Studies on acrylamide in foods: Effect of precursors on acrylamide formation in real and model systems

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June 2017

Dedicated to my husband & family…….

Declaration

I hereby declare that thesis entitled **"Studies on acrylamide in foods: Effect of precursors on acrylamide formation in real and model systems"** includes the results of investigations carried out by me, at the Agroprocessing and Technology Division, National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram as a full time research scholar under the supervision of Dr. P Nisha and the same has not been submitted elsewhere for any other degree.

Shamla. L

Thiruvananthapuram
June 2017

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Certificate

This is to certify that the work embodied in the thesis entitled **"Studies on acrylamide in foods: Effect of precursors on acrylamide formation in real and model systems"** is a bonafide record of research work carried out by **Ms. Shamla. L**, under my supervision at Agroprocessing and Technology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Thiruvananthapuram, in partial fulfillment of the requirements for the award of Degree of Doctor of Philosophy in Chemistry, under the Faculty of Science, Cochin University of Science and Technology, Cochin and the same has not been submitted elsewhere for any other degree. All the relevant corrections, modifications and recommendations suggested by the audience and the doctoral committee members during the pre-synopsis seminar of **Ms. Shamla. L** have been incorporated in the thesis.

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SHAMLA. L

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CHAPTER 1

Introduction

1.1. Thermal Processing of foods

Thermal processing are frequently employed in food manufacturing to improve safety, to increase the nutritional quality and also to improve the sensory properties of foods, their palatability and to extend the range of colors, tastes, aromas and texture. Thermal processing is an important treatment for food preservation, especially in the manufacture of shelf-stable foods with specific nutritional properties. The thermal processing like baking, toasting, frying, roasting and sterilization results in desired and undesired effects due to various chemical reactions viz., Maillard reaction (MR), caramelisation and lipid oxidation and a plethora of new molecules are generated. The chemicals that are formed in food as a result of food processing/preparation that exert adverse toxicological effects or create a potential or real risk to humans are called process induced toxicants. These process induced toxicants are also called thermally generated toxicants. As a result of thermal processing of foods different food contaminants such as heterocyclic aromatic amines (HAA), polycyclic aromatic hydrocarbons (PAH), N-nitrosamines, ethyl carbamate, furan, 3-monochloro propranediols (MCPD) and acrylamide are formed. Among this acrylamide is our area of interest that has gained reasonable attention in recent years due to its widespread occurrence in foods.

1.2. Acrylamide as a chemical compound

Acrylamide (2-propenamide) is an unsaturated amide having a molecular formula C_3H_5NO. This compound is produced commercially by the hydration of acrylonitrile [ATSDR, 2009; Castle & Erikson, 2005; Medeiros et al., 2012; Elbashir et al., 2014]. It is an versatile industrial chemical which was first synthesised in 1893. It is a white, odourless, crystalline solid having a molecular weight of 71.08 $gmol^{-1}$. It has a melting point of 84.5°C [Friedman, 2003], high boiling point of 136ºC at 3.3 kPa/25 mmHg and low volatility of vapor pressure

of 0.007 mmHg at 25ºC [Norris, 1967; American Cyanamid, 1969; Habermann, 1991]. It is stable at room temperature but violently undergoes polymerization at its melting point or under UV light [ATSDR, 2009]. Acrylamide readily dissolves in water and other polar solvents such as methanol, ethanol, dimethyl ether & acetone and less soluble in benzene and heptane. However it is insoluble in nonpolar solvents [NTP, 2011]. Acrylamide slowly sublimes at room temperature [Girma et al., 2005]. The chemical structure of acrylamide is given in fig 1.1. Acrylamide possess an amide group and an electron deficient vinylic double bond. This compound is not having a strong chromophore and hence it does not fluoresce [Eriksson, 2005]. The electron-withdrawing carboxamide group activates the double bond and undergoes nucleophilic reactions especially via a Michael addition [JIFSAN/NCFST, 2002]. Acrylamide can form complexes with transition metals which inturn can be used for the production of water soluble polymers and co-polymers having industrial and scientific applications [Girma et al., 2005].

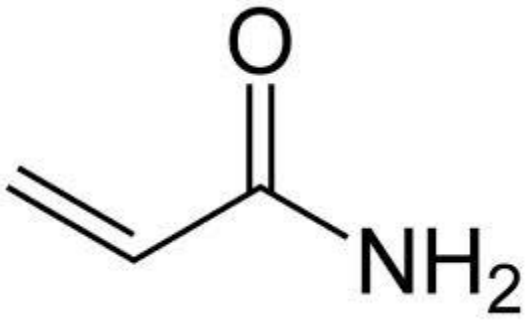

Fig 1.1. Chemical structure of acrylamide

1.3. Possible sources of acrylamide exposure

Acrylamide is used as a monomer in the production of polyacrylamide which finds various industrial applications since 1950. It is a chemical which is used in the production of plastic,

paint & paper industry. Polyacrylamide has numerous applications in biomedical and research laboratories which include the preparation of gels for electrophoresis, separation of proteins by electrophoresis etc [Smith and Oehme, 1991]. It is used as a grouting agent for the construction of buildings, dam foundations etc. So occupation may be a source of acrylamide exposure mainly through inhalation and dermal contact. Polyacrylamide is used as a coagulant for purifying water inorder to make it potable. The polymer is not toxic but there is always some residue of the monomer remaining in the drinking water. In waste water treatment it is used as a flocculant in improving the process of sludge thickening and dewatering. Acrylamide also finds application as a soil conditioner [Tornquist, 2005]. Cigarette smoke also contains acrylamide and the estimated exposure resulting from average smoking is about 3μg/kg of body weight per day. It is also used in the formulation of several type of personal care and grooming products such as lotions, cosmetics, deodorants, soaps, shampoos etc. Cosmetics contain residual monomeric acrylamide due to the fact that polyacrylamide is used in shampoos as a foaming agent and as a lubricant in make up, soaps and lotions [IPCS, 1985; Smith et al., 2000; Tareke et al., 2000; Vattem and Shetty, 2003; Zangrando et al., 2012]. It was found that another possible source of acrylamide exposure may be due to the consumption of processed foods. Scientists have found that acrylamide is formed as a by-product of heat induced reactions between amino acid asparagine and the reducing sugars [Stadler, 2002; Mottram, 2002].

1.4. A brief history of detection of acrylamide in foods

While building a tunnel in Hallandsas (Sweden), Rhoca-Gel was used which was part of a sealing agent to prevent the leakage of water, but the polymerisation was not completed entirely and hence acrylamide leaked into water [Reynolds, 2002]. Shortly after cows

showed neurotoxicological symptoms and fishes in ponds died, both being exposed to this contaminated water. The blood samples of the tunnel workers were examined and they revealed high levels of acrylamide-Hb adducts which are the biomarkers of acrylamide exposure in the workers blood [Hagmar et al., 2001]. Surprisingly the control group without known exposure to acrylamide also showed levels of acrylamide-Hb adducts. The ubiquity of this acrylamide-Hb adducts led to the hypothesis that acrylamide might be ingested through diet. Thus in 2002, Swedish National Food Administration and Stockholm University jointly reported the findings of acrylamide in carbohydrate rich foods when subjected to high temperature processing such as baking and frying. These findings were soon confirmed by other research groups and together with the stakeholders efforts were carried out to build greater understanding of acrylamide, concerning the mechanism of its formation in foods, the risk associated with the consumers and the possible strategies to lower the levels of acrylamide in foods. Thus the discovery of acrylamide in cooked foods particularly in starch based foods raised an alarm on the safety of such foods. Further studies found out that acrylamide is formed in food, as a by-product of high temperature cooking processes of food usually greater than 120ºC [Friedman, 2003]. Acrylamide has not been reported in uncooked foods and is present in low or undetectable levels in foods cooked at lower temperatures, such as boiling. Acrylamide is a process induced contaminant that is formed during high temperature processing (e.g., frying, baking and roasting) of carbohydrate and protein rich foods [Tareke et al., 2002]. The reducing sugars and asparagine are believed to be the major precursors responsible for acrylamide formation in plant-based foods [Zyzak et al., 2003]. The Maillard reaction which involves the interaction between the free amino group of amino acids/proteins and the carbonyl group of

sugars/carbohydrates has an important role in the formation of acrylamide along with temperature-time combinations [Lee & Shibamoto, 2002; Martins et al., 2000).

1.5. Occurrence of acrylamide in foods and dietary exposure

After the discovery of acrylamide in foodstuffs since 2002, many European countries were engaged in acrylamide monitoring programs. The member states from both competent authority and the food industry have submitted the data on the occurrence of acrylamide in food products to the Joint Research Centre of the European commission (EC). Thus in 2007, the EC recommended that the member states should perform monitoring of acrylamide in foodstuffs that are known to contain high acrylamide levels and contribute significantly to human exposure. On the basis of the results obtained in monitoring the Member States from 2007–2011, the EC has set certain "indicative values" for acrylamide in various foodstuffs. The 'indicative values" are not safety thresholds, but only intended to indicate the need for an investigation if the values are exceeded in order to explore whether appropriate measures have been taken to control the formation of acrylamide [European Commission Recommendation, 2011].

Acrylamide is formed during high temperature processing such as frying, roasting & baking and is not typically found in boiled or microwaved foods. The highest acrylamide levels have been found in fried potato products, bread and bakery wares, and coffee. Acrylamide has been found however, in food products other than biscuits, bread, breakfast cereals, cereal-based baby foods, coffee, french fries, jarred baby foods, potato crisps, home cooked potato products, such as hazelnuts and almonds [Amrein et al., 2005], olives and recently, in foods not subjected to severe heating, such as dried fruits (e.g. plums, pears, apricots) [Amrein et al., 2007]. Although the concentrations of acrylamide in such products are very

high, their contribution to the overall acrylamide intake is marginal. Animal derived heat treated foods such as meat and fish, generally exhibit low or negligible levels of acrylamide [Swedish National Food Administration, 2002; EFSA, 2009; European Commission, 2006]. All the same, a great variability in acrylamide levels between different products of each food category as well as between different brands of the same product has been reported. The difference in the concentration of precursors (free asparagine and reducing sugars) in raw materials, difference in food composition and in process conditions applied can easily explain the observed variability [Boon et al., 2005].

Several international groups have estimated the dietary exposure to acrylamide being the highest reported by JECFA, which concluded that acrylamide mean dietary exposure estimates were 1μg/kg body wt. per day, and 4 μg/kg body wt. per day for a consumer at a high percentile of the distribution [JECFA, 2010]. The World Health Organization estimates a daily dietary intake of acrylamide in the range of 0.3–2.0 μg/kg body wt. for the general population and up to 5.1 μg/kg body wt. for the 99th-percentile consumers [WHO, 2005]. Children eat more acrylamide than adults probably because of their higher caloric intake relative to body weight as well as their higher consumption of certain acrylamide-rich foods, such as French fries and potato crisps [Dybing et al., 2005]. Heudorf et al., [2009] assessed the dietary exposure to acrylamide in 5-6 years aged children by means of urinary excretion of mercapturic acids as biomarker. They reported a median (95th percentile) daily uptake of acrylamide in children of 0.54 (1.91) μg/kg body wt. thus confirming that children are a vulnerable subgroup of population. Arribas-Lorenzo and Morales [2009] estimated the dietary exposure from potato chips in the Spanish population. The authors reported a daily dietary exposure (based on a 3-days food record) from potato crisps of 0.053 μg/kg body wt.

for the adult population (17-60 years) and of 0.142 μg/kg body wt. for children (7-12 years). In most of the populations, the major contributors to acrylamide intake are potato crisps and chips, bread and coffee each accounting for nearly one third of the total intake [WHO, 2005]. Other food products can account for up to 10% of the total intake of acrylamide. The EFSA reported that the acrylamide in food [2013] was disclosed recently for public consultation and the contribution to acrylamide dietary exposure was assessed for ten food groups and six food subgroups for different food surveys and age groups. The acrylamide exposure patterns were presented for infants, toddlers, children, adolescents and adults, elderly and very elderly according to European surveys collected and analyzed by EFSA Consulting Panel. Taking into account these results, it is clear that children and adults have different exposure patterns. For adults, coffee and fried potatoes are important contributors to acrylamide intake with values up to 33% and 49%, respectively. For children and adolescents, cereal-based and potato-based products are the main sources of the compound with values up to 51 and 60%, respectively. Although bread presents low amounts of acrylamide its contribution to acrylamide intake is around 22% for adults due to its high consumption in a typical diet.

1.6. Different pathways of acrylamide formation in foods

The presence of acrylamide in a broad range of foods has led to a huge amount of studies that intended to determine the formation pathway of the compound. The most accepted route of formation is the Maillard reaction that occurs between reducing sugars and amino acids [Mottram et al., 2002; Stadler et al., 2002]. This extremely complex reaction between amino compounds (principally amino acids) and reducing sugars has been the subject of much research by food scientists seeking to identify compounds that provide the flavor and color

characteristics of heated foods [Hodge, 1967; Nursten, 1981; Mauron, 1980; Hurrell, 1982; Mottram, 1994]. Studies suggest that the presence of acrylamide was detected when foods are heated at temperatures higher than 120ºC, for moderately long periods of time under limited presence of water.

Many reported works explained the different aspects of acrylamide formation mainly by identifying the intermediates and other by-products of the reaction. It was found that some important and direct precursors contributing to the formation of acrylamide were 3-aminopropionamide [Granvogl et al., 2004], decarboxylated Schiff base [Zyzak et al., 2003], decarboxylated Amadori product [Yaylayan et al., 2003] acrylic acid [Becalski et al., 2003; Stadler and Scholz, 2004] and acrolein [Yasuhara et al., 2003]. On the other hand, other reaction routes for the formation of acrylamide in foods were also discussed in order to explain the presence of the compound matrices with low amounts of asparagine and/or reducing sugars. A brief review on the different possible pathways for acrylamide formation is presented.

1.6.1. The Maillard Reaction: The asparagine Route

Pyrolysis experiments of model systems composed of amino acids and sugars suggested the formation of acrylamide through the Maillard reaction. The crucial participant in the production of acrylamide was the free asparagine. The pyrolysis of asparagine alone may release acrylamide by thermally initiated decarboxylation and deamination. The yield increased a few hundredfold in the presence of reducing sugars. This was confirmed by the fact that acrylamide is formed in heated potato and cereal products, which contain reducing sugars and are particularly rich in free asparagine. By using ^{15}N-labelled asparagine and ^{13}C-labelled glucose in mass spectral studies, it was unambiguously demonstrated that the amide

nitrogen and the three-carbon backbone of acrylamide originated both from the corresponding positions in the asparagine molecule. Thus sugar backbone and α-amino group of asparagine was not incorporated in the acrylamide molecule [Mottram et al., 2002; Weisshaar and Gutsche, 2002; Stadler et al., 2002].

The early stages of the Maillard reaction involve the condensation of an amino acid (e.g. asparagine) with the carbonyl group of a reducing sugar to afford a *N*-glycosyl adduct (e.g. *N*glycosyl asparagine), which is in equilibrium with the Schiff base (Figure 1.2.). *N*-glycosyl asparagine generated high amounts of acrylamide, suggesting that the early Maillard reaction is a major source of acrylamide [Stadler et al., 2002]. This reaction usually proceeds to form Amadori compounds (pathway **I**). These Amadori products will degrade through the classical Maillard reaction pathways leading to the formation of flavour and colour compounds, instead of acrylamide. Since the Amadori compound does not easily decarboxylate, it can be concluded that the acrylamide formation pathway starts to follow another route prior to the Amadori rearrangement step [Stadler et al., 2004; Taeymans et al., 2004].

More concrete evidence on how the Schiff base can generate acrylamide was provided by several studies [Yaylayan et al., 2003; Zyzak et al., 2003; Stadler et al., 2004]. According to Zyzak et al., [2003], the Schiff base undergoes heat-induced decarboxylation (Figure 1.2.). This may proceed via the zwitterionic form to generate the azomethine ylide (pathway **II a**). Yaylayan et al., [2003] suggested a decarboxylation via intramolecular cyclization to the oxazolidin-5-one intermediate (pathway **II b**). A 1, 2-prototropy determines the final location of the proton in the neutral imines [Stadler et al., 2004]. The so-formed decarboxylated Schiff base (imine 1) can hydrolyze (pathway **III a**) upon heating to form

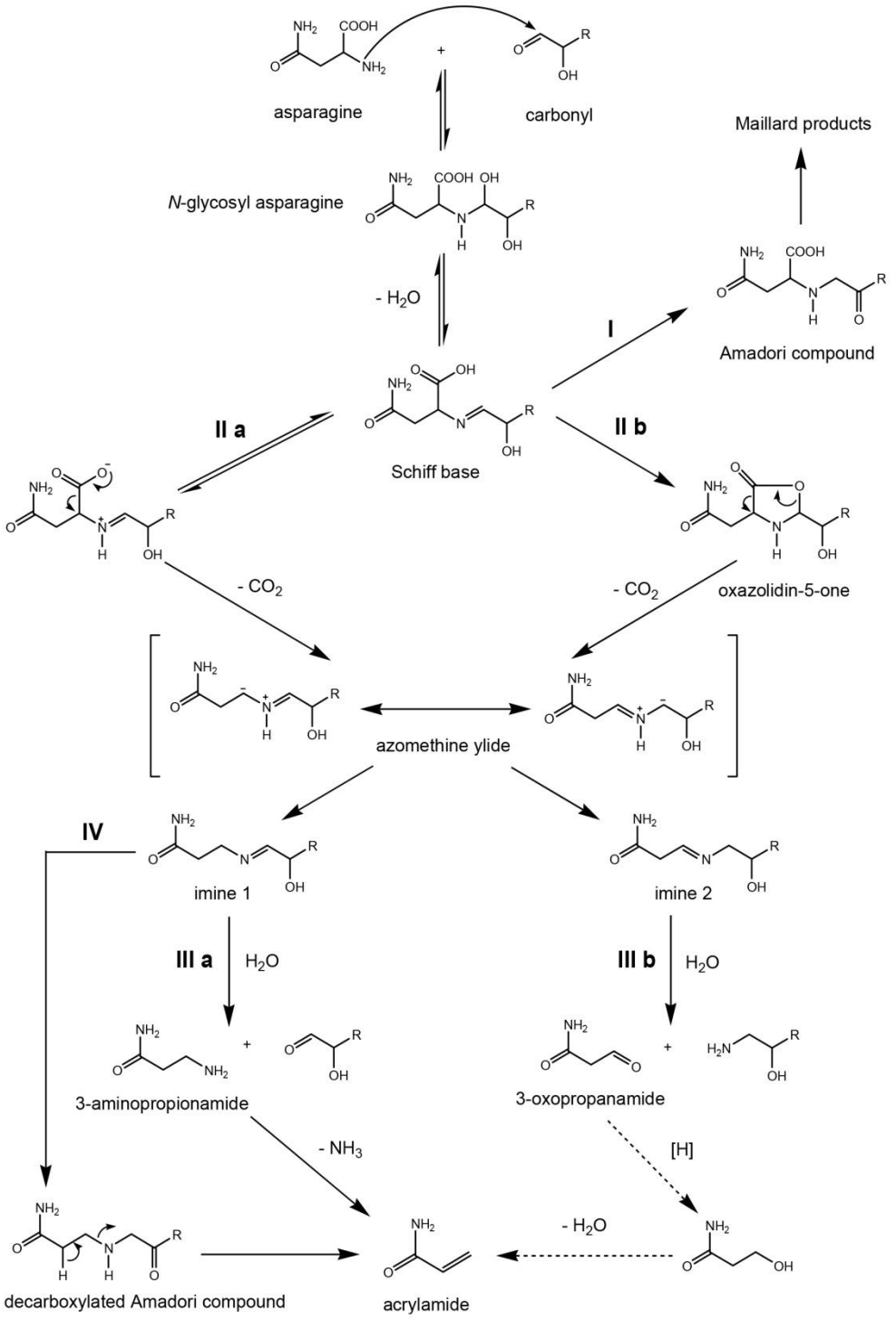

Fig 1.2. Proposed mechanisms of acrylamide formation through the Maillard reaction, starting from asparagine and a carbonyl source (Yaylayan et al., 2003; Zyzak et al., 2003; Stadler et al., 2004; Yaylayan and Stadler, 2005)

3-aminopropionamide which was proven to form acrylamide very efficiently via the elimination of ammonia [Granvogl and Schieberle, 2006]. After tautomerization of imine 1 (pathway **IV**), the carbon- nitrogen covalent bond may break as a consequence of a β-elimination reaction. Although no direct evidence of the decarboxylated Amadori compound has been provided, the β- elimination reaction was proven to occur by means of experiments using model Amadori compounds [Stadler et al., 2004].

The 1, 2-prototropic H-shift may also lead to the imine 2, which upon hydrolysis (pathway **III b**) furnishes the Strecker aldehyde of asparagine (3-oxopropanamide). However, this aldehyde did not release high amounts of acrylamide [Stadler et al., 2004; Blank et al., 2005]. Not only reducing sugars may act as a carbonyl source to form acrylamide. Also α-dicarbonyls or even any other carbonyl compound might generate acrylamide in the presence of asparagine [Mottram et al., 2002; Rydberg et al., 2003; Zyzak et al., 2003; Schieberle et al., 2005; Amrein et al., 2006]. A Strecker-type degradation of asparagine, initiated by α-dicarbonyl compounds and similar to pathway **II a** and **III a** (Figure 1.2. and 1.3.) was reported [Schieberle et al., 2005; Granvogl and Schieberle, 2006]. Yet, the type of carbonyl significantly affects the yields of acrylamide. The α-hydroxycarbonyl compounds, such as reducing sugars, generate much higher amounts of acrylamide compared to α-dicarbonyls or aldehydes. This can be explained by the fact that the presence of a hydroxyl group in β-position to the nitrogen atom (imine 1) favours the rearrangement (tautomerization) to the decarboxylated Amadori product (pathway **IV**). With α-dicarbonyls as precursor, it was however postulated that pathway **III b** is preferred above **III a**, due to the tendency of the carbonyl group in β-position to the nitrogen atom to delocalize the negative charge. As

mentioned above, pathway **III b** does however not release high amounts of acrylamide [Stadler et al., 2004; Blank et al., 2005].

Thus in conclusion, it may be assumed that the route via asparagine in the presence of reducing sugars is the major pathway to form acrylamide in foodstuffs. But the yield is however not very high. On molar basis, less than 1% of free asparagine is converted to acrylamide [Mottram et al., 2002; Stadler et al., 2002; Becalski et al., 2003; Biedermann and Grob, 2003; Stadler and Scholz, 2004; Surdyk et al., 2004; Amrein et al., 2004]. It is reported that ammonium hydrogen carbonate might increase the conversion rate up to 5% [Biedermann and Grob, 2003; Amrein et al., 2004; Weisshaar, 2004a; Amrein et al., 2006]. The simultaneous degradation of acrylamide through polymerization reactions and Michael type addition reactions indicates a difficulty in predicting the reaction yield [Mottram et al., 2002; Stadler et al., 2002; Stadler et al., 2004]. In this respect, kinetic modelling of acrylamide formation has been used as a way to quantify the amount of acrylamide formation and degradation, based on a simplification of the complex formation and degradation pathways [Knol et al., 2005; Wedzicha et al., 2005; Claeys et al., 2005a; Claeys et al., 2005b; Claeys et al., 2005c; Corradini and Peleg, 2006; De Vleeschouwer et al., 2006].

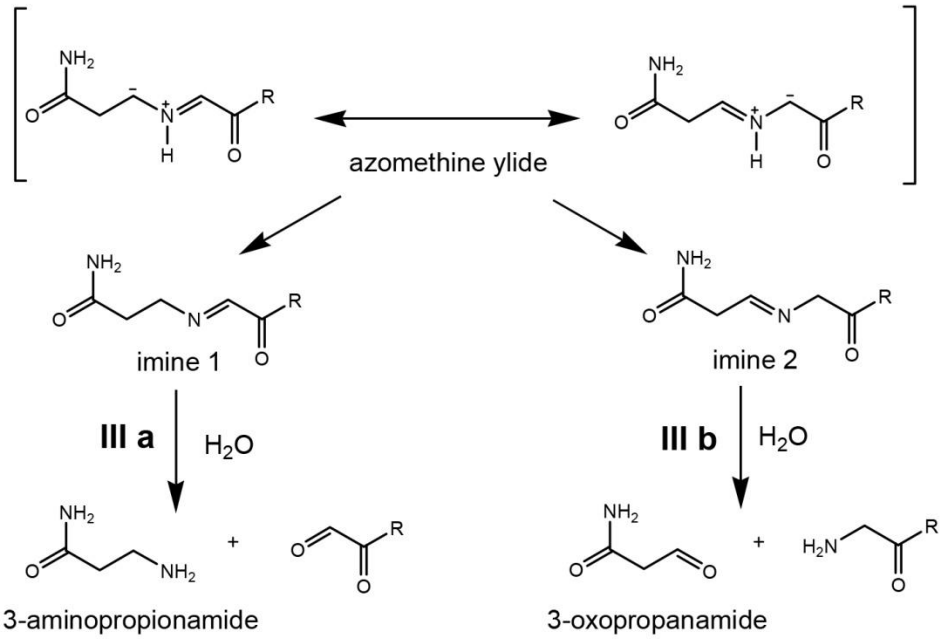

Fig 1.3. Resonance stabilized structures of the azomethine ylide after the condensation of free asparagine with α-dicarbonyls

1.6.2. Pyrolytic acrylamide formation from other aminoacids, proteins and peptides

In addition to asparagine, other free amino acids such as aspartic acid, glutamine, methionine, cysteine and lysine might also liberate acrylamide to a much lower extent upon pyrolysis with a reducing sugar [Mottram et al., 2002; Weisshaar and Gutsche, 2002; Stadler et al., 2002; Becalski et al., 2003]. This formation was initially ascribed as small asparagine impurities in the used amino acids. It was however demonstrated that β-alanine and aspartic acid can generate acrylic acid during their thermal decomposition (Fig 1.4.), which can subsequently react with free ammonia to form acrylamide [Yaylayan et al., 2005; Ehling and Shibamoto, 2005].

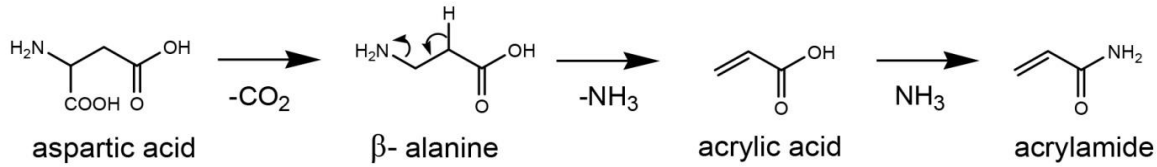

Fig 1.4. Formation of acrylamide starting from aspartic acid or β-alanine (Yaylayan et al., 2005)

Peptides having asparagine at the C-terminus can also produce acrylamide, but to a much lower extent than free asparagine [Schieberle et al., 2006]. Also the dipeptide carnosine (*N*-β-alanyl-L-histidine) produced acrylic acid and acrylamide. It was postulated that the dipeptide bond hydrolyzes to release β-alanine, which can form acrylamide as discussed above. Also 3-aminopropionamide can be released from carnosine to yield acrylamide after its subsequent deamination. The presence of creatine (a major constituent of meat) however greatly suppressed the formation of acrylamide, which was expected since meat is not known to contain high levels of acrylamide [Friedman, 2003; Yaylayan et al., 2005].

Another acrylamide formation pathway in protein-rich foodstuffs was proposed by Claus, [2006]. In this study, acrylamide was generated from purified wheat gluten by thermal degradation of an alanine-containing peptide (fig 1.5.). Thus it was found that more than one amino acid, and even bound amino acids, can generate acrylamide. The efficiency of the conversion of acrylic acid to acrylamide is however limited by the availability of free ammonia. Yet, this ammonia is extremely volatile at the applied heating temperatures [Yaylayan et al., 2005]. Consequently, it is clear that these formation pathways are far less important compared to the ones described under the previous section. Furthermore, acrylamide, generated in these protein-rich foodstuffs, may readily react with nucleophilic amino acids present through Michael type addition reactions.

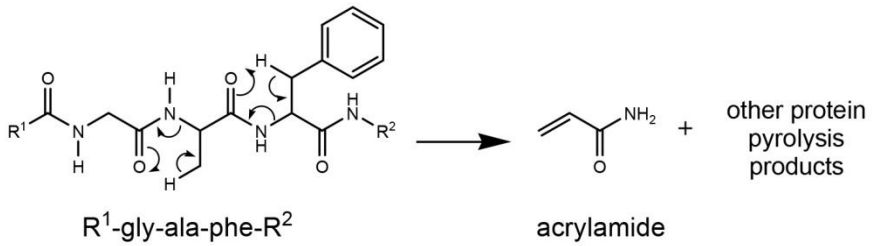

Fig 1.5. Proposed mechanism of acrylamide formation upon protein pyrolysis (Claus et al., 2006)

1.6.3. Acrylamide formation through 3-aminopropionamide

Other than a possible intermediate during the Maillard reaction, 3-APA can also be formed in raw foodstuffs by enzymatic decarboxylation of asparagine. It was explained, that in the presence of 3-APA in food matrix, acrylamide can be formed easily, even under aqueous conditions during heating. In this reaction pathway, the Maillard reaction is not taking place and acrylamide can be formed even if there are no reducing sugars in the system via direct deamination of 3-aminopropionamide (Fig 1.6.). This explains that why acrylamide is found in raw materials containing low amounts of asparagine [Granvogl et al., 2004].

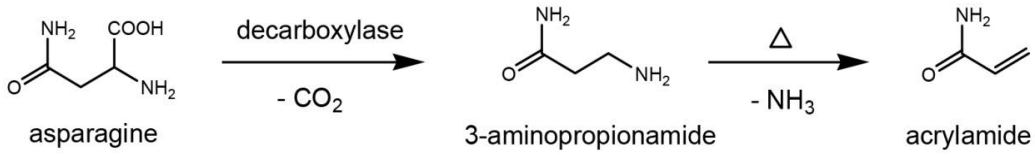

Fig 1.6. Enzymatic decarboxylation of asparagine leading to 3-aminopropionamide, which deaminates to acrylamide upon heating (Granvogl et al., 2004; Granvogl and Schieberle, 2006)

More studies regarding the effect of 3-aminopropionamide and 3-(alkylamino)propionamides (aminopropionamides that can be naturally present in food) on acrylamide formation was given by Zamora et al., [2009]. It was found that there are

possibly diverse pathways by which 3-aminopropionamide and 3-(alkylamino)propionamides are converted into acrylamide depending on water activity and presence of other carbonyl compounds. The proposed pathway (fig1.7.) suggested the existence of different competitive pathways by which 3-aminopropionamide and 3-(alkylamino)propionamides are converted into acrylamide.

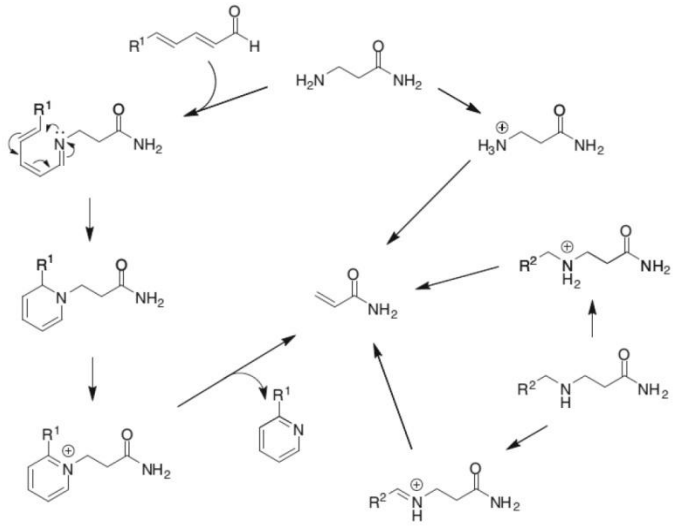

Fig 1.7. Possible pathways for the degradation of 3-aminopropionamide and 3-(benzylamino)propionamide (Zamora et al. 2009)

It was concluded that at low water activity and high temperatures, both types of amino compounds are converted rapidly into acrylamide to high extent (40–50%) and carbonyl compounds apparently do not play a significant role, at least in relation to the amount of acrylamide produced. No significant change was observed when water activity increases in the conversion of 3-(alkylamino)propionamides into acrylamide. Thus the amount of acrylamide produced from 3-aminopropionamide decreases and carbonyl compounds have a positive effect in the formation of acrylamide. It was concluded that the type of precursor

involved is likely to play a major role in the amount of acrylamide produced [Zamora et al., 2009].

1.6.4. Acrylamide formation through acrolein pathway

Researchers proposed the importance of oil hydrolysis products in the formation of acrylamide, particularly in lipid-rich foodstuffs [Gertz and Klostermann, 2002; Lingnert et al., 2002; Gertz et al., 2003]. Multistep heat degradation processes of triacylglycerols to acrolein and acrylic acid were suggested as the formation pathway of acrylamide. Acrolein was shown to be formed from lipids in large amounts [Umano and Shibamoto, 1987] and may provide the reactive carbonyl function which generates acrylamide in the presence of asparagine [Becalski et al., 2003; Yasuhara et al., 2003; Weisshaar, 2004b]. Another possible route is the oxidation of acrolein to acrylic acid, which reacts with ammonia to form acrylamide (Fig 1.8.). But it is still unclear about the importance of these reaction pathways in foodstuffs relative to that of asparagine [Stadler and Scholz, 2004].

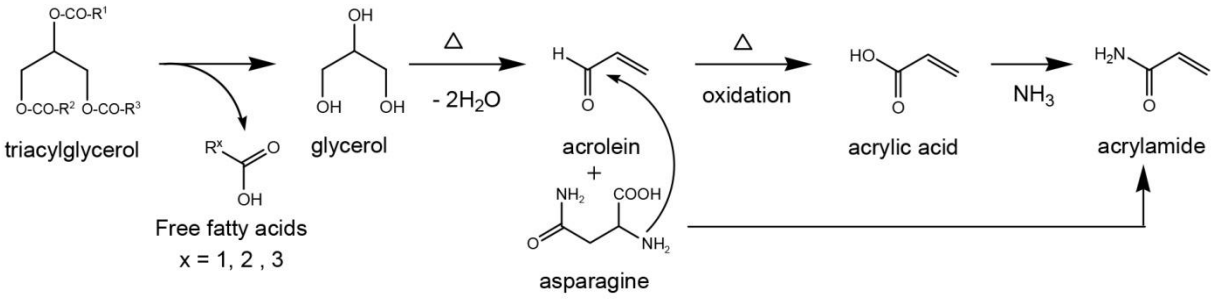

Fig 1.8. Formation of acrylamide after hydrolysis of triacylglycerol (Gertz and Klostermann, 2002; Gertz et al., 2003; Yasuhara et al., 2003)

1.7. The factors which influence the formation of acrylamide in foods

1.7.1 Precursor concentrations and processing conditions employed

Acrylamide formation was influenced by different factors. Based on our earlier studies it was found that the parameters such as heating time, heating temperature, the ratio of amino

acid and reducing sugar, etc., has an influence on acrylamide formation. Various studies were performed in model systems to study acrylamide formation with pure precursors and in actual food matrices during various heat processing methods such as frying, baking and roasting it was found that formation of acrylamide in food systems is more complex [Robert et al., 2004]. Meanwhile, the control of acrylamide content and maintenance of original food quality need to be simultaneously considered during heat processing [Zhang et al., 2009]. Fiselier et al., [2006] summarized the effect of frying temperature on the formation of acrylamide and demonstrated that the temperature during the second half of the process is the most important since acrylamide is formed toward the end of frying. Romani et al., [2008] indicated that the increase of frying time becomes a key factor in terms of the quantity of acrylamide and its formation rate when the temperatures of the potato surface and the oil bath reach 120 and 140ºC, respectively, after around 4 min of frying. Besides, other factors related to the formation of acrylamide include precursor levels and water content in raw materials, pretreatment, pH, etc. The precursor concentrations including asparagine, glucose, and fructose play an important role in the generation of acrylamide. Since the asparagine concentration was found as a prerequisite for the heat-induced formation of acrylamide, control of free asparagine could turn out to be a useful approach to mitigate acrylamide formation. A direct relationship between the acrylamide contents and asparagine levels was demonstrated in baked/toasted wheat and rye breads [Granby et al., 2008]. Vivanti et al., [2006] reported the reducing sugars glucose and fructose as important contributors for acrylamide formation. The correlation of formation of acrylamide and Maillard reaction as function of different glucose/fructose ratios in French fries were investigated using color measurement method [Mestdagh et al., 2008]. Even non-reducing

sugar, sucrose was found as an efficient reactant, leading to the release of reducing sugars that are then available to react with the R-NH2 group of asparagine via the Maillard pathway after thermally induced hydrolysis [Zhang et al., 2009]. Other factors such as water content and pretreatments were also investigated. During the baking of white bread, the temperature of the crust in combination with water content had a significant effect on acrylamide formation. Ahrne et al., [2007] reported that the concentration of acrylamide decreased at very high temperatures and lower water contents.

The choice of raw material is usually related with the amount of precursors present. The agronomic factors that affect raw material composition mainly include fertilization methods, harvest season and climatic conditions. A reverse correlation between the amount of fertilizer applied in potato cultivation and the acrylamide content in the edible products has been revealed, since reducing sugar contents are elevated while crude protein and free amino acids decrease when less nitrogen-fertilizer is given [De Wilde et al., 2006]. When wheat was grown under conditions of severe sulfate depletion or sulfur deficiency, dramatic increase in the concentration of free asparagine was found and subsequently enhancement of acrylamide content during baking was observed [Granvogl et al., 2007]. Other factors such as harvest year and climatic conditions also influence acrylamide formation in addition to fertilization. The temperature conditions and the favourable light during the cultivation period enhance the amino acid and protein contents, thus promoting the formation of acrylamide during baking [Amrein et al., 2004].

Along with the climatic conditions, the variety of the raw material as well as storage conditions also influences the precursor composition, particularly in potatoes [Vicklund et al., 2008; Grob et al., 2003]. It is reported that the acrylamide level in potato chips made

from tubers stored at low temperature is much higher than that from those stored at high temperature. Thus the storage of raw materials at low temperatures should be avoided for the control of precursor content. However, the storage at too high temperatures can reduce the shelf life and sensory attributes of the raw materials. Thus the variety and storage conditions of raw materials are overall contributing to the level of amino acids and reducing sugars. Zhang et al., [2009] reported that the recommendation of heat processing methods such as low temperature vacuum frying, short time heating, and avoiding the use of palm olein as for modification of processing can effectively control the formation of acrylamide.

Color is considered as one of the most visually important parameter in defining the quality of fried products and is formed as a result of Maillard reaction, which depends on the concentrations of reducing sugars and amino acids or proteins at the surface, and the temperature and time of frying [Pedreschi et al., 2007]. Many studies reported high correlation between the acrylamide formation and color development of products [Lukac et al., 2007; Pedreschi et al., 2007; Gokmen and Senyuva, 2006]. Further researches are going on to find new and improved factors contributing to the formation of acrylamide.

1.8 Analytical methods for the detection of acrylamide in foods

Different chromatographic techniques, such as gas chromatography (GC) or liquid chromatography (LC) in combination with appropriately selective and specific detectors have been used for the quantification of acrylamide in various food matrices. However, analysis of acrylamide is generally performed by one of two methodologies using LC-MS/MS and GC-MS. HPLC coupled with MS is the most preferred method for separation and quantification of acrylamide in foods, due to its sensitivity, selectivity, and versatility. An LC method for the acrylamide detection by HRMS in full scan mode was compared to

LC coupled to tandem mass spectrometry method and it was found that the results were perfectly in line with those obtained by LC-MS/MS [Triose et al., 2014]. The Orbitrap mass analyzer was described for the first time in the year 2000 by Makarov and has now reached the status of a mainstream mass spectrometry technique. It shows an insignificant trade-off in sensitivity versus resolving power, distinguishing it from other high-resolution analyzers [Makarov & Scigelova, 2010].

An alternative to the conventional HPLC is ultra-performance liquid chromatography (UPLC) which requires higher pressures and relies on lower flow rates. A good separation of mixture components in a shorter time and high sensitivity are the main advantages of ultra performance liquid chromatography. Such a separation is achievable by using reversed-phase columns with much thinner film and particle size than in typical HPLC columns. Chromatographic separation of acrylamide on a traditional column filled with 5 μm particles suffers from weak retention and separation of polar compounds such as acrylamide and deformation of the peak shapes. The introduction of a UPLC method solved these problems, because it improved the analyte peak symmetry. The smaller size of bed particles (1.7 μm) renders the analysis faster, but not less efficient. Other advantages of UPLC include the minimized solvent consumption in separations resulting from low flow rates (0.2 ml min^{-1}), substantially shorter time of separation compared to traditional liquid chromatography techniques, more symmetric peaks of separated compounds and considerably smaller sample volumes [Oracz et al., 2011].

Most researchers use reversed-phase chromatography for the chromatographic separation of acrylamide. Because of the high polarity of acrylamide, it is difficult to choose an appropriate mobile phase to achieve good analyte elution with a reasonable retention time.

Most chromatography experts have experienced problems retaining and separating polar compounds, similar to acrylamide, when using conventional reversed-phase chromatography. These difficult-to-analyze compounds either pass through the column unretained or, if retained at all, co-elute at the beginning of the chromatogram. Waters Atlantis columns (Waters, Milford, MA, USA) are designed for these types of challenging separations. Atlantis dC18 columns are a silica-based line of difunctionally bonded C18 columns that provide the optimal balance of retention for polar and non-polar compounds in reversed-phase chromatography [Zhang et al., 2005].

An alternative to reversed-phase columns is ion-exchange chromatography. In this case, an IonPac ICE-AS1 column (Dionex, Sunnyvale, CA, USA) could be used that combines ion exchange with size exclusion chromatography. The advantage is that there is a significant increase of the *k'* value compared to reversed-phase columns, leading to a good separation of acrylamide from matrix compounds even for untreated sample extracts [Zhang et al., 2005]. However, hydrophilic interaction liquid chromatography (HILIC) is one of the most successful approaches for the retention and separation of polar compounds. The primary advantage of HILIC as a separation technique is the strong retention of polar, hydrophilic compounds that are unretained under conventional reversed phase conditions, without the requirement for an ion-pair additive in the mobile phase. Quantitative assays of acrylamide or its derivatives contained in foods were also performed by gas chromatography coupled with mass spectrometry. In the case of gas chromatography, derivatization of the analyte is required in order to increase volatility, selectivity, sensitivity and retention time. The most popular method of acrylamide derivatization is its bromination prior to the analysis [Zhang et al., 2011]. This technique is highly selective and improves the assay precision, it also

effectively compensates for the difficult and time-consuming derivatization process. Another method of acrylamide derivatization consists of its silylation followed by solid phase microextraction (SPME), which can be used in the analysis of polar and non-polar compounds in gases, liquids, and solids [Oracz et al., 2011].

GC based methods with and without derivatization was developed which showed a satisfactory agreement with LC-MS for the detection of acrylamide in various foods. A GC method coupled with MS following the derivatization was proposed for the detection of trace levels (<50 μg kg^{-1}) of acrylamide in cereal-based foods due to its high sensitivity (LOD 2 μg kg^{-1}) and great recoveries (93-104%) [Pittet and Perisset, 2004]. It is important to note, however, that the derivatization is not necessarily essential and some scientific groups have chosen to eliminate this lengthy procedure using a more polar GC phase, although this approach does not provide sufficiently low limit of detection in comparison to the derivatization techniques [Soare et al., 2006, Wenzl et al., 2006, Becalski, 2005]. The main drawback of GC–MS without derivatization is the lack of characteristic ions in the mass spectrum of underivatized acrylamide and the interference caused by matrix decomposition. Due to the high background noise, a low limit of detection is impossible to obtain. The application of gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) allows to decrease the interference, which results in a larger acrylamide peak area. The values of LOQ and LOD in the GC–MS/MS method for baby foods are below 5 μg kg^{-1} and 1.5 μg kg^{-1}, respectively [Oracz et al., 2011].

Lee et al., (2007) detected acrylamide in aqueous matrices using direct immersion solid-phase microextraction (SPME) coupled with gas chromatography positive chemical ionization tandem mass spectrometry, which showed very high sensitivity (0.1 μg kg^{-1}).

The other detectors used in a tandem with gas chromatographs to quantify acrylamide are flame-ion detectors (FID) or electron capture detectors (ECD). A GC–ECD method with prior derivatization by $KBrO_3$ and KBr was used to determine the amounts of brominated acrylamide derivative in fried foods. Electron capture detectors are selective and very sensitive. For a GC–ECD method, the values of LOD and LOQ for potato crisps, potato chips and fried chicken wings were around 0.1 $\mu g\ kg^{-1}$ and 3 $\mu g\ kg^{-1}$ respectively, which provides evidence of a good precision when working with this relatively new method. Besides, the cost of instruments in a GC–ECD system is lower compared to GC–MS/MS, while their sensitivity is comparable [Oracz et al., 2011].

Capillary electrophoresis is a powerful alternative for analyzing organic compounds based on charge-to-mass ratio differences with high separation efficiency. Two in-line preconcentration capillary zone electrophoresis methods (field amplified sample injection and stacking with sample matrix removal) have been evaluated for the analysis of acrylamide in foodstuffs after being derivatized with 2-mercaptobenzoic acid, both of which showed similar sensitivity and precision compared to chromatography-based methods [Bermudo, 2006; Bermudo, 2007]. A laser-induced fluorescence detection method mediated by capillary electrophoresis was studied for the detection of acrylamide in potato crisps with good recovery (90-95%) and precision (RSD <5.7%) [Chen, 2011].

Enzyme-linked immunosorbent assay (ELISA) is a rapid method based on the recognition of antigen-antibody binding with high specificity and affinity, as well as the signal readout through optically detecting colored products catalyzed by enzyme labels. Because of the high specificity/affinity of antigen-antibody recognition and the high efficiency of enzymatic catalysis, ELISA has many advantages, such as good sensitivity, selectivity, high

throughput, and the ability of coupling to other technologies, for example, biotin-avidin amplification and chemiluminescence. The application of ELISA for acrylamide quantification requires synthesis of specific antibodies, which has been troublesome because of the low molecular mass of this compound. In general, substances with molecular mass are not immunogenic and do not elicit synthesis of antibodies. Preston (2008) reported to use polyclonal antibodies for this purpose. The isolation of acrylamide binding antibodies enabled quantitative determination of acrylamide in foods. Because of the low molecular mass and the lack of strong epitope groups, acrylamide cannot by itself elicit synthesis of specific antibodies, however, its coupling (as a hapten) to immunostimulating carrier proteins was found to be an effective method for the stimulation of antibody synthesis. Then, the antibody of acrylamide can be produced via immunoreactions stimulated by the complex antigen [Qiniqin et al., 2015].

Therefore, ELISA has attracted an increasing attention for detecting acrylamide in foods. In this new field, the two key issues are the development of appropriate antigens to obtain high affinity antibodies, and signal amplification. Compared to the common analytical techniques, ELISA is a simpler method with similar sensitivity, lower cost, shorter detection time, and does not require expensive equipment or complex sample preparation. Therefore, ELISA kits available for commercial application are of significant interest. For example, an ELISA kit was tested and gave good recovery (from 92% to 96%) with a LOD and a linear range of 5 $\mu g\ kg^{-1}$ and 10-10000 $\mu g\ kg^{-1}$, respectively [Franek, 2014]. However, ELISA still needs to be improved and the analytical results should be confirmed by other robust methods. Meanwhile, the ways to obtain stable antibodies of acrylamide with high affinity are still the key issue in this field.

Electrochemical biosensing methods have been proposed to detect acrylamide in foods or complex matrices only in the past few years. A biosensor is a device used to detect an analyte that combines a biological component (bioreceptor) with a physicochemical detector (transducer). Electrochemical biosensors, with electric signal (such as current and potential) output, have shown the advantages of speed, simplicity, suitability for automation, and sensitivity, leading to broad applications in food safety. The first trial for determining acrylamide in wastewater was based on biocatalysis from microbial metabolism, including respiration and enzyme reactions. Ignatov [1997] first quantified acrylamide by defining specific respiratory activity as the difference between the rate of oxygen consumption of *Brevibacterium sp.* and endogenous cell respiration after the introduction of acrylamide. The reduction of oxygen concentration and the current created from the metabolism of acrylamide could be used to detect acrylamide. Silva [2011] designed an electrochemical biosensor based on the electron transfer of a direct biochemical interaction between acrylamide and whole cells of *Pseudomonas aeruginosa* containing intracellular amidase, which catalyzed the hydrolysis of acrylamide, producing ammonium ions and acrylic acid. The use of biochemical reactions from microbial metabolism can lead to real-time detection of acrylamide. Good stability, a long working life, and the presence of various enzymes in cells are the advantages of microbial biosensors. However, the dependence on a living organism elicits long response time. Also, protecting the bacteria against aggressive environments is another problem.

Recently, a novel fluorescent sensing method based on acrylamide polymerization and the unique photophysical properties of quantum dots (QDs) was proposed to detect acrylamide [Hu, 2014]. In this study, QDs containing carbon-carbon double bonds after modification of

N-acryloxysuccinimide polymerized under UV irradiation, resulted in the decrease of the distance between QDs and the fluorescence intensity [Liu, 2011; Noh et al., 2010]. In the presence of acrylamide, the distance of QDs became larger due to the participation of acrylamide in the polymerization reaction, resulting in an increase of the fluorescence intensity. Therefore, a correlation was established between the concentration of acrylamide and changes of fluorescence intensity after UV irradiation. The linear interval and LOD were in the range of 35-350000 μg kg^{-1} and 35 μg kg^{-1}, respectively. Compared to the standard methods and electrochemical biosensing, the lower sensitivity of this method limits its applicability to the detection of acrylamide in various food samples. For LC-MS/MS and GC-MS methods, the general procedures of pretreatment include homogenization, spiking with an internal standard, extraction, defatting, deproteinization, purification by SPE cartridges and derivatization (for GC-MS).

The addition of an internal standard in food samples can allow the compensation for recovery and to keep track of the possible loss during the whole sample preparation procedure, which improves the accuracy, precision, and repeatability of measurements. Most of the published studies used $^{13}C_3$-acrylamide as an internal standard for acrylamide detection, which has very similar properties to acrylamide. However, D_3-acrylamide, $^{13}C_1$-acrylamide, N, N-dimethylacrylamide, propionamide, and methacrylamide also have been used as internal standards [Gokmen et al., 2009; Zhang, 2007].

Extraction is a critical procedure in food pretreatment prior to the detection of acrylamide. The hydrophilic character of acrylamide allows for an application of water or organic solvents, such as methanol and acetonitrile, for its extraction from foods [Rosen and Hellenas, 2002; Tateo and Bononi, 2003]. Water can minimize the co-extraction of

hydrophobic compounds from foods, but other hydrophilic interferences still remain that should be removed afterwards [Zhang et al., 2005]. In comparison to water, organic solvents have the advantages of easier separation even without centrifugation and more convenient evaporation. The combination of water (salt solutions) and organic solvents improved the extraction efficiency in some studies [Zhang, 2007; Yamazaki, 2012]. Depending on the food matrix, a defatting step with an organic solvent or a deprotonating step could be necessary to remove interfering components [Delatour et al., 2004]. For protein-rich food samples, Carrez reagents ([I] potassium ferricyanide and [II] zinc sulfate), acetone, ethanol, or methanol were used to precipitate and remove proteins [Bagdonaite, 2008].

Solid phase extraction (SPE) has been used extensively in purifying the extracts of food samples due to its simplicity, stability, suitability for automation, accuracy, precision, and the repeatability of instrumental analysis. Depending on the selection of SPE cartridges used for purification of acrylamide from the extracts of food samples, two strategies have been developed. One strategy relies on acrylamide absorption from the complex extract by the cartridges through hydrogen bonding, π-π interaction, and cation exchange, followed by elution of acrylamide using other polar solvents. Some common cartridges are Oasis HLB, Oasis MCX, Isolute Multi-Mode, ENVI-Carb, and Isolute ENV+. The second strategy is applied to retain the interferences and collect the eluent containing acrylamide by using Oasis HLB cartridges coupled with Bond Elut-Accucat and a custom made SPE column filled with a mixture of C18, SCX, and SAX sorbents. Among these cartridges, Oasis HLB and Isolute Multimode are the most preferred. Recently, novel filling materials such as carbon nanotubes, magnetic chitosan, and molecule imprinted polymers were used, resulting in an effective purification [Bortolomeazzi et al., 2012; Xu, 2013; Xu, 2012].

Selection of an appropriate analytical methodology for the determination of acrylamide is mostly based on the type of food matrix. Its quantification in food is difficult because of the low molecular mass, lack of chromophores or fluorescent functional groups, high polarity, very good water solubility, high reactivity, and low volatility. Quantification of acrylamide in complex matrices rich in interfering compounds is an especially demanding task. The analysis of coffee samples to determine the acrylamide content has high importance because the exposure to acrylamide from coffee has been found to contribute substantially to the overall dietary acrylamide exposure. Several papers describe the determination of acrylamide in coffee samples using LC-MS/MS or GC-MS, indicating the existence of significant problems related to the matrix suppression phenomenon and the presence of interfering co-extractives [Alves et al., 2010; Andrejewski et al., 2004], therefore a challenge still remains with respect to the development of stable, reliable, and robust methods for difficult food matrices, such as cocoa powders, coffee, and high salt flavorings. High-resolution mass spectrometry (HRMS) is particularly suitable for the detection of low molecular weight food contaminants and it can provide some analytical advantages over other MS techniques.

1.9. Health risks of dietary acrylamide

Acrylamide most pertinent health issue related to its widespread occurrence in food is its potential to cause cancer in humans. The International Agency for Research on Cancer classified acrylamide as “Group 2A” carcinogen which is probably carcinogenic to humans due to its neurotoxicity, carcinogenicity and genotoxicity in 1994 [Lyon, 1994; Erkekoglu & Baider, 2010; Hogervorst, 2010]. It is classified as ‘Category 2 carcinogen’ and ‘Category 2 mutagen’ by European Commission as well as a substance of “very high concern” by

European Chemical Agency in 2010 [EC, 2002; ECHA, 2010]. Recently ESFA [2013] has presented a scientific report on acrylamide giving the analytical results of the food commodities collected since 2010. Daily exposures to acrylamide across surveys and age groups were estimated at 0.4 to 1.9 μg kg^{-1} body wt. per day and 0.6 to 3.4 μg kg^{1} body wt. per day, respectively, far below the threshold of acute toxicity. Therefore, the health concern lies mainly upon the possible mutagenic or carcinogenic effects of lower dose and long term exposure [ESFA, 2015].

The acrylamide genotoxicity arise due to two reasons. First, acrylamide can be converted to its metabolite glycidamide, which is three times stronger mutagen compared to acrylamide and can induce point mutations in various systems [Fazendeiro, 2013]. Secondly, acrylamide can act as a Michael acceptor to form adducts with thiol, hydroxyl, and amino groups in DNA, which leads to DNA damage [Watzek, 2012]. Workers who experience occupational exposure to acrylamide suffer from the damage of both peripheral and central nervous systems, since the neurotoxic effects of acrylamide are cumulative and chronic [Pennisi, 2013]. Acrylamide is toxic to humans at various levels via inhalation, dermal absorption, and ingestion. Rodent studies have proven a high level exposure to be neurotoxic and even deadly [Friedman, 2003]. The no-observed-adverse-effect level (NOAEL) for morphological changes in the nervous systems of rats was 200 μg kg^{-1} body wt. per day. Tardiff et al., [2010] reported that the tolerable daily intake of acrylamide was 40 μg kg^{-1} per day for neurotoxicity, and 2.6 μg kg^{-1} per day for carcinogenicity.

1.10. Metabolism of acrylamide

The metabolic pathways for acrylamide in living system is widely studied in rats and mice, and several studies indicate that acrylamide is rapidly metabolized and excreted

predominantly in the urine as metabolites [Sumner et al., 1992, 1999, 2003; Twaddle et al., 2004]. The metabolic pathway for the biotransformation of acrylamide is given in fig 1.9. In Phase I metabolism, acrylamide is partly converted to an epoxide, glycidamide through in vivo oxidation by cytochrome P450 2E1 (CYP2E1) enzyme [Calleman et al., 1990; Settels, 2008]. Another initial step, catalyzed by the enzyme CYP2E1, involves oxidation of acrylamide to its epoxide derivative, glycidamide. Glycidamide may also undergo hydrolysis, perhaps catalyzed by epoxide hydrolases, leading to the formation of 2,3-dihydroxypropionamide and 2,3-dihydroxypropionic acid which is a less important pathway for the detoxification process. Orally ingested acrylamide undergoes conjugation with glutathione in Phase II metabolism.

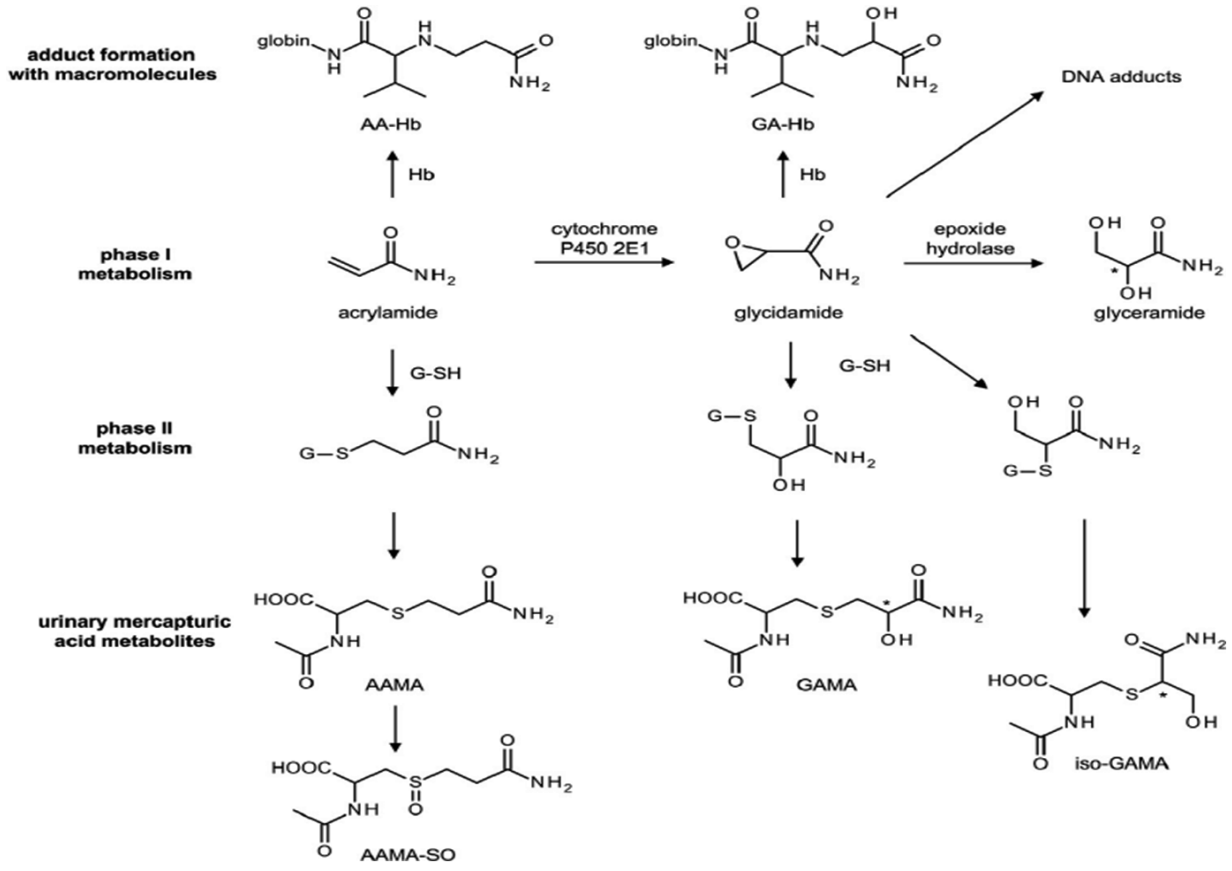

Fig 1.9. Metabolic pathway for the biotransformation of acrylamide

After degradation and acetylation of the conjugate, the resulting mercapturic acid N-acetyl-S-(2-carbamoylethyl)-1-cysteine (AAMA) is excreted with urine. Glycidamide can also be conjugated with glutathione and is excreted with urine after metabolic conversion to the mercapturic acids N-acetyl-S-(2-carbamoyl- 2-hydroxyethyl)-l-cysteine (GAMA) and N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-l-cysteine (iso-GAMA). Glycidamide is more reactive than acrylamide towards DNA and proteins. However, conversion of acrylamide to glycidamide has been shown to be less important in humans than in rodents [Fuhr et al., 2006; Fennell & Friedman, 2005; Fennell et al., 2005]. The amount of GAMA excreted in urine was about 9%–29% of the oral dose in rodents, and it was between 0.7% and 6% in human. Both acrylamide and glycidamide react with nucleophilic sites in macromolecules (including hemoglobin and DNA) in Michael-type additions [Bergmark et al., 1991, 1993].

1.11. Use of Response surface methodological approach for explaining the formation of acrylamide

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). RSM can be used in problems that have ingredients and/or processing conditions as variables [Arteaga et al., 1994]. Many researchers have successfully applied RSM for optimizing food processing operations [Frank, 2001; Luciane et al., 2001; Pietrasik & Li-Chan, 2002; Lee et al., 2006; Zhang et al., 2007]. Farah and Zaibunnisa, [2012] reported the optimization of cocoa beans roasting process based on concentration of pyrazine and acrylamide with the optimized roasting conditions being able to produce high quality cocoa beans with low concentration of acrylamide. Also, Madihah et al., [2013]

studied the formation of acrylamide in Arabica coffee beans by optimizing the roasting conditions consisting of temperature and time. High quantity of flavour compounds with low level of acrylamide resulted based on optimized roasting temperature and time. Therefore use of RSM has been an effective method in improving product quality. Li et al., [2016] investigated the effect of garlic powder addition on the formation and elimination of acrylamide in asparagine/glucose low moisture model system using RSM. The effect of garlic powder on bread and bread quality were also reported.

1.12. Objectives of the study

The objectives of the present study are

(1) Development and optimization of HPLC method for the survey of acrylamide in deep fried chips.

(2) Correlation of acrylamide formation with ripening – A case study with respect to plantain & jack chips as part of mitigation strategies.

(3) The effect of process parameters on acrylamide formation in model systems (glucose/asparagine & fructose/asparagine) by RSM and use of empirical models for explaining the formation and degradation of acrylamide

1.13. Significance of the study

Acrylamide found in heat treated foods has led to an intensive and persistent research effort, since it is a neurotoxic, genotoxic and probable carcinogenic compound to humans. Acrylamide is a by-product of the Maillard reaction and can be generated from food components during heat treatment, as a result of the reaction between the reactive carbonyl group on the sugar and amino group of the amino acid [Mottram et al. 2002]. Acrylamide in food is a concern because it can cause cancer in laboratory animals at high doses, and is

reasonably accepted to be a human carcinogen [NTP, 2011]. In 2013, the FDA called attention to acrylamide in foods by publishing a draft guidance for industry, providing information to help growers, manufacturers, and food service operators on how to reduce the concentration of acrylamide in certain foods [FDA, 2013]. Dietary exposure of acrylamide over a long period causes genotoxicity and mutation which leads to development of tumors in many organs [Besaratinia & Pfeifer, 2004].

The consumption of processed foods has been on rise and most of these processed foods are exposed to high temperature processing. Following guidelines from FAO/WHO, a detailed investigation was carried out by FDA (US), EFSA (Europe) and Health Canada (Canada) to find out the acrylamide exposure to their respective population and to make necessary recommendations to reduce the exposure. Indian subcontinent with its vast diversity consumes a variety of heat processed food products from staple foods to snack products. However, there is compete lack of data with respect to acrylamide content in Indian traditional products, except a few reports. It was found that there is a need for an extensive study to estimate the occurrence of acrylamide in various heat processed products consumed in India and this information can be used for assessing intake assessment and also for planning mitigation strategies to reduce the human exposure. However, no such attempts have been reported from India. This alarming health risk and lack of data relating our traditional fried products with acrylamide draws the background of this study.

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CHAPTER 2

Acrylamide in deep fried snacks of India

2.1. Introduction

Acrylamide is a process induced food toxicant found in various carbohydrate rich food matrices exposed to very high temperature. The International Agency for Research on Cancer [IARC, 1994] classified acrylamide as probably carcinogenic to humans and the evidence for the carcinogenic, reproductive and genotoxic effects are reported on experimental animals [Dearfield et al., 1995]. Meanwhile a consultation regarding the risk assessment of acrylamide was jointly held by the Food and Agriculture Organization and the World Health Organisation in 2002 [FAO/WHO, 2002]. Thus the detection of the high concentrations of acrylamide in food products has become a serious concern among the regulating authorities, the food industry and the consumers. Various survey studies indicated that the levels of acrylamide in potato products such as French fries and potato crisps were the highest of the foodstuffs investigated. These findings caused worldwide concern about the possible public health risks from dietary exposure to acrylamide.

The first breakthrough in acrylamide research was the simultaneous discovery by several groups that the acrylamide is formed from the reducing sugars and asparagine in the Maillard reaction in a complex mechanism [Mottram et al., 2002; Stadler et al., 2002; Yaylayan et al., 2003; Zyzak et al., 2003]. Later additional formational mechanisms from peptides, proteins and biogenic amines were also identified. [Claus et al., 2006].

Many studies have been carried out for quantifying the levels of acrylamide in various foods of different countries. A study on various food categories in Japan and Hongkong were carried out by Ono et al., [2003] and Leung et al., [2003]. A survey has been conducted on the occurrence of acrylamide in several carbohydrate-based foods available on the Australian market [Croft et al., 2004]. Acrylamide levels in selected baked and fried foods in

Jordan have been investigated by Al-Dmoor et al., [2004]. A survey of retail Turkish foods was conducted for the evaluation of acrylamide [Senyuva and Gokmen, 2005]. Analysis of acrylamide in Chinese foods was done by Wei-Chih Cheng et al., [2006]. Tateo et al., [2007] evaluated the acrylamide level in foods which are common in the Italian market. Another report regarding the survey of foods in the Turkish market was done by Olmez et al., [2008]. The analysis of acrylamide levels in popular Iranian potato and corn products were reported by Mohammad et al., [2010].

Online public databases have been set up in Europe by the European Commission's Joint Research Centre [JRC, 2005] and in the USA by the Food and Drug Administration [FDA, 2005], showing that data on acrylamide levels from North America and European countries have been well established. A toxicological evaluation of acrylamide was carried out by FAO/WHO Expert Committee on Food Additives [JECFA] in 2005. The committee suggested to extend the survey studies on the occurrence of acrylamide in foods consumed in developing countries, as this information is valuable for conducting intake assessment as well as considering mitigation approaches so as to reduce human exposure.

India with its diverse food habits, consumes a wide range of deep fried and high temperature processed foods (chips of different kind, puri, tandoori products etc). Chips are the most popular variety of snacks in India, especially Kerala. Potato, jack, sweet plantain and plantain chips are the popular processed food items resulting in substantial value addition. It is reported that the prolonged exposure of these kinds of snacks may lead to many of the life style associated diseases like diabetes, cardiovascular diseases and cancer which inturn is a major cause for the mortality in India [Dikshit et al., 2012]. To our knowledge, there is no study regarding the levels of acrylamide in fried Indian snacks. Considering the importance

of generating such data, the objective of this study was to survey and compare the occurrence of acrylamide in four different deep fried chips viz. potato, jack, ripe plantain and raw plantain chips to provide an overview of the acrylamide content in these foods.

Numerous reviews have been reported on different analytical techniques for the determination of acrylamide in foods [Castle and Erikson, 2005]. Both MS and non-MS methods have been reported for the quantification of acrylamide in various food matrices. Non-MS methods include capillary zone electrophoresis, liquid chromatography (LC) coupled with pulsed electrochemical detection and time-of-flight MS, ion-exclusion and ion-exchange LC [Wenzl et al., 2003]. The use of water as extraction medium and different SPE phases renders emphasis on clean-up steps to avoid interference from co-extractives. These methods require expensive instruments and complicated, long extraction procedures for the acrylamide analysis, even though they show good analytical performance. In our present study, we have modified a sample extraction protocol for HPLC-DAD quantification of acrylamide in fried Indian snack foods (potato, jack, ripe plantain and raw plantain chips).

2.2. Materials and methods

2.2.1. Materials

Standard acrylamide (>99%) was purchased from Sigma Aldrich (St. Louis MO, USA). Water was obtained from a water purification system, Millipore; Milli-Q (Bangalore). All solvents used in the experiments were of high performance liquid chromatography (HPLC) grade. Oasis HLB (30 mg, 1 ml) solid phase extraction (SPE) catridges were obtained from Waters Corp. (Milliford, Massachusetts USA). Syringe filters Dismic-13 CP (0.45µm, hydrophilic) with cellulose acetate membrane were supplied by Toyo Roshi Kaisha Ltd, Japan. Stock solutions of 10µg/ml acrylamide were prepared in Milli-Q water which was

diluted with water to give a series of standard solutions (0.2, 0.4, 0.6, 0.8 and 1µg/ml) respectively. All these solutions were stored at 4ºC for about 3 months.

2.2.2. Food samples

All samples selected for this survey study were purchased randomly from the different local production sites of the Southern province of Kerala. A total of 80 samples of four different kinds of chips (20 samples of each kind) including potato, jack, sweet plantain and plantain chips which are the common popular snacks in India. The samples were homogenised and the subsamples of the homogenate were stored at -20ºC in centrifuge tube with plastic screw-capped lids until analysis.

2.2.3. Sample extraction

10g of the sample was defatted twice by adding 15 ml of hexane each. Then it was filtered and the residue was evaporated under vacuum. 25 ml acetone and 500 µl of water was added and ultrasonicated for about 20 min at 40ºC. Subsequently, the mixture was centrifuged at 3000 x g for 10 minutes; the resulting supernatant was removed and filtered through 0.45 µm cellulose acetate syringe filter. The filtrate was evaporated under vacuum and re-dissolved in minimum amount of water. Final filtrate was introduced to solid phase clean-up step, in which the cartridge was previously conditioned using 1 ml of methanol and 1 ml of Milli-Q water, respectively. 1ml of the filtrate was passed through SPE catridge and the first five drops discarded and the remaining filtrate collected for the HPLC injection.

2.2.4. HPLC-DAD analysis

Calibration standards and the sample extracts were injected into a Schimadzu HPLC system (Japan) consisting of two LC-8A chromatography pump units, column oven (CTO-10AC VP), system controller (SCL-10A VP), rheodyne injector (USA) with a loop of 20 µl

volume, diode array detector (DAD; SPD-M10A VP). An isocratic elution pattern was adopted for the separation of the analyte, and 100% Milli-Q water was used as the mobile phase. The column temperature was set at 25°C; the flow rate was maintained at 0.5 ml/min, while the detection was performed at 206nm. The injection volume was 20 µl. In this condition the retention time for acrylamide was 6.8min.

2.3. Statistical analysis

The experimental results were expressed as mean ± SD (standard deviation) of triplicate measurements. The data were subjected to one way analysis of variance (ANOVA) and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for windows, standard version 7.5.1, SPSS and the significance accepted at $p \leq 0.05$.

2.4. Results and Discussion

Gas chromatography–mass spectrometries (GC/MS), liquid chromatography with tandem mass spectrometry (LC–MS/MS) [Bortolomeazzi et al., 2012; Mojska et al., 2012] are the most commonly used techniques for the detection of acrylamide in foods. Analysis of acrylamide by GC-MS requires pre-derivatization which is time consuming and involves handling of chemicals such as bromine. Since acrylamide is a simple molecule, it lacks characteristic ions in the mass spectrum and this causes interferences by the matrix which increases background noise and hence a low limit of detection is impossible [Oracz et al., 2011]. Detection of acrylamide by HPLC-MS/MS requires more sample cleanup prior to analysis for preventing peak interferences and enhancing sensitivity. Hence we focussed on the development of a modified HPLC method which would unambiguously verify the detection of acrylamide in a range of deep fried snack foods.

In the present study, the linearity plot was linear covering five different concentration levels of acrylamide. Each calibration solution was analyzed in triplicate and average value of the results was used as the representative for each point. The regression equation was determined as: $y = 227311.5x + 12304.1$ where y is the peak area and x is the concentration of acrylamide (μg/ml). The peak area is linearly related to the concentration of acrylamide. The limit of detection (LOD) and the limit of quantification LOQ was calculated by LOD = (3.3 * SD/S), LOQ = (10 * SD/S) where SD = the standard deviation of the response and S = the slope of the calibration curve. The LOD and LOQ were 1.04 μg/ml and 3.17 μg/ml respectively based on the signal-to-noise of 3 and 10, respectively. The mean value, median and range of acrylamide content in potato, jack, sweet plantain and plantain chips are presented in Table 2.1. The average levels of acrylamide in these fried food items ranges from 426.23 to 1456.54 μg/kg and the values are significantly different from each other ($p \leq 0.05$). Precision was determined in terms of intra-day repeatability and inter-day reproducibility as relative standard deviation (RSD) %. Among the four products, sweet plantain chips had the lowest coefficient of variation (CV) of 3% and plantain chips had 4.8% CV (Table 2.2.). The data indicated that the repeatability of the method was acceptable and the reproducibility ranged from CV of 2.5 to 6.4%. Thus our proposed HPLC- DAD method has very good intra- and inter-day precision and is able to determine acrylamide at ppm level and should be very useful for food analysis. In addition, the recovery tests were conducted by adding known amount of acrylamide standard (500μg/kg) to the homogenised potato, jack, ripe plantain and plantain chips, followed by the same sample preparation procedure and HPLC analysis. The recoveries ranged from 90 to 103% with CV less than 10 (Table 2.3.).

Table 2.1. Acrylamide levels in food samples from the South Indian market

Food Sample (n=20)	**Mean AA* (µg/kg)**	**Median (µg/kg)**	**Range (µg/kg)**
Potato Chips	1456.54 ± 22.12[a]	1533.86	82.05-4245.59
Jack Chips	587.82 ± 30.41[b]	210.80	46.18-2431.37
Sweet Plantain Chips	455.30 ± 22.27[c]	227.77	24.81-1959.80
Plantain Chips	426.23 ± 12.66[d]	197.63	14.73-1690.48

** mean ± SD (n=3)*

[a-d] *Values are significantly different from each other and significance accepted at p≤0.05*

Table 2.2. Repeatability Test of the HPLC-DAD method

Test No	Acrylamide Concentration (µg/kg)			
	Potato Chips	Jack Chips	Sweet Plantain Chips	Plantain Chips
1	2855	1934	1944	1576
2	2757	1899	1805	1445
3	2684	1832	1852	1517
4	2913	1749	1834	1645
5	2932	1880	1810	1575
Mean ± SD	2828 ± 106	1859 ± 72	1849 ± 56	1552 ± 75
CV (%)	3.7	3.9	3	4.8

Table 2.3. Recovery test of the HPLC-DAD method*

Sample	**Acrylamide added (µg/kg)**	**Recovery (%)**	**CV (%)**
Potato Chips	500	90	7.1
Jack Chips	500	94	3.9
Sweet Plantain Chips	500	103	1.9
Plantain Chips	500	94	8.0

**n=3(triplicates)*

There are very few reports available on quantification of acrylamide in food products by HPLC. Geng et al., [2011] developed an HPLC-DAD method for the detection of acrylamide in starch-based foods and the analysis was carried out by precolumn derivatization bromination. The LOD of the method was 15µg/kg and the recoveries are in the range 89.6-102%. Another HPLC-UV method using reversed-phase C18 column was put forward by Xu et al., [2012] for the detection of acrylamide in crust and potato chip samples using methanol/water system. The LOD was found to be 66ng/L and recoveries ranged from 88.9-89.5%. In the present method, we have used acetone/water system for the extraction of acrylamide which improved the extraction efficiency thereby reducing extraction time (~ 40 min). It was reported that the recovery of acrylamide is improved when a mixture of acetone and water was used as extraction solvent [Takatsuki et al., 2003]. Good recovery was obtained by using Oasis HLB catridge rather than simple filtration techniques [Zhang et al., 2005, 2007]. Highly resolved chromatograms with minimum interferences are obtained when pure water was used as eluent. We used minimum organic solvents for the extraction steps making it more eco-friendly.

In the present study a total of 80 samples (20 samples each for potato, jack, ripe and raw plantain chips) were procured from different local production sites of Kerala. Acrylamide from samples were extracted using the above mentioned procedure followed by HPLC quantification and all the extracts gave clear chromatograms at the desired retention time. A representative chromatogram showing acrylamide peaks in standard and four samples are shown in Figure 2.1. The variation in the acrylamide content among different batches within a product type may be due to the difference in the chemical composition of the raw material and the processing conditions applied. Studies have been performed on the level of AA in

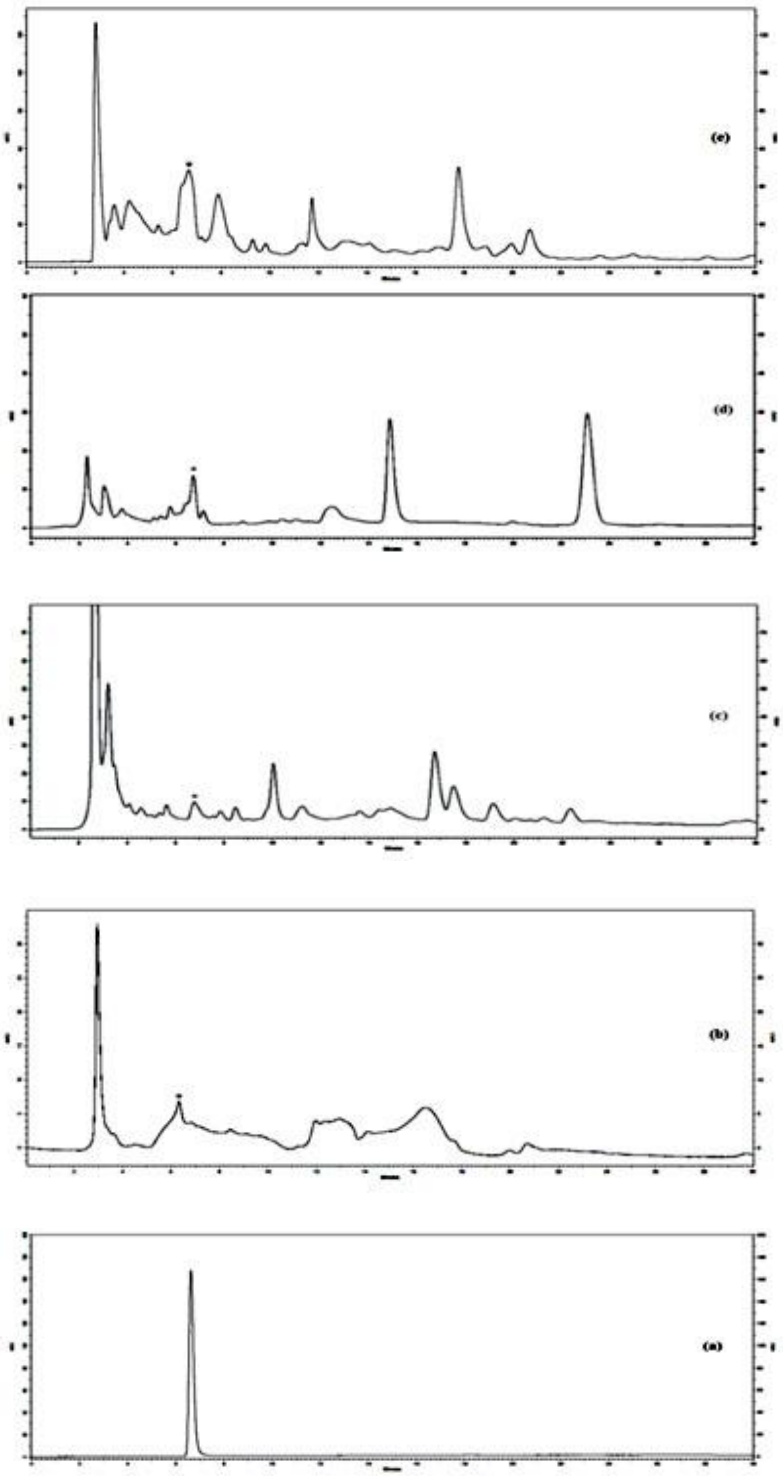
various fried foods worldwide. Reported level of acrylamide in potato products like chips, crisps and French fries varies from 81-26784 µg/kg [Shepherd et al., 2010; Chen et al., 2011]. Chinese deep fried foods like paicha, youtiao, yougao, crisp mahua and mahua contain acrylamide levels in the range 212-248 µg/kg [Wang et al., 2013]. Fried instant noodle samples from the Hebei province showed acrylamide concentrations of 930 µg/kg [Yang et al., 2012]. In deep fried Thai conventional foods like sweet potato chips, taro crisps, banana fritters, durian chips etc acrylamide levels ranged from 150-500 µg/kg [Komthong et al., 2012]. Carribean food samples such as banana chips, banana fritters and dumplings (white flour and whole wheat) contain 100-430 µg/kg, 1090 µg/kg, 2440 µg/kg & 3360 µg/kg respectively [Bent et al., 2012]. Among the samples selected for our study, potato chips were found to contain highest level of acrylamide ranging from 82.05-4245.59 µg/kg.

Levels of asparagine, total free amino acids and sugar (mg/g of wet weight) varied in raw potato tubers from 1.5 to 11.4, from 11 to 31.5, and from 0.86 to 23, respectively [Becalski et al., 2004]. These findings suggest the greater dependence of reducing sugar on acrylamide formation in potato tubers than the aminoacid asparagine [Amrein et al., 2003]. Certain other factors which have a direct effect on the amount of precursors in potatoes are variety [Hebeisen et al., 2005], storage temperature/time [Noti et al., 2003] and also the level of nitrogen and phosphorus in the soil, which in turn had a direct influence on acrylamide formation in the cooked products.

The concentration of acrylamide in jack chips selected for our study ranged from 46.18-2431.37 µg/kg. It is reported that the total soluble amino acids in jack fruit range from 270 to 670 µg and its concentration decreases during ripening [Ghosh, 1996]. In the present study

jack chips is having lower levels of acrylamide when compared to potato chips and this could be due to the lower concentration of precursors in raw jackfruit as compared to that of potato. Indeed there is a lack of data on the asparagine content in raw jackfruit which is very important as far as the formation of acrylamide in jack chips is concerned. This is the first investigation regarding the quantification of acrylamide in deep fried jack products. Acrylamide levels of the raw and ripe plantain chips in the present study ranged from 14.73-1690.48 µg/kg and 24.81-1959.80 µg/kg respectively. Very few studies are reported on the occurrence of acrylamide in banana/plantain products. Daniali et al., [2010] reported acrylamide levels ranging from 268-3585 µg/kg in banana fritters made from over ripe banana. Acrylamide levels upto 900 µg/kg were reported in plantain products [Bassama et al., 2011**]** and found that its formation in plantain is in the same magnitude as that of rye and wheat products.

Investigations on reactions favouring the formation of acrylamide revealed that the process is initiated by the reaction between reducing sugar and amino acid at high processing temperature indicated it to be by-product of the Maillard reaction. The variation in the occurrence of acrylamide in the different samples studied may be explained by the variation in the concentration of precursor in the raw materials, its maturity stage, storage conditions & changes in the climatic and geographical conditions etc.



***Acrylamide peaks**

Fig 2.1. HPLC Chromatograms of (a) standard acrylamide, (b) deep fried plantain chips, (c) sweet plantain chips, (d) jack chips & (e) potato chips

2.5. Summary

The present investigation is the first report on occurrence of acrylamide in deep fried snacks of India. As can be seen, there is a wide variation in the level of acrylamide in each of the samples studied. The variation of acrylamide level in different product types depends mainly on the level of precursors in the raw materials used. It is reported that the concentration of precursors for acrylamide formation vary with respect to maturity, variety, climatic, geographic and post harvest handling conditions and processing conditions (time and frying temperature etc) employed. As there was no history of such conditions available for the product studies, it is advisable to perform additional experiments on the effect of above parameters on precursors and the formation of acrylamide in various products. Detailed analysis covering more number of samples including home cooked food groups should also be included for giving supporting information on the widespread potential risk from acrylamide for public health experts, food policy makers and consumers.

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CHAPTER 3

3.1. Introduction

It was observed from the previous chapter that the occurrence of acrylamide is greater in chips made from ripened banana. Considerable levels of acrylamide were also found in jackfruit chips. The study also indicated that selection of proper maturity can limit the formation of acrylamide in the same (Shamla and Nisha, 2014). Studies suggests that many factors such as the nature of phenolic compounds, processing conditions, and type of food matrices may contribute to the increase or decrease in levels of acrylamide formation [Jin et al., 2013].

Antioxidants particularly phenolic compounds have been reported to inhibit the formation of acrylamide in model and various food systems. It is reported that the phenolic compounds present in plantain extract could act as natural antioxidants which could help in mitigating the toxicity of acrylamide consumed by the body through an antioxidant protective mechanism [Jun et al., 2008]. Morales et al., [2014] reported that green tea, oregano & cinnamon extracts reduced the acrylamide formation in fried potatoes whereas thyme and bougainvillea extracts had no effect on the formation of acrylamide. Alternatively, these additives constitute a group of phytochemicals which are highly beneficial to human health because of their antioxidant properties.

The modification of food processing parameters which could limit the formation of acrylamide is one of the strategies adopted for the mitigation of acrylamide in foods. Another strategy is by selection of better raw materials which have low contents of the precursors of acrylamide formation namely, asparagine and reducing sugars (FDE, 2014). Thus considering mitigation as a matter of concern and to evaluate the chemical composition of the plantain and jackfruit at different maturity, we tried to elucidate the proximate composition, sugar and amino acid profiling and phenolic compounds composition at different stages of maturity. The precursors were further correlated with

formation of acrylamide in deep fried plantain and jack chips at different ripening stages. The chapter is divided into two, Part A & Part B deals with acrylamide formation in chips made from plantain and jack at various maturity stages, respectively.

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CHAPTER 3 -Part A

Acrylamide formation in plantain (*Musa paradisiaca*) chips influenced by different ripening stages: A correlation study with respect to reducing sugars, amino acids and phenolic content

3.A.1. Introduction

Banana/plantain is the fourth most important food crop in the world after rice, wheat, and maize followed by potato and India is the largest producer of banana in the world [Singh et al., 2016]. Since plantain is a good source of carbohydrate, the formation of acrylamide during high-temperature processing such as frying and baking is unavoidable. A study by Daniali et al., [2010] on the acrylamide content of five popular Malaysian fried and baked banana based snacks revealed that the acrylamide content ranged from 74.0 to 7468.8 μg/ kg for banana fritter (pisang goreng), 28.9 to 243.7 μg/kg for banana chips (kerepek pisang), 160.7 to 500.4 μg/kg for sweet banana chips (kerepek pisang manis), not detected to 154.4 μg/kg for banana cake (kek pisang) and 31.7 to 609.1 μg/kg for banana balls (cekodok pisang). Bent et al., [2012] reported that banana chips (green & ripe) and banana fritters made from bananas were found to contain 100-430, 180 and 1090 μg/kg of acrylamide, respectively. Effect of maturity on the formation of acrylamide in banana fritters made from Musa paradisiaca variety, Awak and Abu has also been reported [Daniali et al., 2013]. They indicated a strong correlation between the reducing sugar content and acrylamide formation as on increasing fruit maturity. But no correlation was found with asparagine and acrylamide formation in banana fritters.

Chips made by deep frying of raw matured plantain are a popular snack product in many parts of the world. The chips prepared by deep frying the core of unripe plantain, variety-Nendran (*Musa paradisiaca*) at a stage of maturity II, peel still completely green, is one of the most favorite snack items among children as well as adults, especially in South Asian countries. The plantain/banana ripening is usually divided into seven ripening stages depending on its maturity [Soltani et al., 2011] and the colours were reproduced and translated to a numerical scale where stage I-entirely green; II-green with a trace of yellow;

III-more green than yellow; IV-more yellow than green; V-yellow with a trace of green; VI-entirely yellow; VII-entirely yellow with brown speckles. Ripe plantain (Nendran variety) at maturity III & IV are also used for making chips that will be slightly sweeter in taste. A preliminary survey on the market samples carried out by the authors revealed reasonable levels of acrylamide in plantain chips (unripe & ripe) which were high in ripe plantain chips [Shamla and Nisha, 2014]. The survey warranted a detailed investigation on the influence of different ripening stages on acrylamide formation in plantain chips as there were no reports on how the maturity affects the sugar & amino acid profile and the phenolic content of plantain. Recent studies on the mitigation of acrylamide formation in foods revealed that when polyphenols are added as antioxidants to food systems; it will influence the Maillard reaction affecting formation of acrylamide in food systems [Cheng et al., 2015, Xu et al., 2014]. A good documentation of the precursors such as reducing sugars and asparagine in plantain along with the total phenolic & flavonoid content could be helpful in understanding the formation of acrylamide in plantain chips. Therefore, the present study was undertaken with an objective to investigate the changes in the composition of plantain fruit during different stages of ripening and its correlation with the formation of acrylamide in deep fried chips. Thus, the proper information regarding maturity will help to understand the level of precursors, total phenolic & flavonoid content thereby we can reduce the levels of acrylamide in the final product to a greater extend.

3.A.2. Materials and methods

3.A.2.1. Reagents and chemicals

Standard acrylamide (>99%), D-(+) glucose, D-(-) fructose, sodium phosphate, sodium carbonate, aluminium chloride hexahydrate, potassium acetate, sodium hydroxide, gallic acid, quercetin, aluminium chloride, Folin-Ciocalteau reagent, amino acid standards and o-

phthalaldehyde (OPA) were purchased from Sigma-Aldrich (St. Louis MO, USA). HPLC water was purified on a Milli-Q system (Millipore India Pvt Ltd, Bangalore, India). Solvents used were methanol, acetic acid & acetonitrile of high-performance liquid chromatography (HPLC) grade. Oasis HLB (30 mg, 1 ml) solid phase extraction (SPE) cartridges were obtained from Waters Corp. (Milliford, Massachusetts USA). Minigen syringe filters (0.22 μm diameter) were obtained from Genetix Biotech Asia Pvt. Ltd, New Delhi, India.

3.A.2.2. Sample preparation

A bunch of plantain with around twenty tiers, variety Nendran (*Musa Paradisiaca*) at stage I maturity (green), was harvested from an authenticated agricultural farm in Trivandrum, Kerala, India and was kept at ambient conditions. Chips were made from plantain using maturity stages I-V. Two days of subsequent intervals were given for each ripening stages two, three, four and five respectively, after harvesting. Plantains were peeled manually, and the remaining fruit was used for making chips. The proximate composition, reducing sugars, amino acids, and phenolic compounds of the peeled fruit were estimated. Fig 3.A.1. represents maturity stages I-V of plantain, plantain fruit after peeling and the chips prepared from it.

3.A.2.3. Preparation of plantain chips

As chips cannot be made from fully ripened plantain, only stages of I-V were selected for preparing plantain chips in the present study. After peeling the plantain manually, the remaining fruit was sliced into thin rounds with thickness of approximately 1 mm. The sliced samples (500 g) were deep fried in coconut oil (3 L) using a stainless steel electrical deep fat fryer (NOVA, Flomatic Industries PTE Ltd, Singapore) at 165ºC for 7 min. The temperature and time of frying adopted for the study was optimized earlier based on product quality in terms of texture (by rupture test of banana chips using texture analyser, TA- HDi,

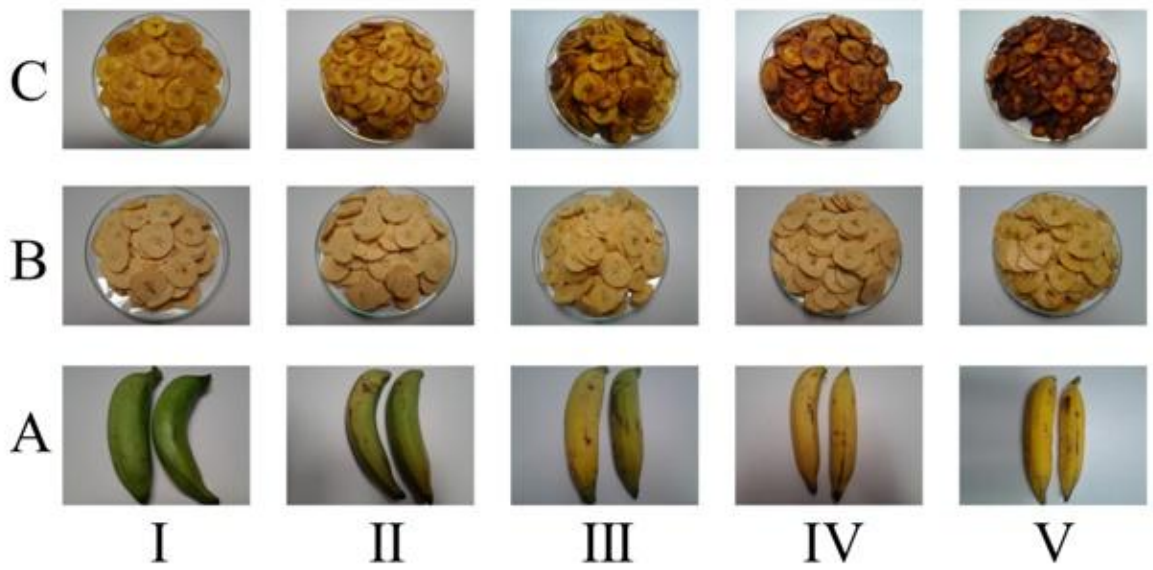

Fig 3.A.1. Different ripening stages of Nendran (I-V) used in the study
A-raw plantain with skin; B- plantain fruit after peeling and C- fried chips

Stable Microsystems, UK, using a ball probe) and colour (*L*, *a* and *b* values using ColourFlex EZ, HunterLab Instruments, Virginia, USA) and sensory analysis in comparison with the commercial samples. The temperature was monitored by the use of a thermometer TTX 110 type T temperature prob (Ebro, Germany). For each stage, chips were prepared in triplicates, using the fruit from different tiers. Freshly prepared chips from each ripening stage were analyzed for the acrylamide content using the procedure given under 3.A.2.9.

3.A.2.4. Chemical compositional analysis

The standard procedures of AOAC [2005] were used for the determination of moisture, ash, crude fat and protein contents of all stages of raw plantain. Triplicate samples of all the five stages of plantain were oven-dried at 100°C transferred to a desiccator, and allowed to cool at room temperature for moisture content. The sample weights were recorded before and after heat treatment in a muffle furnace (550°C for 12 h) for the ash content determination. Micro-Kjeldahl method was used for the protein estimation with

nitrogen to protein conversion factor of 6.25 and fat content was determined using Soxhlet extraction. Total carbohydrate was calculated using the difference method.

3.A.2.5. Determination of reducing sugars by HPLC

The reducing sugars, glucose and fructose of plantain fruit (fresh samples after peeling as mentioned under 3.A.2.2.), were measured using Shimadzu HPLC (Kyoto, Japan) technique using a reversed phase Supelcosil LC-NH_2 column (25cm × 4.6mm, 5µm) equipped with a Refractive Index detector. The standards of glucose and fructose in the concentration range 5-15 mg/ml were used for quantification. The sample extraction was carried out according to Vivanti et al., [2006] with slight modifications. For the sugar analysis, the sample preparation was as follows. 1 g of plantain fruit (after peeling) paste was mixed with 10 ml acetonitrile/water (8.5:1.5 v/v) and stirred for 5-15 min. The suspension was centrifuged at 1700 x g for 10 min, and the supernatant was passed through 0.22 µm syringe filter. The mode of elution used was isocratic with the mobile phase consisting of (85:15 v/v) acetonitrile/water at a flow rate of 1 ml/min.

3.A.2.6. Determination of amino acids by HPLC

Ten amino acids namely asparagine, glutamine, serine, glycine, threonine, valine, isoleucine, leucine, phenylalanine and lysine were detected and quantified by the HPLC method [Georgi et al., 2005]. The amino acid analysis was performed using Shimadzu HPLC system (Kyoto, Japan) containing a binary pump delivery system (LC-20AD), robotic autosampler (SIL-10AP), column thermostat (CTO-20A) and a fluorescence detector (RF-10 AxL). Briefly, the standard amino acids and the samples were automatically derivatized with OPA by programming the robotic autosampler. The derivatized amino acids were detected by the fluorescence detector by an excitation emission wavelength λ = 340/450 nm using Zorbax Eclipse-AAA column, 5 µm, 150 × 4.6 mm (Agilent), at 40°C. Mobile phase A was 40 mM

NaH_2PO4, adjusted to pH 7.8 with NaOH, while mobile phase B was acetonitrile/methanol/water (45/45/10 v/v/v). The separation was obtained at a flow rate of 2 ml/min with a gradient program that allowed 0-1.9 min at 0% B, followed by an 18.1 min step that raised eluent B to 57%, 18.6 to 22.3 min at 100 % B and then equilibration at 0% B was performed in a total analysis time of 30 min.

3.A.2.7. Total Phenolic (TPC) & Flavonoid Content (TFC)

Nendran fruit after peeling, at different ripening stages, was freeze dried (VirTis, Genesis, U.S.A), powdered and extracted with methanol (1:10 w/v) at ambient temperature until the solvent become colorless. The extracts were filtered through Whatman No.1 filter paper and were evaporated to dryness in a rotary evaporator (Buchi, Switzerland) under reduced pressure at 35°C. Finally, the extracts were washed with 10 ml of methanol and stored at 4°C for further analysis.

Folin-Ciocalteau reagent was used for the total phenolic content measurement as described by Singleton & Rossi [1965]. The results were expressed as mg GAE/g dry weight of extract by measuring the absorbance at 760 nm using a multimode reader (Synergy, Biotek, USA). TFC was measured based on the aluminum chloride colorimetric method described by Chang et al., [2002] with quercetin as standard. The absorbance was measured at 415 nm using multimode reader (Synergy, Biotek, USA) and the results were calculated as milligram quercetin equivalents (mg QE/g dry weight of extract).

3.A.2.8. Quantification of polyphenols by HPLC

The freeze dried samples as mentioned under 3.A.2.7. was extracted using methanol (1:10 w/v) at ambient temperature until the solvent become colorless. These methanolic extracts and eleven reference compounds (gallic acid, catechol, chlorogenic acid, syringic acid, p-coumaric acid, ferulic acid, ellagic acid, cinnamic acid, quercetin, kaempferol & apigenin in

1mg/ml concentration) were prepared in methanol and were filtered through 0.45µm PTFE syringe filter. Filtrate (20 µl) was injected into an HPLC system (Shimadzu, Japan) containing two LC 20AD preparative liquid chromatography pump units, a reverse-phase Phenomenex, Luna® C18 column (250×4.6mm i.d.; 5 mm), a column oven (CTO-20 AC VP), a system controller (SCL-20A VP), a Rheodyne injector (USA) with a loop of 20 µl volume and a diode array detector (DAD; SPD-M20A VP) was used for the analysis of polyphenols.

The HPLC analysis was performed according to Arun et al., [2015] with some modifications. Two solvent systems were used as mobile phases for the analysis in which solvent A was a mixture of methanol, acetic acid & water in the ratio 10:2:88 and methanol-acetic acid-water in the ratio 90:2:8 was used as solvent B with the gradient program 0–15 min 15% B, 16–20 min 50% B, 21–35 min 70% B, 36–50 min 100% and finally the column was regenerated in 10 min. 20 µl was used for the HPLC analysis and the flow rate was set at 1ml/min. The phenolic acids were detected at a wavelength of 280 nm. Comparing the retention time of peaks for the standards, the sample peaks were identified for the individual phenolic acids. The compounds were confirmed by spiking with corresponding authentic phenolic standards. Shimadzu CLASS-VP version of 6.14 SP1software is used for the data acquisition and analysis.

3.A.2.9. Analysis of acrylamide by HPLC

The analysis of acrylamide was performed as reported by Shamla and Nisha, [2014]. Finely ground fresh chips samples (4.0 g) were defatted four times using hexane (15 ml) followed by vigorous shaking (5 min) and was filtered. The residue was dried under vacuum and was analysed for acrylamide. Acrylamide was extracted with acetone using ultrasonication (Elma, Germany) at 40°C for about 30 min. It was filtered and the filtrate was evaporated to

dryness and the residue was dissolved and made up to 2 ml with water. The cleanup of the extracts were done by passing through Oasis HLB catridge (Waters Corporation, Milford, Massachusetts USA) and finally through 0.45 µm syringe filter before HPLC analysis. A known amount of standard acrylamide was used as the internal standard.

The HPLC used for the analysis of acrylamide was a Shimadzu HPLC system (Japan) consisting of two LC-8A chromatography pump units, column oven (CTO-10AC VP), system controller (SCL-10A VP), rheodyne injector (USA) with a loop of 20 µl volume, diode array detector (DAD; SPD-M10A VP). The HPLC column used was Atlantis ® dC 18 (4.6X 250 mm i.d.; 5µm). An isocratic elution pattern was adopted for the separation of the analyte, and 100% Milli-Q water was used as the mobile phase. The column temperature was set at 25°C; the flow rate was maintained at 0.5 ml/min while the detection was performed at 206 nm. The injection volume was 20 µL and the retention time for AA was 6.8 min.

3.A.3. Statistical analysis

Statistical analysis of all data was performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL). Duncan's post-hoc comparison of means was carried out to determine significant differences for proximate compositional analysis along with precursor compositions which include reducing sugars, amino acids, total phenolic & flavonoid content determinations for the respective ripening stages of Nendran. The term significant is used to indicate differences for which $p<0.05$. The correlations between reducing sugars, amino acids, total phenolic & flavonoid content in all the five stages of Nendran variety were studied using Pearson's correlation coefficient. Three independent analysis were performed for each stage wise analysis of acrylamide. Values are expressed as mean± standard deviation of minimum three experiments.

3.A.4. Results and Discussion

3.A.4.1. Proximate compositional analysis of plantain at different stages of ripening

Table 3.A.1. summarizes the proximate composition of plantain at five different ripening stages. The statistical significance of each value was noted by Duncan's test. The moisture content was increased during the process of ripening, from stage one (55.20 g/100 g) to stage five (63.95 g/100 g). Stover and Simmonds [1987] reported that during fruit ripening the sugar concentration increases in the pulp than in the peel thus promoting a difference in osmotic pressure. As a result of transpiration to the atmosphere and osmosis to the pulp, the peel loses water causing an increase in the fresh weight of the pulp as the fruit ripens. A significant increase in protein, ash, and fat content were observed during different ripening stages of plantain. Baiyeri et al., [2011] reported higher ash content in ripe plantain which was due to the release of mineral elements as a result of the tissue breakdown during ripening. The carbohydrate content decreases while the sugar content increases from stage I to V of plantain. This is because as the plantain ripens, the starch content decreases due to the conversion of starch to sugar.

3.A.4.2. Reducing sugars

Table 3.A.2. summarizes the concentrations of glucose and fructose in the Nendran variety of plantain at five different stages of ripening. The HPLC profiling of reducing sugars (glucose and fructose) in all the five ripening stages of plantain is given in Fig 3.A.2. Results from our study indicated that both glucose and fructose content increased during ripening, as expected. The glucose and fructose content increased from 4.83 to 15.15% and 4.76 to 15.70%, respectively, from stages I-V. As a result of fruit ripening associated with plantains, the cell wall gets ruptured, and the fruit becomes very soft, resulting in an increase in starch breakdown and a simultaneous increase in the concentration of soluble sugars. The liberation

of these soluble sugars as on ripening may be attributed to the activities of various enzymes such as starch phosphorylase and amylase [Mohan et al., 2014].

Table 3.A.1. Proximate composition (% of nutrient) in the pulp of raw plantains (Nendran) from stages I-V

Stages	**I**	**II**	**III**	**IV**	**V**
Moisture	55.20±0.30[a]	55.87±0.03[b]	57.40±0.04[c]	60.76±0.02[d]	63.95±0.04[e]
Total ash	3.47±0.03[a]	4.86±0.02[b]	5.22±0.00[c]	5.85±0.02[d]	6.18±0.08[e]
Crude fat	0.24±0.05[a]	0.45±0.04[b]	0.68±0.01[c]	0.86±0.01[d]	1.20±0.03[e]
Crude protein	2.73±0.02[a]	2.89±0.05[b]	2.90±0.04[b]	2.95±0.01[b]	3.08±0.02[c]
Carbohydrate	55.23±0.09[a]	43.98±0.07[b]	35.58±0.03[c]	32.48±0.02[d]	28.95±0.05[e]

*Values are means ± SE (*n = *3). Means values in the same row followed by a different superscript letter are significantly (*$p \leq 0.05$*) different*

3.A.4.3. Amino acids

The amino acid concentrations found in all the five ripening stages of plantain are listed in Table 3.A.2. Asparagine is the major amino acid which is reported to influence the formation of acrylamide. The asparagine content ranged from 1.80, 2.98, 1.37, 1.42 & 1.89 mg/g respectively for the ripening stages of I to V. The amino acid glutamine increased up to stage III and then decreased. The amino acids serine, isoleucine, leucine & phenylalanine exhibited an increase up to stage four and then decreased. The glycine content did not show any significant differences in the concentration during ripening. Threonine increased from stage one to three and then decreased. There is a slight increase found for the amino acid valine from stage one to two and then decreased. Ketiku [1973] reported that during fruit ripening, the concentration of lysine, methionine, histidine, proline, phenylalanine was

Table 3.A.2. Reducing sugars (g/100gm), free amino acids (mg/g) and polyphenol (mg /g) (fresh wt.)contents of Nendran fruit at five different maturity stages (n=3)

Precursors	Stage I	Stage II	Stage III	Stage IV	Stage V
Reducing Sugars (g/100gm)					
Glucose	4.83±0.28^{a}	6.24±0.10^{b}	8.42±0.08^{c}	9.41±0.06^{d}	15.15± 0.15^{e}
Fructose	4.76±0.05^{a}	6.28±0.10^{b}	8.65±0.09^{c}	9.75±0.17^{d}	15.70±0.07^{e}
Amino acids (mg/g)					
Asparagine	1.80±0.05^{a}	2.96±0.02^{b}	1.37±0.04^{c}	1.45±0.09^{c}	1.89±0.04^{d}
Glutamine	0.15±0.07^{a}	0.50±0.03^{b}	0.59±0.02^{c}	0.43±0.04^{d}	0.06±0.05^{a}
Serine	1.34±0.09^{a}	1.37±0.02^{a}	1.78±0.03^{b}	1.78±0.03^{b}	1.23±0.03^{c}
Glycine	2.00±0.08^{a}	2.01±0.02^{a}	2.00±0.02^{a}	2.00±0.03^{a}	2.00±0.03^{a}
Threonine	1.64±0.05^{a}	2.09±0.02^{b}	2.91±0.01^{c}	2.70±0.02^{d}	2.59±0.02^{e}
Valine	1.27±0.01^{a}	1.36±0.03^{b}	1.29±0.01^{c}	1.31±0.04^{c}	1.28±0.01^{a}
Isoleucine	2.38±0.01^{a}	2.69±0.01^{b}	2.95±0.03^{c}	3.04±0.02^{d}	2.57±0.08^{e}
Leucine	1.25±0.02^{a}	1.30±0.02^{b}	1.36±0.05^{b}	1.47±0.02^{b}	1.40±0.09^{b}
Phenylalanine	1.10±0.02^{a}	0.93±002^{b}	1.46±0.07^{c}	1.62±0.03^{d}	0.76±0.01^{e}
Lysine	0.60±0.02^{a}	0.70±0.01^{b}	0.52±0.09^{c}	0.55±0.01^{c}	0.42±0.04^{d}
Phenolic acids (mg/g)					
Gallic acid	11.26±0.509^{a}	4.08±0.170^{b}	1.23±0.448^{c}	0.21±0.056^{d}	0.13±0.063^{e}
Chlorogenic acid	12.20±0.777^{a}	10.82±0.462^{b}	0.38±0.10^{c}	1.33±0.285^{d}	0.80±0.063^{e}
Syringic acid	3.61±0.213^{a}	1.19±0.190^{b}	0.37±0.171^{c}	0.036±0.008^{d}	-
p-Coumaric acid	3.01±0.859^{a}	0.41±0.063^{b}	0.12±0.037^{c}	0.038±0.009^{d}	0.028±0.010^{e}
Quercetin	9.38±0.438^{a}	7.45±0.478^{b}	1.85±0.581^{c}	0.210±0.119^{d}	0.086±0.026^{e}

*Values are mean ± SE (*n = *3). Mean values in the same row followed by a different superscript letter are significantly different (*$p \leq 0.05$*)*

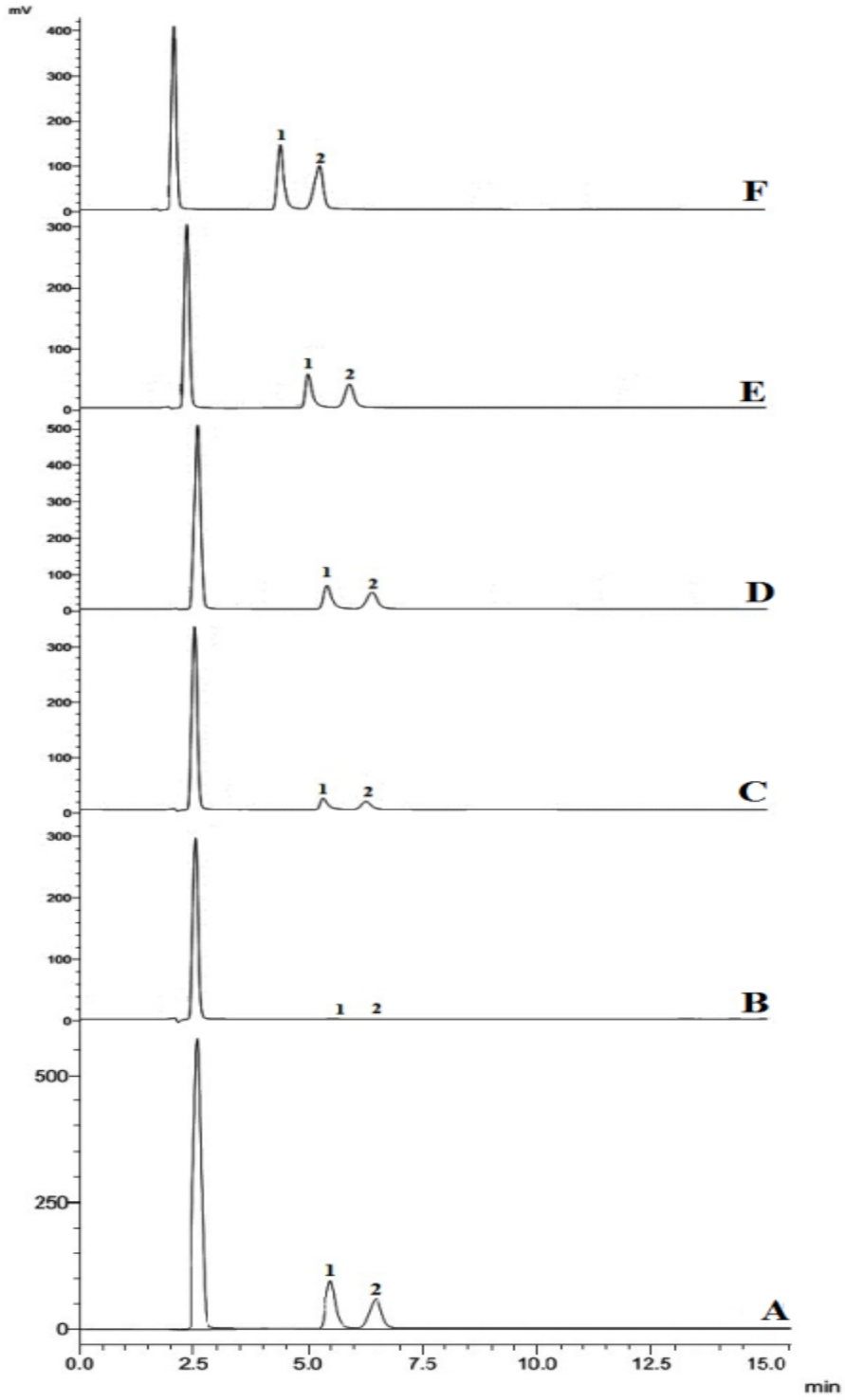

Fig 3.A.2. HPLC profiling of sugars (1 - fructose & 2 - glucose) in all the five ripening stages of Nendran (A represents profiling of standards and B-F represents five different ripening stages from I to V)

increased. Khawas et al., [2014] reported that there is a decline in the essential amino acid content as on increasing fruit maturity of culinary banana (Musa ABB). However, in the present study, the change in amino acids during ripening did not follow a pattern, as reported earlier.

3.A.4.4. TPC & TFC contents in five different ripening stages of Nendran

Many factors such as variety, cultivation, species, area, ripeness, harvesting time, climatic conditions, storage time and environment directly contribute to the phenolic content of fruits. Among these, the environmental factors which include agronomic (biological culture, greenhouses or fields, hydroponic culture, fruit yield per tree, etc.) or climatic (sun exposure, soil type, rainfall) have a significant role in the total phenolic content. However, the important factor that affects the concentration of polyphenols is the fruit maturity [Iqbal and Bhanger, 2006]. In the present study, it was noted that the total phenolic content decreased significantly with increase in the ripeness of the plantain (Fig 3.A.3.). The total phenolic content showed a decline from 275.34 to 31.92 mg GAE/g dry weight of extract from stage I to V of plantain with a statistical significance of $p \leq 0.05$. The increase of polyphenol oxidase activity together with the increase in the polymerization of leucoanthocyanidins and the hydrolysis of astringent arabinose ester of hexahydrodiphenic acid contributes to the loss of astringency may be attributed to the decrease in the total phenolic content upon fruit ripening [Parr and Bolwell, 2000]. The TFC exhibited a similar decline upon fruit ripening. The flavonoid content in the unripe stage is significantly higher (Fig 3.A.3.) when compared to the ripened stage of plantain (7.505 to 0.573 mg QE/g dry weight of extract from stage I to V, respectively). This may be because of the fact that during ripening, different phenolic compounds condenses forming complex phenolic compounds such as tannins and lignin, etc,

which cannot be determined by the present analytical method employed [Ben-ahmed et al., 2009].

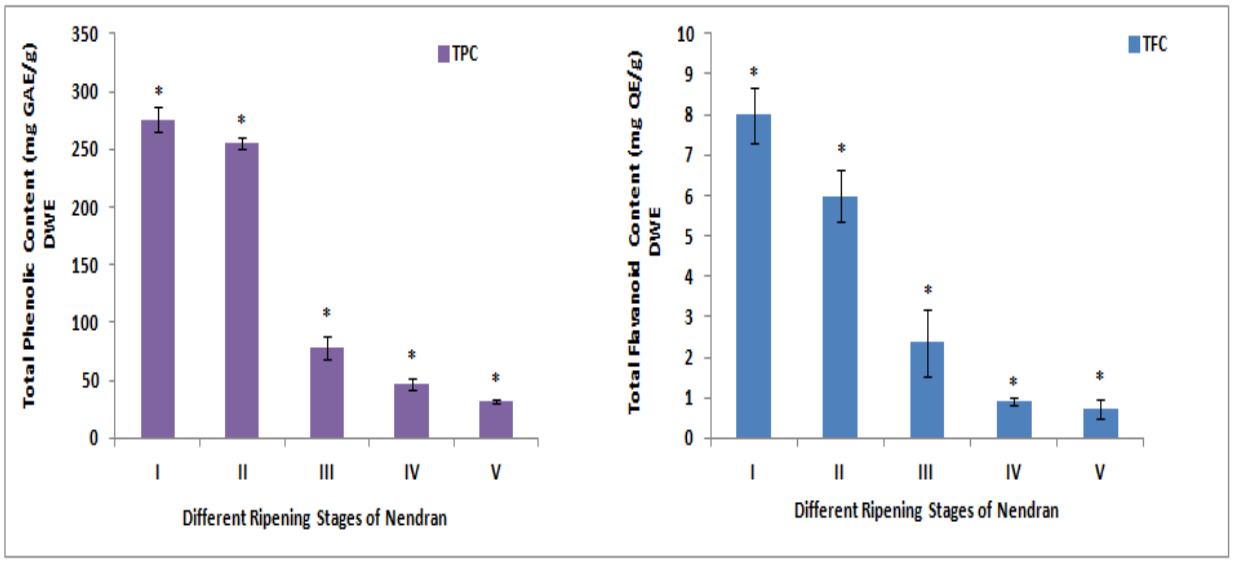

Fig 3.A.3. TPC& TFC contents of Nendran pulp extract as on increasing ripening (* indicates the values are significantly different)

3.A.4.5. HPLC quantification of polyphenols in different stages of Nendran ripening

The individual phenolic compounds in each stage were quantified by HPLC (Fig 3.A.4.). Gallic acid & chlorogenic acid was present in higher amounts in Nendran variety of plantain. As can be seen, the concentration of the phenolic compounds decreased significantly ($p \leq 0.05$) upon fruit ripening (Table 3.A.2.), which is in accordance with the increase in acrylamide content.

From the above studies, it was found that there was a change in the concentration of the precursors of acrylamide formation viz., reducing sugars, amino acid and asparagine, during the ripening of Nendran. Phenolic compounds are found to decrease during the ripening process. In order to understand how the chemical changes during the ripening influence the formation of acrylamide when exposed to high-temperature processing like deep frying,

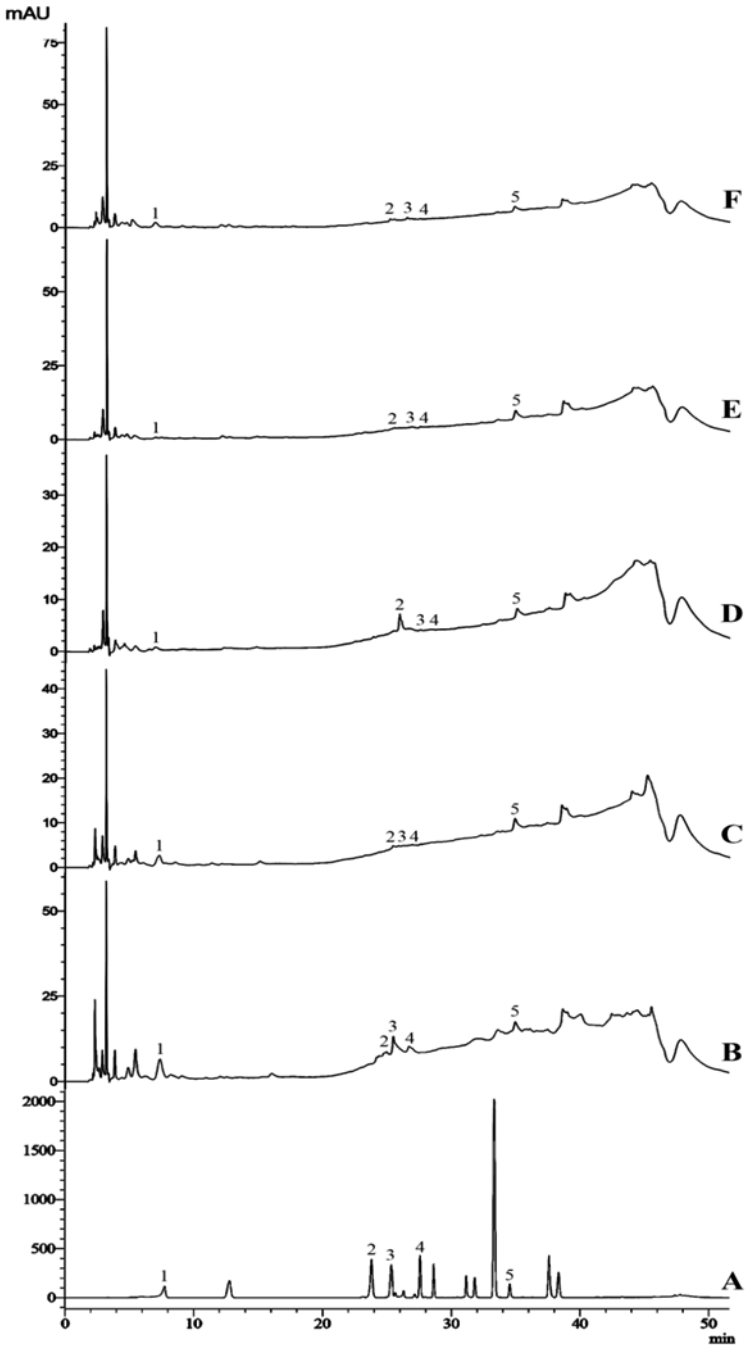

Fig 3.A.4. HPLC chromatogram of (A) authentic standards (1) gallic acid, (2) chlorogenic acid (3) syringic acid (4) p-coumaric acid and (5) quercetin; (B) stage I; (C) stage II; (D) stage III; (E) stage IV and (F) stage V of plantain (Nendran variety)

deep fried chips were prepared from plantain stages I-V, as a model. The occurrence of acrylamide in the chips was estimated and correlated with the precursors as well as the phenolic content, to understand the effect of ripening on the formation of acrylamide.

3.A.4.6. Estimation of Acrylamide

Deep fried chips were prepared from plantain at different stages of ripening and the formation of acrylamide during the deep frying process was estimated using HPLC. It was interesting to note that the acrylamide content in the chips were increased with ripening. The bar graph showing acrylamide concentrations in plantain chips from stage I to stage V of ripening is given in Fig 3.A.5. and the values are 49.8 ± 2.4, 185.6 ± 3.5, 240.2 ± 3.6, 315.6 ± 24.1 and 2062.0 ± 26.9 µg/kg respectively. The acrylamide concentrations were significantly different ($p \leq 0.05$) for each ripening stages of Nendran variety of plantain. There are very few reports on the occurrence of acrylamide in banana/plantain products. Acrylamide levels in banana fritters made from over ripe banana are reported to range between 268-3585 µg/kg [Daniali et al., 2010]. Another study by Bassama et al., [2011] reported the acrylamide levels up to 900 µg/kg in plantain-based products. The acrylamide content in commercial samples of chips made from green and ripe banana, collected from Caribbean market, was reported to be 100 - 430 and 180 µg/kg [Bent at al., 2012]. Mulla et al., [2016] reported a higher level of acrylamide content in chips made from ripe banana, which is correlated to its higher reducing sugar content. They also reported that the formation of acrylamide in plantain is in the same magnitude as that of potato, rye and wheat products. The results from the present study are in accordance with our previous study on market samples where the occurrence of acrylamide in deep fried chips prepared from raw and ripe plantain was found to range from 14.73-1690.48 and 24.81-1959.80 µg/kg respectively [Shamla and Nisha, 2014].

The results from the study indicated that the acrylamide content in the product was increased with increase in ripening. As discussed earlier, the increase in the level of acrylamide in the chips with the increase in the ripening of plantain may be correlated to the change in the precursors. Further to confirm this, statistical correlation were carried out to find out the correlation between the levels of reducing sugars, amino acids, and phenolic compounds.

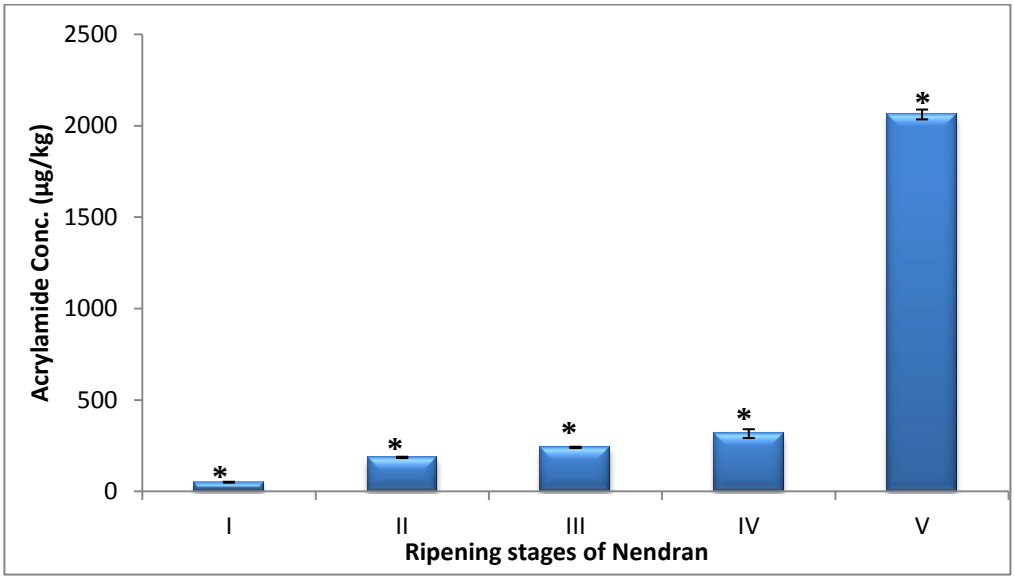

Fig 3.A.5. Bar graph showing acrylamide concentrations for five different ripening stages(I-V) of plantain (Nendran) [* represents the values are significantly different]

3.A.5. Correlating acrylamide formation with reducing sugars, amino acids, total phenolic & flavonoid content in different ripening stages of plantain

The correlation between the acrylamide content in the final products with reducing sugars, amino acids and the five major phenolics in the five ripening stages of plantain (Nendran) were examined using the Pearson correlation (r), and the results are shown in Table 3.A.3. The correlation between reducing sugar contents (glucose and fructose) and acrylamide levels in the Nendran variety yielded high positive r values of 0.95 and 0.94 respectively for

glucose and fructose. Positive correlation was reported earlier between the formation of acrylamide and reducing sugars in banana fritters made from Abu & Awak varieties of banana [Daniali et al., 2013]. Reducing sugars are also correlated with acrylamide formation in chips [Mulla et al., 2016]. The results from the present study indicated that the increase in acrylamide content correlates positively with the reducing sugar content as on increasing fruit ripening. The reducing sugars glucose and fructose are important for acrylamide formation via Maillard reaction pathway, and glucose was found to generate more acrylamide than fructose [Robert et al., 2004]. Correlation between the precursors and acrylamide formation has been reported for potato and potato products. The highly reactive aldehyde group in glucose is reported to be responsible for the greater acrylamide formation compared to ketohexose group in fructose. In contrary to this, Rydberg et al., [2005] reported that fructose plays a major role in the formation of acrylamide in potato system. Pollien et al., [2003] reported in a study that, fructose generate more acrylamide than glucose when heated with asparagine at 150ºC, in a model system. It is reported that glucose and fructose concentrations in the tubers significantly correlated with acrylamide formation in the products [Wicklund et al., 2006]. Becalski et al., [2004] reported that the acrylamide formation in French fries can be effectively controlled by the use of potatoes with low levels of sugar.

Table 3.A.3. Pearson correlation between acrylamide formation and precursor concentrations in Nendran variety of all the five stages

Precursors	Pearson correlation (r)	P-value	R^2
Reducing Sugars			
Glucose	0.947	0.015	0.78
Fructose	0.936	0.019	0.87
Aminoacids			
Asparagine	-0.034	0.957	0.00
Glutamine	-0.560	0.326	0.31
Serine	-0.492	0.392	0.24
Glycine	-0.030	0.960	0.00
Threonine	0.320	0.599	0.10
Valine	-0.257	0.675	0.06
Isoleucine	-0.210	0.735	0.04
Leucine	0.342	0.573	0.11
Phenylalanine	-0.572	0.313	0.33
Lysine	-0.728	0.163	0.53
Polyphenols			
Gallic acid	-0.490	0.402	0.24
Chlorogenic acid	-0.594	0.290	0.35
Syringic acid	-0.974	0.005	0.24
p-Coumaric acid	-0.400	0.504	0.16
Quercetin	-0.500	0.316	0.33

It was found that the asparagine which is reported to be one of the major precursors for acrylamide formation demonstrated a weaker correlation with acrylamide formation. All the other amino acids except, threonine and leucine, exhibited a negative correlation with the formation of acrylamide. However, the correlation coefficient 'r' for threonine and leucine were, much below the acceptable range. State of maturity and storage conditions influences sugar levels which directly affects the formation of acrylamide in fried potato products [Abong et al., 2009]. Friedman and Levin [2008] reported that the concentration of asparagine does not have a significant effect on acrylamide formation in potatoes. Yoshida et al., [2005] reported that among reducing sugars and asparagine, reducing sugars in potato tubers play the limiting factors for acrylamide formation in potato chips. As reported in the case of potatoes by various researchers, it was observed from the present study that the changes of precursors during ripening of plantain influence the formation of acrylamide during the deep frying process for making chips and thus the formation of acrylamide can be controlled by selecting proper stage of maturity.

Previous studies suggest that phenolic compounds can influence the acrylamide formation [Zhu et al., 2009; Morales et al., 2014]. Considering the influence of polyphenols on acrylamide formation, the TPC & TFC contents were evaluated for all the ripening stages of Nendran. It was found that the acrylamide formation correlated negatively with TPC and TFC with r values of -0.585 & -0.575 respectively. Five major phenolic compounds in plantain viz gallic acid, chlorogenic acid, syringic acid, p-coumaric acid & quercetin quantified at stages I-V were also correlated with acrylamide content in the chips prepared. It was found that all the five compounds exhibited a negative correlation with the acrylamide content in chips indicating that the decrease in the phenolic content during ripening may be one of the reasons for promoting the formation of acrylamide in chips during frying.

Earlier reports suggest that plant polyphenols comprise a large group of compounds having different structures having a greater influence on the formation of acrylamide on heating [Zhu, et al., 2009]. Polyphenols act as free radical scavengers thereby inhibiting the chain reaction by the combination of the hydrogen atom of phenolic –OH with free radicals. Besides the antioxidant capacity of polyphenols, some other mechanistic pathway may be influencing the formation of acrylamide along with structure, concentration & reaction conditions which have not been given a correct explanation so far. In a study by Constantinou and Koutsidi [2016], it is reported that, in Maillard reaction systems, phenolic antioxidants may react with sugar fragments and/or reactive carbonyl compounds, forming adducts through electrophilic aromatic substitution reactions and inhibits acrylamide formation. The number and position of phenol hydroxyls of flavonoid compounds play an important role in controlling the formation of acrylamide. Furthermore, corresponding parameters, including the number, positions and spatial steric effects of functional hydroxyls, and the related mode-of-actions remain largely uncertain [Zhang et al., 2016]. In general, the more phenolic hydroxyls are present in the chemical structures of flavanols and derivatives, the greater the change in the antioxidant attributes of the Maillard reaction products, no matter whether the formation of acrylamide is reduced or promoted. The flavanols and derivatives may contribute their active phenolic hydroxyl groups to the Maillard reaction, thus affecting the final antioxidant activity of the MRP [Huang et al., 2017].

The study, therefore, documents the chemical changes during ripening of plantain and also highlight how these changes in reducing sugars, amino acids, polyphenol & flavonoid content during ripening affect the formation of acrylamide during the preparation of deep-fried chips. The changes in the concentration of phenolic compounds during ripening and its

effect on acrylamide formation have not been reported earlier, as per the knowledge. Thus our study recommends stages I and II, which possess less reducing sugars and higher phenolic content, for making chips with lower acrylamide content.

3.A.6. Summary

Formation of acrylamide during deep frying of plantain showed a positive correlation with respect to reducing sugars, whereas a poor correlation were observed with amino acids. Five major phenolic compounds namely gallic acid, chlorogenic acid, syringic acid, p-coumaric acid & quercetin were quantified in all the stages of plantain ripening and found to decrease during ripening. All these five compounds also exhibited a negative correlation with acrylamide formation in chips indicating that higher phenolic content in raw plantain along with reduced levels of reducing sugars in the initial ripening stages minimized the formation of acrylamide in deep fried plantain chips. Higher the phenolic content, more the antioxidant activity which would act as free radical scavengers thereby inhibits the chain reaction minimizing acrylamide formation. Thus by selecting the proper ripening stage of raw material with lesser sugar content and higher phenolic content, the formation of acrylamide in the final product can be reduced to a reasonable level. The study also warrants the importance of similar studies that could help to formulate strategies and guidelines for reducing acrylamide in a variety of high temperature processed food products that can bring down the exposure of this toxicant to the population.

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CHAPTER 3 –Part B

Evaluation of reducing sugars, amino acids & polyphenolics in different maturity stages of jackfruit and its influence on acrylamide formation in deep fried chips

3.B.1. Introduction

Jackfruit (*Artocarpus Heterophyllus L.*) is a large sized tree belonging to the family Moraceae bearing the largest fruit among edible fruits [Baliga et al., 2011]. The jackfruit is indigenous to India and is widely grown in Bengladesh, Burma, Sri Lanka, Malaysia, Indonesia, Philippines, Brazil and other countries [Narasimham, 1990; Othman, & Subhadrabandhu, 1995].

Jackfruit is gaining lots of research attention in the recent times for its nutritional and health benefits [Swami et al., 2012]. Ruiz-Montanez et al., [2015] reported that jackfruit is a rich source of phenolic compounds and phytochemicals that can be used for preparing value added products, nutraceutical and health benefits for enhancing human health. Swami et al., [2012] reported that jackfruits are good sources of essential nutrients. It has been reported by Jagtap and Bapat, [2010] that jackfruits are widely used as a traditional folk medicine in South-East Asian parts, Indonesia, western part of Java and India, researches in this area suggest that jackfruit possess many biological activities, including anti-bacterial, anti-diabetic, anti-inflammatory, antioxidant and anti-helmintics activities [Shanmugapriya et al., 2011; Biworo et al., 2015]. Polysaccharides present in jackfruit have received much attention because of their wide potential biological activities which include anti-tumor [Jin, 2012], immune-regulation [Wang et al., 2015], antioxidant activity [Samavati & Manoochehrizade, 2013], hypoglycemic effect [Zhu et al., 2013] and antibacterial effect [Nie et al., 2013]. Fernando et al., [1991] reported that jackfruit is rich in potassium and vitamin B6 which helps in lowering the blood pressure and decreases homocysteine levels in blood reducing the risk of heart disease respectively. Jackfruit is reported to reduce stomach cancer, increasing digestion, preventing bone related disorders, maintaining proper blood circulation & also in the production & distribution of thyroid gland [Swami et al., 2012].

Jackfruit can be consumed as vegetable and fruit depending upon the maturity. Tender and unripe jackfruit is widely consumed as vegetable where as the ripened fruit is enjoyed as a delicacy and hence it is known as “poor man's” fruit in eastern & southern parts of India [Rahman et al., 1995]. However, there are no reports in the chemical composition of jackfruit during various maturity stages. Variety of products are made from jackfruit depending on the maturity and jackfruit chips are the most popular and commonly consumed snack food items in the Asian countries [Swami et al., 2012]. Deep fried chips are made out of unripe matured and ripe jackfruit. These snacks are found to contain reasonable levels of acrylamide, ranged from 46.2 to 2431.4 µg/kg [Shamla and Nisha, 2014]. Acrylamide, a heat induced food toxicant, is formed in foods when subjected to high temperaure processing. The formation pathway of acrylamide is directly linked to Maillard reaction between the reducing sugars (glucose and fructose) and aminoacid asparagine at temperatures above 120ºC [Pedreschi et al., 2006]. The modification of food processing parameters which could limit the formation of acrylamide, is one of the strategy adopted for the mitigation of acrylamide in foods. Another strategy is by selection of better raw materials which have low contents of the precursors of acrylamide formation namely, asparagine and reducing sugars [FDE, 2014]. Previous study by our group on plantain chips indicated that selection of proper maturity can limit the formation of acrylamide in the same [Shamla and Nisha, 2017]. Thus considering mitigation as a matter of concern and to evaluate the chemical composition of the jackfruit at different maturity, we tried to elucidate the proximate composition, sugar and amino acid profiling and phenolic composition analysis of jackfruit at different stages of maturity. The precursors were further correlated with formation of acrylamide in deep fried jack chips.

3.B.2. Materials and methods

3.B.2.1. Reagents and chemicals

Standard acrylamide (>99%), sucrose, D-(+) glucose, D-(-) fructose, sodium phosphate, sodium carbonate, aluminium chloride hexahydrate, potassium acetate, sodium hydroxide, gallic acid, quercetin, aluminium chloride, Folin-Ciocalteau reagent, amino acid standards and o-phthalaldehyde (OPA) were purchased from Sigma-Aldrich (St. Louis MO, USA). HPLC water was purified on a Milli-Q system (Millipore India Pvt Ltd, Bangalore, India). Solvents used were methanol, acetic acid & acetonitrile of high-performance liquid chromatography (HPLC) grade. Oasis HLB (30 mg, 1 ml) solid phase extraction (SPE) cartridges were obtained from Waters Corp. (Milliford, Massachusetts USA). Minigen syringe filters (0.22μm diameter) were obtained from Genetix Biotech Asia Pvt. Ltd, New Delhi, India.

3.B.2.2. Collection of samples

Jackfruits (*Artocarpus heterophyllus), soft variety (koozha)* were collected from NIIST campus (Trivandrum). It takes normally 20 days for the flowers to undergo the process of anthesis and become 6 inch fruit. Usually, 95 days are required for the development of the 6 inch fruit to the fully matured fruit ready to get harvested. Four individual stages of fruits were identified and selected based on the fruit maturity cycle, i.e., 45, 65, 75 and 95 days from the day once the fruit is set. All the four stages were taken from the same plant and are harvested on their maturity basis. Each fruit was opened, bulbs were separated from the rind, the seeds were removed. A portion of the bulbs were pulped for proximate composition, reducing sugars, amino acids, and phenolic compounds analysis and the remaining bulbs were used for preparing jack chips.

3.B.2.3. Chemical compositional analysis

The standard procedures of AOAC [2005] were used for the determination of moisture, ash, crude fat and protein contents of all stages of jackfruit. Triplicate samples of all four stages of jackfruit were oven-dried at 100°C transferred to a desicator, and allowed to cool at room temperature. The sample weights were recorded on a digital balance (Mettler Toledo) before and after heat treatment in a muffle furnace (550°C for 12 h) for the ash content determination. Micro-Kjeldahl method was used for the protein estimation with nitrogen to protein conversion factor of 6.25 and fat content was determined using Soxhlet extraction. Total carbohydrate was calculated using the difference method. Total carotenoids (TC), fibre content & titrable acidity was detected by the method described by Ranganna [1979].

3.B.2.4. Determination of sugars by HPLC

Glucose, fructose and sucrose in different maturity stages of jackfruit were measured using HPLC (Shimadzu, Kyoto, Japan) technique using a reversed phase Supelcosil LC-NH_2 column (25cm × 4.6mm, 5µm) equipped with a refractive index detector. The standards of glucose, fructose and sucrose in the concentration range 5-15 mg/ml were used for quantification. The sample extraction was carried out according to the method adopted by Vivanti, Firotti, and Friedman [2006] with slight modifications. For the sugar analysis the sample preparation was as follows. Jackfruit pulp (1 g) was mixed with 10ml acetonitrile/water (8.5:1.5 v/v) and stirred for 5-15 min. The suspension was centrifuged at 1700 x g for 10 min, and the supernatant was passed through 0.22 µm syringe filter. The mode of elution used was isocratic with the mobile phase consisting of (85:15 v/v) acetonitrile/water at a flow rate of 1 ml/min.

3.B.2.5. Determination of amino acids by HPLC

Ten amino acids namely asparagine, glutamine, serine, glycine, threonine, valine, isoleucine, leucine, phenylalanine and lysine were detected and quantified by the HPLC method [Georgi et al., 2005]. The amino acid analysis was performed using Shimadzu HPLC system (Kyoto, Japan) containing a binary pump delivery system (LC-20AD), robotic autosampler (SIL-10AP), column thermostat (CTO-20A) and a fluorescence detector (RF-10 AxL). Briefly, the standards and samples were automatically derivatized with OPA by programming the robotic autosampler. The derivatized amino acids were detected by a fluorescence detector by an excitation emission wavelength λ = 340/450 nm using Zorbax Eclipse-AAA column, 5 µm, 150 × 4.6 mm (Agilent), at 40°C. Mobile phase A was 40 mM NaH_2PO_4, adjusted to pH 7.8 with NaOH while a mixture of acetonitrile,methanol & water in the ratio 45:45:10 (v/v/v) was taken as mobile phase B. The separation was obtained at a flow rate of 2 ml/min with a gradient program that allowed 0-1.9 min at 0% B, followed by an 18.1 min step that raised eluent B to 57%, 18.6 to 22.3 min at 100 % B and then equilibration at 0% B was performed in a total analysis time of 30 min.

3.B.2.6. Total Phenolic (TPC) & Flavonoid Content (TFC)

The jackfruit pulp at four different maturity stages was freeze dried (VirTis, Genesis, U.S.A), powdered and extracted with methanol (1:10 w/v) at ambient temperature until the solvent become colorless. The extracts were filtered through Whatman No.1 filter paper and were evaporated to dryness in a rotary evaporator (Buchi, Switzerland) under reduced pressure at 35°C. Finally, the extracts were washed with 10 ml of methanol and stored at 4°C for further analysis.

The total phenolic content was measured using Folin-Ciocalteau reagent as described by Singleton, and Rossi [1965]. The results were expressed as mg GAE/g dry weight of extract

by measuring the absorbance at 760 nm using a multiplate reader (Synergy, Biotek, USA). TFC was measured based on the aluminium chloride colorimetric method described by Chang et al., 2002] with quercetin as standard. The absorbance was measured at 415 nm using multimode reader (Synergy, Biotek, USA) and the results were calculated as milligram quercetin equivalents (mg QE/g dry weight of extract).

3.B.2.7. Quantification of polyphenols by HPLC

The freeze dried samples were extracted using methanol (1:10 w/v) at ambient temperature until the solvent become colorless. These methanolic extracts and eleven reference compounds (gallic acid, catechol, chlorogenic acid, syringic acid, p-coumaric acid, ferulic acid, ellagic acid, cinnamic acid, quercetin, kaempferol & apigenin in 1mg/ml concentration) were prepared in methanol and were filtered through 0.45μm PTFE syringe filter. Filtrate (20 μl) was injected into an HPLC system (Shimadzu, Japan) containing two LC 20AD preparative liquid chromatography pump units, a reverse-phase Phenomenex, Luna® C18 column (250×4.6mm i.d.; 5 mm), a column oven (CTO-20 AC VP), a system controller (SCL-20A VP), a Rheodyne injector (USA) with a loop of 20 μl volume and a diode array detector (DAD; SPD-M20A VP) was used for the analysis of polyphenols.

The HPLC analysis was performed according to Arun et al. [2015] with some modifications. Two solvent systems were used as mobile phases for the analysis in which solvent A was a mixture of methanol, acetic acid & water in the ratio 10:2:88 and methanol-acetic acid-water in the ratio 90:2:8 was used as solvent B with the gradient program 0–15 min 15% B, 16–20 min 50% B, 21–35 min 70% B, 36–50 min 100% and finally the column was regenerated in 10 min. 20 μl was used for the HPLC analysis and the flow rate was set at 1ml/min. The phenolic acids were detected at a wavelength of 280 nm. Comparing the retention time of peaks for each of the standards, the sample peaks were identified for the individual phenolic

acids. The compounds were confirmed by spiking with corresponding authentic phenolic standards. Shimadzu CLASS-VP version of 6.14 SP1software is used for the data acquisition and analysis.

3.B.2.8. Preparation of jack chips

The bulbs were manually sliced to obtain longitudinal pieces (approx 1 cm × 5 cm). The sliced bulbs were fried using a stainless steel electrical deep fat fryer (NOVA, Flomatic Industries PTE Ltd, Singapore) with 3 L of coconut oil. 500 gm of jackfruit slices were fried at a temperature of 165ºC for about 7 minutes (The frying conditions were optimized prior to the experiments in comparison with market samples and based on sensory characteristics, as discussed in Part A). The temperature and time of frying adopted for the study was optimized earlier in terms of texture (by rupture test of banana chips using texture analyzer, TA- HDi, Stable Microsystems, UK, using a ball probe) and colour (*L*, *a* and *b* values using ColourFlex EZ, Hunter Lab Instruments, Virginia, USA) and sensory analysis in comparison with the commercial samples. The temperature was observed by the use of a thermometer TTX 110 type T temperature prob (Ebro, Germany). The jack chips were air cooled for 5 min and was stored under vacuum for extraction & quantification of acrylamide. For each stage, chips were prepared in triplicates, using the bulbs from four maturity stages. The sliced jackfruit and the deep fried chips prepared from four different ripening stages were given in fig 3.B.1. Freshly prepared chips from each maturity stage were analyzed for the acrylamide content using the procedure given under 3.B.2.9.

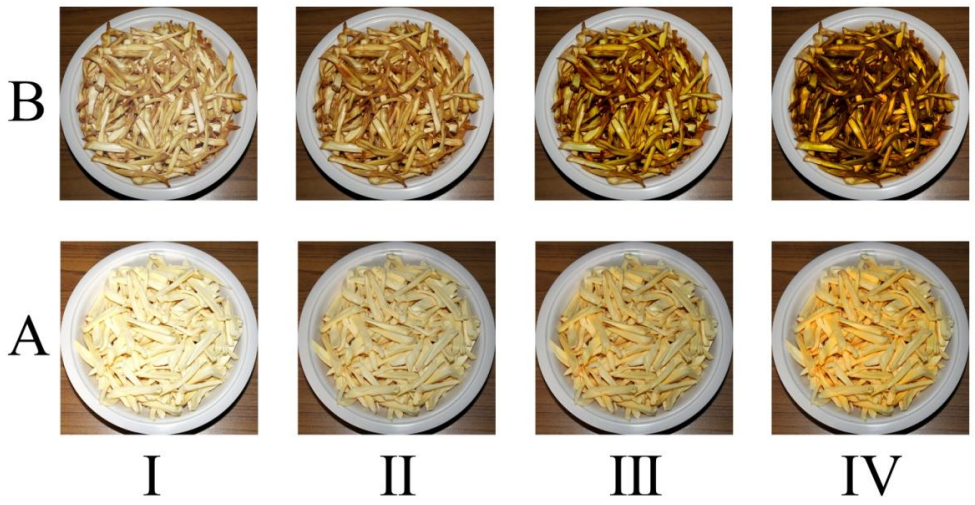

Fig.3.B.1. Different ripening stages of jackfruit (I-IV) used in the study A-sliced jackfruit & B- deep fried jack chips

3.B.2.9. Analysis of acrylamide by HPLC

The analysis of acrylamide was performed as reported by Shamla and Nisha, [2014]. Finely ground fresh chips samples (4.0 g) were defatted four times using hexane (15 ml) followed by vigorous shaking (5 min) and was filtered. The residue was dried under vacuum and was analysed for acrylamide. Acrylamide was extracted with acetone using ultrasonication (Elma, Germany) at 40°C for about 30 min. It was filtered and the filtrate was evaporated to dryness and the residue was dissolved and made up to 2 ml with water. The cleanup of the extracts were done by passing through Oasis HLB catridge (Waters Corporation, Milford, Massachusetts USA) and finally through 0.45 µm syringe filter before HPLC analysis. A known amount of standard acrylamide was used as the internal standard.

The HPLC used for the analysis of acrylamide was a Shimadzu HPLC system (Japan) consisting of two LC-8A chromatography pump units, column oven (CTO-10AC VP), system controller (SCL-10A VP), rheodyne injector (USA) with a loop of 20 µl volume, diode array detector (DAD; SPD-M10A VP). The HPLC column used was Atlantis ® dC 18

(4.6X 250 mm i.d.; 5µm). An isocratic elution pattern was adopted for the separation of the analyte, and 100% Milli-Q water was used as the mobile phase. The column temperature was set at 25°C; the flow rate was maintained at 0.5 ml/min while the detection was performed at 206 nm. The injection volume was 20 µl and the retention time for AA was 6.8 min.

3.B.3. Statistical analysis

Statistical analysis of all data was performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL). A Duncan's post-hoc comparison of means was carried out to determine significant differences for proximate compositional analysis along with precursor compositions which include reducing sugars, amino acids, total phenolic & flavonoid content determinations for the respective maturity stages of jackfruit. The term significant is used to indicate differences for which p less than 0.05. The correlations between reducing sugars, amino acids, total phenolic & flavonoid content in all the four stages of jackfruit pulp were studied using Pearson's correlation coefficient. Three independent analysis were performed for each stage wise analysis of acrylamide. Values are expressed as mean ± standard deviation of minimum three experiments.

3.B.4. Results and Discussion

3.B.4.1. Compositional analysis of jackfruit on maturation

The proximate compositional analysis which include moisture, ash, fat, protein, carbohydrate content and crude fibre of jackfruit at four different maturity stages were estimated and are listed in Table 3.B.1. The moisture content of jackfruit pulp increased from 49.80 g/100g to 65.90 g/100g from stages one to four respectively during the process of maturation. An increase in ash, protein and fat content was observed on maturation of

jackfruit. The crude fibre content was found to decrease on ripening. The yellow to orange colour of the bulbs is attributed to the presence of carotenoids and the total carotenoid content of the bulbs increased from 0.06 to 0.63 from stage I to stage IV which is in accordance with what was reported earlier [Selvaraj and Pal, 1989; Ong et al., 2006; Jagadeesh et al., 2007]. It is also reported that the total acidity of jackfruits were lower in the ripened stage [Bhatia et al., 1955].

Table 3.B.1 Proximate composition (% of nutrient) in the pulp of raw jackfruit (Artocarpus heterophyllus) from stages I-IV

Stages	**I**	**II**	**III**	**IV**
Moisture	49.80±0.30[a]	53.34±0.02[b]	59.83±0.01[c]	65.90±0.02[d]
Ash	0.59±0.02[a]	0.81±0.01[b]	1.06±0.02[c]	1.86±0.06[d]
Crude fat	1.08±0.10[a]	6.81±0.22[b]	8.72±0.10[c]	9.24±0.30[d]
Crude protein	1.03±0.02[a]	2.28±0.12[b]	2.98±0.03[b]	3.59±0.06[b]
Carbohydrate	61.34±0.09[a]	28.41±0.17[b]	18.97±0.10[c]	18.37±0.26[d]
Crude fibre	3.97±0.03[a]	2.77±0.05[b]	1.44±0.03[c]	0.96±0.10[d]
Total Carotenoid	0.06±0.02[a]	0.13±0.01[b]	0.17±0.04[c]	0.63±0.03[d]
Titrable acidity	0.26±0.04 [a]	0.13±0.02[b]	0.13±0.01[c]	0.06±0.02[d]

*Values are means ± SE (*n = *3). Means values in the same row followed by a different superscript letter are significantly (*$p \leq 0.05$*) different*

The sugars glucose, fructose and sucrose in jackfruit during various maturity stages are summarized in Table 3.B.2 and the corresponding HPLC profiling is given in Fig 3.B.2. The sugars increased during maturation, as expected. The glucose, fructose and sucrose content increased from an initial level of 4.98, 4.79, and 3.08 at stage I to 7.21, 5.51 and 5.71%

respectively, at the stage IV. It was reported that the total free sugar content of jackfruit increased with ripeness from 0.99 g/100 g fresh fruit to 6.91 g/100 g fresh fruit [Chowdhury et al., 1997).

The amino acid profile of jackfruit at four maturity stages is listed in Table 3.B.2. The major amino acids in jackfruit were leucine, isoleucine, asparagine and threonin followed by valine, serine, phenylalanine, lysine and glutamine. There was a significant increase in the level of isoleucine; glutamine increased up to stage III and then reduced drastically at stage IV. We have observed similar results for ripening of *Musa paradisiaca* in our earlier study [Shamla & Nisha, 2017]. There was no significant difference in the concentration of glycine, serine and valine on maturation. The concentration of all the other amino acids was found to decrease during maturation. Zhu et al. [2017] reported the presence of asparagic acid, glutamic acid, valine, leucine & lysine amino acids in jackfruit pulp.

3.B.4.2. Evaluation/Quantification of phenolic/antioxidant compounds/composition in jackfruit

Reports suggest that polyphenols, carotenoids, anthocyanins and flavonoids present in fruits and other plants act as very good natural antioxidants by scavenging free radicals and thus protects cellular damage [Benvenuti et al., 2004; Pietta, 2000]. Therefore, the total phenolic content (TPC), fIavonoid content (TFC) and the individual phenolic compounds were estimated in the present study. It was noted that the total phenolic content decreased significantly with increase in the maturity of jackfruit (Fig 3.B.3.), from 202.46 to 47.81 mg GAE/g dry weight of extract at stage I to IV with a statistical significance of $p \leq 0.05$. A similar trend was also observed in the case of TFC. The flavonoid content in the jackfruit at stage I is significantly higher (Fig 3.B.3.) when compared to stage IV (1.744 to 0.302 mg QE/g dry weight of extract from stage I to IV, respectively) with a statistical significance of

p≤0.05. The TPC and TFC of pulp from Malaysian jackfruit is reported to be 10.34 ± 0.16 (mg GAE/g DM) and 2.27 ± 0.31 (mg QE/g DM) respectively). The TPC and TFC of methanol extract of the pulp from fully ripened jackfruit is reported to be 0.21±0.012 (mg GAE/g) and 0.24±0.012 (mg QE/g) [Jagtap et al., 2010].

Table 3.B.2 Reducing sugars (g/100gm), free amino acids (mg/g) and polyphenol (mg/g) contents (fresh wt.) of jackfruit pulp at four different maturity stages (n=3)

Precursors	Stage I	Stage II	Stage III	Stage IV
Reducing Sugars (g/100gm)				
Glucose	4.98 ± 0.04^{a}	5.23 ± 0.03^{b}	5.71 ± 0.04^{c}	7.21 ± 0.08^{d}
Fructose	4.79 ± 0.01^{a}	4.84 ± 0.02^{b}	5.12 ± 0.07^{c}	5.51 ± 0.01^{d}
Sucrose	3.08 ± 0.02^{a}	3.09 ± 0.01^{a}	3.15 ± 0.03^{b}	5.71 ± 0.02^{c}
Amino acids (mg/g)				
Asparagine	2.92 ± 0.05^{a}	2.29 ± 0.03^{b}	2.19 ± 0.01^{c}	1.58 ± 0.03^{d}
Glutamine	0.65 ± 0.01^{a}	0.92 ± 0.04^{b}	1.38 ± 0.02^{c}	0.25 ± 0.01^{d}
Serine	1.32 ± 0.04^{a}	1.24 ± 0.05^{a}	1.22 ± 0.09^{a}	1.24 ± 0.05^{a}
Glycine	2.02 ± 0.08^{a}	2.01 ± 0.05^{a}	2.00 ± 0.11^{a}	2.01 ± 0.02^{a}
Threonine	2.90 ± 0.06^{a}	2.00 ± 0.09^{b}	1.72 ± 0.01^{c}	2.07 ± 0.02^{d}
Valine	1.36 ± 0.01^{a}	1.29 ± 0.07^{a}	1.31 ± 0.06^{a}	1.28 ± 0.03^{a}
Isoleucine	2.97 ± 0.02^{a}	2.99 ± 0.01^{a}	3.31 ± 0.05^{b}	3.70 ± 0.02^{c}
Leucine	3.90 ± 0.02^{a}	3.46 ± 0.05^{b}	1.44 ± 0.03^{c}	1.08 ± 0.02^{d}
Phenylalanine	1.13 ± 0.01^{a}	1.02 ± 0.05^{b}	0.98 ± 0.02^{b}	1.03 ± 0.01^{b}
Lysine	0.68 ± 0.02^{a}	0.66 ± 0.05^{b}	0.60 ± 0.01^{b}	0.53 ± 0.09^{b}
Phenolic acids (mg/g)				
Gallic acid	0.492 ± 0.076^{a}	0.184 ± 0.007^{b}	0.133 ± 0.009^{c}	0.092 ± 0.010^{d}
Chlorogenic acid	1.302 ± 0.089^{a}	0.983 ± 0.004^{b}	0.791 ± 0.006^{c}	0.694 ± 0.007^{d}
p-Coumaric acid	0.063 ± 0.007^{a}	0.043 ± 0.004^{b}	0.038 ± 0.001^{b}	0.023 ± 0.002^{c}
Quercetin	0.250 ± 0.013^{a}	0.160 ± 0.012^{b}	0.129 ± 0.005^{c}	-

*Values are mean ± SE (*n = *3). Mean values in the same row followed by a different superscript letter are significantly different (*p ≤ *0.05)*

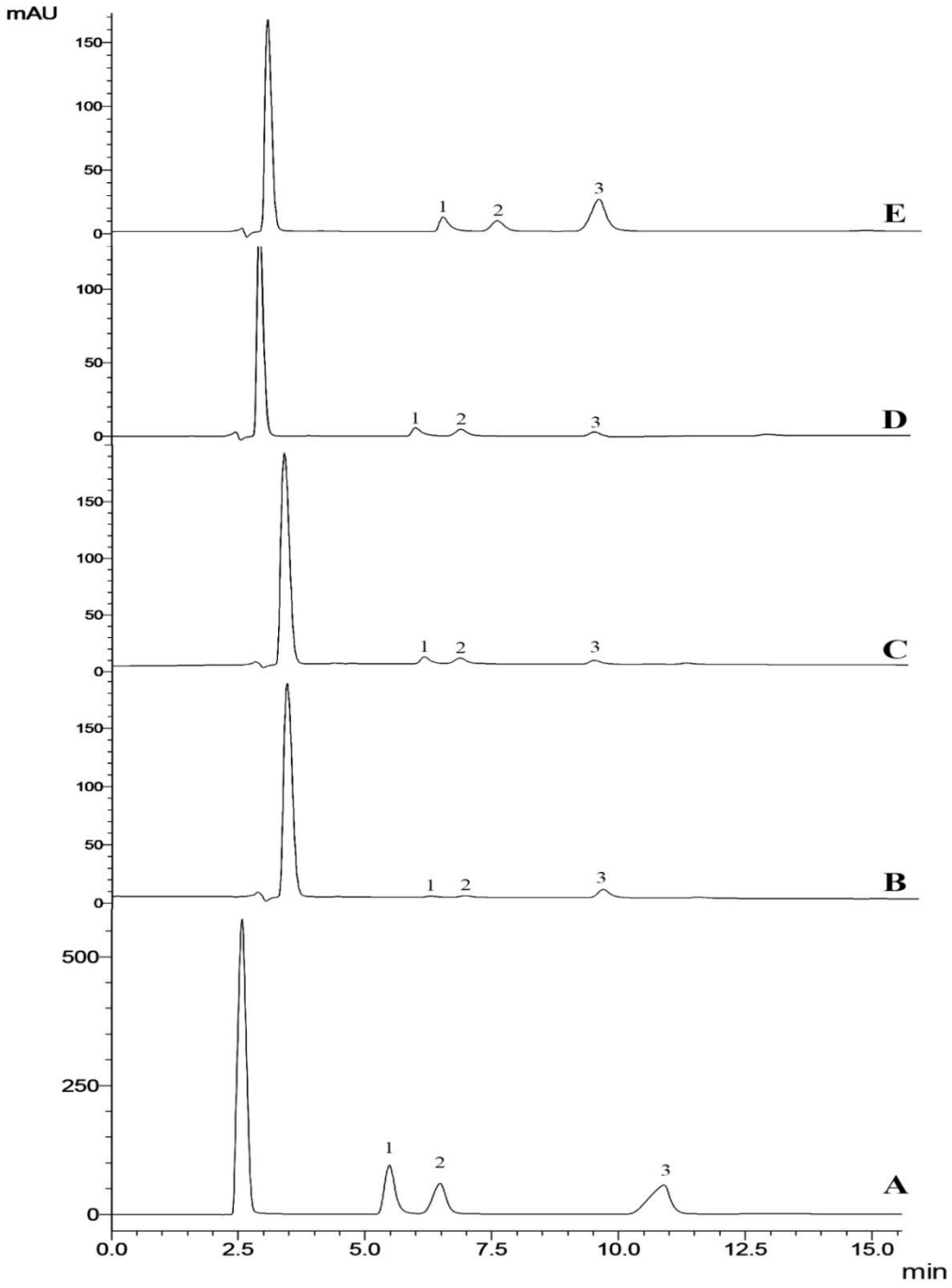

Fig 3.B.2. HPLC profiling of sugars (1 – fructose, 2 – glucose& 3 –sucrose) in all the four maturity stages of jackfruit (A represents profiling of standards and B-E represents four maturity stages from I to IV

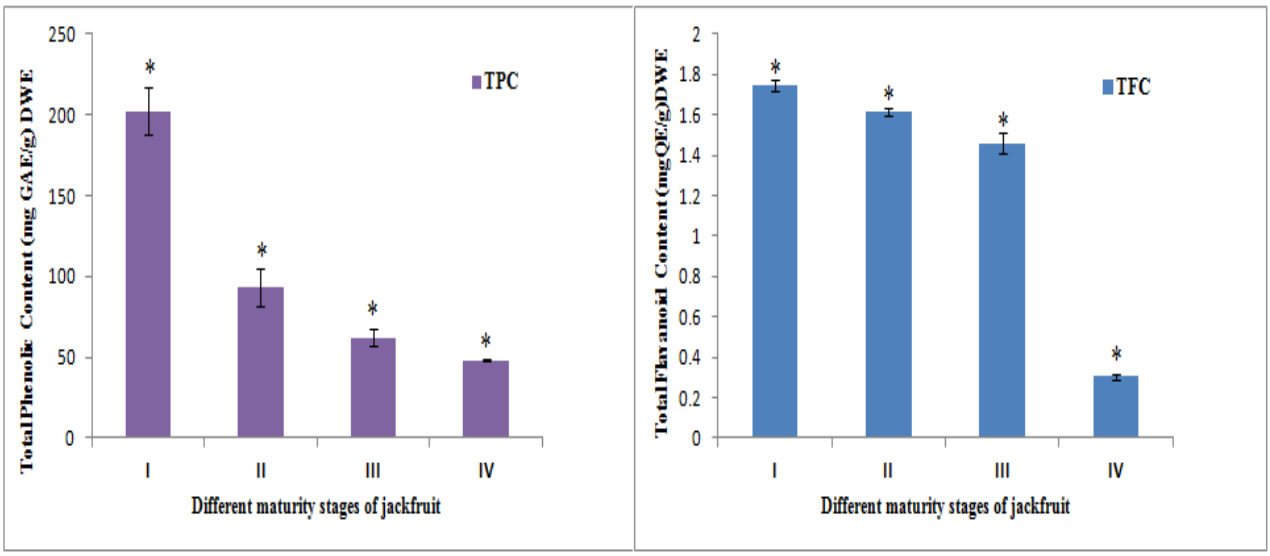

Fig. 3.B.3. TPC & TFC contents of jackfruit pulp extract as on increasing maturity (* indicates the values are significantly different)

A similar decrease in TPC & TFC was observed during the ripening of *Musa paradisiaca*, in our earlier study [Shamla & Nisha, 2017]. The decrease in phenolic and flavonoid content may be attributed to the increased polyphenol oxidase activity during ripening as well as formation of complex molecules by the condensation of phenolics/flavonoids [Parr and Bolwell, 2000; Ben-ahmed et al., 2009].

The individual phenolic compounds in the pulp (bulbs) during ripening of jackfruit were quantified by HPLC technique (Fig 3.B.4.). As can be seen, the concentration of the phenolic compounds decreased significantly ($p \leq 0.05$) on fruit maturity (Table 3.B.2.) in accordance with the decrease in TPC and TFC. Chlorogenic acid was found to be the major phenolic compound in jackfruit followed by gallic acid, quercetin, syringic acid and p-coumaric acid at all the stages of ripening. A study by Shrikanta et al., [2015] reported resveratrol in jackfruit pulp, where as gallic acid and coumaric acid were not detected.

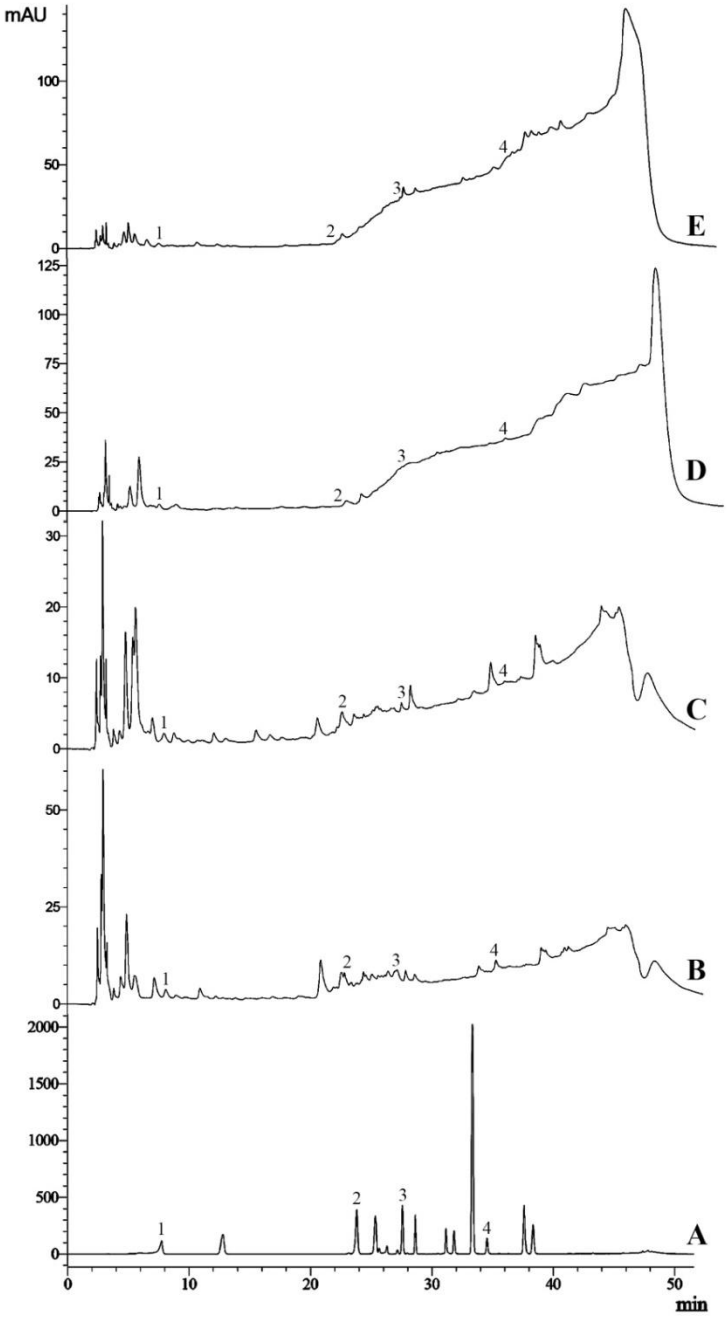

Fig.3.B.4. HPLC chromatogram (A) of authentic standards (1) gallic acid, (2) chlorogenic acid (3) p-coumaric acid and (4) quercetin; (B) stage I; (C) stage II; (D) stage III and (E) stage IV of jackfruit

3.B.4.3. Correlating change in composition of jackfruit on maturation with acrylamide formation in deep fried chips

Chips made from jackfruit is one of the popular snacks in Asian and African countries [Swami et al., 2012]. A preliminary survey done by our group reported significant level of acrylamide in deep fried jack chips [Shamla & Nisha, 2014]. Acrylamide is a heat induced toxicant formed mainly by the reaction between reducing sugars and asparagine. Since acrylamide is genotoxic and carcinogen in rodents, and confirmed to be neurotoxicant in human, efforts have been undertaken to reduce the levels of acrylamide in foods, thus by reducing the human exposure [IARC, 1994]. Change of precursors (reducing sugars and asparagine) in the raw materials has been reported to be one of the reduction strategies proposed. This may be achieved by modification of cultivar techniques, storage temperature, selection of raw materials with minimum content of precursors etc, without affecting the physico chemical and sensory characteristics of the foods.

It was found on the present study that asparagine is one of the predominant amino acid in jackfruit, at the same time the reducing sugars, another important precursor for acrylamide formation, were also increased on ripening. Considering all these facts we thought it would be worthwhile to investigate the effect of ripening on the formation of acrylamide which may be important for formulating mitigation strategies. Therefore, deep fried chips were made from jackfruit at different stages of maturity and the acrylamide content was estimated. It was interesting to note that the acrylamide content in jack chips increased with maturity. The acrylamide concentrations of chips at each stages maturity were significantly different from each other ($p \leq 0.05$) and were 234.82 ± 15.23, 539.02 ± 10.41, 591.63 ± 12.30 and 812.81 ± 9.52 μg/kg respectively from stages I to IV (Fig 3.B.5.). As discussed earlier the increase in the precursors could be correlated to the increase in acrylamide content during

maturation of jackfruit. In order to validate this hypothesis, statistical correlations were carried out between the levels of reducing sugars, amino acids, and phenolic compounds in different maturity stages of jackfruit and acrylamide content.

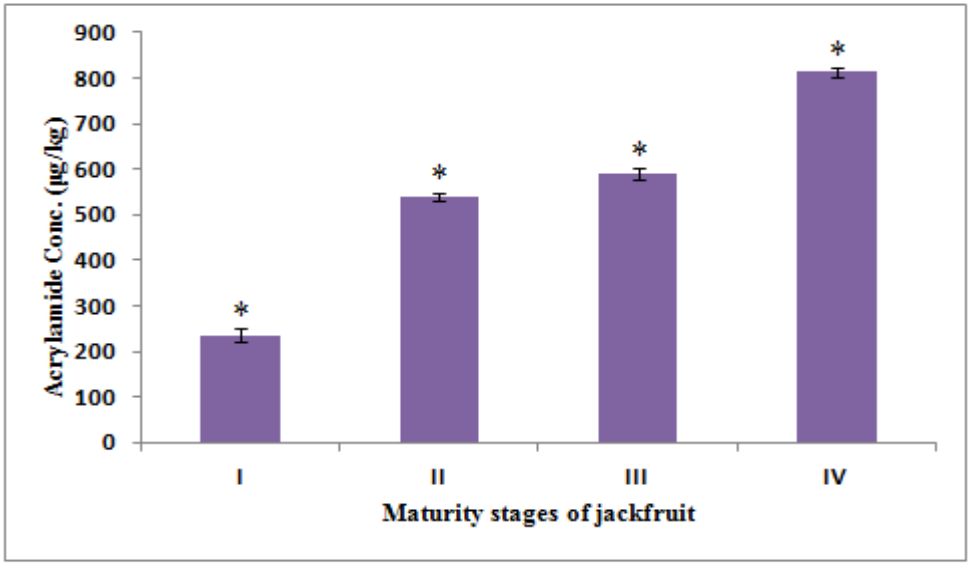

Fig. 3.B.5. Bar graph showing acrylamide concentrations for four different maturity stages (I-IV) of jackfruit (* represents the values are significantly different)

The correlation between the acrylamide content in the final products with reducing sugars, amino acids and the five major phenolics in four maturity stages of jackfruit were examined using the Pearson correlation (r) (Table 3.B.3.). The results indicated that the increase in acrylamide formation was positively correlated with reducing sugar content as on increasing fruit maturity of jackfruit. The correlation between glucose, fructose and sucrose with acrylamide levels in jackfruit yielded positive r values of 0.89, 0.88 & 0.76 respectively. However, asparagine which is reported to be one of the major precursors for acrylamide formation demonstrated a weaker correlation with acrylamide formation. All the other amino acids except, isoleucine exhibited a negative correlation with the formation of acrylamide.

Table 3.B.3 Pearson correlation between acrylamide formation and reducing sugars, amino acids & polyphenolic concentrations in jackfruit pulp at four different maturity stages

Precursors	Pearson correlation (r)	P-value	R^2
Reducing Sugars			
Glucose	0.886	0.114	0.80
Fructose	0.883	0.120	0.78
Sucrose	0.762	0.238	0.58
Aminoacids			
Asparagine	-0.502	0.498	0.25
Glutamine	-0.218	0.781	0.05
Serine	-0.826	0.174	0.68
Glycine	-0.559	0.441	0.31
Threonine	-0.758	0.242	0.57
Valine	-0.257	0.675	0.06
Isoleucine	0.861	0.139	0.74
Leucine	-0.893	0.107	0.80
Phenylalanine	-0.727	0.273	0.53
Lysine	-0.908	0.092	0.83
Polyphenols			
Gallic acid	-0.943	0.057	0.89
Chlorogenic acid	-0.968	0.032	0.93
p-Coumaric acid	-0.999	0.001	0.99
Quercetin	-0.978	0.022	0.96

Similar results are reported for potato & potato products [Rydberg et al., 2005; Pollien et al., 2003] and tubers [Wicklund et al., 2006]. Becalski et al., [2004] reported that the acrylamide formation in French fries can be effectively controlled by the use of potatoes with low levels of sugar. Daniali et al., [2013] reported a good correlation between the reducing sugars and the acrylamide content as on increasing fruit maturity in the case of banana. Even though the reports suggest a positive correlation between the acrylamide formation and asparagine content, the same was not established in the present study.

Polyphenolic compounds have been reported to influence the formation of acrylamide [Liu et al., 2015, Huang et al., 2017]. Considering the influence of polyphenols on acrylamide formation, the TPC & TFC contents were evaluated for all the maturity stages of jackfruit and correlated with acrylamide formation in chips. The TPC and TFC of the jackfruit was found to decrease on maturation, and was found to correlate negatively with acrylamide formation, with r values of -0.950 & -0.844 respectively. Four major phenolic compounds in jackfruit viz gallic acid, chlorogenic acid, p-coumaric acid & quercetin were quantified at stages I-IV and were also correlated with acrylamide content. It was found that all the four compounds exhibited a negative correlation with the acrylamide content ($p \leq 0.05$) in chips indicating that decrease in the phenolic content during maturation process may be one of the reasons for promoting the formation of acrylamide in chips during frying.

It was observed from our present study that the maturity of jackfruit had an impact on the concentrations of precursors, influencing acrylamide formation in deep fried chips. Thus the proper selection of the maturity stage of raw jackfruit will be an added advantage for mitigating acrylamide formation in jack chips.

3.B.6. Summary

Acrylamide formation during deep frying of jackfruit showed a positive correlation with respect to reducing sugars, whereas a poor correlation were observed with amino acids. Four major phenolic compounds namely gallic acid, chlorogenic acid, p-coumaric acid & quercetin were quantified in all the stages of jackfruit and found to decrease during maturation. The compounds also showed negative correlation with acrylamide indicating that higher phenolic content in raw jackfruit along with lower levels of reducing sugars in the initial stages minimized the formation of acrylamide in deep fried jack chips as observed in the case of plantain chips. Thus by selecting the proper maturity stage of raw material

with lesser sugar content and higher phenolic content, the formation of acrylamide in the final product can be reduced to a reasonable level.

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CHAPTER 4

Response surface methodological approach for studying the effect of parameters on acrylamide formation and use of empirical models for explaining the formation and degradation of acrylamide

4.1. Introduction

The discovery of acrylamide in heat treated foods by Swedish National food Administration and Stockholm University in 2002 focussed on the renewed attention on Maillard reaction. The Maillard reaction or the non-enzymatic browning reaction was found to be the main source of acrylamide formation in foods, with reducing sugars and asparagine as the major precursors [Mottram et al., 2002; Stadler et al., 2002; Yaylayan et al., 2003; Nurtsen, 2005; Mottram, 2007; Halford et al., 2012; Oral et al., 2014; Tamanna and Mahmood, 2015]. Many efforts have been carried out by researchers for finding the various factors contributing for acrylamide formation in certain matrixes like potato, cereals, bread, almonds & coffee [Zhang and Zhang, 2008]. The reaction yield of acrylamide was affected by so many factors such as heating temperature, heating time, type of sugars and the water activity [Mestdagh et al., 2006]. Various studies have been conducted for understanding the kinetics of acrylamide formation in aqueous and dry model systems [Capuano et al., 2009; Vleeschouwer et al., 2006]. This mechanistic approach cannot be easily used in the complex food matrix as in the case of model systems. In food systems, reactants cannot meet each other readily and their activities will be different from model experiments [Knol et al., 2005; Kim et al., 2005; Matthaus et al., 2004; Williams, 2005]. Also there are temperature and concentration gradients within the food during heating. So recommendation of a model giving mathematical description of the formation or degradation of acrylamide in food avoids the problem of considering all the mechanisms that occur during processing of foods. Thus the formation and degradation of acrylamide could be successfully modelled using mathematical functions by Corradini and Peleg [2006], where the heat and mass transfer effects are taken up in empirical constants.

Response surface methodology (RSM) is commonly used as an optimization technique in food science, probably because of its comprehensive theory, high efficiency and simplicity. RSM is an empirical statistical modeling technique employed form multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously. RSM encompasses a group of techniques used to study the relationship between one or more measured responses and input variables [Bezerra et al., 2008]. RSM is appropriate experimental design in applications where several responses are measured for each set of experimental conditions and a model is fitted for each response. Wu et al., [2007] reported that in the case of RSM, less number of experiments are needed to evaluate multiple variables and their interactions making it less laborious and time consuming. The main objective of the present work was to optimize the formation of acrylamide in two model systems glucose-asparagine & fructose-asparagine by RSM. The study investigated the effect of heating temperature, heating time and concentrations of the sugars on acrylamide formation in glucose/asparagine and fructose/asparagine model systems using response surface methodology. After optimizing the condition for the formation of acrylamide in two model systems the formation and degradation of acrylamide was statistically verified by fitting the obtained data in two empirical models viz. Logistic-Fermi and Logistic-Exponential equations.

4.2. Materials and methods

4.2.1. Chemicals

Standard acrylamide (>99%), D-(+) glucose, D-(-) fructose, L-asparagine were purchased from Sigma-Aldrich (St. Louis MO, USA). HPLC water was purified on a Milli-Q system (Millipore India Pvt Ltd, Bangalore, India). Oasis HLB (30 mg, 1 ml) solid phase extraction (SPE) cartridges were obtained from Waters Corp. (Milliford,

Massachusetts USA). Minigen syringe filters (0.22µm diameter) were obtained from Genetix Biotech Asia Pvt. Ltd, New Delhi, India. Pyrex glass vials with septum screw caps were obtained from Agilent Technologies (USA).

4.2.2. Preparation of Reaction Mixtures

The procedure for preparing the reaction mixtures was adapted from Knol et al., [2010] with slight modifications. Each glucose-asparagine and fructose-asparagine systems were taken in three different combinations of concentrations 0.5, 1 & 1.5M, so as to make the total concentration of reducing sugar and asparagine as 2M each in hermetically closed pyrex screw-capped glass tubes (Agilent Technologies (USA) at 140, 150, 160, 170, 180, 190 and 200°C in a silicone oil bath (JULABO, 220V, 50 Hz). 2ml of milli-Q water was added and ultrasonicated (Elma, Germany) for about 15 minutes. The tubes were then immersed in the oil up to the cap. At predetermined heating times (4, 8, 12, 16, 20, 24, 28 and 30 min), samples were taken and immediately cooled in ice and stored at -20 °C prior to analysis. Experiments were carried out in triplicate.

4.2.3. Analysis of acrylamide

An HPLC-DAD method used in our previous study was used for the quantification of acrylamide in the reaction mixtures [Shamla and Nisha, 2014; Shamla and Nisha, 2017]. The reaction mixtures were diluted with Milli-Q water (1:10) and filtered through 0.45 µm syringe filter and finally the cleanup of the extracts were done by passing through Oasis HLB catridge (Waters Corporation, Milford, Massachusetts USA) before HPLC analysis. A known amount of standard acrylamide was used as the internal standard. The analysis was carried out by a Shimadzu HPLC system (Japan) consisting of two LC-8A chromatography pump units, column oven (CTO-10AC VP), system controller (SCL-10A VP), rheodyne injector (USA) with a loop of 20 µl volume, diode array detector (DAD; SPD-M10A VP). Atlantis ® dC 18 (4.6X 250 mm i.d.; 5µm) column was used

for the HPLC analysis with an isocratic elution pattern for the separation of the analyte. The mobile phase used was 100% Milli-Q water. The temperature of the column was set at 25°C; the flow rate was set at 0.5 ml/min and 206 nm wavelength was used for the detection. The volume of injection was 20 µl and the retention time for AA was 6.8 min.

4.2.4. Doehlert experimental design

A response surface methodological approach was used in this experiment to study the effect of the reaction temperature (X_1), reaction time (X_2) and concentration of reducing sugar (X_3) on acrylamide formation in two model systems viz. glucose-asparagine and fructose-asparagine. The experiment was carried out based on a Doehlert matrix design. The description of a region around the optimal response was given by Doehlert design and contains k^2 + k +1 experiments where k is the number of factors [Ferreira et al., 2007]. A set of 13 experiments were generated for three variables along with additional 4 replications at the central point were also performed. So a total of 17 experiments were carried out in triplicate. Table 4.1. represents the coded values given by the Doehlert design and the corresponding real values for each experiment and the experimental responses for the two model systems glucose/asparagine and fructose/asparagine. Natural values were deducted from the matrix using the formula given in equation (1)

$$U_j = x_j \Delta U_j + U_j^0 \quad (1)$$

And the variation step ΔU_j was calculated with equation (2)

$$\Delta U_j = \frac{\left(U_j \max - U_j^0\right)}{x_j \max} = \frac{\left(U_j \min - U_j^0\right)}{x_j \min} \quad (2)$$

U_j and x_j were respectively the natural and coded values, max and min being the maximum and minimum value of the variable j (natural or coded), while U_j^0 was the natural value at the center of the range for each factor. The effect of each factor in the

mathematical model represented by the linear, quadratic and interaction effects were interpreted by a second degree polynomial equation as given in equation (3).

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum \sum_{i<j}^{k} \beta_{ij} x_i x_j + \varepsilon \qquad (3)$$

Where Y is the response of the experiment, x_i and x_j are the levels of variables, β_0 is a constant term, β_j represents the coefficients of linear terms, β_{jj} are the coefficients of quadratic terms, βij are the coefficients of the interaction terms and i or j represent the independent variables. The response surfaces were given by the model equations. Multiple regression analysis based on the least square method was performed using STATGRAPHICS Centurion software (XVII-X64) for the analysis and interpretation of the results. Analysis of the variance (ANOVA) and p-value significance levels ($p < 0.05$) were used for understanding the significance of the effects. The experimental response subjected to optimization was the formation of acrylamide in two model systems: glucose/asparagine & fructose/asparagine.

Table 4.1. Doehlert design: coded variables, real variables and experimental responses for the formation of acrylamide in glucose/asparagine and fructose/asparagine model systems

Exp. No.	Coded matrix values			Real matrix values			Experimental Response (Acrylamide formation µg/mL)	
	X_1	X_2	X_3	X_1 (Temp)	X_2 (Time)	X_3 (Conc. of sugar)	Glucose/ asparagine model	Fructose/ asparagine model
1	0	0	0	170	17	1	540.80	488.49
2	1	0	0	170	30	1	932.46	**588.44**
3	-1	0	0	170	4	1	39.65	234.14
4	0.5	0.866	0	200	23.5	1	**939.91**	478.03
5	-0.5	-0.866	0	140	10.5	1	50.35	114.45
6	0.5	-0.866	0	140	23.5	1	917.50	496.26
7	-0.5	0.866	0	200	10.5	1	734.21	522.11
8	0.5	0.289	0.816	180	23.5	1.5	722.93	256.87
9	-0.5	-0.289	-0.816	160	10.5	0.5	367.43	29.96
10	0.5	-0.289	-0.816	160	23.5	0.5	583.67	188.61
11	0	0.577	-0.816	190	17	0.5	687.60	159.64
12	-0.5	0.289	0.816	180	10.5	1.5	374.75	494.53
13	0	-0.577	0.816	150	17	1.5	749.61	447.63
14	0	0	0	170	17	1	540.80	488.49
15	0	0	0	170	17	1	521.05	488.49
16	0	0	0	170	17	1	540.80	488.49
17	0	0	0	170	17	1	521.05	488.49

4.3. Empirical mathematical modeling

Previous reports suggest that acrylamide is not only formed but also undergoes degradation when heating at above 160° C [Knol et al., 2005; Gokmen and Senyuva, 2006; Stadler et al., 2004; Wedzicha et al., 2005]. Corradini & Peleg [2006] & Claeys et al., [2005] studied the formation and degradation of acrylamide using empirical mathematical models. The empirical models were used by Knol et al., [2008] for studying the formation of acrylamide in potato crisps. Li et al., [2016] reported that the empirical models were used for studying the effect of garlic powder on acrylamide in low moisture asparagine/glucose model system and bread baking study. Two empirical models Logistic-Fermi and Logistic-Exponential models were used for the study of

acrylamide in two model systems: glucose/asparagine & fructose/asparagine. The Logistic-Fermi model describes the formation of acrylamide by a Logistic function and the degradation of acrylamide by a Fermi function. Equation (4) represents the Logistic-Fermi function.

$$Y= (1/ (1+\exp (k_d*t-k_d*t_d)))*(A*(1/ (1+\exp (k_g*t_g-k_g*t))))-A*(1/ (1+\exp (k_g*t_g))) \quad (4)$$

Where Y is the concentration of acrylamide and the parameters are A, k_g, k_d, t_g & t_d. Here 'A' is the temperature dependent "scale factor" for acrylamide concentration, 'k_g' and 'k_d' are the temperature dependent steepness parameter for acrylamide generation and degradation, 't_g' and 't_d' are the temperature dependent time characteristics for points in generation and degradation. Logistic-Exponential model differs from Logistic-Fermi model in that part which describes the degradation of acrylamide at higher temperatures for prolonged heating times. Logistic-Exponential function is given by equation by (5)

$$Y= \exp (-t/\tau)*(A*(1/ (1+\exp (k_g*t_g-k_g*t)))) A*(1/ (1+\exp (k_g*t_g))) \quad (5)$$

Where Y is the concentration of acrylamide and the parameters are A, τ, k_g, t_g. 'A' is the temp dependent "scale factor" for acrylamide concentration, 'τ' is the characteristic time, 'k_g' is the temperature dependent steepness parameter for acrylamide generation and 't_g' is the temperature dependent time characteristics for points in generation. The two functions predicts the concentration of acrylamide at prolonged heating times differently. Logistic-Fermi model predicts that the concentration of acrylamide becomes zero at prolonged heating times and Logistic-Exponential model predicts that a residual concentration of acrylamide remains after prolonged heating times.

4.3.1. Statistical analysis and validation of models

The variations of responses (y_i) were evaluated by the analysis of variance (ANOVA). The data were analyzed using STATGRAPHICS Centurion software (XVII-X64). The

fitting of the mathematical models was evaluated by a lack of fit test. The accuracy (A_f) and the bias factors (B_f) were used to estimate the over or under prediction.

$$A_f = 10^{\frac{1}{n}\sum_{i=1}^{n}\left|\log\left(\frac{y_{i,c}}{y_{i,e}}\right)\right|}, \quad B_f = 10^{\frac{1}{n}\sum_{i=1}^{n}\log\left(\frac{y_{i,c}}{y_{i,e}}\right)}$$

Ross [1996], n is the number of experiments

A bias factor value between 0.9 and 1.1 was considered as a criterion for validation in order to have a model with less than 10% under and over-estimation.

4.4. Results and discussion

4.4.1. Optimization of acrylamide formation in glucose/asparagines model system using RSM

The coefficients of variables and their interaction effects were summarized in Table 4.2. The validity of the model was evaluated based on the analysis of variance at a significance level of $p<0.05$, indicating that they are significantly different from zero at the 95.0% confidence level. Here the temperature, time and the product of temperature & time are having greater effect on the formation of acrylamide with $p<0.05$. The R-Squared statistic indicates that the model as fitted explains 94.36% of the variability in acrylamide. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 87.10%. These results indicated that the regression equation of the fitted model can be used for predicting the actual experimental study without going for the lab experiment. The resulting second degree polynomial equation for acrylamide concentration (Y) is given as $Y = 532.898 + 427.864*X_1 + 154.543*X_2 + 42.54*X_3 - 46.842*X_1^2 - 381.898*X_1*X_2 + 216.098*X_1*X_3 + 185.751*X_2^2 - 209.963*X_2*X_3 + 37.5535*X_3^2$. The effect of temperature, time and concentration of sugar on acrylamide formation was explained by the Pareto chart given in fig 4.1. It was found that the temperature and time are having positive influence on

acrylamide formation. The product of temperature and time are having negative influence on acrylamide formation. Fig 4.2. is the response surface plot which indicates that the formation of acrylamide was found to be maximum at equimolar concentration of sugar at all temperatures and time. So we have optimised from the response surface methodology that the formation of acrylamide in glucose/asparagine system was maximum at equimolar concentration of glucose at all temperatures and time used in the study.

Table 4.2. Analysis of variance for acrylamide formation in glucose/asparagine model system

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
X1:Temperature	732271.	1	732271.	80.88	0.0000 *
X2:Time	95528.7	1	95528.7	10.55	0.0141 *
X3:Concentration	7229.81	1	7229.81	0.80	0.4012
X1*X1	3134.53	1	3134.53	0.35	0.5747
X1*X2	109378.	1	109378.	12.08	0.0103 *
X1*X3	27978.4	1	27978.4	3.09	0.1222
X2*X2	49284.8	1	49284.8	5.44	0.0524
X2*X3	26423.1	1	26423.1	2.92	0.1313
X3*X3	2281.36	1	2281.36	0.25	0.6311
Total error	63374.0	7	9053.43		
Total (corr.)	1.12309E6	16			

**Significant for p 0.05 or 5 % at 95 % confidence level*

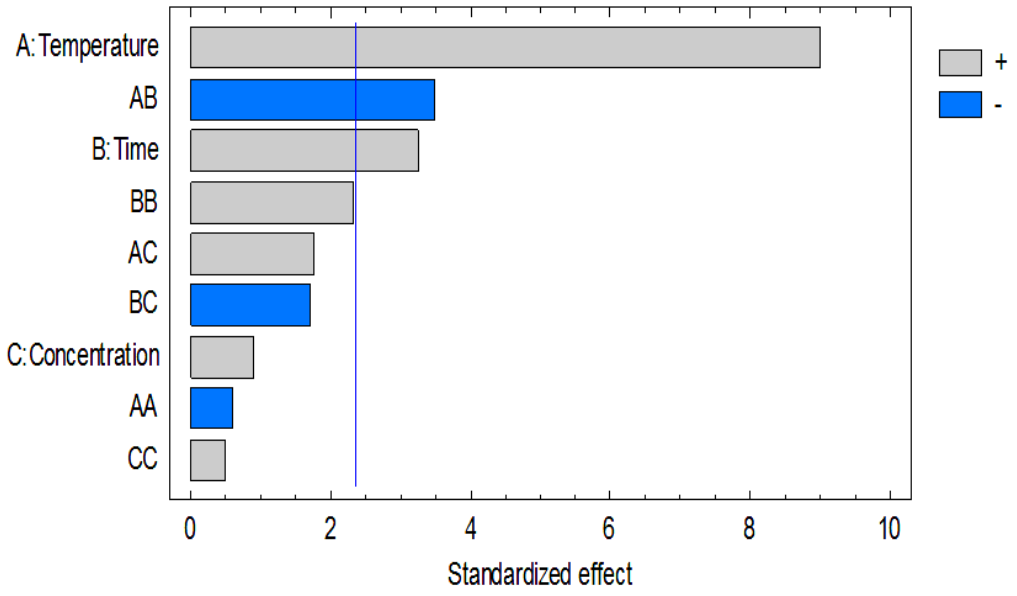

Fig 4.1. Standardized Pareto chart for acrylamide formation in glucose/asparagine model system (+ positive influence and – negative influence)

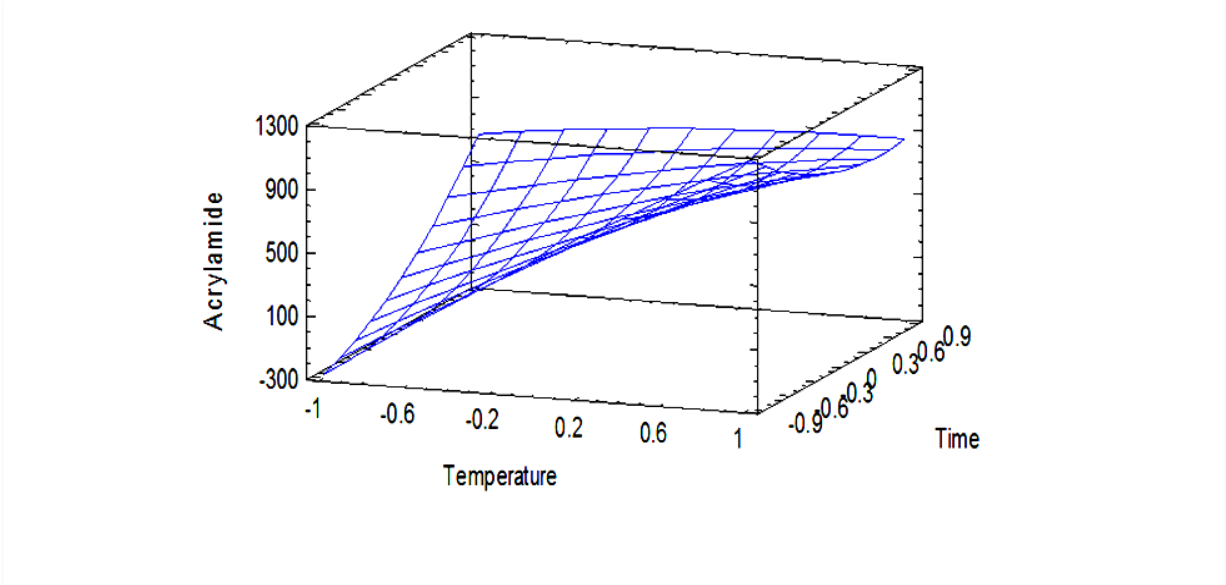

Fig 4.2. Estimated Response Surface graph at equimolar concentration of glucose in glucose/asparagine model system

4.4.2. Optimization of acrylamide formation in fructose/asparagine model system using RSM

The coefficients of variables and their interaction effects were summarized in Table 4.3. The validity of the model evaluated based on the analysis of variance at a significance

level of $p<0.05$, indicated that they are significantly different from zero at the 95.0% confidence level. Here the temperature, time and the product of temperature & time are having greater effect on the formation of acrylamide with $p<0.05$. The R-Squared statistic indicates that the model as fitted explains 90.36% of the variability in acrylamide. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 77.97%. These results indicated that the regression equation of the fitted model can be used for predicting the actual experimental study without going for the lab experiment. The resulting second degree polynomial equation for acrylamide concentration (Y) is given as $Y = 488.489 + 120.915*X_1 + 81.2014*X_2 + 167.618*X_3 - 77.198*X_1^2 - 245.895*X_1*X_2 - 155.755*X_1*X_3 - 88.6422*X_2^2 - 90.4964*X_2*X_3 - 297.289*X_3^2$. The effect of the three parameters temperature, time and concentration of sugar on acrylamide formation was explained by the Pareto chart given in fig 4.3. It was found that the temperature and time are having positive influence on acrylamide formation. The product of temperature and time are having negative influence on acrylamide formation. Fig 4.4 is the response surface plot which indicates that the formation of acrylamide was found to be maximum at equimolar concentration of sugar at all temperatures and time. So we have optimised from the response surface methodology that the formation of acrylamide in fructose/asparagine system was maximum at equimolar concentration of fructose at all temperatures and time used in the study.

Table 4.3. Analysis of variance for acrylamide formation in fructose/asparagine model system

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
X1:Temperature	58481.3	1	58481.3	8.89	0.0205 *
X2:Time	26373.1	1	26373.1	4.01	0.0853
X3:Concentration	112246.	1	112246.	17.06	0.0044 *
X1*X1	8513.62	1	8513.62	1.29	0.2927
X1*X2	45345.8	1	45345.8	6.89	0.0341 *
X1*X3	14534.6	1	14534.6	2.21	0.1808
X2*X2	11223.6	1	11223.6	1.71	0.2328
X2*X3	4908.63	1	4908.63	0.75	0.4163
X3*X3	142972.	1	142972.	21.73	0.0023 *
Total error	46053.5	7	6579.07	8.89	0.0205
Total (corr.)	477975.	16			

**Significant for p 0.05 or 5 % at 95 % confidence level*

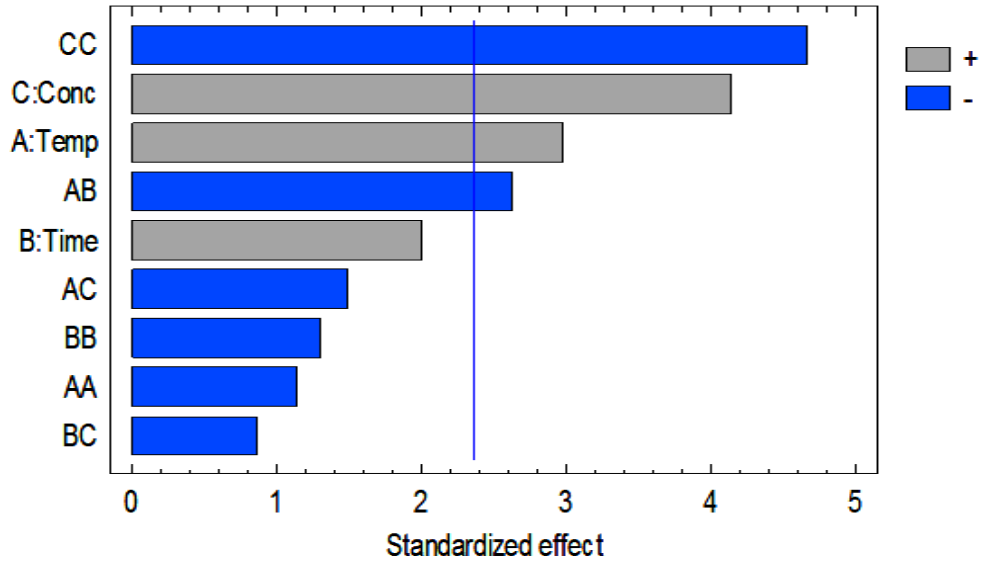

Fig 4.3. Standardized Pareto chart for acrylamide formation in fructose/asparagine model system (+ positive influence and – negative influence)

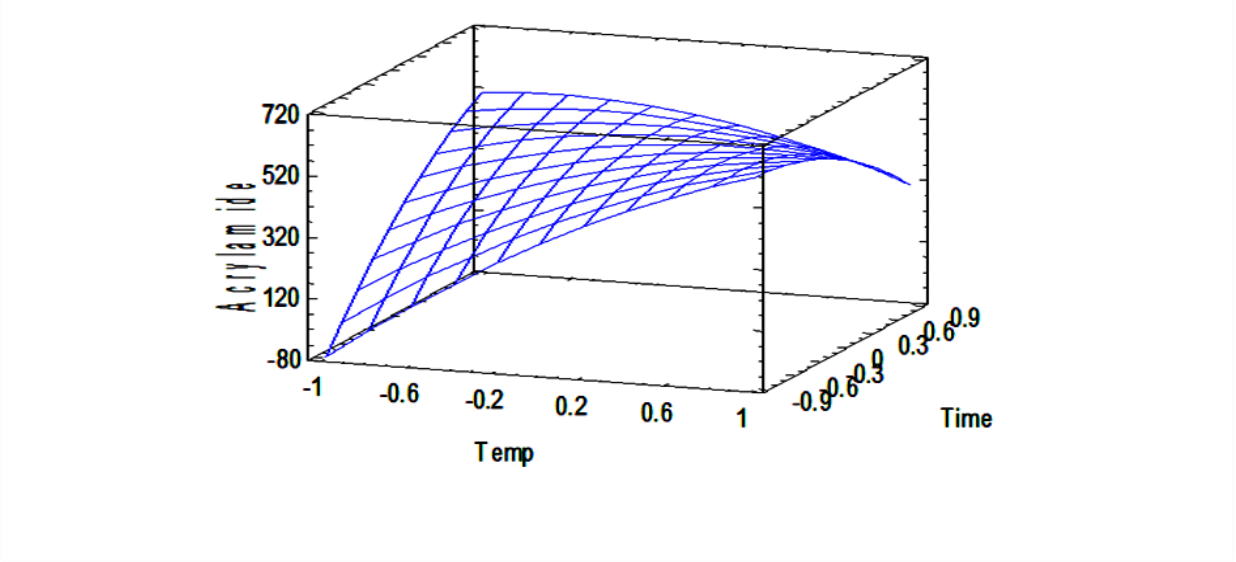

Fig 4.4. Estimated Response Surface graph at equimolar concentration of fructose in fructose/asparagine model system

4.5. Experimental study of acrylamide formation at all temperatures and time using equimolar concentration of sugars in glucose/asparagine and fructose/asparagine model systems

In glucose/asparagine model system after optimizing the condition for the formation of acrylamide using RSM, we have gone for the full experimental study at temperatures of 140, 150, 160, 170, 180, 190 & 200°C & heating times of 4, 8, 12, 16, 20, 24, 28 & 30 minutes keeping equimolar concentration of glucose. The acrylamide formation at all temperatures and time for glucose/asparagine model system was given in fig 4.5. It was found that the concentration of acrylamide increases up to 30 min for 140, 150, 160 & 170 °C. For 180 and 190°C the concentration of acrylamide increases up to 20 min and then decreases. At 200°C the formation of acrylamide increases up to 8 min and then starts decreasing.

In the case of fructose/asparagine model system after optimizing the condition for the formation of acrylamide using RSM, we have gone for the full experimental study at

temperatures of 140, 150, 160, 170, 180, 190 & 200°C & heating times of 4, 8, 12, 16, 20, 24, 28 & 30 minutes keeping equimolar concentration of fructose. The acrylamide formation at all temperatures and time for fructose/asparagine model system was given in fig 4.6. It was found that the concentration of acrylamide increases up to 30 min for 140, 150, 160, 170 & 180°C. At 190°C the concentration of acrylamide increases up to 8 min and then decreases, again shows a gradual increase and then decreases. For 200°C the formation of acrylamide increases up to 24 min and then decreases. So it was found that along with the formation of acrylamide there occurs a degradation of acrylamide at higher temperatures for prolonged heating time. Thus the formation and degradation of acrylamide was verified by fitting the obtained data in two empirical mathematical models: Logistic-Fermi & Logistic-Exponential.

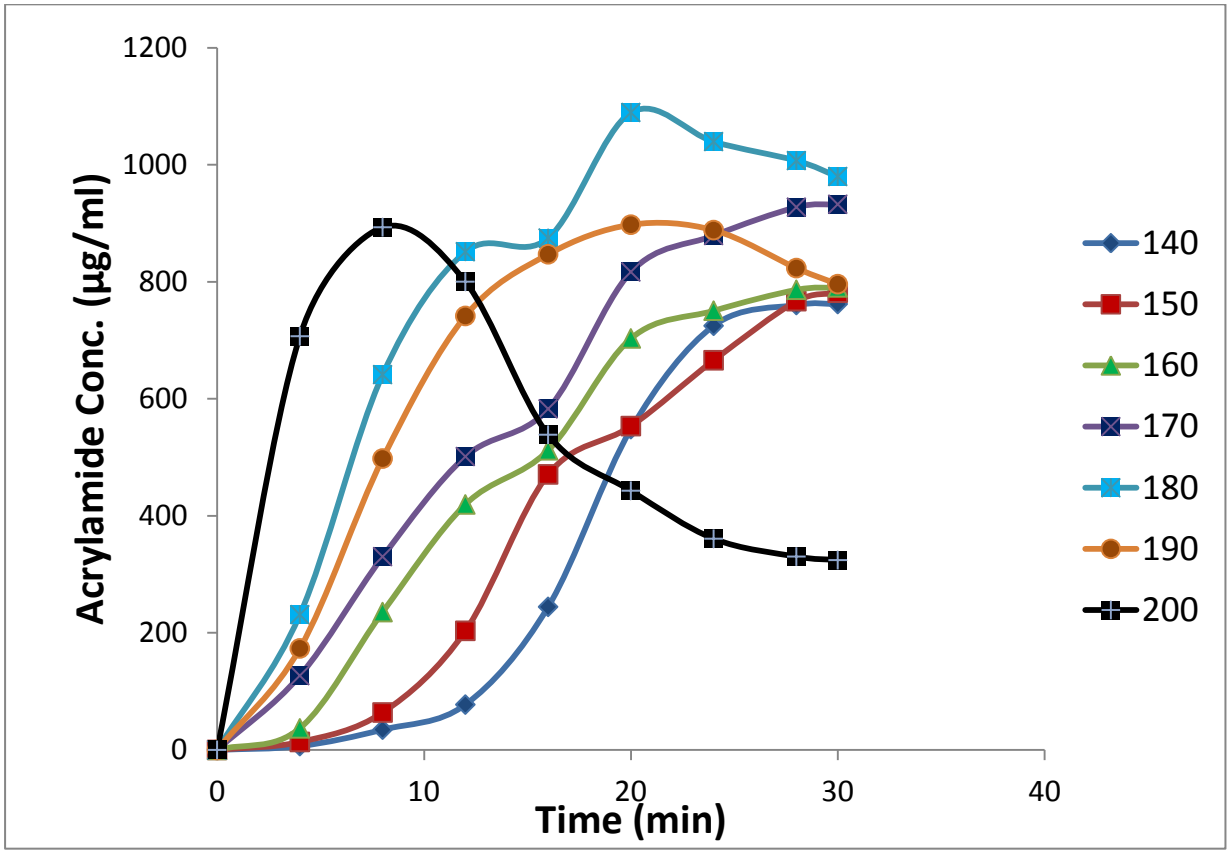

Fig 4.5. Formation of acrylamide in glucose/asparagine model system at temperatures of 140, 150, 160, 170, 180, 190 & 200°C at heating times of 4, 8, 12, 16, 20, 24, 28 & 30 mins

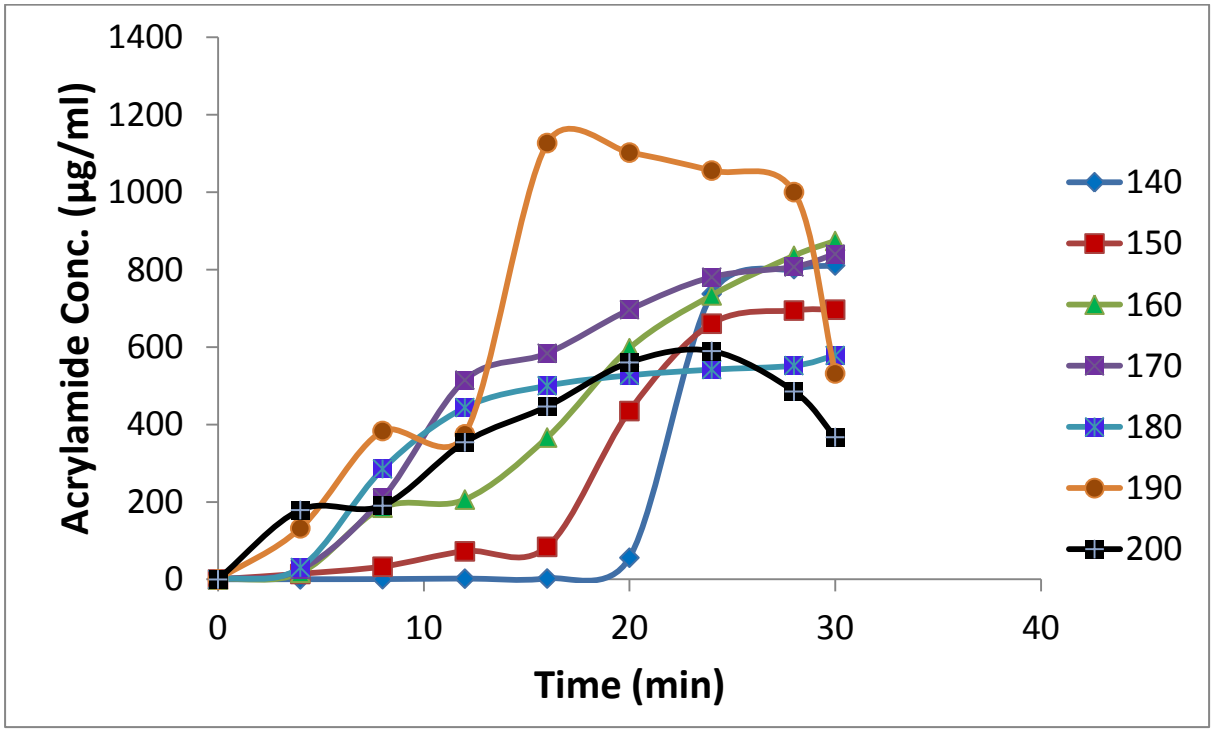

Fig 4.6. Formation of acrylamide in fructose/asparagine model system at temperatures of 140, 150, 160, 170, 180, 190 & 200°C at heating times of 4, 8, 12, 16, 20, 24, 28 & 30 mins

Obtained data were fitted into the two above mentioned empirical model equations, Logistic-Fermi and Logistic-Exponential as discussed in section 4.5 and statistically analyzed using OriginPro 8.5.1 software. The parameters obtained for the Logistic-Fermi model for both glucose/asparagine & fructose/asparagine model system is given in Table 4.4. When we are fitting the obtained data in Logistic-Fermi model equation, the parameters A, k_g, k_d, t_g or t_d should not become negative. If any of the parameter is coming negative then that model could not satisfactory explain the formation of acrylamide. Here for both glucose/asparagine and fructose/asparagine model systems, some of the parameters for Logistic-Fermi model are coming negative. And hence this rules out the prediction of the Logistic-Fermi model which implies that the concentration of acrylamide becomes zero at prolonged heating times at higher temperatures. So we have gone for the fitting of the obtained data in Logistic-Exponential model equation for the glucose/asparagine and fructose/asparagine model systems. The parameters obtained for Logistic-Exponential equation for glucose/asparagine and fructose/asparagine is given in Table 4.5.

Table 4.4. Parameters obtained by fitting the obtained data in Logistic-Fermi model equation

Temp (ºC)	A		k_g		k_d		t_g		t_d	
	Glu/Asp	Fru/Asp	Glu/Asp	Fru/Asp	Glu/Asp	Fru/Asp	Glu/Asp	Fru/Asp	Glu/Asp	Fru/Asp
140	838.72	3136.16	3.65	0.115	-3.99E-01	-1.36	0.684	-8.61	17.80	21.82
150	1603.60	814.75	0.249	2.709	3.10E-12	-0.56	15.49	0.67	-1.97E+06	19.16
160	953.63	1034.90	0.176	0.172	-3.27	-34.52	10.91	17.69	4.15	- 205380
170	5274.80	876.72	0.039	0.242	0.135	-1.25E-01	30.68	10.90	35.07	-2100
180	1316.73	598.03	0.253	0.775	0.755	-1.03E-01	5.53	6.90	3.22E+01	0.483
190	1010.79	1192.18	0.321	0.328	0.253	0.97	7.02	11.70	37.33	29.83
200	1.33E+07	39834	0.733	0.008	0.055	0.376	3.18	-263.17	-163.68	29.43

Table 4.5. Parameters obtained by fitting the obtained data in Logistic-Exponential model equation

Temp (ºC)	A		t_g		τ		k_g	
	Glu/Asp	Fru/Asp	Glu/Asp	Fru/Asp	Glu/Asp	Fru/Asp	Glu/Asp	Fru/Asp
140	2500	2500	20.49	22.91	25.70	25.70	0.325	1.002
150	2500	2500	20.77	21.22	29.64	23.44	0.195	0.404
160	2500	2500	18.16	22.10	31.82	37.67	0.154	0.171
170	2500	2500	16.27	17.40	48.92	33.66	0.114	0.147
180	2500	2500	8.39	16.24	41.48	25.35	0.164	0.120
190	2500	2500	10.80	14.29	30.57	37.50	0.176	0.524
200	2500	2500	1.41	15.32	16.80	17.02	0.388	0.208

The parameters A, t_g, τ and k_g are getting positive values when the fitting of the Logistic-Exponential model equation was carried out. For both glucose/asparagine and fructose/asparagine model systems, the values obtained at all temperatures succeeded in fitting with the Logistic-Exponential equation. It was observed from our study that a decrease in concentration of acrylamide occurs for both model systems at higher temperatures and may be due to the fact that degradation of acrylamide occurs with a residual amount of acrylamide remaining after prolonged heating times. The fitting of the Logistic-Exponential plot for the glucose/asparagine and fructose/asparagine model system at all heating temperatures and time is given fig 4.7. & fig 4.8. respectively.

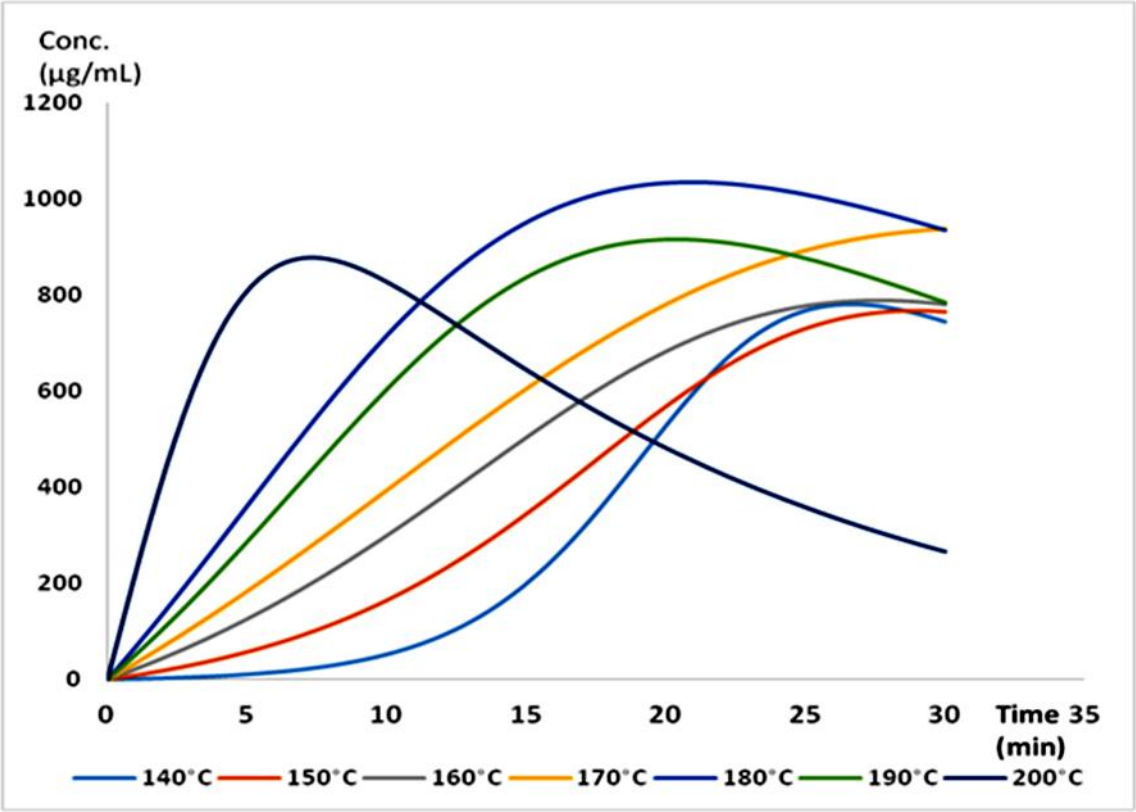

Fig 4.7. Fitting of the Logistic-Exponential plot for the glucose/asparagine model system

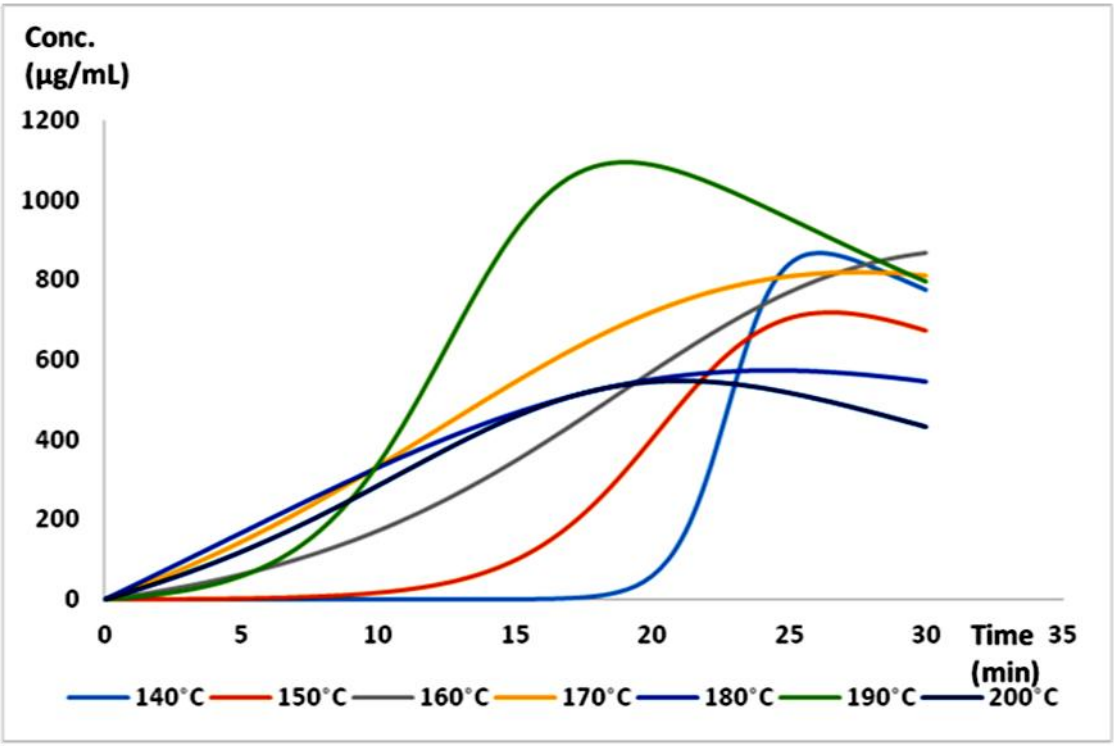

Fig 4.8. Fitting of the Logistic-Exponential plot for the fructose/asparagine model system

4.6. Summary

In the present study RSM was used to optimize the formation of acrylamide in two model systems glucose/asparagine & fructose/asparagine. The reaction temperature, reaction time and the concentration of reducing sugar are the factors which are taken for the RSM study. It was found that at equimolar concentration of reducing sugar the formation of acrylamide could be successfully explained at the corresponding temperature and time. Further as the reaction temperature increased after prolonged heating times, a decrease in the concentration of acrylamide was found. We have used two empirical mathematical models Logistic-Fermi and Logistic-Exponential for studying the formation and degradation of acrylamide. The experimental values were fitted into these empirical equations and it was found that the Logistic-Exponential model could successfully explain the degradation of acrylamide with a residual amount of acrylamide remaining after prolonged heating times. In future, if we could establish the relationship between these model parameters and the precursor contents and also

the temperature dependence of model parameters it would be helpful for the food manufacturers and industries to develop new mitigation strategies.

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CHAPTER 5

Summary and Conclusion

5.1. Summary and Conclusion

Thermal processing are frequently employed in food manufacturing to improve safety, to increase the nutritional quality and also to improve the sensory properties of foods, their palatability and to extend the range of colours, tastes, aromas and texture. Thermal processing is an important treatment for food preservation, especially in the manufacture of shelf-stable foods with specific nutritional properties. The thermal processing like baking, toasting, frying, roasting and sterilization results in desired and undesired effects due to various chemical reactions viz., Maillard reaction, caramelisation and lipid oxidation and a plethora of new molecules are generated. The chemicals that are formed in food as a result of food processing/preparation that exert adverse toxicological effects or create a potential or real risk to humans are called process induced toxicants. The final concentrations of thermal-process contaminants in foods depend on the concentration of their corresponding precursors as well as the severity of thermal processing conditions.

Acrylamide is a heat induced toxicant that gained attention by the scientific community and public in the recent years. The Maillard reaction between the reducing sugars and the amino acid asparagine at temperatures above 120ºC has been suggested as the formation pathway of acrylamide in foods. Acrylamide found in high temperature treated foods has led to an intensive and persistent research effort, since it is a neurotoxic, genotoxic and probable carcinogenic compound to humans. The most important health issue related to the occurrence of acrylamide in foods is its ability to induce cancer in humans and it is classified as 'probable human carcinogen' by the International Agency for Research on Cancer (IARC). In order to understand the exposure to the population, it is very important to assess the occurrence of acrylamide in various food products. Therefore the objective of the work is to

study the occurrence of acrylamide in popular snack foods of India mainly potato, jack, sweet plantain and plantain chips and to understand the occurrence of acrylamide by the consumption of these snacks. The effect of precursor concentrations in raw materials and its correlation to acrylamide formation in different stages of fruit maturity with respect to banana and jackfruits were studied. The influence of phenolic and flavonoid contents in raw material and its effect on acrylamide formation was also studied. This study focuses on the proper selection of the raw material for the chips preparation so that we can reduce the formation of acrylamide in these snack food products. Response surface methodology (RSM) was attempted to understand the role of precursors and processing conditions on formation of acrylamide.

The first chapter provides updated literature review on acrylamide, possible sources of acrylamide exposure, history of how acrylamide was first detected in foods, occurrence of acrylamide in foods and dietary exposure, different formation pathways of acrylamide, various factors influencing the formation of acrylamide in foods, analytical methods for acrylamide quantification in foods, health risks and metabolism of acrylamide and use of RSM for explaining the formation of acrylamide.

The second chapter describes a modified sample extraction protocol for HPLC-DAD quantification of acrylamide in fried Indian snack foods (potato, jack, ripe plantain & raw plantain chips). The HPLC-DAD method was standardised and a survey was conducted for quantifying the levels of acrylamide in deep fried snacks from 20 different production sites (80 samples representing four important product categories). The limit of detection and the limit of quantitation for the method were 1.04µg/ml and 3.17µg/ml respectively. Acrylamide concentrations in four groups of snacks ranged from 82.05- 4245.59 µg/kg for potato chips,

46.18-2431.37 µg/kg for jack chips, 24.81-1959.80 µg/kg for sweet plantain chips and 14.73-1690.48 µg/kg for plantain chips respectively. The results revealed reasonable levels of acrylamide in the products studied which indicated the general risk of consumer exposure to acrylamide from Indian foods.

The third chapter comprises of two parts. Part A and part B explain our efforts in bringing out the influence of reducing sugars, amino acids and polyphenolics in raw fruit on acrylamide formation in deep fried chips with different ripening stages (I-V) in the case of banana and different maturation stages (I-IV) in jackfruit, respectively. Both glucose and fructose increased during ripening and demonstrated a positive correlation on formation of acrylamide, correlation coefficient of r = 0.95 & 0.94 for banana and r = 0.89 & 0.88 for jackfruit respectively ($p<0.05$), whereas asparagine, was poorly correlated ($p>0.05$). The decreased levels of phenolic content during ripening of plantain & jackfruit were negatively correlated with acrylamide formation in the deep fried chips prepared. Thus the selections of proper stage of raw material renders reduced formation of acrylamide in banana chips and jack chips to a reasonable extent.

The fourth chapter describes acrylamide formation in two model systems consisting of glucose & asparagine and fructose & asparagine at temperatures between 140 and 200°C by Response surface methodology (RSM). Acrylamide content was chosen as the response for investigation and the independent variables are heating temperature (X_1), heating time (X_2) and concentration of sugars (X_3). A three factors Doehlert matrix was used as the experimental design. Natural values were deducted from the matrix and the obtained system responses were fitted into a second order polynomial equation. The variation of responses was evaluated by the analysis of variance (ANOVA) for the two model systems studied. The

obtained data were analyzed using Statgraphic centurion XVII-X64 version software. The conditions favouring the formation of acrylamide were determined by RSM for glucose/asparagine and fructose/asparagine systems. After the optimization of parameters for the acrylamide formation by RSM, the full experimental study was conducted keeping equimolar concentrations of glucose and fructose at all temperatures and time. It was observed that the acrylamide under go degradation at higher temperatures for prolonged heating times. The verification of the decrease in acrylamide concentration at higher temperatures was done by fitting the obtained data in two empirical models *viz* Logistic-Fermi and Logistic- Exponential. The Logistic-Exponential model was best suited for explaining the degradation of acrylamide at higher temperature and a residual acrylamide remains after prolonged heating time which is in accordance with our experimental result in both glucose/asparagine and fructose/asparagine model systems.

The study on acrylamide is very important as it is a process induced toxicant (carcinogen) and India with vast cultural diversity consumes lot of processed snack products. The present study brought out the need for an extensive study to estimate the occurrence of acrylamide in various heat processed products consumed in India and this information can be used for assessing intake assessment and also for planning mitigation strategies to reduce the human exposure. As there is no data on acrylamide content of Indian products, Food Safety and Standards Authority (India) has taken initiative to carry out the risk assessment studies and to further come up with data generation. The evidences from present study will help the regulatory agencies to address the issue and awareness creation. Data base on acrylamide in foods is important for the regulatory authority to draft strategies to control the exposure**.**

Abbreviations

3-APA	3-Aminopropionamide
AA	Acrylamide
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
ATSDR	Agency for toxic substances and disease registry
CV	Coefficient of variation
CYP2E1	Cytochrome P4502E1 enzyme
DAD	Diode array detector
DM	Dry matter
DNA	Deoxyribonucleic acid
EC	European commision
ECD	Electron capture detector
EFSA	European food safety authority
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and agriculture organization
FDA	Food and drug administration
FID	Flame ionization detector
GAE	Gallic acid equivalents
GC	Gas chromatography
HAA	Heterocyclic aromatic amines
Hb	Hemoglobin
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
IARC	International agency for research on cancer
JECFA	Joint FAO/WHO expert committee on food additives

KBr	Potassium bromide
$KBrO_3$	Potassium bromate
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar
MCPD	3-Monochloro propanediols
MR	Maillard reaction
MRP	Maillard reaction products
MS	Mass spectrometry
MS/MS	tandem mass spectrometry
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NOAEL	No-observed-adverse-effect-level
OPA	o-phthalaldehyde
PAH	Polycyclic aromatic hydrocarbons
PTFE	Polytetra fluoro ethylene
QDs	Quantum Dots
QE	Quercetin equivalents
RSD	Relative standard deviation
RSM	Response surface methodology
SD	Standard deviation
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SPSS	Statistical Program for Social Sciences
TFC	Total flavonoid content
TPC	Total phenolic content

UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
WHO	World Health Organization

List of Publications in SCI Journals

- **L. Shamla** and P. Nisha (2014). Acrylamide in deep fried snacks of India. Food Additives and Contaminants Part B: Surveillance. 7(2014), 220-225.

- **L. Shamla** and P. Nisha (2017). Acrylamide formation in plantain (Musa Paradisiaca) chips influenced by different ripening stages: A correlation study with respect to reducing sugars, amino acids and phenolic content. Food Chemistry. 222(2017), 53-60.

- **L. Shamla** and P. Nisha (2017). Evaluation of reducing sugars, aminoacids & polyphenolics in different maturity stages of jackfruit and its influence on acrylamide formation in deep fried chips. Food Research International. (Communicated)

- **L. Shamla** and P. Nisha. Response surface methodological approach for studying the effect of reaction temperature, reaction time and concentration of sugars on acrylamide formation in glucose/asparagine & fructose/asparagine model systems and use of empirical models for explaining the formation and degradation of acrylamide. (Under preparation)

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- Mitigation studies for acrylamide formation in banana chips. XXI ICFOST (International) on Innovations in Food Science and Technology to Fuel the Growth of the Indian Food Industry, Pune, India, 2012.

- Fruit maturity & Acrylamide Formation – A case study with respect to jack chips. National conference on emerging avenues in Food technology for better health and safety. Thangal Kunju Musaliar Institute of Technology, Kollam**.** 2013.

- Attended National Workshop on Mass Spectrometry in the DST-PURSUE sponsored National Workshop on Mass Spectrometry held at Mahatma Gandhi University, Kottayam, Kerala, 2013.