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Gas Chromatographic Investigation of Acrylamide Formation in Browning Model Systems

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Acrylamide formed in browning model systems was analyzed using a gas chromatograph with a nitrogen−phosphorus detector. Asparagine alone produced acrylamide via thermal degradation at the level of 0.99 μg/g of asparagine. When asparagine was heated with triolein—which produced acrolein at the level of 1.82 ± 0.31 ($n = 5$) mg/L of headspace by heat treatment—acrylamide was formed at the level of 88.6 μg/g of asparagine. When acrolein gas was sprayed onto asparagine heated at 180 °C, a significant amount of acrylamide was formed (114 μg/g of asparagine). On the other hand, when acrolein gas was sprayed onto glutamine under the same conditions, only a trace amount of acrylamide was formed (0.18 μg/g of glutamine). Relatively high levels of acrylamide (753 μg/g of ammonia) were formed from ammonia and acrolein heated at 180 °C in the vapor phase. The reaction of acrylic acid, which is an oxidation product of acrolein and ammonia, produced a high level of acrylamide (190 000 μg/g of ammonia), suggesting that ammonia and acrolein play an important role in acrylamide formation in lipid-rich foods. Acrylamide can be formed from asparagine alone via thermal degradation, but carbonyl compounds, such as acrolein, promote its formation via a browning reaction.

KEYWORDS: Acrylamide; acrolein, asparagine; browning reaction; gas chromatography

INTRODUCTION

The presence of acrylamide in foods has been recently reported by European researchers (*1*, *2*). After these papers, many institutions have begun to analyze acrylamide in food products. For example, the U. S. Food and Drug Administration reported the analysis of acrylamide in 286 commercial food products (*3*). Their results ranged from none detected to 1184 ppb (Lipton Recipe Secrets Onion Soup and Dip Mix).

The method commonly used for acrylamide analysis is the high-performance liquid chromatography (HPLC)/mass spectrometry (MS) method. A commercial LC/MS instrument became available in 1980. However, it is still a very expensive instrument. The price of LC/MS today ranges from $200,000 to $300,000. Therefore, it is difficult to study acrylamide in food products in individual laboratories. In the present study, a less expensive gas chromatographic method for acrylamide analysis was developed using browning mode systems.

Significant amounts of acrylamide (221 mg/mol of amino acid) formation were reported in a browning model system that consisted of an equimolar of asparagine and glucose heated at 185 °C (*4*). Tremendous numbers of chemicals are formed in a browning reaction, which occurs from the interaction between carbonyl compounds (aldehydes, ketones, sugars, carbohydrates, and lipids) and amine compounds (ammonia, alkylamines, amino acids, proteins, peptides, and phospholipids) upon heat treatment (*5*, *6*). Some of these browning reaction products, which contain a three carbon unit (e.g., acrolein, propionaldehyde, propanenitrile, propioamide, and methylglyoxal), can be precursors of acrylamide. In addition, a series of alkyl amides (e.g., propionamide—which are closely related to acrylamide in their structure) have been found in many sugar/amino acid browning model systems. For example, *n*-alkylamides and N-alkyl *n*-amides were formed in beef fat heated with glycine at 200 °C (*7*). In the present study, therefore, simple chemicals such as acrolein and ammonia, in addition to asparagine and triolein, were used for the preparation of browning reaction mixtures.

MATERIALS AND METHODS

Chemicals and Reagents. Triolein, L-asparagine monohydrate, L-(+)-glutamine, glycerol, and acrylic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acrylamide and acrolein were bought from Tokyo Kasei Kogyo, Co., Ltd. (Tokyo, Japan). Ammonium solution (special reagent grade) was from Kanto Kagaku, Co., Ltd. (Tokyo, Japan).

Measurement of Detection and Quantitative Limits of Acrylamide. A standard solution of acrylamide was prepared with ethyl acetate (1 mg/L). Detection and quantitative limits were determined using a

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method previously reported (*8*). A standard solution of 1 μL of acrylamide (1 ng of acrylamide) was injected into GC. The injection was duplicated six times.

Recovery Tests on Acrylamide from Silica Gel Column Chromatography. Silica gel chromatography was used to clean up samples. Acrylamide (400 μg) in 1 mL of dichloromethane was placed in a glass column (185 mm × 15 mm o.d.) packed with 10 g of silica gel and subsequently developed with 100 mL each of dichloromethane, methanol/ethyl acetate (1/19, v/v), and methanol/ethyl acetate (1/19, v/v) in series. Acrylamide was analyzed by a gas chromatograph/mass spectrometer (GC/MS).

Recovery Tests on Acrylamide from Triolein. Triolein (5 g) spiked with acrylamide (100 μg) was placed in a 300 mL separatory funnel with 130 mL of a hexane/methanol (10/3) solution, and then, the solution was shaken for 10 min. After the methanol layer was removed, the residual hexane solution was extracted with 30 mL of methanol. The methanol layer was added to the original methanol solution. After the methanol solution was filtered with a glass fiber, the methanol filtrate was condensed with a rotary evaporator under reduced pressure. The methanol was removed further with a purified nitrogen stream. The brown viscous material obtained was dissolved into 100 mL of dichloromethane. This dichloromethane solution was transferred into a glass column (185 mm × 15 mm o.d.) packed with 10 g of 75−150 μm mesh silica gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and anhydrous sodium sulfate (1 g) was placed on the top of the silica gel. After all of the dichloromethane was eluted, acrylamide was eluted with a 100 mL methanol/ethyl acetate (5/95) solution. The eluate was condensed with a rotary evaporator, and then, it was condensed further with a purified nitrogen stream. The residual brown viscous material was dissolved in ethyl acetate, and then, the volume of the sample solution was adjusted to exactly 5 mL with ethyl acetate. The ethyl acetate solution was analyzed for acrylamide by a GC/nitrogen phosphorus detector (NPD).

Recovery Tests on Acrylamide from Solid Matrix. Soy protein (50 g), starch (50 g), and acrylamide (1 mg) were mixed well with 125 mL of water in an aluminum pan (245 mm × 180 mm × 30 mm depth). After water was removed from the mixture in an electric dryer at 200 °C, a part of the residual solid material (10 g) was ground and then added into 50 mL of methanol in a 100 mL flask. The methanol solution was sonicated for 20 min. After the solution was filtered, methanol was removed from the filtrate by a rotary evaporator. Methanol was further removed by a purified nitrogen stream. The residual material was treated with silica gel column chromatography and analyzed for acrylamide as described above. The blank sample was prepared with the same mixture without acrylamide. The recovery values were adjusted to the blank values.

Sample Preparation of Reaction Mixtures from Thermal Degradation of Asparagine and Glutamine. Two grams of asparagine monohydrate (1.76 g as asparagine) was heated in a 50 mL recovery flask (unsealed) at 180 °C for 30 min in an oil bath. Glutamine (2 g) was placed in a watch glass in a stainless steel beaker and then heated at 180 °C for 30 min in an oil bath—a watch glass was used for glutamine because the solid formed after heating was very hard and difficult to remove from a flask. The reaction mixture became solid after it was cooled to room temperature. The solid materials were transferred into a mortar and then ground into powder.

After 50 mL of methanol was added to the reaction mixture (from asparagine) or the powder (from glutamine), the solution was sonicated for 20 min. After the methanol solution was filtered, the methanol filtrate was condensed with a rotary evaporator under reduced pressure. The methanol was further removed with a purified nitrogen stream. The brown viscous material obtained was treated with silica gel column chromatography as described above for acrylamide analysis.

Analysis of Acrolein Formed in Headspace of Heated Triolein. Triolein (5 g) was heated in a three-necked round bottom flask at 180 °C for 30 min. Acrolein formed in the headspace was collected by a 100 μL gastight syringe (Supelco, Bellefonte, PA), and then, 10 μL of the headspace gas was injected into a GC/flame ionization detector.

Sample Preparation of Reaction Mixtures of Triolein and Amino Acids. Triolein (5 g) and 2 g of asparagine or 2 g of glutamine were mixed in a 50 mL recovery flask. The mixture was heated at 180 °C for 30 min in an oil bath. The reaction mixture was transferred into a 300 mL separatory funnel with a 130 mL hexane/methanol (10/3) solution. The solution was subsequently treated in exactly the same way as the method described for Recovery Tests on Acrylamide from Triolein.

Sample Preparation of Reaction Mixtures of Amino Acids and Ethylene Glycol or Glycerol. Asparagine or glutamine (2 g each) and 5 mL each of ethylene glycol or glycerol were mixed in a 50 mL recovery flask, which was subsequently heated at 180 °C for 20 min. The reaction mixtures (1 mL each) were treated with silica gel column chromatography as described above for acrylamide analysis.

Sample Preparation of Reaction Mixtures of Amino Acids and Acrolein. Amino acid (2 g of asparagine or glutamine) was heated to 180 °C in a 50 mL recovery-flask, into which acrolein gas was subsequently sprayed for 30 min using a gas washing bottle purging with a highly purified nitrogen stream (acrolein concentration in a gas phase = 117 mg/L). After 50 mL of methanol was added to the reaction mixture, the solution was sonicated for 20 min. The solution was filtered and then treated with silica gel chromatography as described above for acrylamide analysis.

Sample Preparation of Reaction Mixtures of Acrolein and Ammonia. Silica gel (2 g) in a 50 mL recovery flask was sprayed with ammonia gas using a gas washing bottle purging with air, and simultaneously, it was sprayed with acrolein gas using the method described above at various temperatures in an oil bath for 30 min. After 50 mL of methanol was added to the flask, the samples were treated using the procedure described above for acrylamide analysis. A final sample obtained from this experiment was also analyzed for acrylamide using LC/MS to confirm the GC/MS method.

Sample Preparation of Reaction Mixtures of Acrylic Acid and Ammonia. Silica gel (2 g) and 2 g of acrylic acid in a recovery flask was sprayed with ammonia gas using the method described above for 30 min at 180 °C in an oil bath. After 50 mL of methanol was added to the reaction mixture, the samples were treated using the same procedure as described above for acrylamide analysis.

Sample Preparation for LC/MS Analysis. A 1 g aliquot of the ethyl acetate sample prepared above was weighed into a 15 mL disposable Corning centrifuge tube (concurrent recoveries were fortified at this point with D_3-acrylamide), and 10 mL of water was added. The sample was placed on a platform shaker for 20 min at 150 rpm, followed by centrifugation for 30 min at about 2800 rpm. The supernatant was removed (5 mL) and filtered through a Whatman 5.0 μm nylon with glass fiber disposable filter into a 12 mL tube.

An Oasis HLB solid phase extraction (SPE) cartridge (0.2 g/6 mL, Waters Corp, Milford, MA) was conditioned with 1 column volume of methanol followed by 1 column volume of water. Each conditioning solvent was allowed to flow by gravity. An aliquot of the sample (0.2 g) was then cleaned with the SPE. The eluant was discarded. Acrylamide residues were eluted with 3 mL of water into a 15 mL graduated centrifuge tube. The final sample volume was adjusted, and an aliquot was filtered through a 0.2 μm Acrodisc (Pall Corp., Ann Arbor, MI) into an autosampler vial.

Acrylamide Analysis by GC/NPD and GC/MS. A Hewlett-Packard (HP) 5890A GC equipped with a 30 m × 0.25 m i.d. (d_f = 0.25 μm) DB-WAX fused silica capillary column (J & W Scientific, Folsom, CA) and a NPD was used for routine acrylamide analysis. The oven temperature was held at 50 °C for 1 min and then programmed to 180 °C at 30 °C/min and to 210 °C at 5 °C/min. Injector and detector temperatures were 250 and 280 °C, respectively. Linear velocity of helium carrier gas was 27 cm/s in splitless mode.

An Agilent 6890 Series GC interfaced to an Agilent MS with an Agilent 5973 N mass selective detector was used to confirm acrylamide in samples. The GC was equipped with a 30 m × 0.25 mm i.d. (d_f = 0.30 μm) DB-FFAP (J & W Scientific). The oven temperature was held at 40 °C for 2 min and then programmed to 250 °C at 5 °C/min and held for 10 min. The injector temperature was 280 °C. The linear velocity of helium carrier gas was 36 cm/s in splitless mode.

Acrylamide Analysis by LC/MS Sample Analysis. Sample analysis was conducted with a Perkin-Elmer Series 200 autosampler and micropump (Perkin-Elmer, Shelton, CT) coupled to a PE Sciex API 2000 tandem mass spectrometer via an atmospheric pressure chemical

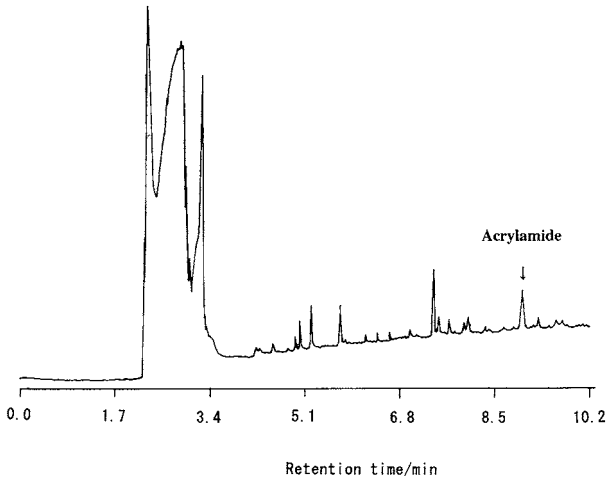

Figure 1. Typical gas chromatogram of a sample obtained from asparagine heated at 180 °C. Refer to the Materials and Methods for GC conditions.

ionization (APCI) source (PE Biosystems, Walnut Creek, CA). The APCI source was operated in positive ionization mode at 475 °C with nitrogen gas. The MS was operated in multiple reactant monitoring mode to observe the transition of *m*/*z* 72 to *m*/*z* 55 for acrylamide and *m*/*z* 75 to *m*/*z* 58 for deuterated acrylamide (via collision-induced dissociation with nitrogen gas). Chromatographic separation was accomplished with a Thermo Hypersil-Keystone Hypercarb (catalog no. 35005-052131, 50 mm × 2.1 mm i.d., 5 μm particle size). The autosampler was programmed to inject 5 μL. The mobile phase condition was isocratic at 99/1 acetic acid (0.1%)/methanol with a flow rate of 400 μL/min.

RESULTS AND DISCUSSION

Because acrylamide is highly soluble in water (215.5 g/100 mL) and less soluble in organic solvents (155 g/100 mL methanol, 12.6 g/100 mL ethyl acetate, and 0.0068 g/100 mL hexane), sample preparation steps for GC analysis are significantly difficult. Therefore, many methods were tested for sample preparations and the method reported in the Materials and Methods was found to be the best method at this point.

The detection and quantitative limits of GC/NPD were 0.20 and 0.67 ng, respectively, in the present study. **Figures 1** and **2** show typical gas chromatograms of samples obtained from asparagine alone and from an asparagine/triolein model system, respectively.

The recovery of acrylamide from silica gel chromatography was 0% by 100 mL of dichloromethane (fraction 1), 100% by 100 mL of methanol/ethyl acetate (1/19, v/v; fraction 2), and <0.01% by methanol/ethyl acetate (1/19, v/v, fraction 3). Therefore, the optimum condition for silica gel cleanup was to wash a column with 100 mL of dichloromethane and then to elute acrylamide with 100 mL of methanol/ethyl acetate (1/19, v/v).

Recovery efficiencies of acrylamide from triolein and solid matrix were 90 and 85%, respectively. The results indicate that the method developed in the present study is satisfactory.

Table 1 shows the results of acrylamide analysis in various browning model systems. The values are average of two experiments. It was hypothesized that asparagine and glutamine alone produced acrylamide upon heat treatment because they contain an amide moiety in their molecule. Asparagine alone produced acrylamide (0.99 μg/g of asparagine) upon thermal degradation, while glutamine produced 0.17 μg/g under same conditions. When asparagine was heated at 180 °C with glucose, a large amount of acrylamide (1200 μg/g of asparagine) was formed. The result was similar to the previously reported value

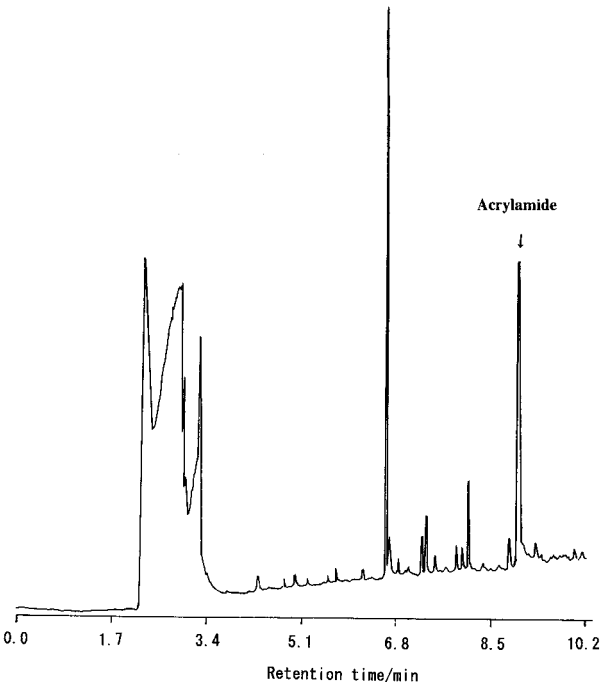

Figure 2. Typical gas chromatogram of a sample obtained from an asparagine/triolein model system. Refer to the Materials and Methods for GC conditions.

Table 1. Results of Acrylamide Analysis in Browning Model Systems

reactants			
amines (amount)	carbolys (amount)	temp (°C)	amount of acrylamide (μg/g of amine)
L-asparagine (1.76 g)		180	0.99
L-asparagine (1.76 g)	triolein (5 g)	180	88.6
L-asparagine (1.76 g)	glucose	180	1200
L-(+)-glutamine (2 g)		180	0.17
L-(+)-glutamine (2 g)	triolein (5 g)	180	3.53
ammonium chloride (1 g)	triolein (5 g)	180	0.51
L-asparagine (1.76 g)	ethylene glycol (5 g)	180	<0.01
L-asparagine (1.76 g)	glycerol (5 g)	180	4.42
L-asparagine (1.76 g)	acrolein (0.878 g)[a]	180	114
L-(+)-glutamine (2 g)	glycerol (5 g)	180	0.34
L-(+)-glutamine (2 g)	acrolein (0.878 g)	180	0.18
ammonium chloride (2 g)	acrolein (0.878 g)	180	<0.01
ammonia (0.329 g)	acrylic acid (2 g)	180	190 000

[a] Acrolein was supplied as a gas phase, whose concentration was 117 mg/L in nitrogen, at a flow rate of 250 mL/min for 30 min. [b] Ammonia was supplied as a gas phase, whose concentration was 73.0 mg/L in air, at a flow rate of 150 mL/min for 30 min.

(1672.7 μg/g of asparagine) (*4*), suggesting that acrylamide formation was promoted by glucose via the browning reaction. As mentioned above, the browning reaction occurs from interaction between an amine and a carbonyl compound. Many cooking practices involve the use of oils, which can be a carbonyl source for the browning reaction. Therefore, triolein was used as one of the carbonyl reactants in the present study. When asparagine was heated with triolein at 180 °C, acrylamide formed at the level of 88.6 μg/g of asparagine. Formation of ammonia and glycerol was observed in this reaction mixture. It is well-known that α-amino acid produces ammonia via Strecker degradation in the presence of a carbonyl compound (*9*). **Figure 3** shows the hypothesized formation mechanisms of acrylamide from amino acids and lipids. When asparagine was reacted with glycerol, acrylamide formed at the level of 4.42 μg/g of asparagine, whereas asparagine did not produce

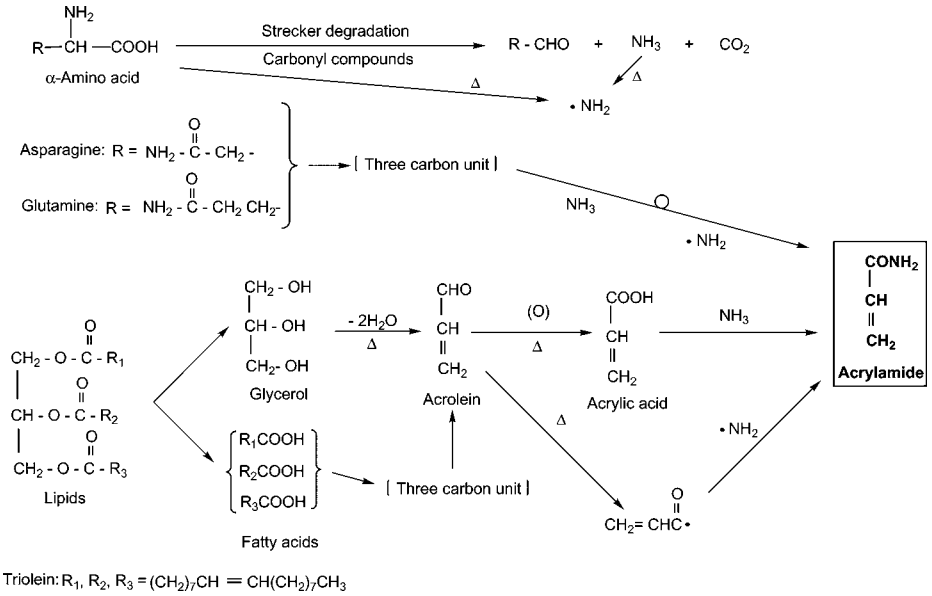

Figure 3. Hypothesized formation mechanisms of acrylamide from an amino acid and a lipid.

detectable amounts of acrylamide with ethylene glycol. The results suggest that the acrylamide formation is accelerated by a carbonyl compound and that a three carbon unit, such as glycerol, is required for acrylamide formation. Also, when ammonium chloride was heated with triolein as an ammonia source, acrylamide formed at the level of 0.51 μg/g of ammonium chloride. From these results, it is hypothesized that glycerol produced from lipids, such as triolein, forms acrolein via a dehydration reaction. Acrolein was oxidized to give acrylic acid, which subsequently reacted with ammonia from asparagine to yield acrylamide (*10*).

It is well-known that lipids (triglycerides) produce a large amount of acrolein by heat treatment. For example, when various cooking oils were heated at 300 °C for 2 h, large amounts of acrolein formed (*11*). Acrolein in the headspace of heated corn oil increased considerably when the oil was heated above 200 °C (*12*). When triolein was heated at 180 °C for 30 min, acrolein formed at the level of 1.82 ± 0.31 ($n = 5$) mg/L of headspace gas in the present study. The reaction of acrylic acid and ammonia produced a great amount of acrylamide (190 000 μg/g of ammonia), suggesting that ammonia and acrolein play an important role in acrylamide formation in lipid-rich foods upon heat treatment.

When acrolein gas was sprayed onto asparagine heated at 180 °C, significant amounts of acrylamide were formed (114 μg/g of asparagine). On the other hand, when acrolein gas was sprayed onto glutamine under the same conditions, acrylamide was formed only at the level of 0.18 μg/g of glutamine. Even though both amino acids contain an amide moiety, asparagine produced much more acrylamide than glutamine did in the model systems used in the present study. This may be due to differences in the amount of ammonia formation from asparagine and glutamine rather than due to their amide moiety. Also, asparagine may produce a three carbon unit more readily than glutamine does.

Figure 4 shows the amounts of acrylamide formed from the reaction of ammonia and acrolein at various temperatures. Acrylamide formed even at room temperature. Acrylamide formation increased with temperatures up to 180 °C and then reduced at 200 °C. The results suggest that acrylamide formation does not require very high temperatures. It is also reported that acrylamide formation from a reaction of asparagine and glucose reached its highest at a temperature around 170 °C (*4*).

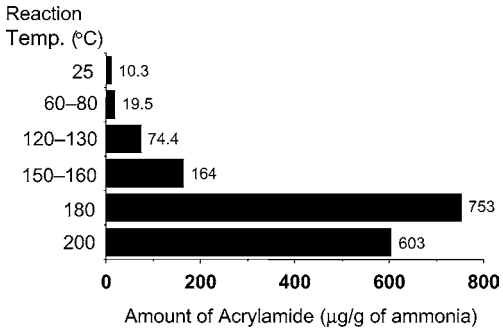

Figure 4. Acrylamide formed from ammonia and acrolein at various temperatures.

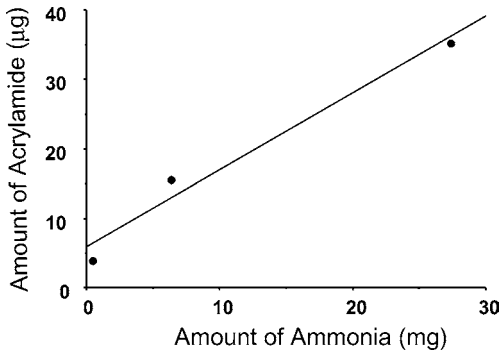

Figure 5. Amount of acrylamide formed from different amounts of ammonia in an ammonia/acrolein model system.

Figure 5 shows the amount of acrylamide formed from different amounts of ammonia (0.42, 6.56, and 27.0 mg) in an ammonia/acrolein model system. The values are the average of two experiments. In this system, the amount of acrolein (100 mg) was kept constant. The total amount of acrylamide formed was proportional to the amount of ammonia reacted. The relationship between the amount of ammonia (X, mg) and the amount of acrylamide formed (Y, μg) was written as $Y = 1.107X + 5.8846$, $R^2 = 0.971$.

The results obtained in the present study suggest the following formation pathways for acrylamide (refer to **Figure 3**): (i) Acrylamide forms from an amino acid alone upon thermal degradation. In this case, a three carbon unit is provided by an

amino acid. (ii) Ammonia produced from α-amino acids via Strecker degradation in the presence of carbonyl compounds reacts with acrylic acid, which is produced from acrolein, to give acrylamide. Acrolein forms from lipids (triglyceride) at high temperature treatment. (iii) An acrylic radical formed from homolytic fusion of acrolein at high temperatures absorbs an amine radical formed from amino acid at a high temperature treatment to yield acrylamide. In the case of ii and iii, acrolein also forms from a compound with a three carbon unit.

α-Amino acids, in particular asparagine, may play an important role in acrylamide formation in foods (*4*, *13*). However, acrylamide can also form from many different food constituents in addition to amino acids. Many foods and beverages that have undergone certain heat treatments may produce acrylamide via nonenzymatic browning reactions. It should be noted that browning reactions involve numerous types of chemical reactions, such as oxidation, reduction, dehydration, hydrolysis, and dehydrogenation as well as radical reactions. Also, it is very important to consider that formation of acrylamide in foods is at extremely low levels. Therefore, it may not be possible to explain every acrylamide formation by conventional organic reaction mechanisms.

When the sample obtained from an ammonia/acrolein model system heated at 180 °C was analyzed for acrylamide by LC/MS, a level of 507 μg/g of ammonia was found. This level was lower than that obtained by GC. This may be due to additional cleanup performed with the SPE. The result indicates that a GC/NPD method was comparable to a LC/MS method. The gas chromatographic method provided satisfactory results on acrylamide analysis in the present study.

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