Determination of the Kinetics and Mechanism of Decomposition of Tryptophan Amadori Rearrangement Product by RP-HPLC Analysis

Varoujan Yaylayan* and Nazhat G. Forage

Department of Food Science and Agricultural Chemistry, Macdonald College of McGill University, 21111 Lakeshore Road, Ste. Anne de Bellevue, Quebec, Canada H9X 1C0

Tryptophan Amadori rearrangement product (ARP) was synthesized, and its decomposition was studied at two different temperatures and concentrations over a period of 6 h. RP-HPLC analysis of the reaction mixtures indicated the presence of hydroxymethylfurfural (HMF), maltol, tryptophan, indole, norharman and harman. Kinetic analysis showed that the activation energy (E_a) for the decomposition of ARP and for the formation of maltol from ARP depended on the water content of the reaction mixture; on the other hand, the E_a (4.1 kcal/mol) for the formation of HMF from the ARP was independent of the water content. A comparative study of the formation of HMF and maltol from ARP and from glucose alone showed that the Amadori rearrangement provides a low-energy pathway for the formation of these two compounds. The kinetic data were used to propose plausible mechanisms for the decomposition of the ARP.

INTRODUCTION

Detailed knowledge of the chemical behavior of the Maillard reaction intermediates (Finot et al., 1990) and their thermal decomposition products in food and model systems is still lacking; such information, however, could be a useful tool for the food processing and the flavor industries to predict the type of flavor compounds expected to form during thermal processing. In addition, a knowledge of the kinetics of the decomposition of important intermediates can greatly enhance the thermal processing conditions in terms of maximizing the rate of generation of the desired flavor compounds and suppressing the rate of formation of off-flavor or undesirable products.

The type and quantity of the flavors formed in foods or model systems depend on the time and temperature at which the system is heated. Different products can be formed from the same reaction mixture by changing these parameters. Numerous chemical reactions are involved in the generation of flavors from the Maillard reaction, and each of these chemical reactions has its own activation energy (E_a). To ultimately control the Maillard reaction to produce the desired flavor profile, activation energies of these reactions should be determined. However, there are few kinetic data available in the literature on the formation of Maillard products. Leahy and Reineccius (1989), for example, have determined the E_a of formation of some alkylpyrazines during heating of model systems; the values ranged between 33.4 and 44.8 kcal/mol. Generally, the activation energy data for the Maillard reaction products have been reported within a range of 3–50 kcal/mol, depending on the type of products being followed; the water activity (Eichner et al., 1985) and the pH (Lee et al., 1984) seem to affect the value of E_a. Generally, the lower is the water activity, the higher is the E_a; and the lower is the pH, the higher is the E_a.

Amadori compounds are the key intermediates in the nonenzymatic interaction of reducing sugars with amino acids (Maillard reaction); their thermal degradation (Nursten, 1980) is responsible for the formation of numerous compounds important in the formation of characteristic flavors, aromas, and brown polymers. The widespread occurrence of Amadori products in food necessitates development of HPLC separation methods for identification purposes and for study of their decomposition kinetics. Amadori compounds contain both amino and carboxylic acid groups, in addition to a carbohydrate moiety. Bonded aminopropyl columns are effective at separating mixtures with a wide range of polarities. They have been widely used to separate carbohydrates. Attempts to separate Amadori compounds using such columns from amino acids at neutral pH by using acetonitrile–water mobile phases resulted in long retention times and tailing peaks. The ionized carboxyl groups present in the amino acids and in the Amadori compounds interact with the protonated amino groups on the aminopropyl columns to cause the long retention times observed. Decreasing the pH value to 2.3 with phosphate buffer in the mobile phase afforded symmetrical peaks and shortened retention times. Reversed-phase (RP) columns have also been used successfully to separate Amadori compounds. The retention times for the amino acids were found to be affected by the degree of their ionization, that is, by the pH of the mobile phase. As the ionization of the amino acids increased, retention times decreased. Control of mobile phase pH can affect the degree of ionization of weak acids and bases and significantly influence the retention times. For amino acids, retention was minimal at pH levels where the zwitterion predominates. High pH levels (>9) cause the amino group to be less ionized, while low pH levels (<3) cause the carboxyl group to be less ionized. Thus, extreme pH conditions cause longer retention times on an RP column.

Tryptophan Amadori compound was selected as a model to develop an HPLC procedure for the separation and identification of the decomposition products of the ARP and to determine the kinetic parameters of the decomposition. Successful separation of tryptophan Amadori product (t_R = 9 min) from tryptophan (t_R = 15 min) was reported, on a μBondapak C-18 column with 30% acetonitrile–70% aqueous 0.01 M phosphate buffer at pH 2.3 containing 0.02% SDS counterion at a flow rate of 1 mL/min (Takeoka et al., 1979).

Three different solvent systems were developed by using a C-18 RP column to analyze the products formed during the thermal degradation of tryptophan ARP. Maltol,

* Author to whom correspondence should be addressed.

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HMF, tryptophan, indole, and β-carbolines were predicted to be formed in the decomposition mixture, on the basis of the electron impact fragmentation of the tryptophan ARP. This approach of correlating the mass spectrometric data with thermal degradation products could be a useful tool to predict the outcome of other thermal decomposition reactions.

EXPERIMENTAL PROCEDURES

Materials and Methods. Tryptophan, D-glucose, hydroxymethylfurfural (HMF), maltol, indole, and β-carbolines (norharman and harman) were purchased from Aldrich Chemical Co. and used without further purification; all the solvents were of HPLC grade (BDH). Mobile phases were degassed by application of vacuum with gentle agitation for 5 min. Samples were loaded via a Rheodyne injector with a 20-μL loop. C-18 Ultrasphere, 5 μm, 2.0 × 150 mm column from Beckman or C-18 Lichrosphere, 100-RP-18, 5 μm, 2.0 × 150 mm mounted with guard column Licro CART 4-4 from Merck, was used in the analysis. The column was operated at ambient temperature. Repeated injections of 20 μL of the sample were needed to achieve stable peaks for the Amadori product. Water was obtained from a Milli-Q reagent grade water system (Millipore Corp.). Ampules (sealed type) were obtained from Aldrich. The wavelength used to detect the Amadori compound and its decomposition products was 280 nm. Retention times and quantifications represent the average of triplicate injections.

Instrumentation. The modular HPLC system used was a Beckman System Gold consisting of a variable-wavelength UV detector Model 166 and a 110B solvent delivery module controlled by a NEC lap-top computer, connected to a Shimadzu C-R6A integrator–printer operated on 10-mV scale.

Synthesis of Tryptophan Amadori Products (ARP). Amadori product of tryptophan with D-glucose was synthesized according to the procedure of Sgarbieri et al. (1973); 5.0 g of D-glucose and 0.60 g of L-tryptophan were dissolved in 80 mL of methanol and refluxed for 5 h. The concentrate obtained after rotary evaporation was diluted with 3–4 mL of water and then directly applied to a cellulose column (44 × 550 mm). The column was packed with Whatman CF_{11} fibrous powder suspended in water-saturated *n*-butanol and eluted with the same solvent; 20-mL aliquots were collected and stored in the refrigerator overnight to allow crystallization, and the crystals were purified twice with *n*-butanol. A final crystallization was done by suspending it in methanol. The purity of the crystals was ensured by physical, chemical, and thin-layer chromatographic examination.

General Method for Decomposition of the Model Systems. Two sets of six sealed ampules each containing 1.0 mg of tryptophan Amadori product dissolved in 0.75 (43% by weight) or 0.65 mL (39.4% by weight) of water was mixed for 1 min. The ampules were then sealed by oxygen–air flame. The reaction mixtures were heated in a dry bath, obtained from John Scientific Inc., for a period of 6 h at 110 °C for the first set and at 140 °C for the second set. One ampule was removed at the end of each hour, and the contents were diluted with water to 2 mL (final content 500 ppm) and filtered by using filter membrane 0.45 μm, type HA, from Millipore, Waters Scientific, before injection into the column. The following decomposition reactions were also studied by using the above procedure: (a) 0.66 mg of D-glucose dissolved in 0.5 mL of water; (b) 0.2 mg of tryptophan dissolved in 0.5 mL of water.

Identification of Peaks. The peaks were identified by direct comparison of their retention times with that of authentic samples, under identical conditions using different compositions of mobile phases and different wavelengths and by spiking the samples with standards.

Quantification Method. The method used for the quantification was that of two-point calibration curve (Absolute Calibration Curve method), a resident program in the Shimadzu integrator (chromatopack C-R6A). The method is based on obtaining two response factors for each component by injecting two standard samples, the concentrations of whose components are known.

Kinetic Analysis. Regression analyses were performed by using standard nonlinear regression model (Lotus 123 software package); the determination of rate constants and activation energies was accomplished by using standard procedures (Daniels and Alberts, 1975; Atkins, 1982). Disregarding the actual mechanism or the number of steps involved, pseudo-first-order reactions were assumed in determining the kinetics of decomposition of ARP ($\ln R/R_0 = -kt$) or the formation of a single component from the decomposition of ARP ($\ln\{R_0/R_0 - P\} = kt$), where R is the concentration of the reactant (mol/L), R_0 is the initial concentration of reactant, P is the concentration of product at time t, and t is time (h).

Table I. Retention Times of the ARP and Its Decomposition Products in Three Different Solvent Systems

compd	t_R, min	solvent system[a]
ARP	2.7	a (pH 4.5)
ARP	4.7	b (pH 2.7)
ARP	0.8	c (pH 6.5)
HMF	1.6	a
HMF	2.1	b
TRP	3.9	a
TRP	7.4	b
maltol	2.6	b
indole	3.7	b
NOR	2.6	c
HAR	3.0	c

[a] Solvent system a: 30% methanol, 69.5% water, and 0.5% phosphoric acid (0.01 M). Solvent system b: 68% methoxyethanol, 30% phosphoric acid (0.01 M), and 2% methanol. Solvent system c: 60% acetonitrile, 39.9% water, and 0.1% triethylamine.

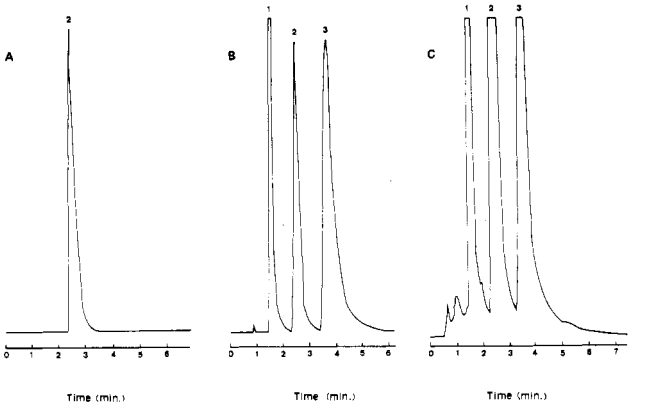


Figure 1. Separation of HMF and Trp from Trp-ARP using solvent system a, flow rate of 1.0 mL/min and wavelength 280 nm. (A) Chromatogram of Trp-ARP; (B) chromatogram of the standard mixture; (C) chromatogram of the heated Trp-ARP solution, after 4 h at 110 °C. (1) HMF; (2) ARP; (3) Trp.

RESULTS AND DISCUSSION

Kinetics of Thermal Decomposition of Tryptophan Amadori Compound. The thermal decomposition of tryptophan ARP (1.0 mg) was studied at two temperatures (110 and 140 °C) and water contents (0.65 and 0.75 mL) as described under Experimental Procedures. The contents of the reaction mixtures were analyzed by RP-HPLC using three different solvent systems, a–c (see Table I); the chromatograms representing the separation achieved by each of the solvent systems are shown in Figures 1–3. Figure 1 shows the separation of HMF and tryptophan from tryptophan ARP using solvent system a. Figure 2 shows the separation of HMF, maltol, indole, and tryptophan from tryptophan ARP using solvent system b. Figure 3 shows the separation of norharman (NOR) and harman (HAR) (β-carbolines) from ARP using solvent system c. Figure 4 shows the plot of concentration of ARP (in 0.65 mL of water) versus time at two temperatures.

On the basis of the quantification of HPLC analysis, the rate constants and the activation energies for the

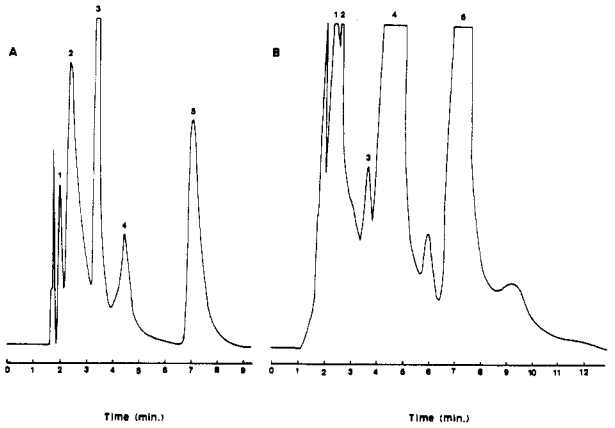


Figure 2. Separation of HMF, Trp, maltol, and indole from Trp-ARP using solvent system b, flow rate of 0.5 mL/min, and wavelength 280 nm. (A) Chromatogram of the standard mixture; (B) chromatogram of the heated Trp-ARP solution, after 2 h at 110 °C. (1) HMF; (2) maltol; (3) indole; (4) ARP; (5) Trp.

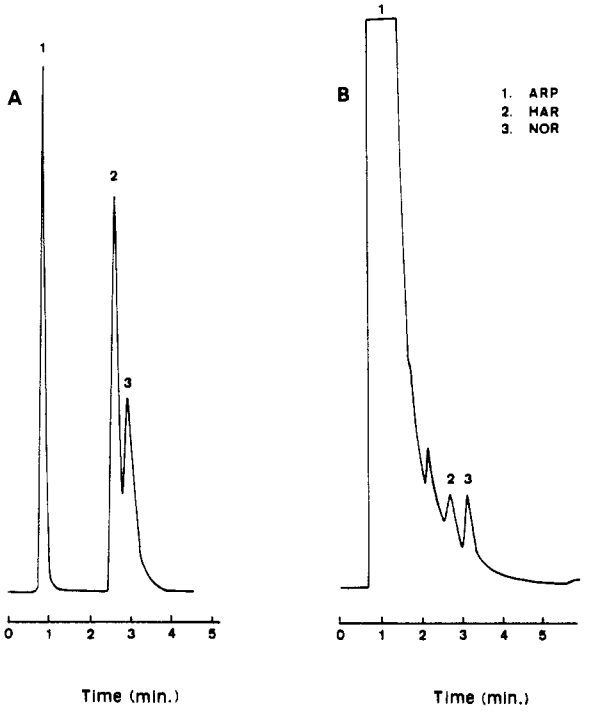


Figure 3. Separation of norharman (NOR) and harman (HAR) from Trp-ARP using solvent system c, flow rate of 1.0 mL/min, and wavelength 280 nm. (A) Chromatogram of the standard mixture; (B) chromatogram of the heated Trp-ARP solution, 6 h at 140 °C.

decomposition of the ARP were calculated; the results are summarized in Table II. According to this table, the rate of decomposition of the ARP is 3.5 times faster (in 0.65 mL of water) at 140 °C than at 110 °C and 4.7 times faster (in 0.75 mL of water) at 140 °C than at 110 °C. Comparing the rates at the same temperature but at different water contents indicates that at 110 °C the rate is 1.4 times faster in the more concentrated solution and at 140 °C the rate is faster only by 1.08 times. These results indicate that decomposition of ARP proceeds by dehydration reactions but that thermally induced nonhydrolytic reactions (such as C–C and C–N bond cleavages) become more important as the temperature is increased.

Activation energies (E_a) for the formation of Amadori products are known to be dependent on the water content of the reaction mixture; Eichner et al. (1985) studied the kinetics of the formation of ARPs in freeze-dried carrots and found that the lower is the water activity, the higher

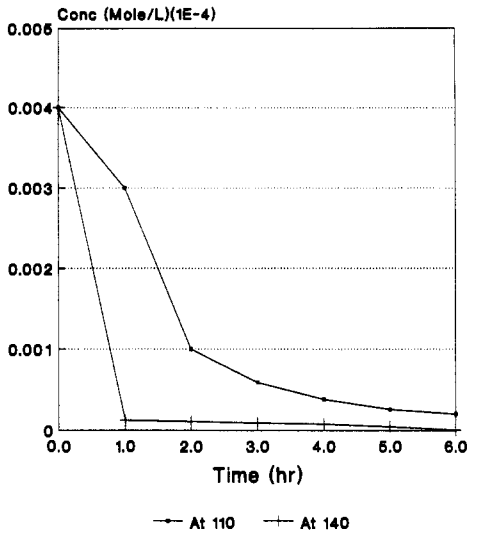


Figure 4. Plot of concentration of ARP (in 0.65 mL of water) versus time at two temperatures.

Table II. Kinetic Parameters for the Decomposition of Trp-ARP at Two Different Concentrations

kinetic parameters	1.0 mg of Trp-ARP/0.65 mL of water	1.0 mg of Trp-ARP/0.75 mL of water
$k^{110°C}$, h^{-1}	0.52	0.36
$k^{140°C}$, h^{-1}	1.83	1.69
E_a, kcal/mol^{-1} K^{-1}	13.2	16.2

is the value of E_a. Consequently, for the reverse of this reaction (decomposition of ARP), this relationship is expected to be also reversed; indeed, the value of E_a was found to depend on the water content of the reaction mixture; the higher is the water content, the higher is the value of E_a. Consequently, dilute solutions of Amadori products are more sensitive to changes in temperature than concentrated solutions.

Kinetics of Formation of HMF and Maltol from ARP. The formation of hydroxymethylfurfural (HMF) in food or in model systems is a useful indicator of the extent of Maillard reaction; maltol, on the other hand, is associated with caramel flavor and is used as flavor potentiater in nonalcoholic beverages. Both HMF and maltol can be formed directly from the degradation of glucose alone (see below).

Figures 5 and 6 show the plot of concentration versus time for the formation of HMF and maltol, respectively, at two temperatures from the decomposition of the ARP. Table III illustrates the kinetic data for these reactions. According to Table III, increasing the temperature by 30 °C increases the rate of formation of HMF by 1.5 times at both water concentrations; however, increasing the water content by 4% increases the rate at both temperatures by 2.5 times, indicating that a hydrolytic reaction is involved in the rate-determining step (see the proposed mechanism below). However, the activation energies at both water concentrations are the same; these observations indicate that the reaction is more sensitive to the water content than to the variations in temperature.

On the other hand, increasing the temperature in the more concentrated solution of the ARP by 30 °C increases the rate of formation of maltol by 1.5 times and by 2.8 times in the less concentrated solution, indicating that thermally induced nonnydrolytic reactions (such as C–C and C–N bond cleavages) might be involved in the rate-determining step (see the proposed mechanism below). However, decreasing the water content by 4% increases

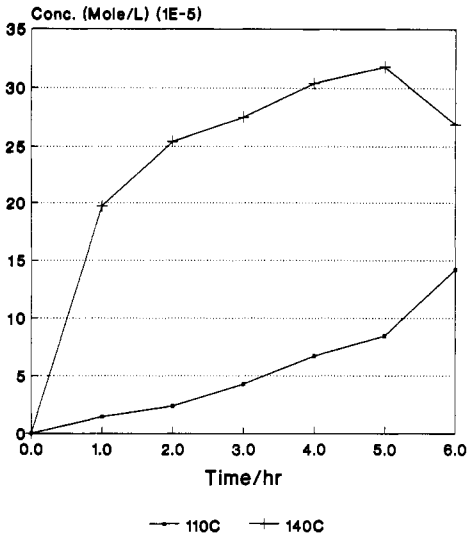


Figure 5. Plot of concentration versus time for the formation of HMF at two temperatures, from the decomposition of the ARP.

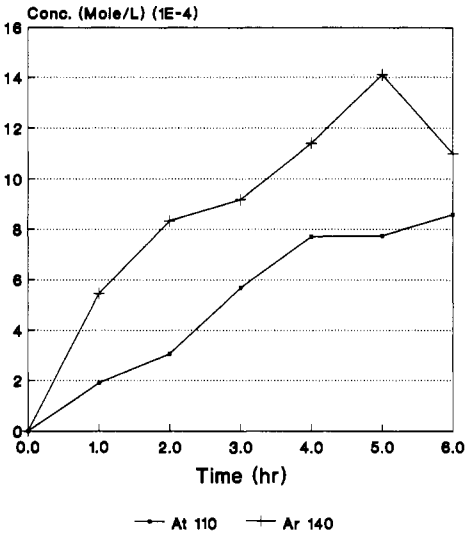


Figure 6. Plot of concentration versus time for the formation of maltol at two temperatures, from the decomposition of the ARP.

Table III. Kinetic Parameters for the Formation of HMF and Maltol from Trp-ARP

	1.0 mg of Trp-ARP/0.65 mL of water, formation of		1.0 mg of Trp-ARP/0.75 mL of water, formation of	
kinetic parameters	HMF	maltol	HMF	maltol
$k^{110°C}$, h^{-1}	0.0049	0.038	0.0128	0.0149
$k^{140°C}$, h^{-1}	0.0074	0.059	0.0189	0.0412
E_a, kcal mol^{-1} K^{-1}	4.15	4.66	4.10	10.65

the rate at 110 °C by 2.5 times and by 1.4 times at 140 °C, indicating that dehydration steps are also involved in the formation of maltol from the ARP. The same conclusion can be reached by comparing the activation energies at two concentrations.

Tryptophan Releases from Trp-ARP. Although the Maillard reaction is known to reduce the nutritional value of foods by effectively removing amino acids through the Amadori rearrangement reaction, some of the amino acid, however, could be released during the thermal decomposition. Figure 7 shows the plot of tryptophan concentration versus time, during the decomposition of ARP. According to this figure the tryptophan concentration

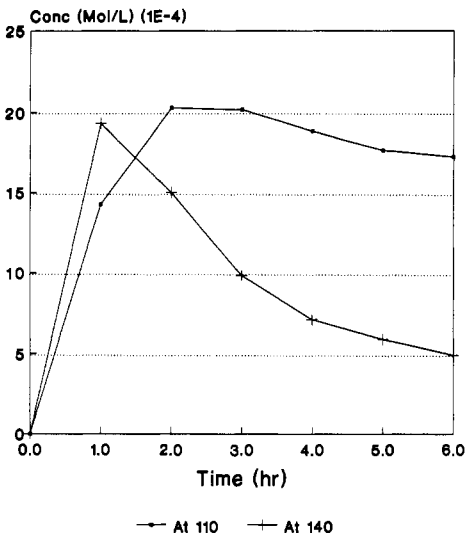


Figure 7. Plot of tryptophan concentration versus time, during the decomposition of ARP.

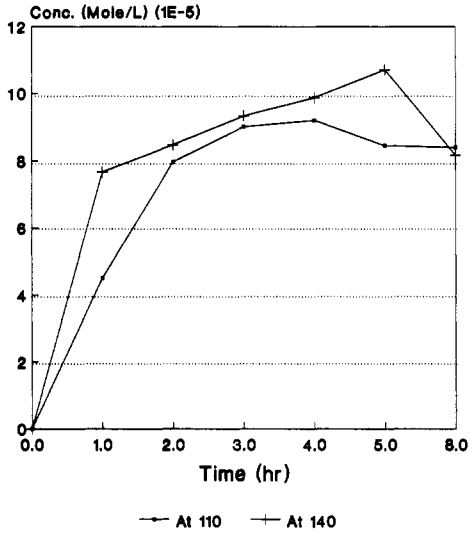


Figure 8. Plot of concentration versus time for the formation of indole during the decomposition of ARP.

Table IV. Kinetic Parameters for the Formation of Indole at Two Concentrations

kinetic parameters	1.0 mg of Trp-ARP/0.65 mL of water (39.4%)	1.0 mg of Trp-ARP/0.75 mL of water (43%)
$k^{110°C}$, h^{-1}	0.0013	0.0037
$k^{140°C}$, h^{-1}	0.0020	0.0048
E_a, kcal mol^{-1} K^{-1}	4.16	2.73

drops after 1 h of its formation at 140 °C and after 2 h at 110 °C, indicating that tryptophan release is accompanied by its subsequent reaction with carbonyl compounds, such as HMF, that are being produced from the decomposition of the ARP.

Kinetics of Formation of Indole from Trp-ARP. Indole is a specific product of tryptophan. It is used widely in perfumery, medicine, and the flavor industry. Figure 8 shows the plot of concentration versus time for the formation of indole during the decomposition of ARP. Table IV shows the kinetic data; according to this table the rate of formation of indole is about 2.5 times faster in the more dilute solution of the ARP. This might be due to the fact that in the more dilute solution other competing reactions that proceed by dehydrations are less predominant due to the presence of more water, and hence at these conditions the rate of formation of indole is faster.

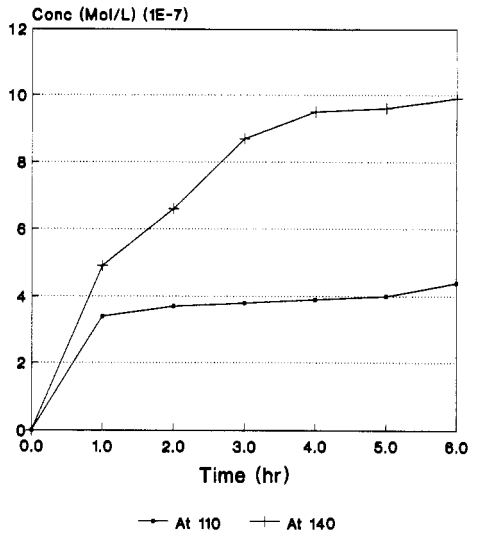


Figure 9. Plot of concentration versus time for the formation of norharman.

Scheme I. Proposed Mechanism of Formation of HMF

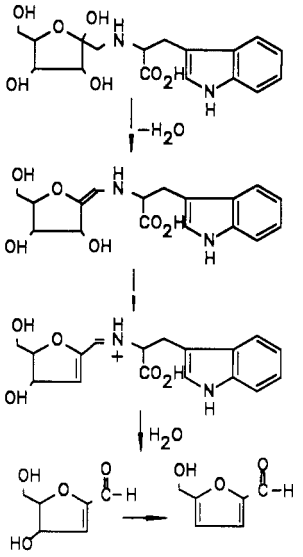
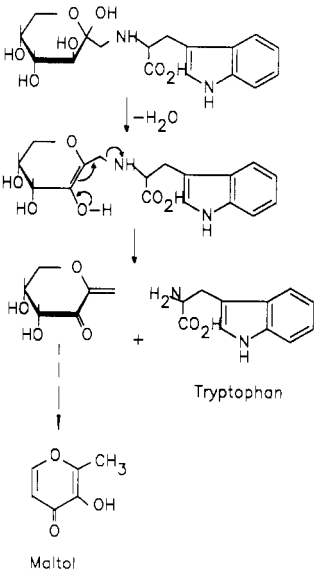


Table V. Kinetic Parameters for Glucose Decomposition in 43% Water

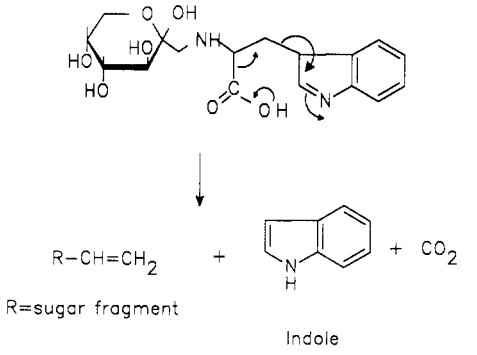
kinetic parameters	HMF formation	maltol formation
$k^{110°C}$, h^{-1}	0.0004	0.0008
$k^{140°C}$, h^{-1}	0.0050	0.0037
E_a, kcal mol^{-1} K^{-1}	25.8	15.8

Formation of Norharman from Trp-ARP. Interest in β-carbolines has recently increased, due to their detection in human tissue (Airaksinen et al., 1981), where they may act physiologically on GABA receptors, possibly being the endogenous ligands at benzodiazepine receptors. It has been shown that β-carbolines are formed in tobacco smoke and in food (Poindexter and Carpenter, 1962); it is possible, therefore, after inhalation or ingestion, these compounds may diffuse into the blood stream through the lungs and the digestive tract. It is therefore important to know the origin of their formation in food. Analysis of heated mixtures of tryptophan and glucose revealed the formation of many β-carbolines (Brautigam and Severin, 1974); Yaylayan et al. (1990), on the basis of electron impact (EI) mass spectrometric studies of tryptophan ARP, suggested that β-carbolines could be formed directly from the Amadori rearrangement product of tryptophan with glucose. Analysis of the thermal decomposition mixtures

Scheme II. Proposed Mechanism of Formation of Maltol and Simultaneous Release of Tryptophan



Scheme III. Proposed Mechanism of Formation of Indole



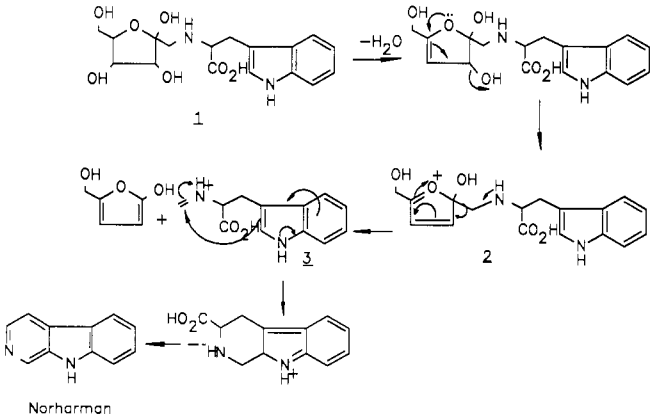
of tryptophan ARP indeed revealed the presence of two commercially available β-carbolines (harman and norharman). The fact that β-carbolines have been detected in the EI mass spectrum and in the aqueous decomposition mixture of tryptophan ARP gives further support to the proposal that EI mass spectral fragmentations might reflect high-temperature decompositions occurring in solution phase and that such spectra could be used as an aide to predict the thermal decomposition products of other ARPs.

Figure 9 shows the plot of concentration versus time for the formation of norharman.

Formation of HMF and Maltol from D-Glucose. Decomposition of D-glucose (0.66 mg) was studied in 0.5 mL of water (43% by weight) at 110 and 140 °C for 6 h to determine the formation of HMF and maltol from glucose alone. Maltol and HMF are known to form during the pyrolysis of sugars in the absence of amino acids. Table V shows the rate constants and activation energies of formation of HMF and maltol. Comparison of the values of rate constants and activation energies of HMF and maltol formation directly from glucose and from ARP indicates that Amadori rearrangement provides a low-energy pathway for their formation by thermal decomposition.

Thermal Decomposition of Tryptophan. Tryptophan (0.2 mg) was dissolved in 0.5 mL of water, and its decomposition was followed at 110 and 140 °C, as described previously. The main objective of this study was to determine whether indole can be produced by the deg-

Scheme IV. Proposed Mechanism of Formation of Norharman



radation of tryptophan alone. According to our results, no indole was present in the decomposition mixture.

Proposed Mechanisms for the Decomposition of ARP. *Formation of HMF.* The proposed mechanism for the formation of HMF is shown in Scheme I. According to this mechanism, tryptophan Amadori product undergoes an initial dehydration step to form a double bond between C-1 and C-2 of the sugar residue, and then the resulting compound undergoes another dehydration reaction assisted by the electron pair of the nitrogen atom, to form the imminium ion; the imminium ion then undergoes hydrolysis (the rate-determining step according to the kinetic analysis) followed by a dehydration step to form the HMF.

Tryptophan Release and Formation of Maltol. Scheme II shows the proposed mechanism of formation of maltol and simultaneous release of tryptophan. In the initial step tryptophan ARP undergoes a dehydration step to form a double bond between C-2 and C-3 of the sugar residue; this is followed by a cleavage of the C–N bond by a thermally induced elimination reaction (possibly the rate-determining step according to the kinetic analysis) to release tryptophan and a sugar residue, which, after dehydration and isomerization, forms maltol.

Formation of Indole. Scheme III shows the proposed mechanism of formation of indole. The tryptophan ARP (shown in one of its tautomeric forms) undergoes a decarboxylation reaction with concomitant deethenylation and concerted transfer of the carboxylic acid proton to the indole nitrogen. Apparently, the sugar residue is essential for this elimination reaction to occur since tryptophan alone did not produce indole under the same decomposition conditions.

Formation of Norharman. Scheme IV illustrates the proposed mechanism of formation of norharman. Tryptophan Amadori product undergoes two dehydration steps to form double bonds between C-4 and C-5 and between C-2 and C-3 of the sugar residue; the second dehydration is assisted by the lone pair electrons of the ring oxygen that leads to the formation of the oxonium ion **2**. Compound **2** then undergoes a C–C bond scission to form the imminium ion **3**, which then undergoes an intramolecular nucleophilic substitution reaction to form the tetrahydrocarboline structure which subsequently decarboxylates and dehydrates to form norharman. This mechanism is based on electron impact (EI) mass spectrometric studies of Trp-ARP, using B/E and B^2/E linked-field scan techniques (Yaylayan et al., 1990).

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