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Analytical Methods

Degradation of asparagine to acrylamide by carbonyl-amine reactions initiated by alkadienals

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ABSTRACT

The degradation of asparagine to acrylamide produced by 2,4-decadienals was analysed in detail in an attempt to understand the reactions pathways involved in the formation of acrylamide by oxidised lipids. Thus, the effects of a_w, pH, oxygen and lipid content, and time and temperature, were studied in different samples of 2,4-decadienal, 2,4-heptadienal, 2,4-hexadienal, 2-octenal, or ethyl 2,4-decadienoate with asparagine, *N*-acetylasparagine, asparagine *t*-butyl ester, glutamine or ammonium chloride. Acrylamide was produced to a higher extent in alkadienal/asparagine mixtures, and the reaction yield increased slightly when the chain length of the oxidised lipid decreased. In addition, the reaction yield was very much reduced when the amino acid was esterified or when an alkenal was employed. Furthermore, acrylamide was not produced when ethyl 2,4-decadienoate, *N*-acetylasparagine, glutamine or ammonium chloride were employed. These results, together with the detection of 3-aminopropionamide and 2-pentylpyridine in 2,4-decadienal/asparagine mixtures, suggested a potential role of the alkadienal in both the decarboxylation of the amino acid and the later conversion of decarboxylated amino acid into acrylamide. This last conclusion was confirmed by studying the effect of the lipid content in the formation of acrylamide in 2,4-decadienal/3-aminopropionamide mixtures.

1. Introduction

Amino acid degradation is a common consequence of the Maillard reaction produced by carbohydrates and it has been related to the formation of both flavour and toxic compounds in addition to be one of the main pathways for the production of compounds responsible for taste and colour during food processing (Didzbalis & Ho, 2001; Hofmann, 2005; Hofmann & Schieberle, 1998; Mottram, 1994). Thus, the Strecker degradation of amino acids is one of the most important reactions leading to final aroma compounds in the Maillard reaction (Whitfield, 1992; Yaylayan, 2003). In addition, asparagine degradation has been related to the formation of acrylamide in heated foodstuffs (Hedegaard, Frandsen, & Skibsted, 2008; Masson et al., 2007; Mustafa et al., 2009; Pedreschi, Kaack, & Granby, 2008; Taeymans et al., 2004; Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2002; Yaylayan, Wnorowski, & Locas, 2003).

Different studies have shown that oxidised lipids compete very efficiently with carbohydrates for these carbonyl-amine reactions and the same products are frequently produced from either carbohydrates or lipids by identical or very similar reaction pathways (Zamora & Hidalgo, 2005). Thus, recent studies have shown that a wide range of oxidised lipids are able to degrade amino acids to their corresponding both Strecker aldehydes (Hidalgo, Gallardo, & Zamora, 2005; Hidalgo & Zamora, 2004; Zamora, Gallardo, & Hidalgo, 2007) and vinylogous derivatives (Hidalgo & Zamora, 2007; Zamora & Hidalgo, 2008), therefore suggesting potential alternative routes for acrylamide formation as a consequence of carbonyl-amine reactions initiated by oxidised lipids.

Among the different oxidised lipids assayed, 2,4-decadienal was found to convert asparagine into acrylamide to a much higher extent than other oxidised lipids, which included both unoxidised lipids and primary, secondary, and tertiary products of lipid oxidation (Zamora & Hidalgo, 2008). However, the reasons for this much higher reactivity of 2,4-decadienal remains to be clarified.

In an attempt to understand the reaction pathways by which lipid oxidation products are able to convert asparagine into acrylamide, this study analyzes in detail the reaction between 2,4-decadienal and asparagine. In addition, the reactions of other oxidised lipids and amino compounds were also studied for comparison purposes.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Aldrich (Milwakee, WI), Sigma (St. Louis, MO), Fluka (Buchs, Switzerland), or Merck

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(Darmstadt, Germany), and were analytical grade. 3-Aminopropionamide was obtained from TCI Europe (Zwijndrecht, Belgium). Labelled [1,2,3-$^{13}C_3$]acrylamide was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). 2,4-Decadienal (93%) was obtained from Aldrich. It was further purified by column chromatography on silica gel 60 using hexane-acetone (9:0.25) as solvent. The aldehyde recovered from the column was chromatographically pure as determined by GC.

2.2. Amino compound/oxidised lipid reaction mixtures

Model reactions were carried out analogously to Granvogl and Schieberle (2006), with the modifications described by Zamora and Hidalgo (2008). Briefly, mixtures of the amino compound (37.5 μmol; 3.75 μmol in some experiments with 3-aminopropionamide) and the oxidised lipid (0–37.5 μmol) were singly homogenised with 0.063–0.200 mm silica gel 60 (300 mg) (Macherey-Nagel, Düren, Germany), 30 μL of 0.3 M buffer (sodium citrate for pH 3–6 and sodium phosphate for pH 6–7) and 0–270 μL (0–45%) of water, and heated under a controlled atmosphere at 180 °C in closed test tubes for 10 min, unless otherwise indicated. The amino compounds assayed were asparagine, *N*-acetylasparagine, asparagine *t*-butyl ester, glutamine, ammonium chloride, and 3-aminopropionamide. The oxidised lipids assayed were 2,4-decadienal, 2,4-heptadienal, 2,4-hexadienal, 2-octenal, and ethyl 2,4-decadienoate. In addition, some experiments also included catalytic amounts of platinum oxide, which was added before homogenisation. The water activity (a_w) of the samples was determined with a Pawkit Decagon analyser (Pullman, WA). The oxygen content in the test tubes was determined with a PAK01P Abiss analyser (Viry-Chantillon, France). The reaction pH was maintained upon heating.

After cooling (15 min at −20 °C), 10 μL of internal standard solution (1 mg/mL of labeled [1,2,3-$^{13}C_3$]acrylamide in methanol) and 2 mL of 0.3 M sodium citrate buffer, pH 2.2, were added. Suspensions were stirred for 1 min, the supernatant was then filtered and its acrylamide content determined. In addition, other heated samples were cooled (15 min at −20 °C) and 50 μL of propionamide (3.6 mg/mL in methanol) added as internal standard. These samples were extracted with methanol (2 × 2 mL), and the extracts were derivatizated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and studied by GC–MS.

2.3. Analysis of acrylamide

Acrylamide was analysed as the stable 2-bromopropenamide by gas chromatography–mass spectrometry (GC–MS) using the method of Castle, Campos, and Gilbert (1991) with the modifications of Andrawes, Greenhouse, and Draney (1987). Briefly, 1 mL of the supernatant was treated with 0.3 g of potassium bromide and 400 μL of saturated bromine solution in water. After 1 h in the dark at 0 °C, the excess of bromine was removed by addition of 1 M sodium thiosulfate until the solution became colourless, and the solution was extracted with 1 mL of ethyl acetate/hexane (4:1). The organic layer was finally dried with sodium sulphate, evaporated until a volume of ~50 μL, treated with 50 μL of triethylamine, and analysed by GC–MS.

The ions monitored for the identification of the analyte, 2-bromopropenamide, were $[C_3H_4NO]^+ = 70$, $[C_3H_4{}^{79}BrNO]^+ = 149$, and $[C_3H_4{}^{81}BrNO]^+ = 151$, using *m/z* 149 for quantification. The ions monitored for identification of the internal standard (2-bromo[$^{13}C_3$]propenamide) were $[^{13}C_2H_3{}^{81}Br]^+ = 110$ and $[^{13}C_3H_4{}^{81}BrNO]^+ = 154$, using *m/z* 154 for quantification. Mass spectra of both compounds are collected, for example, by Pillet, Périsset, and Oberson (2004). The separation of acrylamide analyte after derivatization was performed on GC capillary columns of middle to high polarity. GC–MS analyses were conducted with a Hewlett–Packard 6890 GC Plus coupled with an Agilent 5973 MSD (Mass Selective Detector-Quadrupole type). In most experiments, a 30 m × 0.25 mm i.d. × 0.25 μm HP5-MS capillary column was used. Working conditions were as follows: carrier gas helium (1 mL/min at constant flow); injector, 250 °C; oven temperature: from 50 (1 min) to 240 °C at 5 °C/min and then to 325 °C at 10 °C/min; transfer line to MSD, 280 °C; and ionisation EI, 70 eV.

Quantification of acrylamide was carried out by preparing standard curves of this compound in the 300 mg of silica gel and following the whole procedure described above. For each curve, fifteen different concentration levels of acrylamide (0–200 μg) were used. Acrylamide content was directly proportional to the acrylamide/internal standard area ratio ($r = 0.999$, $p < 0.0001$). Data are mean values of, at least, two experiments. The coefficients of variation at the different concentrations were lower than 10%.

2.4. GC–MS analyses

GC–MS analyses of heated samples derivatizated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide were carried out in an attempt to identify intermediates in these reactions. A Hewlett–Packard 6890 GC Plus coupled with an Agilent 5973 MSD (Mass Selective Detector-Quadrupole type) and a 30 m × 0.25 mm i.d. × 0.25 μm HP5-MS capillary column was used in these experiments. Working conditions were as follows: carrier gas helium (1 mL/min at constant flow); injector, 250 °C; oven temperature: from 40 (10 min) to 240 °C at 5 °C/min and then to 300 °C (10 min) at 10 °C/min; transfer line to MSD, 280 °C; and ionisation EI, 70 eV.

3. Results

3.1. Acrylamide formation in asparagine/2,4-decadienal reaction mixtures

Upon heating, asparagine/2,4-decadienal reaction mixtures were not stable, and the formation of acrylamide – among other compounds – was observed. Acrylamide could be easily identified by GC–MS after derivatization on the basis of its retention index and mass spectra. The yield of this reaction depended on the reaction conditions. Thus, for example, it depended on the water content in the reaction mixture. Fig. 1A shows that an increase in water content increased the amount of the produced acrylamide until achieving a maximum. This maximum was produced when 31% of water was added. In order to compare the results obtained in asparagine/decadienal reaction mixtures with those previously described in phenylalanine/decadienal reaction mixtures (Hidalgo & Zamora, 2007), two different water contents were employed in the different reaction mixtures analysed in this study: 31%, which produced the maximum yield of acrylamide in asparagine/decadienal reaction mixtures, and without addition of water, which is usually employed in most studies on acrylamide formation. Both water contents produced analogous results, but acrylamide was always produced to a higher extent when 31% of water was added than when reactions were carried out without the addition of water. The a_w at these two water contents were 0.95 and 0.60, respectively.

Fig. 1B shows the effect of reaction pH on the amount of acrylamide produced in asparagine/decadienal reaction mixtures. Asparagine/decadienal mixtures produced similar contents of acrylamide at pH 4–6 and the yield of the reaction decreased below pH 4 or above pH 6. Because previous studies (Zamora & Hidalgo, 2008) were carried out using 0.3 M sodium phosphate buffer, pH 6, this buffer was also employed in the present study.

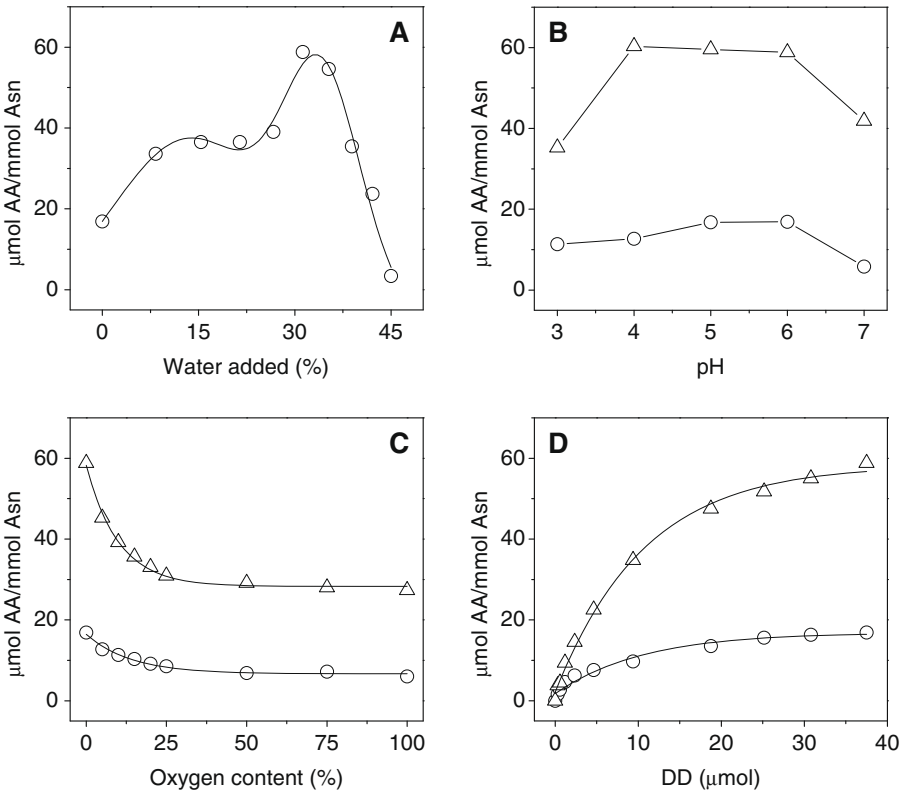

Fig. 1. Effect of: (A) water addition; (B) pH; (C) oxygen content; and (D) lipid content; in the formation of acrylamide in asparagine/2,4-decadienal mixtures heated for 10 min at 180 °C. In panels (B) (C) and (D) two water contents were assayed: without addition of water (○) and 31% of water content (△).

The amount of oxygen content in the atmosphere of the reaction mixture also influenced the amount of acrylamide produced (Fig. 1C). The reaction yield decreased exponentially ($r^2 > 0.96$) as a function of oxygen content. However, even when the reaction was carried out under pure oxygen a significant amount of acrylamide was produced. Thus, when 31% of water was added, the reaction yield decreased from 5.9% to 2.7%, and, without addition of water, it decreased from 1.7% to 0.6%. Because higher acrylamide contents were obtained in the absence of oxygen, a nitrogen atmosphere was always employed in the different asparagine/decadienal mixtures analysed in this study.

3.2. Effect of asparagine/2,4-decadienal ratio in the formation of acrylamide

The alkadienal was the responsible for the formation of acrylamide, which was not produced in the absence of the oxidised lipid (Fig. 1D). However, the formation of acrylamide did not increase linearly as a function of the amount of 2,4-decadienal added. The higher increases in acrylamide content were observed when low contents of 2,4-decadienal were present in the reaction mixture, therefore suggesting that very small amounts of 2,4-decadienal produced significant amounts of acrylamide. On the contrary, 30–37.5 µmol of 2,4-decadienal produced very similar amounts of acrylamide.

3.3. Effect of incubation time and temperature in the amount of acrylamide produced in asparagine/2,4-decadienal reaction mixtures

The reaction rate and the amount of acrylamide produced depended on the incubation time and temperature (Fig. 2). Thus, the amount of acrylamide produced increased linearly ($r > 0.98$, $p < 0.005$) with the incubation time at the different assayed temperatures. After 10 min, the yield of acrylamide formation was 0.6% at 140 °C, 1.2% at 150 °C, 2.2% at 160 °C, 3.5% at 170 °C, and

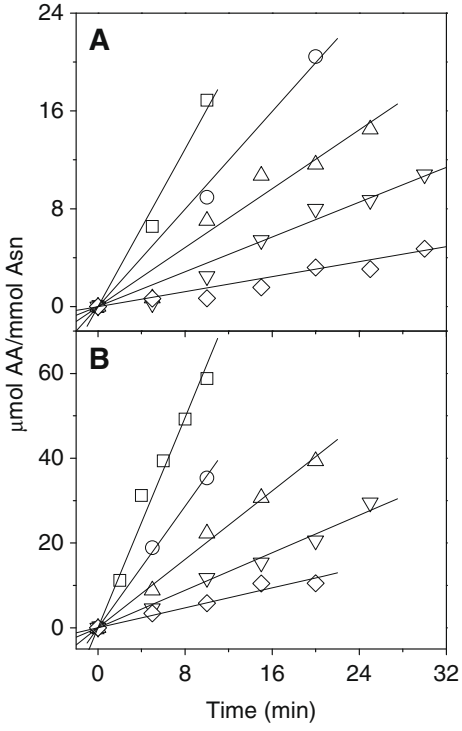

Fig. 2. Effect of time and temperature in the formation of acrylamide in asparagine/2,4-decadienal mixtures. Two water contents were assayed: (A) without addition of water; and (B) 31% of water content. The assayed temperatures were: 140 (◇), 150 (▽), 160 (△), 170 (○), and 180 °C (□).

5.9% at 180 °C when samples were incubated with the addition of 31% of water (Fig. 2B), and 0.07% at 140 °C, 0.2% at 150 °C, 0.7% at 160 °C, 0.9% at 170 °C, and 1.7% at 180 °C when samples were incubated without addition of water (Fig. 2A).

Reaction rates at the different assayed temperatures were calculated using Eq. (1):

$$[\text{acrylamide}] = [\text{acrylamide}]_0 + kt \quad (1)$$

where $[\text{acrylamide}]_0$ represents the intercept, k is the rate constant, and t is the time. As observed in the figure, $[\text{acrylamide}]_0$ was always zero. These rate constants were used in an Arrhenius plot (Fig. 3) for calculation of activation energy (Ea) of acrylamide formation from asparagine in the presence of 2,4-decadienal. The value obtained for Ea was 89.5 kJ/mol for samples incubated without addition of water and 91.6 kJ/mol for samples heated in the presence of 31% of water.

3.4. Effect of the addition of platinum oxide to asparagine/decadienal reaction mixtures

Platinum oxide was added to asparagine/decadienal reaction mixtures to investigate if the acrylamine produced in these reactions was converted into propionamide, analogously to the observed conversion of styrene into ethylbenzene in phenylalanine/decadienal reaction mixtures (Hidalgo & Zamora, 2007). Several asparagine/decadienal reaction mixtures were heated in the presence of platinum oxide and studied by GC–MS. None of the studied reaction mixtures produced propionamide (data not shown).

3.5. Effect of other lipid and amino acid derivatives in the amount of acrylamide produced

Reaction mixtures with different analogues of the lipid and the amino acid were also studied to determine the essential role of the different functional groups, present in both decadienal and asparagine, in the formation of acrylamide (Table 1).

The chain length of the lipid did not have a major influence on the amount of acrylamide produced. Thus, only slight increases were observed when the chain length of the lipid decreased (the reaction yield increased from 5.9% to 6.8% when the chain length decreased from 10 to 6 carbons). On the contrary, any change in the functional group implicated had a major effect in the reaction yield. Thus, the loss of one double bond in the lipid, such as in the 2-octenal, decreased the reaction yield to 0.1%. In addition, the change of the aldehyde group by an ester group, such as in ethyl 2,4-decadienoate, inhibited the reaction and acrylamide was not detected.

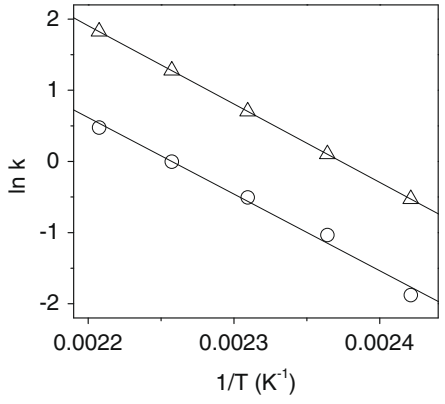

Fig. 3. Arrhenius plot for the formation of acrylamide in asparagine/2,4-decadienal mixtures at two water contents: without addition of water (○) and 31% of water content (△).

Table 1
Influence of the lipid and amino acid derivative in the formation of acrylamide in lipid/amino compound mixtures.[a]

Lipid	Amino acid	μmol AA/mmol Asn
2,4-Decadienal	Asparagine	58.8
2,4-Heptadienal	Asparagine	59.0
2,4-Hexadienal	Asparagine	67.5
2-Octenal	Asparagine	1.2
Ethyl 2,4-decadienoate	Asparagine	n.d.[b]
2,4-Decadienal	*N*-Acetylasparagine	n.d.[b]
2,4-Decadienal	Asparagine *t*-butyl ester	19.0
2,4-Decadienal	Glutamine	n.d.[b]
2,4-Decadienal	Ammonium chloride	n.d.[b]

[a] Mixtures were heated at 180 °C for 10 min.
[b] Not detected.

The α-amino group of the amino acid had an analogous essential role in the acrylamide produced. Thus, when *N*-acetylasparagine was employed, acrylamide was not produced. However, the acid group of the amino acid was not so critical (at least for the *t*-butyl ester assayed). Nevertheless, the reaction yield decreased. Thus, the reaction yield obtained for the *t*-butyl ester of the amino acid was 1.9%. This value was about one third of the acrylamide produced when the free amino acid was employed. Finally, when the amino acid asparagine was replaced by either glutamine or ammonium chloride, acrylamide was not formed, therefore confirming the essential role of asparagine in acrylamide formation by alkadienals.

3.6. 3-Aminopropionamide as an intermediate in the formation of acrylamide in decadienal/asparagine reaction mixtures

3-Aminopropionamide has been proposed as an intermediate in the formation of acrylamide by degradation of asparagine produced by carbohydrates (Granvogl & Schieberle, 2006). Therefore, its potential formation was also studied in decadienal/asparagine reaction mixtures. In these heated reaction mixtures, the formation of different products could be observed. These products were identified by comparison with the retention index and mass spectra of authentic compounds. As expected, the formation of acrylamide was observed [retention index 992, MS *m/z* 215 (6), 214 (8), 200 (13), 174 (6), 147 (100), 131 (7), 73 (41), 66 (7), 59 (5), 45 (14)]. However, the presence of other reaction products was significant. In particular, both 2-pentylpyridine and 3-aminopropionamide were also identified as reaction products. 2-Pentylpyridine had the expected retention index (1192) and mass spectrum [MS *m/z* 136 (1), 120 (22), 106 (24), 93 (100), 92 (9), 78 (9), 65 (8), 41 (6)]. In addition, 3-aminopropionamide produced two derivatives having 3 and 4 trimethylsilyl groups, respectively. 3-Aminopropionamide with three trimethylsilyl groups: retention index 1353, MS *m/z* 304 (0.3), 232 (5), 231 (3), 218 (19), 217 (93), 189 (17), 188 (26), 176 (15), 147 (100), 116 (43), 102 (69), 73 (74), 45 (12). 3-Aminopropionamide with four trimethylsilyl groups: retention index 1582, MS *m/z* 376 (0.1), 303 (5), 290 (29), 289 (100), 248 (23), 189 (14), 188 (15), 174 (72), 147 (56), 133 (16), 130 (17), 116 (14), 100 (12), 86 (12), 73 (68), 59 (11), 45 (10).

These results pointed out to 3-aminopropionamide as a potential intermediate in the formation of acrylamide in decadienal/asparagine reactions. An additional confirmation of this result was obtained by heating 3-aminopropionamide in the presence of different amounts of decadienal (Fig. 4). Thus, when 37.5 μmol of 3-aminopropionamide was heated in the presence of 0–37.5 μmol of decadienal, a linear increase ($r = 0.987$, $p < 0.0001$) in the formation of acrylamide was observed. In addition, the acrylamide yield of heating a mixture of 37.5 μmol of 3-aminopropionamide and 37.5 μmol of decadienal was 13.5% (the yield of heating

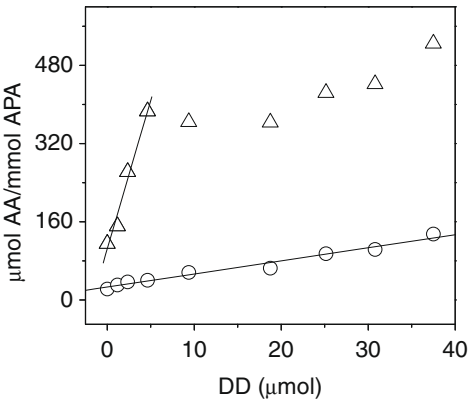

Fig. 4. Effect of lipid content in the formation of acrylamide in 3-aminopropionamide/2,4-decadienal mixtures. Two contents of 3-aminopropionamide were assayed: 3.75 (△) and 37.5 µmol (○).

an analogous equimolecular mixture of asparagine and decadienal was 5.9%, Table 1). When decadienal was present to a much higher extent than 3-aminopropionamide (0–37.5 µmol of decadienal and 3.75 µmol of 3-aminopropionamide), the reaction yield increased, and more than 50% of 3-aminopropionamide was converted into acrylamide.

4. Discussion

A recent study (Zamora & Hidalgo, 2008) has pointed out that some oxidised lipids are able to convert asparagine into acrylamide to a high extent. Among them, 2,4-decadienal was found to be much more reactive than other oxidised lipids for this reaction. The results obtained in the present study suggest that this high reactivity is related to the structure of the lipid, and acrylamide is produced to an extent that depends on the reaction conditions. Furthermore, the detection of 3-aminopropionamide in heated decadienal/asparagine samples points out to the formation of this compound as an intermediate in these reactions.

All these results suggest a mechanism for this reaction like the one indicated in Fig. 5. The reaction of the lipid with the amino acid should produce in a first step the formation of the corresponding imine, which is inhibited when either the carbonyl group of the aldehyde or the free amino group of the amino acid is not present, and which should not be favoured at high water contents. The second step of the reaction should be the decarboxylation of the imine, which would explain the detection of the 3-aminopropionamide. An equilibrium can be supposed between the new imine produced and the corresponding decadienal and 3-aminopropionamide. The a_w is expected again to play a major role in this equilibrium. Finally, an electronic rearrangement in the decarboxylated imine, which may also have place in several steps, would produce acrylamide and an unstable dihydropyridine. This last heterocyclic derivative can be lately transformed into 2-pentylpyridine.

In addition to a_w, reaction pH and content of oxygen should also affect to the reaction yield. Reaction pH is expected to play a major role in the yield of the imine produced, and the presence of oxygen produces the oxidation of the lipid, therefore reducing the yield of the acrylamide formed (Hidalgo & Zamora, 2008).

The structure of the functional group in the oxidised lipid is critical. However, the chain length should not have a major role (except that a shorter chain length should decrease the hydrophobia of the lipid and, therefore, should facilitate its reaction with the amino acid, in accordance to the results shown in Table 1). On the other hand, the loss of one double bond, such as in 2-octenal, should not favour the electronic rearrangement in the decarboxylated imine and, therefore, inhibit the formation of the acrylamide. It is unclear at this time whether the major role of alkadienals is to facilitate the electronic arrangement of the imine to produce acrylamide (as observed in Fig. 5), or if they also play a major role in the decarboxylation of the amino acid to produce 3-aminopropionamide. These studies are being carried out at present in this laboratory and may help to understand the differences in reactivity observed among the different oxidised lipids.

It is somewhat surprising the significant differences observed between the conversion of phenylalanine into styrene (Hidalgo & Zamora, 2007) and the above described conversion of asparagine into acrylamide. These differences are likely related to distinct reaction mechanisms, which should be a consequence of the different structures of both phenylalanine and asparagine. Thus, for example, in decadienal/phenylalanine reaction mixtures, the lower water content, the higher amount of styrene produced. On the other hand, a certain amount of water favoured the formation of acrylamide in decadienal/asparagine reaction mixtures. In addition, the presence of oxygen was more critical in decadienal/phenylalanine reaction mixtures than in decadienal/asparagine reaction mixtures. Furthermore, when decadienal/phenylalanine mixtures were heated in the presence of platinum oxide, ethylbenzene

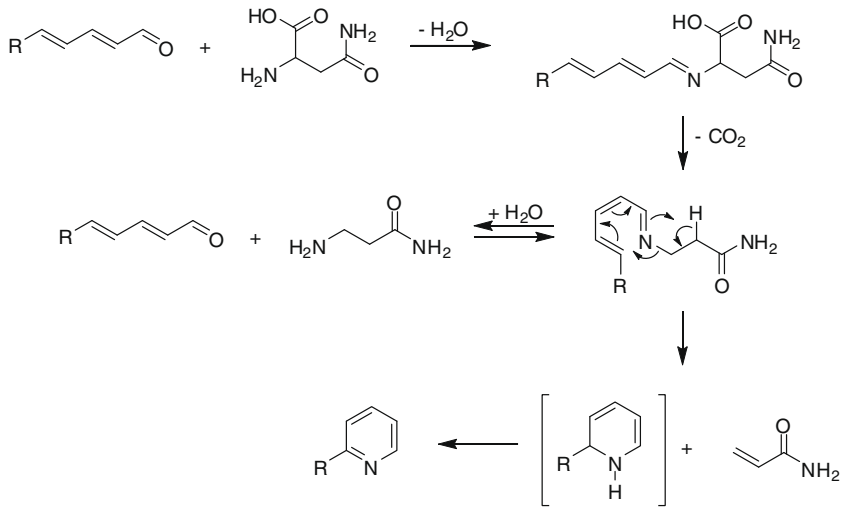

Fig. 5. Proposed pathways for the formation of acrylamide in asparagine degradation produced by alkadienals.

was produced, but propionamide was not detected in decadienal/asparagine reaction mixtures. All these results suggest that the structure of the amino acid is going to play a major role in both the reaction conditions needed and the yield of the amino acid degradation product formed.

The results obtained in this study confirm the high reactivity of some oxidised lipids to convert amino acids into their vinylogous derivatives and point out to the formation of 3-aminopropionamide as a major intermediate in the degradation of asparagine produced by alkadienals.

Acknowledgements

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