including potassium bromide and sodium bromate in an acidic medium. The necessity of additional sample pre-treatment depends upon the matrix. Matrices such as carbohydrate rich foods (e.g.,potato crisps or bread) require additional fractionation steps (Tareke *et al*. 2002; Tareke *et al*. 2000). Usually, the raw extract is subjected to fractionation on a graphitized carbon black cartridge.

Bromination is frequently carried out overnight at 0 °C or slightly above the freezing point of water. It has also been stated that application of isotopically labeled internal standards allowed a reduction in the reaction time from overnight to1 h. This is in accordance with the methods proposed by other scientists (Ono *et al*. 2003; Nemoto *et al*. 2002).

The excess of bromine is removed after the reaction by titration with sodium thiosulfate solution (0.7–1 M) until the solution becomes colorless. The brominated acrylamide is less polar compared with the original compound and, therefore, non-polar organic solvents (usually ethyl acetate or a mixture of ethyl acetate and cyclohexane) are used for the extraction of the analyte from the aqueous phase. Gertz and Klostermann (2002) reported that, on a DB-5 MS column, a transformation of 2,3-DBPA to 2-BPA does not take place, so that it is not necessary to transform the dibrominated compound into the more stable 2- monobromopropenamide by adding triethylamine. However, more recent reports confirm that, in acrylamide analysis with bromination and detection by GC-ECD in HPINNOWAX capillary column, 2-BPA rather than 2,3-DBPA was chosen as the quantitative analyte because the peak response of former was nearly 20 times higher than that of the latter (Zhang *et al*. 2006).

2.2 Wheat:

Wheat (*Triticum* species) is one of the oldest food crops grown in the world. It has been known to exist since 10 000 B.C as an agricultural species intentionally cultivated by man (Feldman, 2001; Gustafson *et al*., 2009). Its popularity stems in part from the adaptability of wheat as a cultivated crop suited for many different soils and climatic conditions. Most significantly, wheat is unique because of the ability of wheat proteins to combine in to the protein mass known as gluten. Wheat is thus the only effective source of flour for bread production in the world (Shellenberger, 1971).

The wheat grain contains 2-3% germ, 13-17% bran and 80-85% mealy endosperm (all constituents converted to a dry matter basis) (Belderok *et al*., 2000).

Bran is rich in B vitamins and minerals it is separated from the starchy endosperm during the first stage of milling. More than half the bran consists of fiber components 53%. Chemical composition of wheat bran fiber is complex, but it contains essentially cellulose and pentosans, polymers based on xylose and arabinose, which are tightly bound to proteins.

The endosperm mainly contains food reserves, which are needed for growth of the seedling, it is rich in energy yielding starch, the mealy endosperm contains fats (1.5%) and proteins (13%) albumins, glubulins and the major proteins of the gluten complex glutenins and gliadins. proteins that will form the gluten at dough making. The contents of minerals (ash) and of dietary fibers are low; 0.5% and 1.5%, respectively (Belderok *et al*., 2000).

The germ lies at one end of the grain. It is rich in proteins (25%) and lipids (8-13%). The mineral level is also rather high (4.5%).Wheat germ is available as a separate entity because it is an important source of vitamin E. Wheat germ has only one half the glutamine and proline of flour, but the levels of alanine, arginine, asparagine, glycine, lysine and threonine are double (Cornell 2003).

2.2.1 Rugag:

Rugag is flaky bakery product, it is considered the major meal in late night in Ramadan of most Sudanese families. It is much similar to corn flakes and breakfast cereal, Rugag is eaten with adding milk and some sugar. The term Rugag came from it is shape, and it is baked in thin sheets.

2.2.2 Homemade biscuits:

Homemade biscuits is one of simple homemade bakery, which is baked in Eid and in special occasions (wedding).It is prepared by using white wheat flour, sugar, milk, oil or ghee, baking powder, ammonium bicarbonate and sometime eggs and vanilla maybe added, it is baked at home or in the local bakery.

CHAPTER THREE

MATERIAL AND METHODS

3.1 Materials and chemicals:

Wheat flour Makhsous (72% extraction) was obtained from Sayga (Khartoum, Sudan), sugar from Safi (Dal group, Khartoum ,Sudan) ,milk and milk powder were from Capo (Dal group, Khartoum, Sudan), ghee (homemade), baking powder (Foster Clarks product LTD. EU). Ammonium bicarbonate and vanilla were obtained from local stores from Omdurman's market,corn flour from Omer company for packaging (Khartoum, Sudan). Acrylamide (purity 99.0%) from Dop organic kimya san ve. Tic. (Turkey). Acetonitrile Carlo Erba Reagents. S.A.S, Dasity group(France), 0.1% formic acid SDFCL S.D FINE. CHEM, UMITED,(Mumbai. India), deionized water was purified by using ultra-pure water system from Heal Force Bio-meditech holding limited (Hong Kong). Anhydrous magnesium sulfate, sodium chloride, PSA(Primary secondary amine) were from Finisterres by Teknokroma Revodixinc. (South Korea).

3.2 Rugag preparation method:

Rugag dough was prepared according to the traditional method employed in Sudanese house hold.

Dough was prepared by thorough mixing of 1 kg of wheat flour,190g of sugar, 100g of milk powder,15mg of vanilla,45g of corn flour,5g of salt, 10 g of baking powder all these ingredients were mixed thoroughly by hand and then water was added gradually(about 2.5 liter) with continuous mixing until reached to very soft dough. Baked in a hot frying pan using spreader (Grgareeb a piece of wooden thin sheet used to spared the dough on the iron plate) to spread small amount of the dough about 100 ml to a thin sheet, which is baked in one side at 165-170˚C for 40 seconds then removed carefully by hand. Samples were kept in plastic container at room temperature.

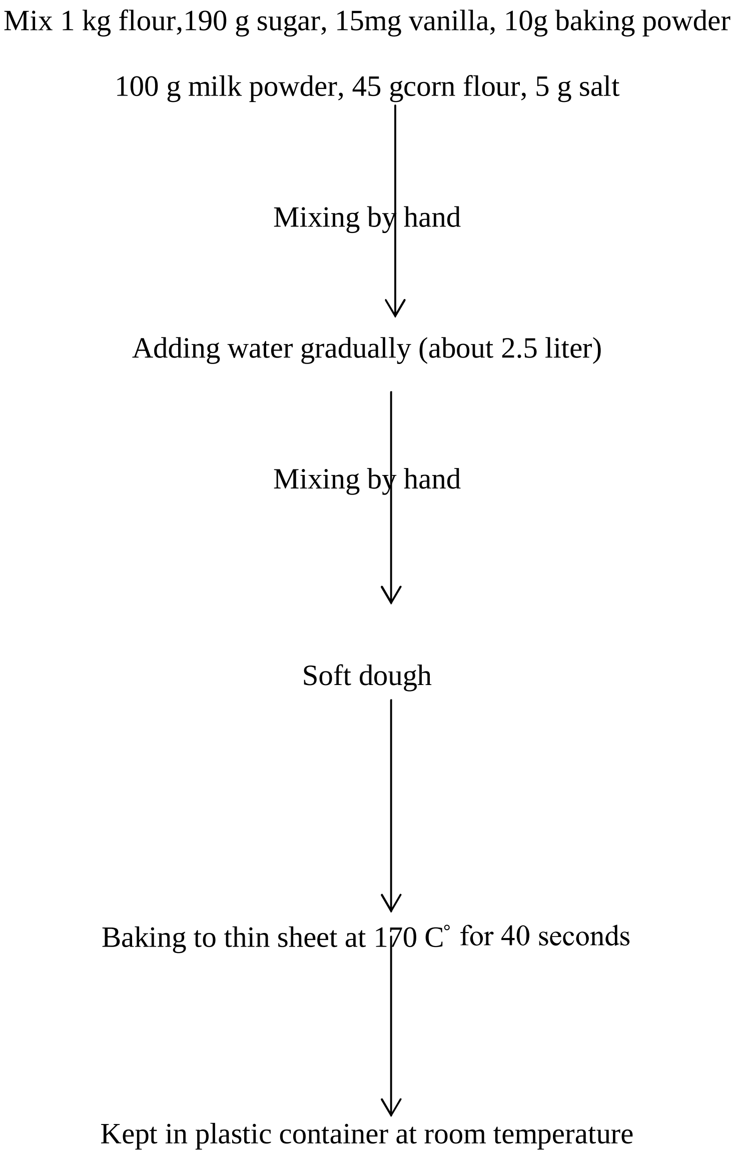


Figure 3: Rugag preparation method.

3.3 Homemade biscuits preparation method:

Homemade biscuits were prepared according to the traditional method employed in Sudanese house hold. Two types of homemade biscuits were prepared, one with added ammonium bicarbonate, and another one prepared using the same quantity of ingredients and processing condition (Time and temperature), but without ammonium bicarbonate.

The recipe used for preparation of biscuits were 200ml of milk with 150ml ghee, 150g of sugar and 45g of corn flour mixed thoroughly in blender (Moulinex, groupe SEB, France),until have a uniform liquid mixture, then other dry ingredients (1kg of flour+ 10g of baking powder+ 15mg of vanilla + 15 g of ammonium bicarbonate and 5g of salt) were mixed thoroughly by hand ,then liquid mixture was added to the dry mixture and mixed together until have a homogenized dough. The dough was shaped by a simple biscuit cutter (local made) to small pieces, placed in greased aluminum trays and baked in oven (Luxell, Tüketim A.S company, Turkey) at 240C$^{\circ}$ for 15 minutes, and the baked biscuit with length 10±0.1 cm ,diameter 3.4±0.2 cm and thickness 1.3±0.2cm was obtained. Samples were kept in plastic container at room temperature.

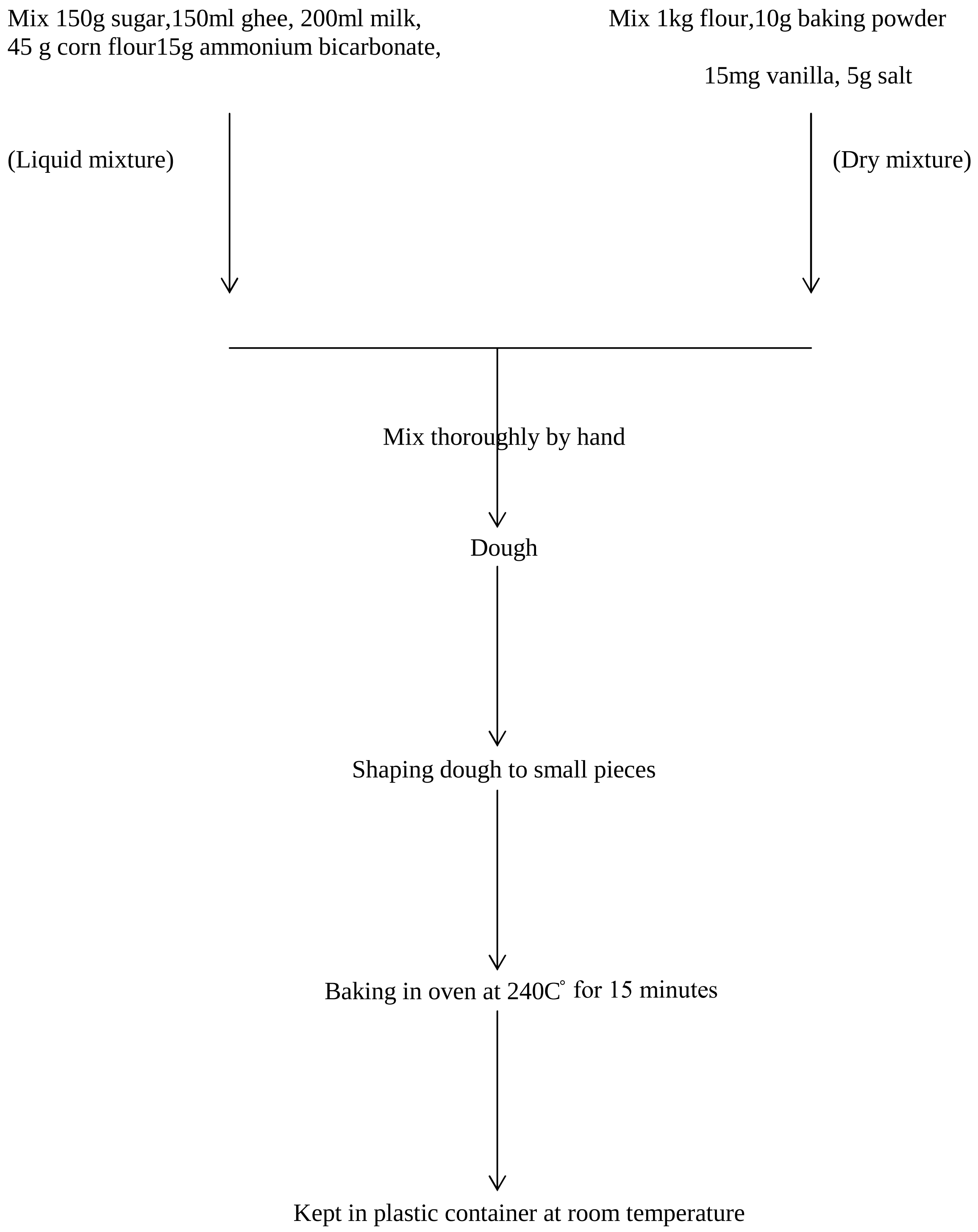


Figure 4: Homemade biscuit preparation method.

3.4 Proximate analysis:

The chemical analyses were carried out according to the standard method of the Association of Official Analytical Chemists (AOAC, 2005).

3.4.1 Moisture content:

Principle:

The moisture of a weighed sample (5 grams) was removed by heating the sample in an oven (under atmospheric pressure) at $105 \pm 1C^{o}$ until a constant weight was obtained. After drying, the covered sample was transferred into a desiccator and cooled to room temperature before reweighing. Triplicate results were obtained for each sample and the mean value was reported to two decimal points according to the following formula:

Calculation:

Moisture content %

$$\text{Moisture content \%} = \frac{(W1-W2)}{W1}\times 100$$

Where:

W_1= Original weight of sample.

W_2= Weight of sample after drying.

3.4.2 Crude protein determination:

The crude protein content was determined in all samples by micro-Kjeldahl method using sodium sulphate catalyst according to the Official Method of the AOAC,(2005).

Principle:

The principle of the method consists of sample oxidation and conversion of nitrogen to ammonia, which reacts with the excess amount of sulphuric acid forming ammonium sulphate. The solution is made alkaline and the ammonia is distilled into a standard solution of boric acid (2%) to form the ammonia-boric acid complex, which is titrated against a standard solution of HCL (0.1N). Accordingly, the crude protein content is calculated by multiplying the total N % by 6.25 as a conversion factor for protein.

Procedure : 0.5gram sample was accurately weighed and transferred together with 2-3 glass pellets, Kjeldahl catalyst (No 33064, BDH, England) and 20ml concentrated sulphuric acid into Kjeldahl digestion flask. After that, the flask was placed into a Kjeldahl digestion unit for about 3hours, until a colorless digest was obtained. Following, the flask was left to cool to room temperature. The distillation of ammonia was carried out in 30 ml boric acid (2%) by using 40 ml distilled water and 60 ml sodium hydroxide solution (33 %). Finally, the distillate was titrated with standard solution of 0.1N HCL in the presence of 2-3 drops of indicator (Bromocreasol green and methyl red) until a brown reddish color was observed. The total nitrogen and protein were calculated using the following formula:

$$N\% = \frac{\text{volume of HCL} \times N \times 14}{\text{weight of sample} \times 1000} \times 100$$

P%= N% × 6.38 (factor)

Where:

N% = crude nitrogen.
P% = crude protein.
N = normality of HCL.
14 = equivalent weight of nitrogen.

3.4.3 Determination of fat content:

Fat content was determined by Soxhlet method of AOAC (2005). Five g of sample was extracted with petroleum either solvent using Soxhelt apparatus for six hours. The crude oil extracted was dried by heating in vacuum oven at 50°C for one hour. The fat content was calculated using the following equation:

$$\text{Fat \%} = \frac{\text{weight of extracted oil}}{\text{weight of sample}} \times 100$$

3.4.4 Determination of crude fiber:

Fiber was determined according to official method of AOAC (2005). About 2g of a defatted sample was placed into a conical flask containing 200ml of H_2SO_4 (0.26N). The flask was fitted to a condenser and allowed to boil for 30 minutes. At the end of the digestion period, the flask was removed and the digestate was filtered through a porcelain filter crucible. After that, the precipitate was repeatedly rinsed with distilled boiled water followed by boiling in 200ml NaOH (0.23N) solution for 30 min under reflux condenser and the precipitate was filtered, rinsed with hot distilled water, 20 ml ethyl alcohol (96%) and 20ml diethyl ether. Finally, the crucible was dried at 105 C^o until a constant weight was obtained and the difference in weight was considered a crude fiber.

Crude fiber % =

$$\frac{\underline{[(\text{Dry residue} + \text{crucible(g)} - (\text{ignited residue} + \text{crucible (g)})]} \times 100}{\text{Sample weight}}$$

3.4.5 Determination of Ash:

The ash content was determined by gravimetric method AOAC (2005). Five grams of the samples were weighed in crucibles, and then placed in a muffle furnace at 550-600 C^o for 3 hours until ashes were carbon free. The crucibles were then cooled in desiccators and weighed. The ash content was calculated using the following equation:

$$\text{Ash\%}=\frac{W_1}{W_2}x100$$

Where:

W_1= Weight of ash
W_2=Weight of sample

3.4.6 Calculation of total carbohydrates:

Total carbohydrates were calculated by difference according to the following:

Total carbohydrates =100% -[Moisture(%)+ Protein (%)+ Fat (%) +fiber (%) +Ash (%)}

3.5 Determination of acrylamide:

3.5.1 Preparation of acrylamide standard solution:

Stock solution of acrylamide standard was prepared by dissolving 0.02mg acrylamide in 1 liter of deionized water in a volumetric flask made up to mark with deionized water.

3.5.2 Sample preparations for LC/MS determination:

The samples were prepared according to original QuEchERS(2003). In 50 ml centrifuge tube five grams of homogenized sample were added to 2 ml of deionized water, then 15ml of acetonitrile were added and mixed thoroughly. Six g anhydrous magnesium sulfate and one g of sodium chloride were added, and shaked vigorously for one minute, using vortex (IKA-works, USA) and then centrifuged (Hettich Mikro 22R, DJB company) at 3700 rpm for one minute. 1ml of aliquot of supernatant was transferred to a 2ml centrifuge tube containing 150mg anhydrous magnesium sulfate and 50 mg PSA, shaken for

30 seconds in vortex, and then centrifuged for one minute at 3700 rpm. An aliquot (2ml) of upper supernatant was dried at room temperature (to evaporate acetonitrile), and then filtered through a 0.45 µm micro syringe filter into a LC/MS auto sampler for analysis.

3.5.3 LC/ MS analysis:

The separation of acrylamide was carried out on LC/ MS (LCMS-2020 SHIMADZU Kyto. Japan). Mobile phase A was 0.1% formic acid and mobile phase B was CAN, mobile phase A:B was 9:1. Pump mode Binary gradient. Total flow was 0.5000 ml\min. Auto sampler model SIL-20AC. Rinsing Speed:35 uL/sec. Sampling speed:15 uL\sec. Oven model CTO-20AC, Maximum temperature:90°C**.** Column name C18. Column length: 150mm. Inner Diameter: 150mm × 4.6mm. MS parameter: Acquisition mode SIM. Polarity positive. Detector voltage +1.20 kV.

3.6 Dietary exposure assessment of acrylamide:

Estimation of the dietary intake of acrylamide is based on the equation for dietary exposure to chemicals (Peter, *et al.*, 2012), while exposure can be defined as:

$$E_i = \frac{\Sigma\, Q_{i,k} \times C_{i,k}}{bw_i}$$

Where:

E_i = The exposure of individual i to some chemical at some specified point in time.

$Q_{i,k}$ = The amount of food k consumed by individual i.

$C_{i,k}$ = The concentration of the chemical of interest in food k consumed by individual i.

bw_i = The body weight of individual i.

While $Q_{i,k}$ assumed as150 g for Rugag and 100 g for biscuit , $C_{i,k}$ in Rugag was 41.73 µg/g (based on 5g of Rugag content 1.391µg/g acrylamide so 150g of Rugag would contain41.73 µg/g acrylamide) and in homemade biscuit was 18.08 µg/g(based on 5g of homemade biscuit content 0.904µg/g acrylamide so 150g of Rugag would contain18.08 µg/g acrylamide), while bw_i the average Sudanese adult body weight is assumed as 60.7 kg for adult (Sarah *et al*., 2012) and child average body weight as 20 kg.

3.7 Statistical analysis:

One- way ANOVA and two sample paired test were performed to examine significant difference between normally distributed data of replicated measurement. Probability level of less than 0.05 was considered significant ($p<0.05$). Data were analyzed using version 17 MINITAB statistical software.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Proximate composition:

Rugag, homemade biscuits with ammonium bicarbonate and homemade biscuits without ammonium bicarbonate, moisture content, protein, fat, fiber, ash results of the proximate composition are presented in Table 2.

Table 2 shows results of proximate analysis of Rugag which indicated that it hasa carbohydrate content of 74.8% resulting mainly from wheat flour which has high amount of carbohydrates. The moisture content was 7.16% due to low thickness of Rugag which enhance water evaporation during the baking at high temperature. It has low fat content (3.7%) as the recipe contained no added oil or ghee, just the fat from milk powder. The content of protein, fiber and ash were 12.31%, 1.0%, 1.0%, respectively. This result is comparable with the result obtained by Suraiami *et al*. (2014) on proximate analysis of cornflakes which has high carbohydrate as content 86% and low moisture content 5.17% while protein, fat and ash were 7.63%, 0.24% and 2.42%, respectively, the variation might be due to the ingredients used and the processing conditions.

As shown in Table 2 there is no significant different (P>0.05) in the proximate composition between homemade biscuits with ammonium bicarbonate and homemade biscuits without ammonium bicarbonate. They showed high carbohydrates content resulting from wheat flour which is a rich source of carbohydrates. The fat content was high due to addition of ghee to the recipe, while moisture content was low due to baking at high temperature 240 ˚C. Fiber and ash content were low since 72% extraction wheat flour was used. The results are in agreement with Mohamed and Ihab (2016) who reported moisture, protein, fat, fiber, ash, carbohydrates of 3.73%,11.75%, 4.46%, 4.44%, 1.0%, 76.67%, respectively.

Table 2: Chemical composition of Rugag, homemade biscuits with ammonium bicarbonate and homemade biscuits without ammonium bicarbonate:

Parameter	**Samples**		
	Rugag	**Biscuit with ammonium bicarbonate**	**Biscuit without ammonium bicarbonate**
Moisture %	7.16 ±.13	7.96±.31	8.03±.07
Protein%	12.31±.12	8.96±.13	9.07±.12
Fat%	3.73±.09	16.45±.23	16.24±.23
Fiber%	1.0±.01	2.43±.15	2.63±15
Ash%	1.0±.01	0.36±.11	0.53±0.1
CHO%	74.79±.23	63.81±.35	63.47±.07
Energy kcal/100g	382±.02	439.23±.5	436.40±.5

*Values are mean ± SD of three replicates.

4.2 Determination of acrylamide content by LC/MS:

4.2.1 Standard acrylamide determination by LC/MS:

Figure 5 shows chromatograph of mass spectra of acrylamide. Figure 6 shows standard choromatogram of acrylamide (0.2 ppm), at retention time 2.350-2.355.

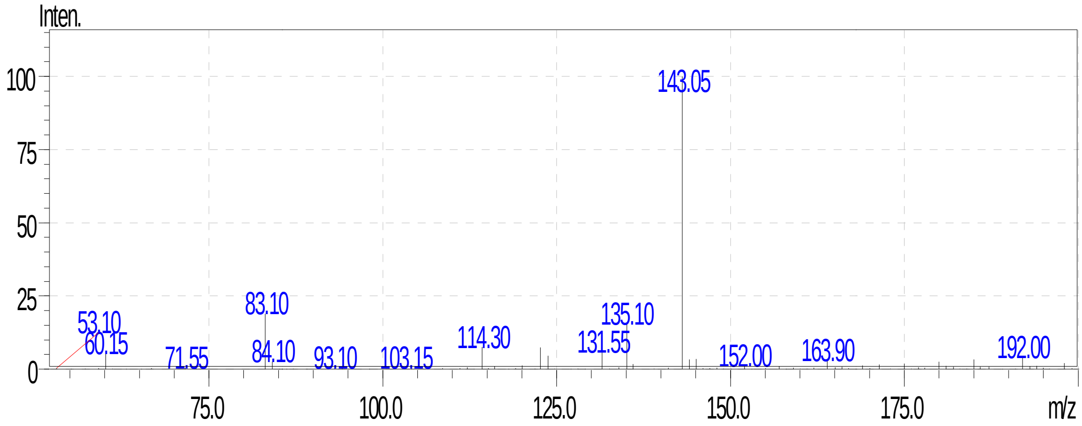


Figure 5:Chromatograph of mass spectra of acrylamide.

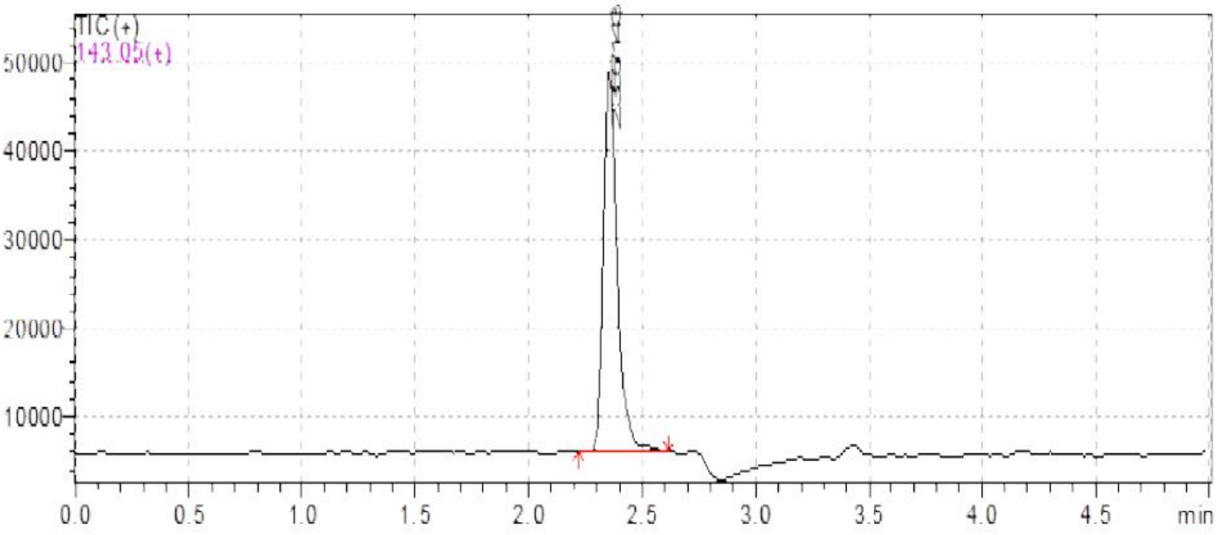


Figure 6:Standard choromatogram of acrylamide (0.2ppm).

4.2.2 Acrylamide content in Rugag:

Figure 6 shows choromatogram of acrylamide in Rugag. The concentration of acrylamide in Rugag was 1.391 ppm (based on the knowing of standard concentration and retention time) as shown in Table 3.This is probably due to the highly content of carbohydrates 74.8% in addition to presence of reducing sugar and free amino groups which are the main precursors of acrylamide in food, besides the low moisture content 7.1% which is more important parameter of acrylamide, therefore a key factor of controlling acrylamide levels, and this could account for much of the variation seen between different types of bakery products (Sadd and Hamlet 2005).

Also baking at high temperature(170˚C) which is considered a suitable temperature for acrylamide formation, as it is well known that acrylamide forms in foods that are cooked at high temperatures (>120°C) (Mottram *et al*.,2002; Tareke *et al*.2002; Becalski *et al*.2003; Biedermann and Grob2003; Rydberg *et al*., 2003), besides the thickness of Rugag which is very thin which enhances heat transfer, which accelerates acrylamide formation in short time (40 seconds), also it is noticed a brown color in the Rugag sheet this correlated with acrylamide formation and concentration as a result of Maillard reaction which is a part of acrylamide pathway. The surface color of the products correlates highly with acrylamide levels in food the darker surface, the more acrylamide it contains (Friedman and Mottram ,2005).

This result is comparable with result obtained by WHO (2002) of acrylamide in breakfast cereals which showed 298µg/kg acrylamide. A study carried out in breakfast cereals in Italy showed that the percentage of acrylamide in samples tested were very high 75% of the breakfast cereals with wide range of contamination from 30µg/kg to 940µg/kg. And their results showed 33% of breakfast cereals exceeded the indicative values recommended by EC 2013/647 set at 200-400 µg/kg (according to the composition) (Lilia *et al*., 2015).

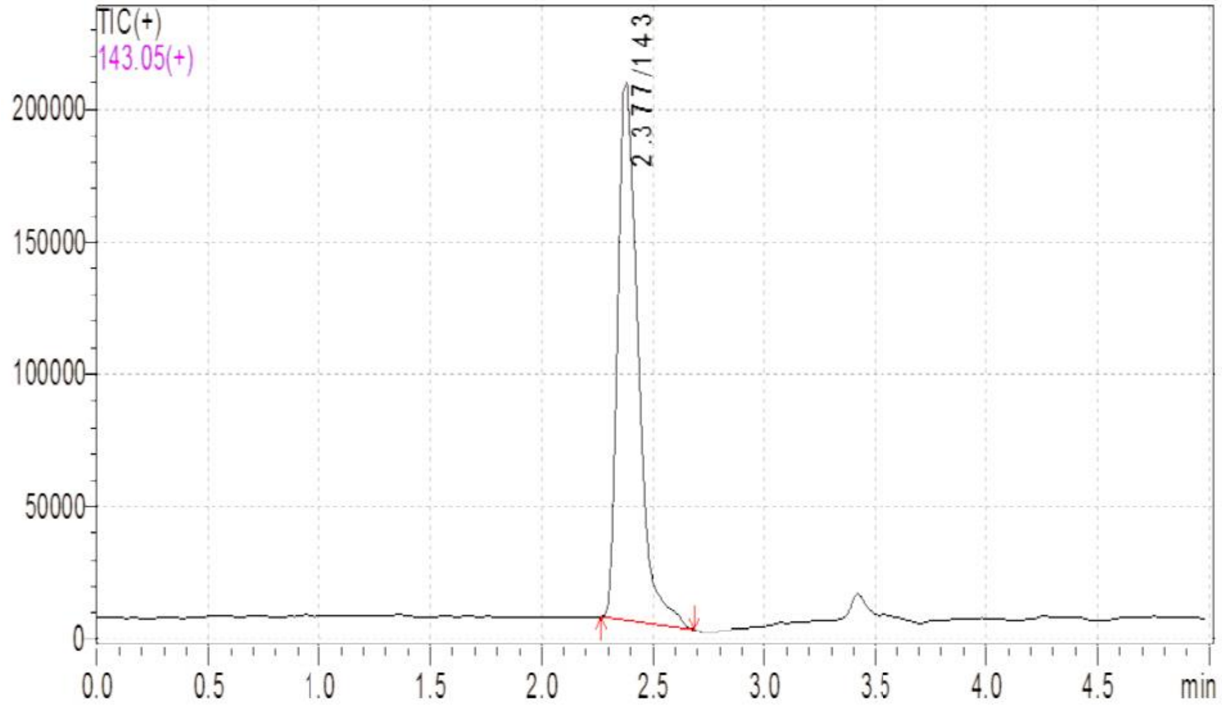


Figure 7:Choromatogram of acrylamide content in Rugag.

4.2.3 Acrylamide in homemade biscuits:

Results in Table 3 showed variation in acrylamide formation between biscuit with ammonium bicarbonate and without ammonium bicarbonate. Figure 7 shows choromatogram for detection of acrylamide in biscuits with ammonium bicarbonate at concentration 0.904 ppm (based on the knowing of standard concentration and retention time) as it is presented in Table 3, this may be due to the addition of ammonium bicarbonates in the presence of reducing sugar besides the high carbohydras content 69.43% which enhance the formation of acrylamide, and the low moisture content 7.5% which promoted the formation of acrylamide at elevated temperature 240 C° of baking which induce the formation of acrylamide. This result is comparable with result obtained by WHO (2002) of acrylamide in biscuits which showed 423µg/kg acrylamide.

It was noticed that both ammonium and added reducing sugar was required to enhance the acrylamide formation mechanism. Another early discovery was that ammonium raising agents could act as accelerants for acrylamide formation, possibly due to increased formation of reactive carbonyls from reducing sugar ammonium reactions (Amrein *et al*. 2004). Graf *et al*. (2006) showed that replacement of ammonium bicarbonate reduced acrylamide by 70% in semi-finished biscuits at an industrial scale.

In a report prepared for the Food Standards Agency (Colin *et al*., 2015) on a survey for 2007- 2014 on biscuits for different ingredients showed that the highest acrylamide content was in biscuits with ammonium bicarbonate in range 27-1573 µg/kg.

As is shown in Figure 8 there is no detection of acrylamide in biscuit without ammonium bicarbonate ,even though there is no significant different in the proximate analysis between the two types of biscuits, and they were baked under the same conditions, but there was detection of acrylamide in biscuit with ammonium bicarbonate while there is no detection of acrylamide in biscuit without ammonium bicarbonate , thus it can be suggested that ammonium bicarbonate promoted the formation of acrylamide.

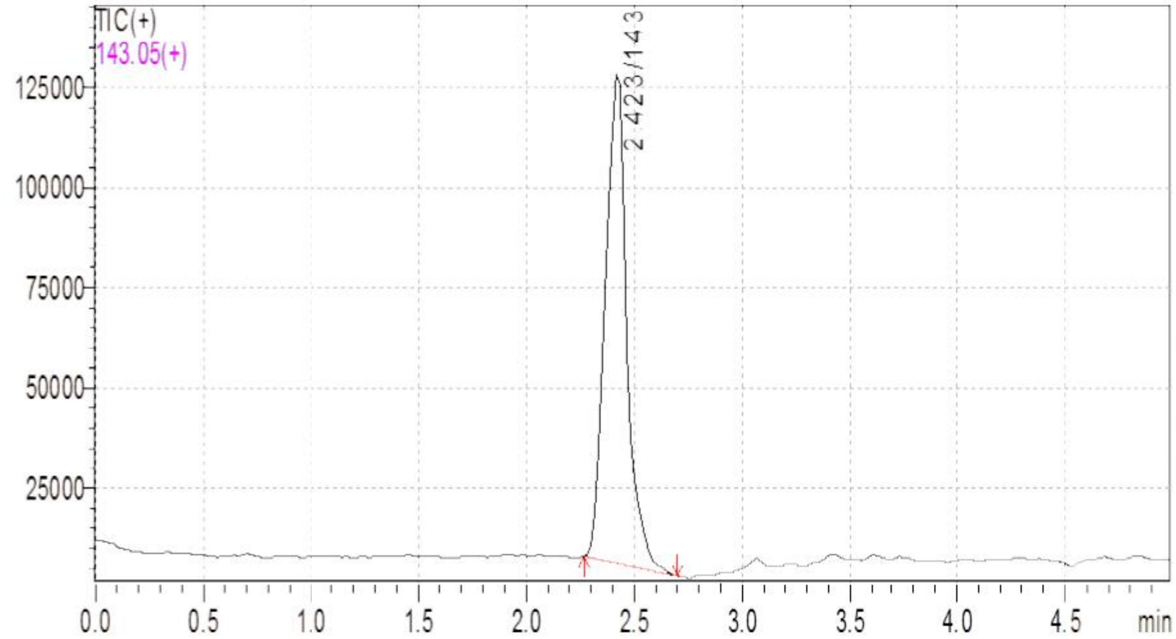


Figure 8: Choromatogram of acrylamide content in homemade biscuit with ammonium bicarbonate:

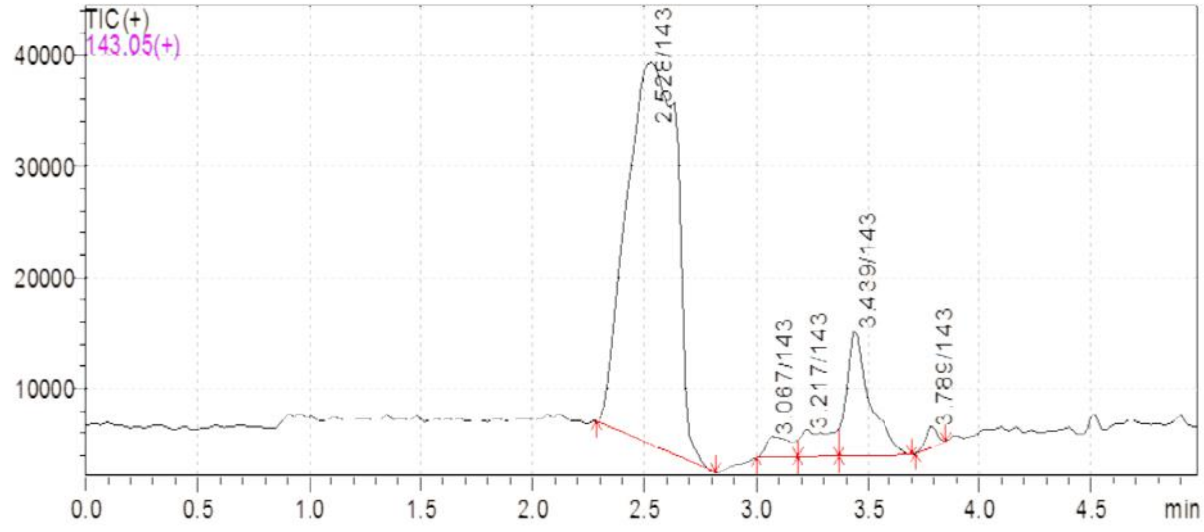


Figure 9: Choromatogram of acrylamide content in homemade biscuit without ammonium bicarbonate.

Table 3: Acrylamide content in Sudanese foods Rugag, homemade biscuit with ammonium bicarbonate and homemade biscuit without ammonium bicarbonate:

Material	Ret. Time	Area	Height	Conc. ppm
Standard	2.355	186306	43232	0.200
Biscuits without ammonium bicarbonate	-	-	-	N.D (W\B)
Biscuit with ammonium bicarbonate	2.423	842089	121896	0.904
Rugag	2.377	1295784	203339	1.391

N.D= Not detected

4.2.4 Acrylamide dietary exposure assessment:

Due to the potential health risks of acrylamide to humans it is necessary to assess the amount of acrylamide and the dietary intake of it to which humans are exposed in some foods. Thus it is attempted to estimate the dietary intake of acrylamide in Sudanese Rugag and homemade biscuit with ammonium bicarbonate.

Based on the equation for dietary exposure to chemicals it is assumed that the dietary intake of acrylamide in Rugag would be 103.12µg/kg.bw/day for adult and 312.97 µg/kg.bw/day for child, and it would be 3093.65 and 9389.25 µg/kg.bw/month for adult and child, respectively.

Whilst in homemade biscuit with ammonium bicarbonate it would be 29.78µg/kg.bw/day for adult and 90.4µg/kg.bw/day for child, consequently in a month it would be 893.4 and 2712µg/kg.bw/month for adult and child, respectively.

Based on these assumptions those values obtained are very high compared with Dybing *et al*. (2005) and Wilson *et al*. (2006)which calculated the average of acrylamide from 0.3 to 0.6 µg/kg.bw/day for adult while children and adolescents 0.4 to 0.6 µg/kg.bw/day. Those values of acrylamide in Rugag and homemade biscuits would cause potential health risk; they reached of levels which cause health risk as stated by EFSA(European Food Safety Authority).EFSA's experts estimated the dose range within which acrylamide is likely to cause a small but measurable tumor incidence (called "neoplastic" effects) or other potential adverse effects (neurological, pre- and post-natal development and male reproduction), the $BMDL_{10}$ (Benchmark Dose Lower Confidence Limit) of acrylamide for tumors is 0.17mg/kg bw/day and for other effects, neurological changes were seen as the most relevant with a $BMDL_{10}$ of 0.43 mg/kg bw/day. While the MOE (margin of exposure) for the cancer-related effects of acrylamide range from 425 for average adult consumers down to 50 for high consuming toddlers, these ranges indicate a concern for public health. For non-genotoxic substances, an MOE of 100 or higher normally indicates no concern for public health. The MOEs for neurological effects range from 1,075 for average adult consumers to 126 for high consuming toddlers (EFSA, 2015).No tolerable daily intake of acrylamide was set since any level of exposure to a genotoxic substance could potentially damage DNA and lead to cancer.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

Conclusion:

It could be concluded that:

The ingredients (carbohydrates, milk proteins and sugar) and processing conditions (high temperature, thin flakes, and low final moisture content) of Sudanese Rugag lead to formation of acrylamide.

Addtion of ammonium bicarbonates as a leavening agent in the processing of homemade biscuits promotes formation of acrylamide as it is one of the precursors.

Acrylamide exposure levels of Sudanese adults and children as results of consumption of Rugag and homemade biscuits made with added ammonium could contribute to a possible health risk which deserves attention as tolerable daily intake of acrylamide was estimated as 40 µg/ day, and 2.6 – 16 µg/ day for neurotoxicity and cancer respectively (Tard.ff *et al*., 2010) since these could be multiple sources of acrylamide in Sudanese foods apart from these two products.

Recommendations:

- Ammonium bicarbonate should not be used as leavening agent, and other leavening agents like sodium raising agent e.g disodium diphosphate, sodium bicarbonate and organic acids or potassium bicarbonate with potassium bitartrate sodium hydrogencarbonate should be used.
- Precursors of acrylamide (asparagine and reducing sugars) should be reduced by selecting cultivars with low levels of precursor, or using asparaginanse and yeast to reduce acrylamide formation.

- Cooking at low temperature as much as possible, and avoid reaching brown color in baked products.
- Further studies to estimate levels of acrylamide in other Sudanese foods should be carried.

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