Acrylamide in Selected Foods and Genotoxicity of Their Extracts

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ABSTRACT

Acrylamide is one of the most important contaminants in the environment. Acrylamide was shown to be a neurotoxicant, reproductive toxicant, and carcinogen in animals. There is consensus among international food safety groups that acrylamide in the diet should be assessed. In the present study, we surveyed by Gas Chromatography-Mass Spectrometry (GC-MS) the levels of acrylamide in 23 foods marketed in Jeddah, Saudi Arabia. The genotoxicity of all food samples, acrylamide and glycidamide were investigated using the Salmonella mutagenicity test. Also, the DNA damaging potency was done for 16 of these foods aqueous and organic extracts beside acrylamide in the comet assay. Acrylamide levels in food ranged from non-detectable to 2200µg/kg. Neither acrylamide nor food aqueous or organic extracts showed mutagenic effects in the Salmonella strains TA98, TA100 and TA1535 in the presence or absence of the metabolic activation system (S9). Also, they did not show DNA damaging effects in the comet assay. Glycidamide showed mutagenicity with TA100 only in the presence of S9 and exhibited extensive DNA breaks in the comet assay. Leukocytes from rat fed Pringles crisps showed extensive DNA damage in comet test. This genotoxicity could not be demonstrated by Pringles in vitro. Continuous surveying of food for acrylamide is necessary to minimize human exposure.
Keywords: Acrylamide, Comet test, GC/MS, glycidamide, mutagenicity, Salmonella.

INTRODUCTION

Much attention and research have been focused on the acrylamide formation in food. This sense of urgency was prompted by the discovery of acrylamide in carbohydrate rich cooked food, by the National Food Administration in Sweden in April 2002. Thus issuing, a warning about the risk management of acrylamide to the food industry and consumers. These researchers found microgram per kilogram to milligram per kilogram levels of acrylamide in foods. Since then researchers have devoted great efforts to measure acrylamide levels in a wide variety of foods such as crisps, French fries, bread and coffee and begun to search for ways to reduce levels of acrylamide in food. Risk assessments of acrylamide, made by United States Environmental Protection Agency (US, EPA) and WHO, imply that dietary intake of acrylamide could be associated with potential health risks. WHO officials stress that the full picture of acrylamide levels in food and its effects on human does not exist.

Acrylamide is formed in the process of Maillard reactions from asparagines in the presence of a carbonyl compound such as reducing sugar. Glucose and fructose concentrations in the tubers were significantly and positively correlated with subsequent acrylamide formation in the products. Glucose, fructose, sucrose, and asparagine concentrations in tubers increased during cold storage, and the acrylamide concentration increased accordingly. The concentrations of asparagine and other amino acids, however, did not change during the cold storage. These changes in the sugar and amino acid contents of potato tubers during short-term storage affect acrylamide level in
chips after frying. Acrylamide content in chips began to increase after 3 days of storage at 2 degrees. (8) A hypothetical mechanism that has been suggested for acrylamide formation from oil is through acrolein which is formed during oil heating from glycerol or as a result of oxidation of polyunsaturated fatty acids and their degradation products. (9) Other, believe that acrylamide is not principally formed from precursors; especially acrolein present in oil itself and that acrylamide formation is non-oxidative in nature. Oxidized fats could compete with other carbonyl compounds for only a trivial role in acrylamide formation through the asparagine/carbonyl pathway. The type of oil, on the other hand, could influence the rate of acrylamide formation. (10)

Acrylamide is known to have neuro-hepato- and geno-toxic effects. Neurotoxicity occurs in both the central and peripheral nervous systems, likely through microtubule disruption, which has been suggested as a possible mechanism for genotoxic effects of acrylamide in mammalian systems. The International Agency for Research on Cancer (IARC 1994) has classified acrylamide as probably carcinogenic to humans; (11) its prolonged exposure has induced tumors in rats. The causative agent in acrylamide - induced carcinogenesis is assumed to be its epoxy metabolite, glycidamide which in contrast to acrylamide gives rise to stable adducts to DNA.(12) Another study suggested that acrylamide itself but not its oxidative metabolite, appears to be involved in acrylamide- induced cellular transformation (13) In the present study, acrylamide level will be surveyed in 23 food items by GC/MS. Genotoxicity of the aqueous and organic extracts of 16 food items beside acrylamide and glycidamide will be assessed by Salmonella mutation test and the comet assay in vitro in human leukocytes. Also, the capacity of Pringles potato
crisps to induce leukocytes DNA breaks will be assessed in vivo in rat fed Pringles potato crisps.

MATERIALS AND METHODS

Source of the carbohydrate food items:
French fries, potato chips, corn based snack chips, breakfast cereals, breads and coffee were purchased directly from fast food restaurants and the local supermarkets in Jeddah, Saudi Arabia. Selections were made for brands which are very popular and commonly used by the public especially among children.

Pringles potato crisps preparation and animal feeding:
One group (3 male and 3 female) of Wistar rats (3 weeks of age 31g for male and 30g for female wt) received diet containing 45% Pringles potato crisps which gives final acrylamide concentration of 0.4µg/gram of the diet for 30 days, the other group received diet containing pure acrylamide (0.4µg/g of diet), and the control group which received standard granulated diet. These diets were prepared weekly. Diet and water was supplied ad libitum. Animals were housed singly in polycarbonate cages. The animal room was maintained on a 12 hours light/dark cycle, the temperature range was 20 to 24°C and the humidity range was 60 to 70%.

Aqueous and organic extraction of acrylamide from food:
Aqueous extraction of acrylamide from food items was initiated by adding 100ml water to 10gm of homogenized backed or fried carbohydrate food and shacked on orbiter shaker for 20min., then subsequently centrifuged at 9,000xg for 30min. at 4°C.(14) The supernatant was filtered through a 0.45µm millipore filter (Millipore Corp., Bedford, MA, USA).
The paste left over from the aqueous extract was further extracted with the organic solvent, dimethylsulfoxide (DMSO) to insure full recovery of all toxic materials in the food. The extraction was done as described by Dutcher et al. (15) Ten gram of the paste were extracted with 12ml of DMSO three times. The three fractions were collected together and diluted with 70ml double-distilled water. The above fraction was then extracted three times with dichloromethane (CH₂CL₂).The organic layer (lower) was washed two times with distilled water and dried under reduced pressure at 40°C. The extract was suspended in an appropriate volume of DMSO and used in this study as the organic solvent extract.

**Acrylamide quantitation by GC/MS:**

Twenty three food items were analyzed for their acrylamide contents using GC/MS. Ten grams of the food was grounded and homogenized; 1µg d³-acrylamide was added as an internal recovery standard and 2µg methacrylamide was added for brominization as a control. Acrylamide was extracted by a mixture of acetone and water. Following that, cleaning was carried and it included, degreasing, several SPE procedures and Carrez clarification which were done by adding bromine. Acrylamide was derivativized to 2, 3-dibrompropionamide overnight, followed by extraction using ethylacetate. After evaporating and another SPE cleaning step a lye-induced elimination was carried out, leading in monobrompropenamide. This was analyzed by means of GC/MS via (at least) two characteristic mass fragments. Quantification was carried out by an 8-point-calibration using d³-acrylamide as the internal standard.
Salmonella mutagenicity test (Ames test):

The *Salmonella typhimirium* mutant strains TA98, TA100 and TA1530 were used for the detection of mutagenicity. The procedure of Maron and Ames (1983) (16) for the preparation of liver microsomal fraction from the liver of rats was followed. Briefly, male Sprague-Dawley rat was injected intra peritoneal with phenobarbital at doses of 30mg/kg for the first 4 days and 60mg/kg on each of the following 3 days prior to killing the animal. β-naphthoflavone, suspended in corn oil, was given by the same route at a dose of 80mg/kg for 2 days prior to killing. The rats were killed by decapitation. The livers were homogenized in ice-cold 0.15M KCl solution in a teflon homogenizer.

After centrifugation (9000x g at 4°C for 10 min.), supernatant S9 was stored at -80°C until used. Mutagenicity test was performed by the plate-incorporation technique. The aqueous and organic extracts were tested on *S. typhimirium* strains with and without the metabolic activation rat liver homogenate S9 (10% S9, v/v). Each extracted sample was assayed in four doses, 1, 10, 50, 100mg/plate using two replica plates per dose. DMSO (100μl) was used as a negative control for organic extracts. Mutagenicity of pure acrylamide was assayed at doses of 4, 8, 20 and 40μg/plate, and glycidamide at doses of 0.08, 0.4, and 0.8μg/plate. Revertant colonies were scored automatically after 66 hours incubation at 37°C using image pro-plus software (media cybernitics, USA). Standard mutagens was used as positive controls included: Ethedium bromide 25μg/plate (with S9); sodium azide 1.5μg/plate (without S9); benzo (a) pyrine 10μg/plate (with S9). Positive response was defined by at least a two fold increase in revertants over the negative control.
Single cell gel electrophoresis (SCGE) or Comet assay:

Genotoxicity of 16 food aqueous and organic extracts, pure acrylamide and glycidamide were tested in vitro in peripheral blood leukocytes. Cells were incubated for four hours at room temperature with food extracts at concentrations of 0.1, 0.5, 1.0 and 5.0 mg/ml of blood, with acrylamide at 0.4, 0.8 and 4 µg/ml of blood and with glycidamide at 0.08, 0.2 and 0.4 µg/ml of blood. After incubation, cells were tested for viability by trypan blue exclusion test. Cells were collected by centrifugation and resuspended in 75 µl low melting point agarose gel for embedding on slides. Also, the genotoxicity was determined in vivo by subjecting the blood cells of rat fed Pringles potato crisps, acrylamide and glycidamide to comet assay.

Blood cells slide preparation and comet scoring:

The technique described by Singh et al. (17) was followed for the detection of DNA strand breaks by way of the alkaline comet assay. For the in vitro experiment, 1 ml of peripheral blood collected from rats tail vein was exposed to extracted materials, 1x10^5 of treated cells were combined with 100 µl of molten low melting agarose and pipetted onto comet slide (Trevigen, USA), then the slides were immersed in freshly prepared cold lysing solution (2.5M NaCl, 100mM Na2 EDTA, 10mM Tris, pH 10, 1% sodium sarcosinate). Just before use, 1% Triton X-100 and 10% DMSO was added to the lysing buffer. Slides then were immersed in the lysing solution for 20 minutes, then electrophoresis was performed at 1 volt/cm for 20 minutes. Slides were rinsed, immersed in 70% ethanol, air dried and stained with ethidium bromide (20 µg/ml), covered and placed in a humidified air-tight container and analyzed within 3-4 hours. Slides were examined at 40x magnifications using a fluorescence microscope equipped
with an excitation filter of 546nm and a barrier filter of 590nm. Images of 100 lymphocytes were randomly selected (50 cells from each of 2 replicate slides) and analyzed from each sample. Cells were automatically analyzed by Loats comet assay software which measures comet image parameters by using the Extended Dynamic Range Imaging (EDRI) technology. The EDRI Comet Analysis system provides a number of powerful analytic measures to characterize and quantify the degree of DNA damage revealed by the comet assay. These measures are automatically generates. These include standardized measures of comet tail length, tail area, tail moment, moment arm and moment of inertia, as well as measures of cellular DNA content, percent of total cellular DNA in the tail and a measure of total cellular DNA fragmentation. Similar procedure was carried for the in vivo experiment to test blood cells from rats fed Pringles potato crisps or pure acrylamide.

RESULTS

Amount of acrylamide in food as determined by GC/MS

In this study, 23 food samples were analyzed for their acrylamide contents. Different brands of French fries, potato chips, bread, crisp bread, biscuits, breakfast cereals and coffee were selected from local supermarkets in Jeddah, Saudi Arabia. Acrylamide levels found are summarized in Table 1. The levels of acrylamide ranged from non detectable to 2200µg of acrylamide per kg of food. The highest level (2200µg/kg) was found in stove roasted bread. Acrylamide level varied within the same food product category. In the French fries and potato crisps category, acrylamide level ranged from 172µg/kg in KFC French fries to 930µg/kg in Pringles potato crisps. Also, in the coffee category
the acrylamide level ranged from non detectable in the light and medium roasted coffee to 950µg/kg in the deep roasted Turkish coffee (Table 1).

Table (1): Amount of Acrylamide in Selected Foods Collected from Local Markets in Jeddah, Saudi Arabia

<table>
<thead>
<tr>
<th>No.</th>
<th>Food type</th>
<th>Acrylamide (µg/Kg of food)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Our findings</td>
</tr>
<tr>
<td>1</td>
<td>Pringles potato crisps</td>
<td>930</td>
</tr>
<tr>
<td>2</td>
<td>Lays potato chips</td>
<td>450</td>
</tr>
<tr>
<td>3</td>
<td>Mc Donald's French fries</td>
<td>273</td>
</tr>
<tr>
<td>4</td>
<td>KFC French fries</td>
<td>172</td>
</tr>
<tr>
<td>5</td>
<td>Tassali potato chips</td>
<td>320</td>
</tr>
<tr>
<td>6</td>
<td>Baik French fries</td>
<td>203</td>
</tr>
<tr>
<td>7</td>
<td>Whole wheat bread</td>
<td>380</td>
</tr>
<tr>
<td>8</td>
<td>White bread</td>
<td>230</td>
</tr>
<tr>
<td>9</td>
<td>Loaf bread</td>
<td>550</td>
</tr>
<tr>
<td>10</td>
<td>Samooli bread</td>
<td>580</td>
</tr>
<tr>
<td>11</td>
<td>Stove roasted bread</td>
<td>2200</td>
</tr>
<tr>
<td>12</td>
<td>Shaboora A (twice –backed bread)</td>
<td>80</td>
</tr>
<tr>
<td>13</td>
<td>Shaboora B (twice –backed bread)</td>
<td>41</td>
</tr>
<tr>
<td>14</td>
<td>Cookies</td>
<td>ND**</td>
</tr>
<tr>
<td>15</td>
<td>Tea biscuits</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>Puffed wheat with honey</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td>Corn flakes</td>
<td>18</td>
</tr>
<tr>
<td>18</td>
<td>Wafer</td>
<td>200</td>
</tr>
<tr>
<td>19</td>
<td>Chocolate cereals</td>
<td>130</td>
</tr>
<tr>
<td>20</td>
<td>Canned bran</td>
<td>ND</td>
</tr>
<tr>
<td>21</td>
<td>Arabic coffee (light roasted)</td>
<td>ND</td>
</tr>
<tr>
<td>22</td>
<td>Turkish coffee (medium roasted)</td>
<td>ND</td>
</tr>
<tr>
<td>23</td>
<td>Turkish coffee (deep roasted)</td>
<td>950</td>
</tr>
</tbody>
</table>

* WHO/FAO Acrylamide in Food Workshop (6)
ND** = Not detected.
Mutagenicity of food extracts, acrylamide and glycidamide in Salmonella test

Aqueous and organic extracts of 23 food samples tested at doses 1, 10, 50, 100mg/plate and pure acrylamide at doses of 10, 100, 250 and 500mM/plate did not show any mutagenicity effect in the Salmonella mutagenicity test, with the three mutant strains TA98, TA100 and TA1535 in presence or absence of the metabolic activation system S9, while glycidamide showed clear mutagenicity at doses of 1, 5 and 10 mM/plate and in the presence of S9 with TA100 strain. The number of revertant colonies increased with the increase of glycidamide concentration (Table 2). Guttman split-half reliability test was also excellent (Chronbach’s alpha = 0.95).

Table (2): Genotoxicity of Glycidamide as Determined by the Salmonella Test Using TA100 Mutant and in the Presence of S9

<table>
<thead>
<tr>
<th>Glycidamide (mM)/plate</th>
<th>Bacterial revertants/plate (spontaneous revertants = 80)</th>
<th>Mean comet tail moment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>305</td>
<td>5</td>
</tr>
<tr>
<td>2.5</td>
<td>Not done</td>
<td>20.5</td>
</tr>
<tr>
<td>5.0</td>
<td>1370</td>
<td>52</td>
</tr>
<tr>
<td>10</td>
<td>3800</td>
<td>75</td>
</tr>
</tbody>
</table>

Acrylamide and glycidamide genotoxicity as determined by comet:

Exposing human peripheral blood leukocytes to pure acrylamide at doses of 5, 10 and 50mM per ml of blood did not cause any DNA breaks as revealed by image analysis and the absence of any comet formation. The comet tail moment for
acrylamide was 0.01 which is similar to the tail moment value for the control (untreated lymphocytes) (Table 3). On the other hand glycidamide induced substantial DNA breaks as indicated by the increase in the comet tail moment. The increase in the tail moment was directly proportional to the increase in the glycidamide concentration as the mean comet tail moment has increased from 5 to 75 with the increase of glycidamide concentration from 1 to 10 mM/plate (Table 2).

**Genotoxicity of food aqueous and organic extracts as determined by comet:**

The extent of DNA damage caused by the aqueous extract of the different food items in the comet assay in vitro is presented as the value of the tail moment (length of the tail x the intensity of EtBr stain). Results in (Table 3a) show that only the aqueous extracts of Lays and Tassali potato chips at doses of 1 and 5 mg exhibited moderate DNA breaks but not with the lower doses (0.1 or 0.5 mg). Three and 5 percent of the cells exposed to Lays exhibited comet with a tail moment of 3 and 4 at extracts of 1 and 5 mg respectively. As for Tassali potato chips it was less genotoxic than Lays, despite the fact that the percentage of cells with comet was almost the same (3 and 4) as Lays, the comet tail moments were only 0.6 and 0.8 at extract doses of 1 and 5 mg respectively. All other food aqueous extracts did not show any DNA breaks and no comet formation at all doses tested (0.1, 0.5, 1, and 5 mg,).
In the organic food extracts, Lays potato chips showed almost similar results to the aqueous extract. On the other hand, cells exposed to Tassali potato chips organic extract did not show any comet, unlike the aqueous extract. Pringles potato crisps showed very small comet tail moment, 0.04 at 1 and 5 mg of extracts. All other food organic extracts did not show any comet and the tail moment was 0.01 which is similar to the value given by the untreated leukocytes control (Table 3b).
Comet formation in vivo in rat cells fed acrylamide and Pringles potato crisps

Acrylamide and Pringles potato crisps induced extensive DNA breaks in rat leukocytes in vivo. The mean comet tail moments in leukocytes from 6 rats fed acrylamide and Pringles potato crisps were 122.15 and 44.32 respectively compared with 0.03 for leukocytes of animal fed normal diet (control). Also the amount of DNA in the comet tails of the animal fed acrylamide and Pringles potato crisps was 87.7% and 45.46% respectively (Fig.1b, c), compared with the control which was 0.86 (Fig.1a). The fact that pure acrylamide and Pringles potato crisps which contains 930µg/kg acrylamide did not induce any DNA breaks in vitro implies that acrylamide is not the direct genotoxicant.

Fig.1a: Photographic Image of Normal Rat Leukocytes Subjected to Single Cell Gel Electrophoresis (Comet) and Analyzed by Image Analysis System, Tail Moment =0.03
Fig. 1b: Photographic Image of Leukocytes from Rat Fed Pure Acrylamide and Subjected to Single Cell Gel Electrophoresis Subsequently Analyzed by Image Analysis System, Tail Moment = 112.35 (Index 17)

Fig. 1c: Photographic Image of Leukocytes from Rat Fed Pringles Potato Crisps and Subjected to Single Cell Gel Electrophoresis Subsequently Analyzed by Image Analysis System, Tail Moment = 35.58 (Index 12)
DISCUSSION

The genotoxic potential of acrylamide in food and its impact on cancer risk in humans is of great concern. Food mutagens cause different types of DNA damage. However, the effect of food mutagens in carcinogenesis can be modified by heritable traits, namely, low-penetrant genes that affect mutagen exposure of DNA through metabolic activation and detoxification or cellular responses to DNA damage through DNA repair mechanisms or cell death.\(^{18}\) Survey for the determination of acrylamide in various Saudi marketed food, showed considerable variations in acrylamide levels within each food group. Similar variations have been reported for the same brands examined elsewhere.\(^{14}\) This high variability is mainly due to the variability in the raw materials source, processing conditions (temperature, time, etc.) and the procedure used for the analysis.\(^{19}\) The presence of high levels of acrylamide in white bread stove toasted bread (2200µg/kg) compared to 230µg/kg and 380µg/kg in white bread and whole wheat bread respectively, demonstrates the influence of cooking temperature and duration on acrylamide formation and verified the original Swedish observation that acrylamide is formed primarily in carbohydrate-rich food prepared or cooked at high temperatures.\(^{20}\) Also, the influence of temperature and roasting time on acrylamide formation in coffee beans is evident from the present results which showed that deep roasted coffee contains 950µg/kg of acrylamide compared to non-detectable value in the light or medium roasted coffee.

Analyses conducted on two samples of twice baked bread (shaboora) and have the same degree of browning showed that shaboora A has twice the amount of acrylamide compared to shaboora B, 80 and 41µg/kg, respectively. This suggests that acrylamide formation in carbohydrate-rich foods is not only
influenced by cooking procedures (cooking temperature and duration) but also by food processing such as fermentation times and the material used for fermentation.

In the present study pure acrylamide did not induce any mutation in *Salmonella typhimirium* strains TA98 TA100 and TA1535, in presence or absence of S9, acrylamide has repeatedly been reported to deliver negative results in bacterial gene mutation assays, in the presence or absence of activating systems (21), despite the fact that it induces sister chromatid exchanges and chromosomal aberrations in mammalian cells in vitro. (22) On the other hand, glycidamide which is one of acrylamide metabolites showed in this study clear mutagenicity with TA100 only and in the presence of S9 but not with strains TA98 and TA1535. Other studies showed that glycidamide induced mutation in the absence of an exogenous metabolic system. (23)

Investigating the DNA damaging potency of acrylamide and its metabolite glycidamide in vitro using normal human leukocytes and the comet assay revealed that acrylamide did not cause any DNA breaks in vitro, on the other hand, we found that glycidamide is substantial genotoxic in human peripheral leukocytes with highly significant induction of DNA strand as indicated by the increase in the comet tail moment. Despite the fact that acrylamide is a known carcinogen in rodents, the underlying mechanism of action is not fully understood. Acrylamide itself is known to react quite slowly with DNA, its metabolite glycidamide formed by CYP2E1, is proposed to represent the ultimate carcinogen. (24) Acrylamide and glycidamide formed DNA adducts at similar specific locations but DNA adduct formation was found to be more pronounced after
glycidamide treatment than after acrylamide treatment and eventually glycidamide was more mutagenic than acrylamide. Accordingly the genotoxicity of acrylamide in human cells is based on the capacity of its epoxide metabolite glycidamide to form DNA adducts.\(^{24, 25}\) In supporting of this, our data showed that Pringle potato crisps aqueous or organic extracts did not induce any DNA breaks in vitro in human leukocytes. However, leukocytes of rat fed Pringles potato crisps showed extensive DNA breaks and large comet tail moment which may imply that the genotoxicity of acrylamide in the food is caused by its metabolites after it is ingested. The in vitro DNA breaks induced by Lays and Pringles potato crisps in this study raise the possibilities that other toxic materials in the food extracts could be causing additional DNA damage and it should not be overlooked.

**CONCLUSION**

The acrylamide content in 23 randomly collected samples of potato crisps, French fries, and various heat-processed foodstuffs varies. In the crisp and fries samples, acrylamide ranged from 172 to 930 microg/kg. The factor determining the differences in acrylamide content in the product was the manufacturer, suggesting the effects of various technological processes and raw material types used. In the coffee samples, acrylamide content ranged from non-detectable in the light and medium roasted coffee to 950 microg/kg in the deep roasted samples. In vitro, neither acrylamide nor food aqueous or organic extracts showed genotoxic effects in the Salmonella or the comet tests. In vivo, leukocytes from rat fed Pringles crisps showed extensive DNA damage in the comet test, suggesting
that acrylamide metabolite is the genotoxicant in blood Leukocytes.

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