Acrylamide Formation from Asparagine under Low-Moisture Maillard Reaction Conditions. 1. Physical and Chemical Aspects in Crystalline Model Systems

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The formation of acrylamide in crystalline model systems based on asparagine and reducing sugars was investigated under low-moisture reaction conditions. The acrylamide amounts were correlated with physical changes occurring during the reaction. Molecular mobility of the precursors turned out to be a critical parameter in solid systems, which is linked to the melting behavior and the release of crystallization water of the reaction sample. Heating binary mixtures of asparagine monohydrate and anhydrous reducing sugars led to higher acrylamide amounts in the presence of fructose compared to glucose. Differential scanning calorimetry measurements performed in open systems indicated melting of fructose at 126 °C, whereas glucose and galactose fused at 157 and 172 °C, respectively. However, glucose was the most reactive and fructose the least efficient sugar in anhydrous liquid systems, indicating that at given molecular mobility the chemical reactivity of the sugar was the major driver in acrylamide formation. Furthermore, reaction time and temperature were found to be covariant parameters: acrylamide was preferably formed by reacting glucose and asparagine at 120 °C for 60 min, whereas 160 °C was required at shorter reaction time (5 min). These results suggest that, in addition to the chemical reactivity of ingredients, their physical state as well as reaction temperature and time would influence the formation of acrylamide during food processing.

KEYWORDS: Acrylamide; asparagine; glucose; fructose; galactose; Maillard reaction; low moisture systems; molecular mobility; crystalline state; crystallization water; HPLC; DSC; PTR-MS

INTRODUCTION

Since the first acrylamide alert in 2002 (*1*) and the discovery of relatively high amounts of acrylamide in carbohydrate-rich foods obtained by thermal processing, numerous studies have been performed to help understand acrylamide formation (reviewed in refs *2* and *3*). Apart from analytical method development and quantification of acrylamide in foodstuffs, research has been focused on elucidation of formation mechanisms from asparagine via Maillard-type reactions (*4–12*) and from lipids (*9*, *13*, *14*), as well as mitigation studies to reduce the acrylamide amounts in the final product (*15–19*). We have also reported on the feasibility of monitoring on-line acrylamide by proton-transfer reaction mass spectrometry (PTR-MS) (*20*). In addition, the formation mechanisms have been elucidated and extended to vinylogous compounds generated from amino acids under low-moisture reaction conditions (*12*, *21*).

The potential of generating acrylamide from suitable precursors has mainly been attributed to the concentration of asparagine, which directly provides the backbone of the acrylamide molecule (*5*, *10*). However, there is some indication in the literature that the type of sugar, or in general the carbonyl compound, may significantly affect the final amount of acrylamide generated through the Maillard reaction (*5*, *10*, *12*, *20*). In our previous work, we found for example that fructose was, by a factor of about 3, more efficient compared to glucose under the same pyrolytic conditions (*12*, *20*). However, no plausible explanation has been provided so far for these findings, which are rather surprising from a purely chemical point of view.

In our recent paper (*12*), we have speculated on the role of physical properties of acrylamide precursors on the formation of this processing contaminant and suggested the melting point of sugars as a possible parameter to consider. To better understand the interplay between chemical reactivity and physical changes in acrylamide formation, we investigated binary mixtures of asparagine monohydrate and reducing sugars, such as glucose, fructose, and galactose, under low-moisture Maillard reaction conditions to elucidate the major factor(s) driving acrylamide formation.

EXPERIMENTAL PROCEDURES

Materials. L-Asparagine monohydrate (Asn·H₂O), D-fructose (Fru), D-glucose (Glc), D-galactose (Gal), and acrylamide were from Fluka/ Aldrich (Buchs, Switzerland). Formic acid, dimethyl sulfoxide (DMSO),

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and water for HPLC were purchased from Merck (Darmstadt, Germany). Acetonitrile was obtained from Baker (Deventer, The Netherlands). The filter units Spartan 13/30 were purchased from Schleicher & Schuell (Dassel, Germany). All other reagents were of analytical grade and were used without further purification. **Caution:** *Acrylamide (CAS 79-06-1) is classified as toxic and may cause cancer. Wear suitable protective clothing, gloves, and eye/face protection when handling this chemical.*

Proton-Transfer Reaction Mass Spectrometry. The PTR-MS from Ionicon Analytik (Innsbruck, Austria) was used to follow on-line generation and release of acrylamide in model systems. The basic experimental setup for the time-resolved measurement of acrylamide by PTR-MS has previously been described (*20*). For all experiments discussed here, the full mass spectrum from m/z 21 to 220 was monitored by PTR-MS on-line, with a 0.2 s dwell time per mass to follow all compounds released from the model reaction mixture. In parallel, the oven and the sample temperatures were monitored as well as the specific m/z 72 corresponding to the protonated acrylamide with a 2 s dwell time to have a more precisely recorded signal. Equimolar mixtures of Fru/Asn·H₂O, Glc/Asn·H₂O, and Gal/Asn·H₂O were heated (from 25 to 190 °C, 5 °C/min) in an oven and the headspace was analyzed by PTR-MS. The ion at m/z 72 was selected as a characteristic signal of acrylamide (*20*).

Differential Scanning Calorimetry (DSC). This was performed with a DSC 220C (Seiko Instruments, Chiba, Japan), which is based on the heat flow principle and cooled with liquid nitrogen. The heat flow and temperature were calibrated with indium and tin, and the low-temperature calibration was checked with water. Samples of sugar/amino acid mixtures (0.1−0.2 mmol) were placed into DSC crucibles (hermetically sealed for the closed system or left open for the open system) and scanned over an appropriate temperature range at a heating scan rate of 5 °C/min, with an empty crucible as reference. DSC measurements were performed on individual precursors and equimolar mixtures in open and closed systems. All thermal analysis experiments were performed in duplicate for 15 mg mixtures. The onset and the endset temperatures of the phenomena observed were determined as the intersection points of the tangents to the heat flow curve step and to the pre- and poststep baselines by use of the DSC software from Seiko.

Gravimetric Analysis. The same experimental procedure was applied as described for PTR-MS measurements. The samples were taken at defined time intervals and weighted at room temperature by using a high-precision balance (±0.0001 g) from Mettler-Toledo (Schwerzenbach, Switzerland).

High-Performance Liquid Chromatography (HPLC). Acrylamide was quantified by liquid chromatography on HPLC equipment series 1100 (Agilent, Palo Alto, CA). The HPLC system consisted of a quaternary pump (G1311A), an autosampler (G1313A), and a diode-array detector (DAD) (UV/vis wavelength 200−900 nm, G1315A). The mobile phase used for the HPLC separation of hydrophilic compounds was a ternary system composed of water and acetonitrile (93/7 v/v) spiked with formic acid (264 μL). The compounds were separated on a Dionex ion-pack ICE-AS1 column: 9 × 250 mm, 75 μm bead diameter, microporous bead type, 8% divinylbenzene (DVB) cross linking, with sulfonic acid ion-exchange groups (Dionex, Olten, Switzerland) at an isocratic flow rate of 1 mL/min. The UV absorbance was monitored from 200 to 900 nm. A complete run required about 90 min.

Standard solutions of acrylamide were prepared in water/acetonitrile (93/7 v/v) from a stock solution. External calibration curves (seven points) were established in the concentration range of interest [(0.5−5) × 10⁻⁵ mol/L]. Data interpretation was performed by use of the HPChem software (Agilent). The detection threshold of acrylamide injected was 10^{-6} mg. The linearity was, for all standard curves, $r^2 >$ 0.999. The standard deviation from one calibration to another was 3.14 × 10⁻⁹ mg. To check the validity of the acrylamide measurement, 10 random experiments were performed six times. The standard deviation (0.10) was not significant compared to the trend observed and allowed proper data interpretation.

Standard Pyrolysis Procedures. *Solid Crystalline Samples.* Asparagine monohydrate (0.375 g, 2.5 mmol) was dry-mixed with fructose (0.45 g, 2.5 mmol), glucose (0.45 g, 2.5 mmol), or galactose (0.45 g, 2.5 mmol) and placed in a reaction vessel that was held at a defined temperature in an oven. After addition of the reactants, the formation of volatile compounds was monitored by PTR-MS. Reaction kinetics was monitored from room temperature up to 190 °C with a 5 °C/min temperature program. Samples for acrylamide quantification in the residual matrix were collected at defined time intervals of the pyrolysis and analyzed by HPLC as described above.

Liquid Samples. Heating of equimolar mixtures of sugar and amino acid (5 mmol each) was performed in DMSO (50 mL). The chemicals of interest were placed in a flask equipped with a reflux condenser and heated at a defined temperature (100 or 150 °C). The solutions (2 mL) were collected regularly over time for acrylamide quantification. Aliquots of the solution (50 μL) were diluted in a water/acetonitrile mixture (950 μL, 93/7 v/v) and sonified for 15 min. The sample was then passed through a 0.2 μm filter (Spartan 13/30) and injected (20−50 μL) as such on the column. The reactions were stopped when they became dark brown.

RESULTS AND DISCUSSION

In the course of our studies on the formation of acrylamide from binary mixtures of asparagine and reducing sugars under low-moisture conditions, we have obtained results that could not be explained by their chemical reactivity. In particular, fructose turned out to be much more efficient than glucose in generating acrylamide, despite the fact that ketose sugars tend to be less reactive compared to aldoses. To understand this phenomenon, the behavior of glucose versus fructose was compared in equimolar sugar/Asn·H₂O models with respect to acrylamide formation. In addition, galactose was studied as another aldohexose sugar under the same reaction conditions.

Role of Sugars in Acrylamide Formation. Equimolar mixtures of crystalline Asn·H₂O and glucose, fructose, or galactose were heated and the formation of acrylamide was monitored on-line in the headspace by measuring its characteristic ion at m/z 72 by PTR-MS (*20*). The general profile of acrylamide release is shown in **Figure 1A**. Higher amounts of acrylamide were produced with fructose (red curve) compared to galactose (blue curve) and glucose (green curve). From a chemical point of view, glucose and galactose were expected, as aldohexose sugars, to generate more acrylamide from asparagine, due to their higher chemical reactivity provided by the more reactive aldehyde group compared to the ketohexose fructose.

Moreover, the magnification of the initial phase (**Figure 1B**) shows that the mixture with fructose generated acrylamide earlier than those containing glucose and galactose. It means that acrylamide is formed at lower temperature, i.e., about 125 °C. On the contrary, in the case of glucose about 140 °C was required to observe acrylamide release. Interestingly, these findings correlate well with the respective melting points of the sugars, as far as fructose and the hexose sugars are concerned, indicating that acrylamide is not released in detectable amounts as long as the sugar has not started to melt.

This hypothesis was further supported by physical changes observed during the reaction of Fru/Asn·H₂O and Glc/Asn·H₂O. The browning reaction of the two samples during the thermal treatment indicated that fructose reacts earlier than glucose (**Figure 2**). First browning was observed at 123−127 °C for the fructose and at 146−152 °C for the glucose system. The melting behavior of the two samples was in line with the browning reaction: the sample with fructose started to melt at 127 °C; the system with glucose, at 150 °C. These data suggest that under low-moisture conditions first physical changes occur (i.e., melting) before the browning reaction can speed up. Thus, the browning (Maillard) reaction is correlated with the melting
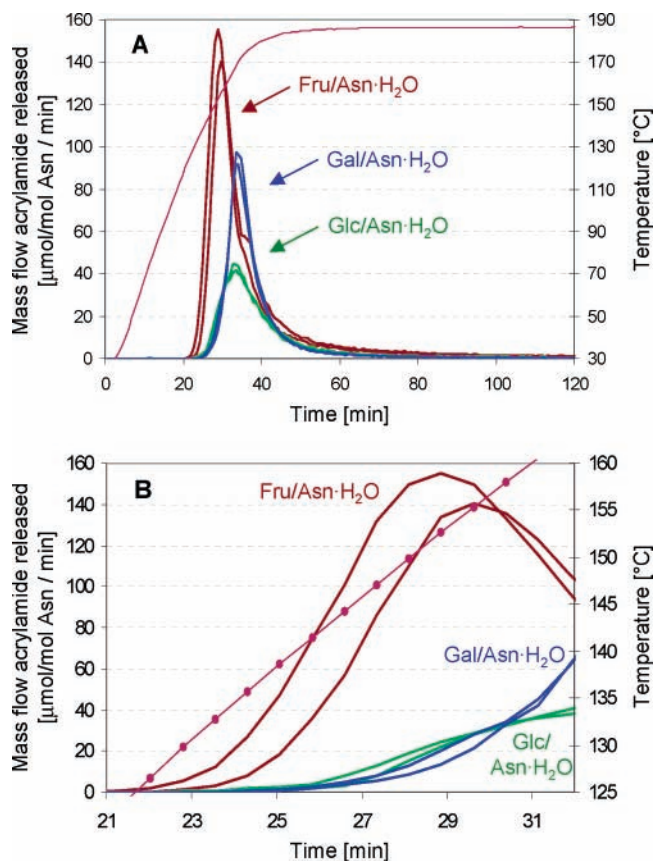
Figure 1. Kinetics of acrylamide release in the headspace during pyrolysis of asparagine monohydrate (Asn·H_2O) with glucose (Glc, green curve), galactose (Gal, blue curve), or fructose (Fru, red curve) monitored on-line by PTR-MS. (A) Entire experiment with a heating rate 5 °C/min from 30 to 190 °C; (B) zoom of the initial acrylamide release zone. Individual release curves of duplicates are shown.

behavior of the precursor system, which is well in line with the kinetics of acrylamide formation obtained by PTR-MS.

Mass Loss upon Pyrolysis. In addition, the loss of mass was determined during the pyrolysis experiment by gravimetric analysis (**Figure 3**). Fructose lost mass earlier and more rapidly, confirming the link between physical state changes and advancement of the Maillard reaction. For both systems, three phases were observed: (i) a first slightly decreasing slope corresponding probably to the release of crystallization water from Asn·H_2O; (ii) a more pronounced slope corresponding to the melting of sugar and the Maillard reaction, releasing additional volatile compounds such H_2O, CO_2, NH_3, and other low molecular weight sugar-derived volatiles; and (iii) a leveling phase at about 22% mass loss, indicating the end of the Maillard reactions. These results led to the hypothesis that molecular mobility of precursors may be a key feature in acrylamide formation under low-moisture conditions, which is triggered by physical properties of the reaction system, such as its fusion behavior for example.

Physical State Changes upon Pyrolysis. The thermal behavior of model reaction systems containing crystalline precursors (sugar/Asn·H_2O) was characterized by DSC to better understand the meaning of the physical changes observed. The thermodynamic behavior of each component individually was studied in open and closed systems. As shown in **Figure 4**, melting of the crystalline sugars and release of crystallization water from Asn·H_2O was generally associated with endothermic phenomena. The nature of the system (open or closed) did not
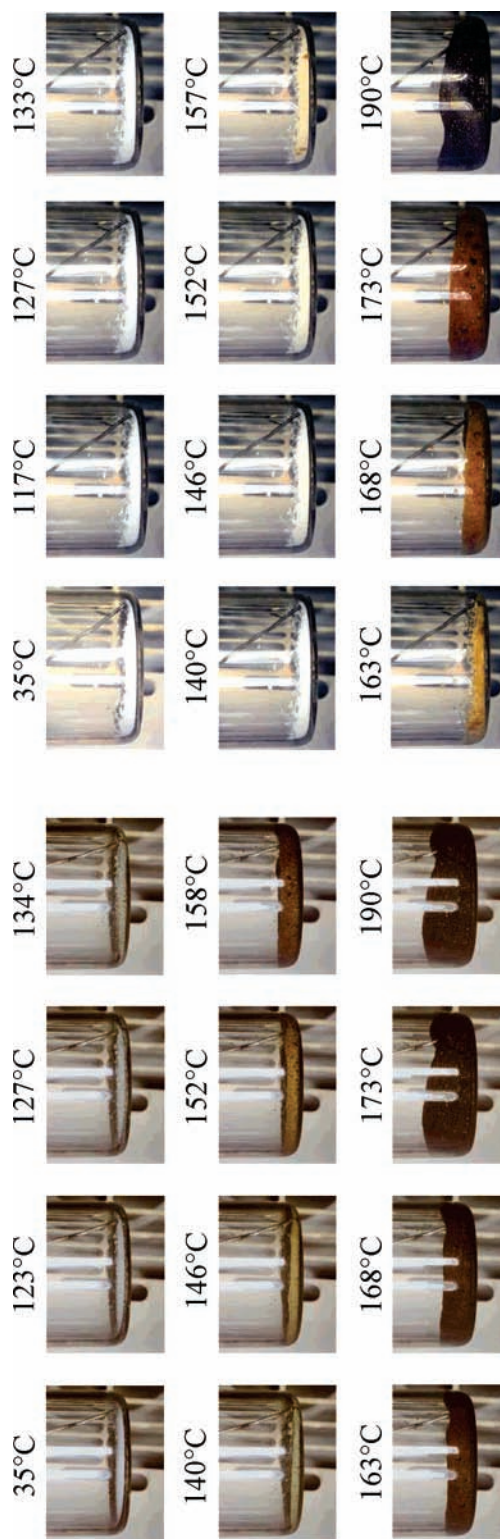
Figure 2. Changes in color and physical state occurring during the pyrolysis of fructose/Asn·H_2O (A) and glucose/Asn·H_2O (B). The pictures were taken during PTR-MS measurements, while acrylamide formation was monitored in parallel.
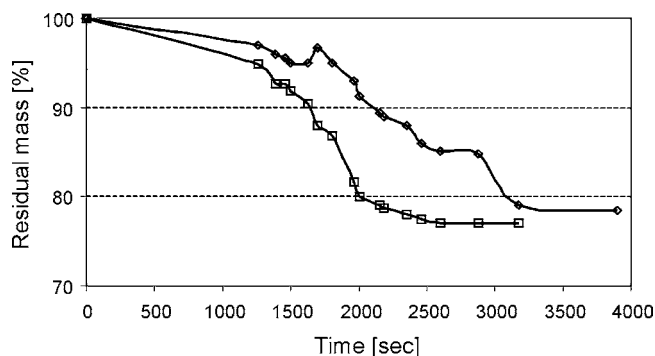
Figure 3. Residual mass obtained upon pyrolysis of crystalline Glc/Asn·H$_2$O (◇) and Fru/Asn·H$_2$O (□) model systems by gravimetric analysis.
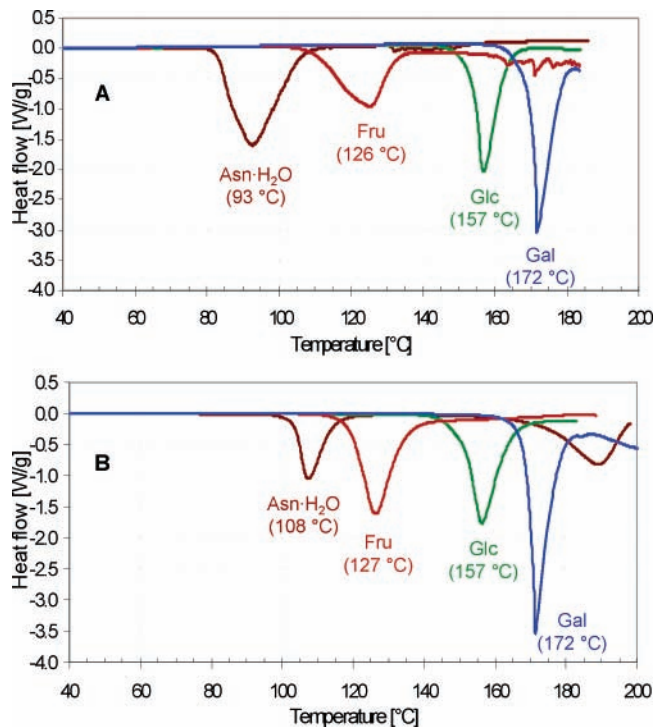

Figure 4. DSC of the individual precursors performed in open (A) and closed (B) systems: asparagine monohydrate (Asn·H$_2$O), brown curve; fructose (Fru), red curve; glucose (Glc), green curve; galactose (Gal), blue curve. The heating rate was 5 °C/min.

affect the melting point of the sugars. Only the temperature of crystallization water release from Asn·H$_2$O was modified in the closed system (brown curve): in the open system, water release occurred at 93 °C (**Figure 4A**), while this phenomenon was observed at 108 °C in the closed system (**Figure 4B**). This may be due to pressure development in the closed cell. Moreover, the presence of free water in the closed system decreased the melting point of asparagine: starting at about 170 °C, the fusion phenomenon was observed at 190 °C (**Figure 4B**), while melting of asparagine in the open system was not apparent in this temperature range.

The results obtained by DSC measurements of binary mixtures of Asn·H$_2$O and reducing sugars are shown in **Figure 5**. In the open systems, release of crystallization water from Asn·H$_2$O occurred at the same temperature in the three reaction systems (93 °C), and evaporated below 105 °C without apparent interaction with the sugar crystals (**Figure 5A**). The Fru/Asn·H$_2$O system (red curve) showed a second endothermic signal at about 124 °C, which corresponds to fructose melting. A third endothermic phenomenon occurred at 142−143 °C, which could
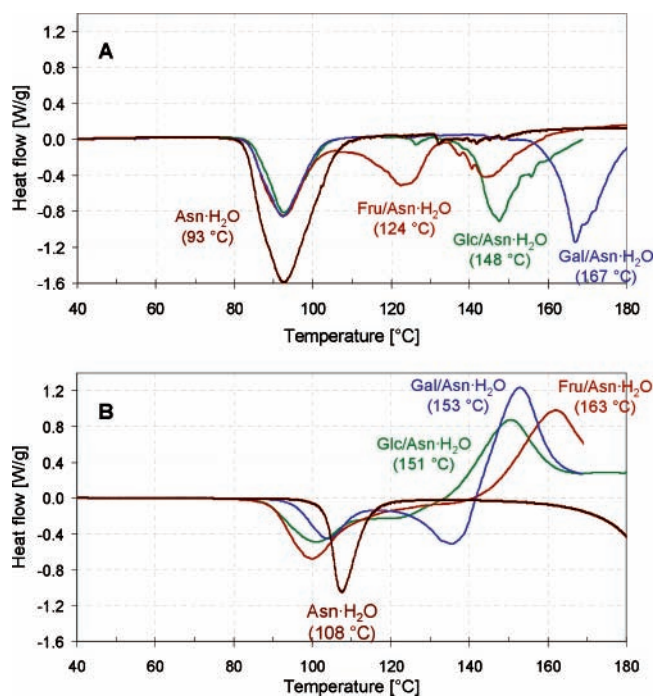

Figure 5. DSC of equimolar mixtures of asparagine monohydrate (Asn·H$_2$O) and fructose (Fru, red curve), glucose (Glc, green curve), or galactose (Gal, blue curve) measured in open (A) and closed (B) reaction systems. The heating rate was 5 °C/min. The brown curve represents pure asparagine monohydrate (Asn·H$_2$O).

be due to fusion or solubilization of asparagine in the liquid water/fructose phase. In the Glc/Asn·H$_2$O (green curve) and Gal/Asn·H$_2$O (blue curve) systems, the fusion phenomena were observed at about 148 and 167 °C, respectively, at lower temperatures compared to the pure sugar systems (**Figure 4A**). This shift of sugar melting is due to the presence of water acting as an impurity or a partial solubilization of sugar during water evaporation from Asn·H$_2$O. In all systems, no exothermic signal was observed although the browning reaction was well visible at the end of the heating period. The absolute value of the Maillard reaction enthalpy is probably lower than that of the physical state change, thus leading to a masking effect of the chemical reaction signal.

In the closed systems (**Figure 5B**), release of crystallization water was again the first thermal event in the three samples, which was slightly affected by the nature of the sugar. The onset of water release was lower than for pure Asn·H$_2$O (brown curve). In the Fru/Asn·H$_2$O system (red curve), a slight positive slope in the temperature range was observed where fructose is usually melting. This observation is most likely due to a masking effect between the melting event and the beginning of the Maillard reaction. From 140 °C on, the slope increased, indicating the well-known exothermic phenomenon, which is linked to the chemical (Maillard) reaction. The Glc/Asn·H$_2$O system (green curve) behaved similarly. However, the signal representing the exothermic reaction occurred earlier (151 °C) compared to Fru/Asn·H$_2$O (163 °C). However, it does not necessarily mean that acrylamide formation from Glc/Asn·H$_2$O could start under milder reaction conditions. In the Gal/Asn·H$_2$O system (blue curve), the beginning of the sugar fusion could clearly be seen (ca. 135 °C), followed by the exothermic signal at 153 °C for Maillard-type reactions.

In general, it is not obvious to interpret the thermal curves shown in **Figure 5B**, representing the physical state changes upon heating of sugar/Asn·H$_2$O binary mixtures in a closed
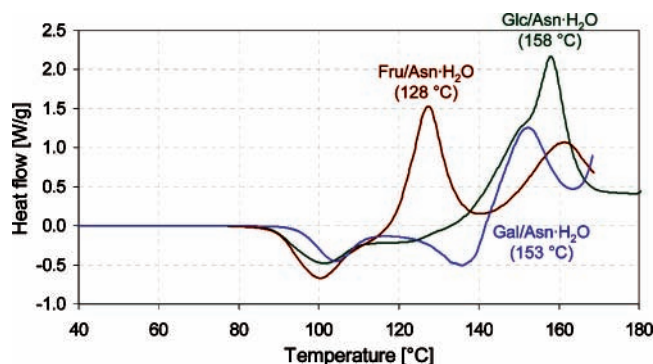
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Figure 6. DSC curves obtained by subtraction from the signals obtained with equimolar mixtures of asparagine monohydrate (Asn·H_2O) and fructose (Fru, red curve), glucose (Glc, green curve), or galactose (Gal, blue curve) in closed reaction systems (**Figure 5B**) the corresponding DSC signals of the respective pure sugars (**Figure 4B**).

Figure 7. Kinetics of acrylamide formation from the binary equimolar mixtures glucose/Asn·H_2O (△, green), galactose/Asn·H_2O (◇, blue), and fructose/Asn·H_2O (○, red) heated in dimethyl sulfoxide at 150 °C (A) and 100 °C (B) as a function of reaction time.

Figure 8. Formation of acrylamide by heating a binary equimolar mixture of L-asparagine and D-glucose for 5 (▲) and 60 (●) min.

system, and to establish a correlation with the chemical reactions. This is most likely due to increased complexity provided by the pressure and/or presence of free water, which may influence the course of the reaction pathways. The physical state changes measured by DSC are resulting curves that correspond to various phenomena, such as melting of the sugar, the chemical (Maillard) reaction, solubilization of asparagine, etc. To facilitate the interpretation of these complex phenomena, the signals obtained with the pure sugars (**Figure 4B**) were subtracted from those of the respective binary mixtures (**Figure 5B**), both collected in closed cells. This representation, shown in **Figure 6**, allows a deconvolution of the thermal event, simplifying data interpretation. Thus, the virtual curve of the Fru/Asn·H_2O system obtained by this treatment exhibits an exothermic signal at a lower temperature (128 °C) compared to Gal/Asn·H_2O (153 °C) and Glc/Asn·H_2O (158 °C), which is in good agreement with the trends observed by PTR-MS measurements (**Figure 1**). According to these results, the higher amounts of acrylamide found in Fru/Asn·H_2O compared to the aldohexose/Asn·H_2O samples may be explained by the increased mobility of the precursors due to early fusion of the reaction system containing fructose. DSC seems to allow clustering of fructose- and aldohexose-based reaction systems with respect to acrylamide formation. Work is in progress with the ketose sugar sorbose, having a melting point of 163 °C, to substantiate these findings. The exothermic signals of Glc/Asn·H_2O and Gal/Asn·H_2O shown in **Figure 6** do not follow the melting behavior of the corresponding sugars (**Figure 4B**), thus suggesting that additional parameters may play a role in solid-state Maillard reaction systems.

Model Reactions in Liquid Systems. To further substantiate the importance of molecular mobility for acrylamide formation under low-moisture conditions, binary mixtures of sugar/Asn were reacted in DMSO as solvent. On the basis of the hypothesis suggesting molecular mobility as a key driver of acrylamide formation under low-moisture conditions, the reaction in liquid systems should follow other rules. In liquid systems, the chemical reactivity of the sugar may be more important, as molecular mobility is not a limiting factor anymore. As shown in **Figure 7A**, the aldose sugars (glucose and galactose) generated more acrylamide than the ketose sugar fructose in DMSO at 150 °C. This is fully consistent with the chemical reactivity of the sugars. Similarly, fructose generates very low amounts of acrylamide at 100 °C compared to glucose (**Figure 7B**).

These results indicate that higher amounts of acrylamide are formed in DMSO, which represents a low-moisture system with high molecular mobility. This is fully consistent with the
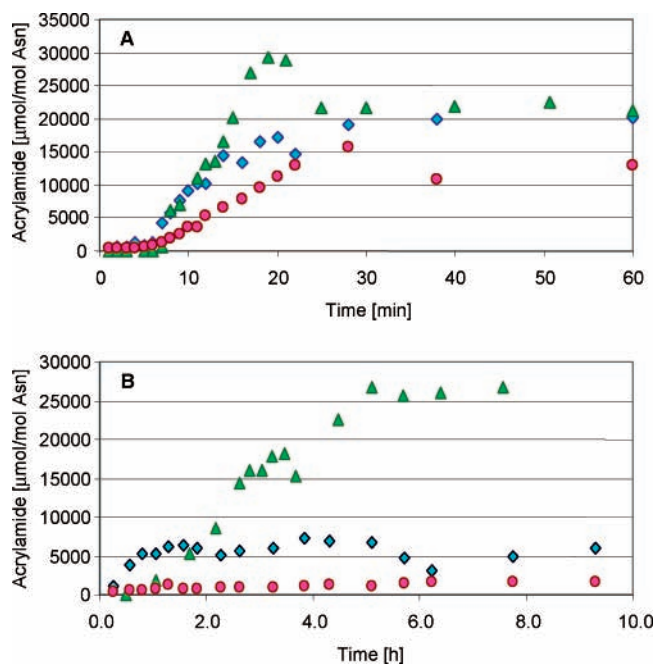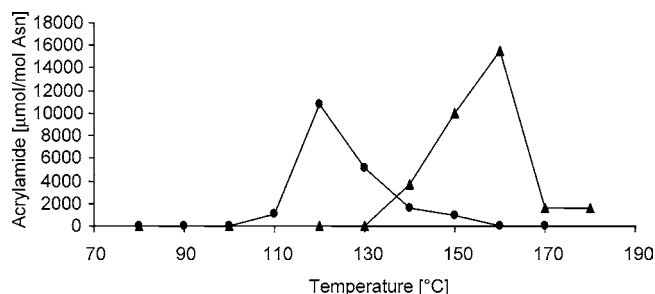
mechanism of acrylamide formation (*12*), as the first step (formation of the Schiff base via the *N*-glycoside) is reversible and gives rise to water as byproduct. Thus, an anhydrous liquid system will lead to high amounts of acrylamide with increasing chemical reactivity of the sugar. When these data are compared with those obtained in crystalline systems, the importance of molecular mobility becomes evident. Liquid systems providing high mobility of precursors generate more acrylamide than solid systems. Therefore, changing phase behavior and thus molecular mobility under low-moisture conditions will significantly influence the kinetics of acrylamide formation. This explains well the observation that glucose generates more acrylamide in DMSO than fructose does, whereas it is the opposite in the solid state.

Effect of Reaction Time and Temperature. Apart from reducing the concentration of potential precursors of acrylamide, reaction time and temperature are obvious parameters to study in order to minimize the formation of acrylamide upon processing. As shown in **Figure 8**, the interplay of these parameters strongly influenced acrylamide formation. Pyrolysis of Glc/Asn at different temperatures for either 5 or 60 min resulted in different curves, indicating high amounts of acrylamide formed already at 120 °C at long reaction times, whereas 160 °C was required to obtain highest amounts of acrylamide at short pyrolysis times. These results are in good agreement with data

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recently published by Taubert et al. (*22*). As shown in this study, reaction time and temperature are covariant parameters that represent a means for controlling acrylamide formation under food processing conditions. The decline of the curves is most likely due to polymerization as recently reported (*12*).

In conclusion, there are several chemical and physical parameters that may help to minimize acrylamide formation. Apart from the nature and chemical reactivity of precursors, their molecular mobility plays a vital role in acrylamide formation of crystalline sugar/asparagine model systems. Under low-moisture conditions, acrylamide formation mainly depends on the physical state of the reaction system (liquid, solid), i.e., melting point of the sugar, as well as the presence of water and its availability, which directly affects molecular mobility. The role of chemical reactivity is more pronounced in liquid reaction systems. Temperature and heating time are covariant and represent another means to influence acrylamide formation. However, as in many food products the ingredients are usually present in the amorphous state, additional investigations are required to bridge crystalline model systems with more complex food matrixes. This work is in progress and will be reported elsewhere.

ACKNOWLEDGMENT

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