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Free glutamine as a major precursor of brown products and fluorophores in Maillard reaction systems

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Summary. Glutamine is one of the most abundant free amino acid found in raw food. In this study, the contribution of free glutamine to nonenzymatic browning and fluorescence was investigated using an aqueous model system with methylglyoxal. The results indicated that glutamine contributed to the Maillard reaction via two pathways. First, the hydrolysis of the amide bond of glutamine led to the release of ammonia which was implicated in the formation of brown color and fluorescence. Among other nitrogen donors tested (asparagine, glutamic acid and urea) our results demonstrated that free glutamine was a major source of ammonia during heating. When heated at 120 and 180 °C, 100% of ammonia was released from glutamine after 60 and 10 min, respectively. The second pathway involved a direct Maillard reaction with the α-amino group of glutamine. Both pathways led to a rapid and complete destruction of glutamine when heated in the model systems. With reference to the Maillard browning (absorbance at 420 nm) glutamine turned out to be the most reactive amine, followed by asparagine, glutamate, ammonia and urea. Maximum fluorescence (excitation and emission wavelengths at 330 and 450 nm, respectively) was also observed with glutamine followed by urea and ammonia. Overall this study suggested that free glutamine predominantly contributes to the color and fluorescence formations of foodstuffs.

Keywords: Glutamine – Ammonia – Maillard reaction – Browning – Fluorescence

Abbreviations: Asn, asparagine; Gln, glutamine; Glu, glutamic acid; MG, methylglyoxal; Pga, pyroglutamic acid; RP-HPLC, reversed phase-high performance liquid chromatography

Introduction

Glutamine (Gln) is one of the most abundant free amino acids present in raw foods. Its concentration was reported to account for up to 25 percent of free amino acids in potato tubers (Davids et al., 2004), 51% in tomatoes (Pratta et al., 2004), 50% in pork (Aristoy and Toldra, 1998) and 25% in cow milk (Agostini et al., 2000). However, Gln is also known to be one of the most unstable amino acids. Its degradation occurs slowly in an aqueous solution at ambient temperature and more rapidly when heated (Snowden et al., 2002). The denaturation of Gln leads to the formation of pyroglutamic acid (Pga) and ammonia, and less abundantly to the formation of glutamic acid (Glu). If Pga and Glu are relatively stable, ammonia can undergo further reactions. It can react with reducing sugars and therefore contributes to the Maillard reaction. The Maillard reaction, which has been thoroughly investigated in food chemistry, affects the overall quality of food when thermally processed, and results in to the development of attractive flavor volatiles, and yellow to dark brown products (melanoidins). The nonenzymatic browning is usually desirable in baked, roasted or fried food, but undesirable in other heat-treated food products such as milk, fruit juices, dry fruits, and tomato sauce.

Reactions between ammonia and reducing carbohydrates in model systems have been investigated to clarify the chemical structures of the related neo-formed compounds. In particular, volatile Maillard reaction products have been identified using gas chromatography coupled to mass spectrometry and mass spectral libraries. Pyrazoles (Yaylayan and Haffenden, 2003) and pyrazines (Chen and Ho, 2002) are the main heterocyclic flavor compounds detected in ammonia model systems. The formation of unidentified brown chromophores, which competes with the synthesis of volatile compounds, was also reported when ammonium carbonate and glucose were added to a model system (Izzo and Ho, 1992).

Except for the ammonium salts used as food additive, amides of acidic amino acids are among the primary sources of ammonia in many food products. For instance,

ammonia can be released by deamidation of asparaginyl and glutaminyl residues in proteins (Riha et al., 1996). Sohn and Ho (1995) who compared the yield of ammonia release from different heated solutions of free amino acids concluded that Gln, asparagine (Asn), cysteine and aspartic acid generated the largest amounts. Urea, a compound with two amide bonds and naturally present in many food products, has been also identified as a precursor of ammonia during heating and microbial degradation (Chen et al., 2000). However nobody, to our knowledge, has evaluated the competition between the thermal decomposition of free Gln to Pga and ammonia, and its degradation via the Maillard reaction.

In the present paper, Maillard reaction systems were therefore used to compare the relative reactivity of Gln versus other free amino acids and urea in the formation of ammonia, brown color products and fluorophores. Methylglyoxal (MG), frequently found in food (Homoki-Farkas et al., 1997) and much more reactive than hexose in the Maillard reaction (Thornalley, 2005), was chosen as the reactant of our model systems.

Materials and methods

Materials

L-glutamine, L-asparagine, L-glutamic acid, *o*-phthaldialdehyde, 2-mercaptoethanol and Brij® 30 were purchased from Fluka (Switzerland), methylglyoxal (40% in aqueous solution), L-pyroglutamic acid and trifluoroacetic acid were obtained from Sigma (St. Louis, MO), urea, ammonia solution, di-sodium hydrogen phosphate, sodium dihydrogen phosphate and 1,4-dioxane from VWR International (France). Acetonitrile HPLC grade, isopropanol HPLC grade and methanol HPLC gradient grade were purchased from Fisher Bioblock Scientific (France). Water for model systems and HPLC was distilled in the laboratory using an Elga Purelab UHQ II system.

Preparation of the model systems and heating procedures

Samples from all system models were dissolved in a 200 mM sodium phosphate buffer pH 9. The buffer was made by slowly adding a monobasic stock solution (NaH_2PO_4) to a dibasic stock solution (Na_2HPO_4) under stirring while measuring pH and stopping when pH 9 was reached. The concentration of each stock solution was 200 mM.

Samples of 3 ml each were distributed into screw capped Pyrex tubes (16 mm in diameter and 100 mm in height) and placed in an oil bath set up at 120 or 180 °C. The oil temperature was monitored and controlled by a Thermocontact ETC-1 electronic temperature controller (Fisher-Bioblock Scientific, Illkirch, France). After various periods of time, the tubes were removed and cooled in ice. They were then either immediately analyzed or stored at −20 °C until analysis.

Free amino acids and urea heating

Equimolar concentrations of Gln, Asn, urea and Glu (100 mM) were heated at 120 °C for 30, 60, 120, 180 and 240 min. The first three molecules were also heated at 180 °C for 10, 20, 30, 60 and 120 min.

Amino compounds – methylglyoxal reaction mixtures

Methylglyoxal (400 mM) was mixed in equal volumes with either Gln, Asn, Glu, urea or ammonia (200 mM) to reach final concentrations of 200 and 100 mM, respectively. The mixtures were immediately heated at 120 °C for 2 h.

Glutamine – methylglyoxal reaction mixtures

Gln solution (100 mM) was heated with increasing concentrations of methylglyoxal (0, 50, 100, 200 and 500 mM) at 120 °C for 240 min. The same model system diluted ten times in a phosphate buffer was heated at 120 °C for 30, 60, 120 and 240 min to study the kinetic of the Maillard reaction.

Ammonia determination

The total ammonia concentration from various reaction mixtures was measured with an ammonia electrode model 95-12 coupled with an Orion Research ion analyzer EA940 system (Thermo Orion, Beverly, MA, USA). The samples were adjusted to pH 12 with NaOH 10 M to measure the un-ionized (NH_3) plus the ionized (NH_4^+) forms of ammonia. A calibration curve was constructed using pure ammonia solutions of increasing concentrations.

Pyroglutamate quantification

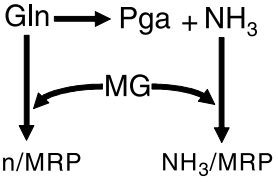
The determination of pyroglutamate (Pga) formation in the Gln solutions was monitored by a reversed phase high performance liquid chromatography (RP-HPLC) with a Thermo Separation Products HPLC instrument coupled to a UV 2000 detector (Thermo Electron Corporation, Courtaboeuf, France). The chromatography was performed using a 4.6 × 250 mm, 5 µm particle size, Alltima HP C18 Amide column (Alltech, France) eluted at a flow rate of 1 ml/min with a linear gradient starting with 100% trifluoroacetic acid (0.1% in water), to 95% trifluoroacetic acid and 5% acetonitrile at 7 min. Under these conditions, the pyroglutamate eluted between 5 and 6 min and was detected at a wavelength of 214 nm. Standard solutions of pyroglutamate were injected into the RP-HPLC to construct the calibration curve. The method presented above was adapted from Snowden et al. (2002), who used a C8 HPLC column and diluted HCl as a mobile phase.

Glutamine quantification

The loss of Gln was determined using HPLC with fluorescence detection after derivatization with *o*-phthaldialdehyde (OPA) as described by Jarrett et al. (1986). Derivatized samples were injected on a 4.6 × 150 mm, 5 µm particle size, Adsorbosphere OPA-HR column (Alltech, France), with a mobile phase of 25 mM sodium acetate (pH 5.9) containing 4.5% (v/v) 1,4-dioxane and 3% (v/v) isopropanol (solvent A), and methanol with 1.5% (v/v) 1,4-dioxane and 1.5% (v/v) isopropanol (solvent B).

Calculation of the concentration of the Maillard reaction products coming from the amide side chain or the α-amino group of glutamine

A simplified Maillard reaction mechanism of Gln and MG can be represented as



where Gln is the glutamine, Pga and NH_3 are the pyroglutamic acid and ammonia resulting from the degradation of Gln, respectively, MG is the reactive methylglyoxal, Gln/MRP are the Maillard reaction products formed between Gln and methylglyoxal, and NH_3/MRP are the Maillard reaction products formed between ammonia and methylglyoxal.

When Gln solutions (100 mM) were heated with increasing concentrations of methylglyoxal, the competition between the direct Maillard reaction of Gln and the reaction of ammonia with methylglyoxal was estimated according to the following equations.

The concentration of the Maillard reaction products coming form the amide side chain, denoted [NH_3/MRP], can be represented by

$$[NH_3/MRP] = [NH_3 \text{ formed}] - [NH_3 \text{ remained}]$$
$$[NH_3/MRP] = [\text{Pga formed}] - [NH_3 \text{ remained}]$$

[Pga formed] and [NH_3 remained] were measured according to the methods described above.

The concentration of the MRP coming form the α-amino group of Gln, denoted [Gln/MRP], can be represented by

$$[\text{Gln/MRP}] = [\text{Gln}]_{t0} - [\text{Pga formed}] - [\text{Gln remained}]$$

In this experiment, the initial concentration of glutamine $[\text{Gln}]_{t0}$ was 100 mM, and this amino acid was totally consumed after 4 h of heating at 120 °C.

$$[\text{Gln}]_{t0} = 100 \quad \text{and} \quad [\text{Gln remained}] = 0$$

Therefore,

$$[\text{Gln/MRP}] = 100 - [\text{Pga formed}]$$

Browning measurements

The formation of brown color products was monitored by absorbance at 420 nm with a Beckman model DU500 spectrophotometer (Beckman-Coulter France, Villepinte, France). When necessary, appropriate dilutions were performed to ensure the linearity of the optical density. The cell path length was 1 cm.

Fluorescence measurements

Fluorescence spectra were recorded with a Varian spectrofluorometer (model Cary Eclipse, Varian, Les Ulis, France). The quantitative measurement of fluorescence intensity was registered at the maximum excitation (330 nm) and emission (450 nm) wavelengths of the Gln-methylglyoxal mixtures.

Statistical analysis

Each sample was prepared in triplicate, and all values were expressed as mean ± SD.

The significance of differences between absorbance or fluorescence of the Gln samples and other samples was analyzed using the Mann-Whitney test (Bialès, 1988).

Results

Kinetic of formation of ammonia from free amino acids and urea

Ammonia formation was measured in solutions of Asn, Gln, Glu and urea. Figure 1 shows the ammonia formation in the four solutions heated as a function of time.

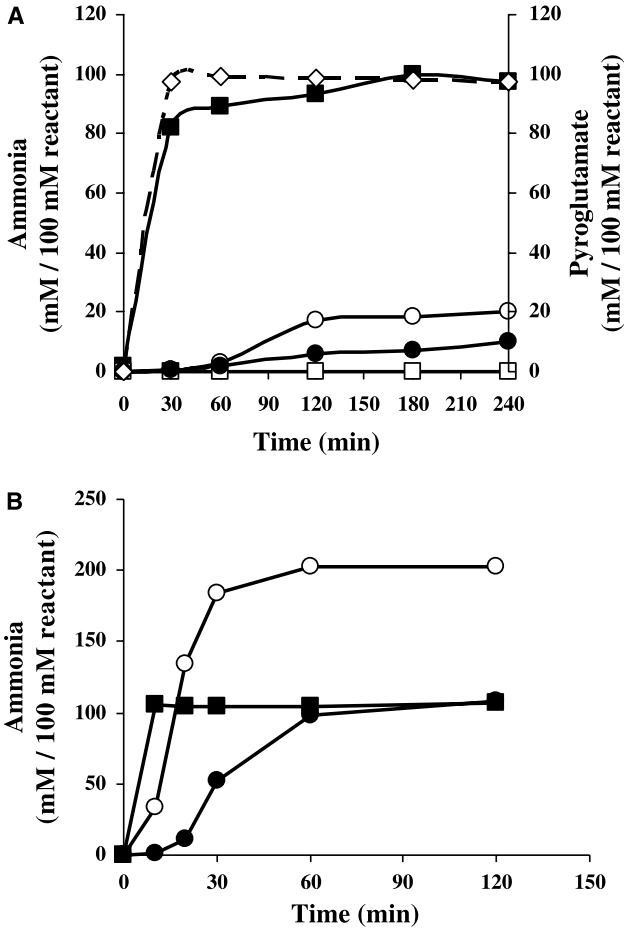


Fig. 1. A Formation of ammonia at 120 °C in aqueous solutions of Gln (■), urea (○), Asn (●) and Glu (□) as a function of time. Formation of pyroglutamate at 120 °C in aqueous solutions of Gln (◇) as a function of time. **B** Formation of ammonia at 180 °C in aqueous solutions of Gln (■), urea (○) and Asn (●) as a function of time

At a temperature of 120 °C (Fig. 1A) Gln was the most important precursor of ammonia with a fast rate of formation. After 60 min of treatment, a plateau was reached with a yield of formation close to 100%. With a similar heat treatment, the kinetic of the ammonia produced by urea reached a plateau at 120 min with a yield of formation close to 20%. The extent of ammonia released from the Asn solution is much lower compared to Gln and urea (∼10% after 240 min). At the end, no ammonia was detected in the Glu solution.

When heated at 180 °C (Fig. 1B) Gln released 100% of ammonia after only 10 min and urea was totally decomposed in ammonia after 60 min, forming two moles of ammonia per mole of urea. Asn which was found relatively stable at 120 °C released up to 100% of ammonia when treated at 180 °C for 120 min.

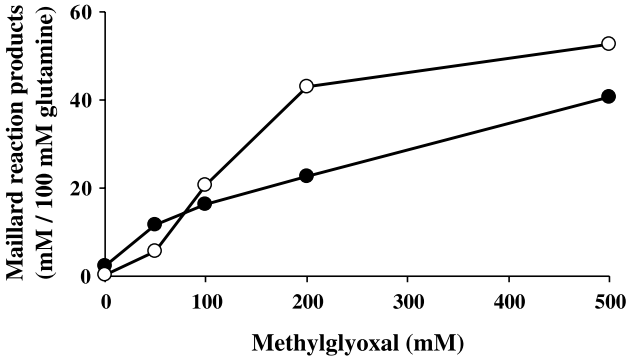


Fig. 2. Gln/MRP (●) and NH_3/MRP (○) formed in aqueous solutions of Gln heated for 4 h at 120 °C as a function of MG concentration

Formation of pyroglutamic acid from glutamine

Gln degradation leads to Pga formation. As shown in Fig. 1A, the formation of Pga followed the same kinetics profile as that observed for ammonia liberation. A plateau was reached after a 60-min heating period, with a yield of formation close to 100% (Pga concentration: 100 mM). Furthermore, there was no loss of Pga over the investigated time of heating treatment.

Effect of methylglyoxal on Gln/MRP and NH_3/MRP formations

Figure 2 shows the time course of Gln/MRP and NH_3/MRP formations in heated Gln solutions (4 h at 120 °C) as a function of MG concentration. In the absence of MG, ammonia and Pga were found at a concentration of 100 mM, which indicates a complete degradation of Gln with no significant loss of ammonia or of Pga after their formation. The OPA analysis of Gln confirmed its total degradation when heated at 120 °C for 4 h independent of the concentration of the MG (data not shown). With increasing concentrations of MG, both concentrations of Gln/MRP and NH_3/MRP appeared to increase in different proportions. Thus, when 50 mM of MG was added to the Gln solution, the formation of Gln/MRP was higher than that of NH_3/MRP. Above 100 mM of MG, a 1/1 Gln/MG molar ratio, the formation of NH_3/MRP appeared to become predominant.

Effect of three amino acids, urea and ammonia on the formation of fluorescence and color in the presence of methylglyoxal

After reaction of Gln, Asn, Glu, urea and ammonia with MG at 120 °C for 2 h, a comparative analysis of browning and fluorescence intensities was carried out.

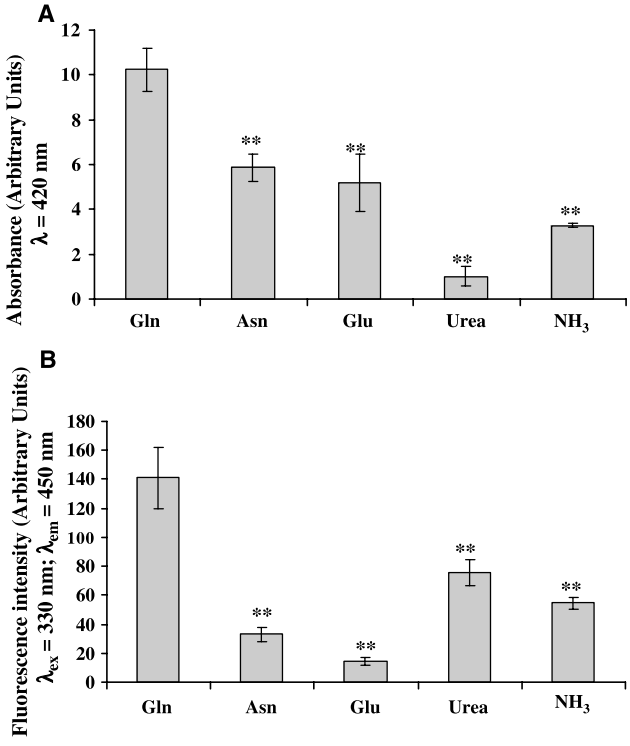


Fig. 3. Formation of Maillard browning as measured by absorption at **A** 420 nm, **B** fluorescence at 330 and 450 nm for the excitation and emission respectively, in aqueous solutions of Gln, Asn, Glu, urea and ammonia heated at 120 °C for 2 h with MG. Statistical significance between Gln solution and each other mixtures was calculated using Mann-Whitney test (**, $p < 0.05$)

As can be seen in Fig. 3A, the formation of brown pigments was significantly more important in the presence of Gln compared to any other amine tested. The Gln reactivity was followed by Asn and Glu, and urea was found to be the least reactive with only ~10% of the browning intensity formed with Gln. The contribution of ammonia to the brown color formation was measured on the ammonia/MG solution and less than one third of the Gln-related browning intensity was observed.

It is also apparent that the maximum fluorescence was reached with Gln (Fig. 3B). However the classification of the other amines according to their contribution to the fluorescence formation is different from the one based on browning. In this case Gln is followed by urea and ammonia, while Asn and Glu exhibited less reactivity.

Kinetics of formation of browning and fluorescence as a function of the glutamine/methylglyoxal (Gln/MG) molar ratio

Gln was reacted with increasing concentrations of MG at 120 °C and the formation of brown color and fluorescence

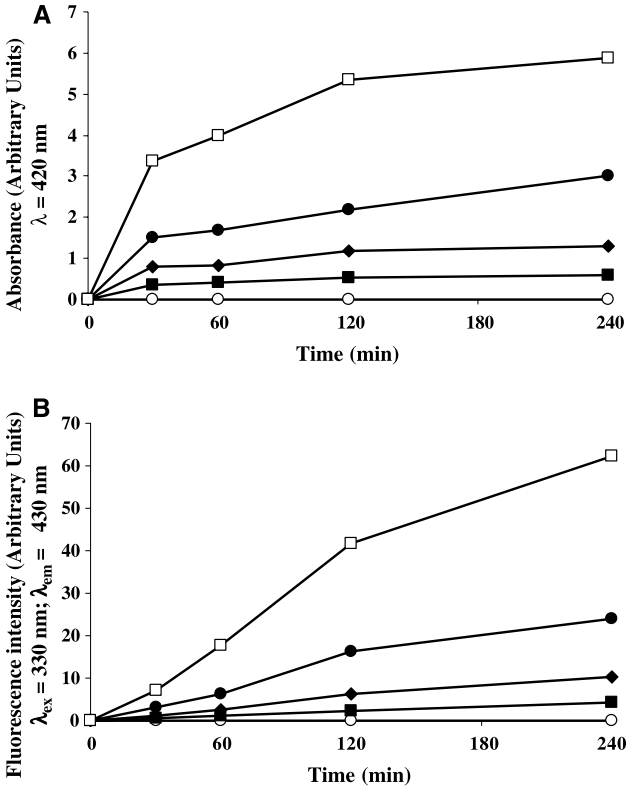


Fig. 4. Formation of Maillard browning as measured by absorption at 420 nm (**A**) and fluorescence at 330 and 430 nm for the excitation and emission respectively (**B**), in heated (120 °C) buffered solutions of Gln and MG as a function of the molar ratio. Gln/MG ratio: 1/5 (□); 1/2 (●); 1/1 (◆); 1/0.5 (■); 1/0 (○)

were recorded from 0 to 240 min (Fig. 4). The samples of the model systems were prepared in a strongly buffered solution (200 mM) to minimize the decrease in the pH during the Maillard reaction. The pH values never decreased below 7.50.

No browning was found in the solution containing Gln alone. The rate of the Maillard browning increased as the concentration of MG became higher in the Gln/MG system (Fig. 4A). No induction period was observed on the curves of browning. A plateau was reached after 30 and 120 min of heating for the lower and the higher concentrations of MG, respectively. After reaching a maximum the browning intensities remained stable until the end of the heating treatment for all samples.

Figure 4B shows that no detectable fluorescence was observed without addition of MG. However, increasing its levels at constant Gln concentration increased the rate of fluorescence. The kinetics of the development of fluorescence can be divided in three phases: (i) an induction period, (ii) a linear increasing rate of formation with time, and (iii) beginning of a plateau period from 120 min. The induction time appeared to be longer when the concentration of MG was lower. The fluorescence then increased with MG concentration and heating time following a zero-order formation until 120 min.

Discussion

In the past many experiments have been carried out with free amino acids to compare the part their Maillard reactivity plays in contributing to the nonenzymatic browning and to the formation of volatile compounds. Classification of these amino acids has commonly been based on browning measurement at 420 nm or quantification of specific Maillard reaction products in simple model systems. Although the different conditions used in the previous studies brought contradictory results, lysine frequently turned out to be the most reactive free amino acid (Kwak and Lim, 2004). More recently, there has been some debate in the literature concerning the interest in such results since free amino acids occur only in lower quantities in foods compared to amino side chains of lysine or arginine bound to the proteins. However the discovery of acrylamide in food became an important example of how a free amino acid can contribute significantly to the Maillard reaction. Therefore, free Asn, which is largely present in plant foods, appeared to be one of the main precursors of acrylamide in heat-processed foods (Stadler et al., 2002).

Besides Asn, free Gln is not only the most prevalent free amino acid of many foods of plant origin, but also one of the most abundant free amino acids in animal food stuffs such as meat or milk. Its role in the formation of Maillard reaction products needs to be clarified. We therefore heated Gln in model systems, and compared its reactivity to either amino acids of closely related chemical structures or urea as an important precursor of ammonia. Our food-related cooking conditions were similar for all experiments. For instance a pH of 9 was selected since it is well established that Maillard reaction is activated under moderately basic conditions (Labuza and Baisier, 1992). This pH is not exactly relevant for common foods. However, in this study we wanted to favor the deprotonation of ammonia and α-amino group of Gln and, therefore, initiated the Maillard reaction by the formation of an imine with MG.

Gln instability was described in aqueous model systems and during heat treatment (Snowden et al., 2002). The cyclization of Gln to Pga takes place with a deamidation. In the present study, the formation of Pga and the loss of ammonia were observed after heating a Gln solution. First

we observed that the Pga formed after degradation of Gln was thermally stable in aqueous conditions. The presence of glucose, fructose or MG had no effect either on the stability of the aqueous solution of Pga (data not shown). Those data indicate that, under aqueous conditions, Pga is an end-product of the thermal degradation of Gln and, therefore, should neither contribute to the non-enzymatic browning of foods with a high moisture content nor to the formation of flavor.

The second degradation product of Gln is ammonia. Its formation was followed and compared to the release from other molecules. At high temperature, we observed that the ammonia released from free Gln was much faster than that from free Asn. Finally, the absence of ammonia when Glu was heated indicates that deamidation and not deamination is involved in the loss of ammonia by Gln. These results are consistent with previously published data from Sohn and Ho (1995), except that these authors reported an additional release of ammonia from the α-amino group of Asn.

Besides Gln, urea can also generate ammonia when heated. Our experiment indicates that the rate of formation of ammonia from urea is dependent on the temperature and time of treatment. They also suggest that in meat foods where Gln and urea are present at similar concentrations, Gln will be the main contributor of ammonia during cooking.

Although the ammonia released from Gln was quite stable in heated solution, an important loss was observed in the presence of MG. The results indicate that under the action of heat the ammonia released from the degradation of Gln attacks the carbonyl groups thus leading to the Maillard reaction. Moreover the data suggest that in the presence of MG, Gln was also directly involved in the Maillard reaction. The reaction of the α-amino group of Gln with the dicarbonyl appeared to be in competition with the ''self-decomposition'' of Gln. Finally, the ammonia released by deamidation was also in competition with reaction of α-amino group of intact Gln to generate Maillard reaction products.

A comparative study of nonenzymatic browning and formation of fluorophores was carried out. Among the five adducts tested, Gln was the major contributor to the Maillard reaction. We tentatively attribute this intensive brown pigment formation and fluorescence to the extreme instability of Gln and to the high reactivity of the free ammonia released. Under mild thermal conditions (120 °C) it appears that the heated solution of urea and MG led to a significantly lower extent of browning and fluorescence than the mixture of Gln and MG. The higher amount of ammonia released from Gln and the additional reactivity attributed to its α-amino-group are two possible explanations for the difference of rate of the Maillard browning observed. The same conclusion can be drawn from the data obtained with Asn. Overall this suggests that, among other nitrogen donors, free Gln predominantly contributes to the overall generation of browning in heated Gln-rich food such as meat and vegetables.

Undoubtedly the rate of the Maillard reaction is affected by the ratio between the two reactants. With reference to color and fluorescence formations we observed a maximum in Maillard reaction at a 1:5 ratio with the Gln/MG model system. Our data are consistent with the previous studies reviewed by Baisier and Labuza (1992) which had concluded that an excess of the carbonyls over amino adducts generally causes an increase in the rate of nonenzymatic browning. Under our conditions, the formation of the fluorophores exhibited an induction phase, but surprisingly the formation of the brown pigments did not. This observation is different from those reported by some authors who attributed the initial induction period to the formation of colorless precursors of the brown pigments (Tanaka et al., 1986). The high temperature selected in the present study might explain the absence of induction period for the brown pigment formation.

In conclusion, the results of this study revealed that Gln, a major free adduct of raw food, significantly influences the extent of brown color and fluorescence in Gln/MG model systems. In order to determine the true contribution of this free amino acid in the Maillard reaction of heat-processed food, the discovery of specific brown products and fluorophores formed from the reaction between Gln and carbonyls is under detailed investigation. Further work will be conducted to estimate the role of Gln and ammonia in the formation of mutagenic compounds.

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