JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008**, *56*, 6075–6080 **6075**

Contribution of Lipid Oxidation Products to Acrylamide Formation in Model Systems

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The reactions of asparagine with methyl linoleate (**1**), methyl 13-hydroperoxyoctadeca-9,11-dienoate (**2**), methyl 13-hydroxyoctadeca-9,11-dienoate (**3**), methyl 13-oxooctadeca-9,11-dienoate (**4**), methyl 9,10-epoxy-13-hydroxy-11-octadecenoate (**5**), methyl 9,10-epoxy-13-oxo-11-octadecenoate (**6**), 2,4-decadienal (**7**), 2-octenal (**8**), 4,5-epoxy-2-decenal (**9**), and benzaldehyde (**10**) were studied to determine the potential contribution of lipid derivatives to acrylamide formation in heated foodstuffs. Reaction mixtures were heated in sealed tubes for 10 min at 180 °C under nitrogen. The reactivity of the assayed compounds was **7** $\gg$ **9** > **4** > **2** $\gg$ **8** ~ **6** $\gg$ **10** ~ **5**. The presence of compounds **1** and **3** did not result in the formation of acrylamide. These results suggested that $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl compounds were the most reactive compounds for this reaction followed by lipid hydroperoxides, more likely as a consequence of the thermal decomposition of these last compounds to produce $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl compounds. However, in the presence of glucose this reactivity changed, and compound **1**/glucose mixtures showed a positive synergism (synergism factor = 1.6), which was observed neither in methyl stearate/glucose mixtures nor in the presence of antioxidants. This synergism is proposed to be a consequence of the formation of free radicals during the asparagine/glucose Maillard reaction, which oxidized the lipid and facilitated its reaction with the amino acid. These results suggest that both unoxidized and oxidized lipids are able to contribute to the conversion of asparagine into acrylamide, but unoxidized lipids need to be oxidized as a preliminary step.

KEYWORDS: Acrylamide; asparagine; carbonyl−amine reactions; decadienal; epoxydecenal; hydroperoxide; lipid oxidation; lipid oxidation products; Maillard reaction; 2-octenal

INTRODUCTION

Acrylamide has been classified by the International Agency for Research on Cancer as probably carcinogenic to humans (*1*). In addition, neurological and reproductive effects of this compound have also been described (*1*). Therefore, the detection of this compound in a broad range of heated foods at concentrations sometimes exceeding 1 ppm (*2*) has led to considerable worldwide activity to investigate its origin and to reduce its content in foods (*3–16*).

Nowadays, acrylamide is broadly accepted to be produced at elevated temperatures and medium to low moisture contents as a consequence of the asparagine degradation produced by the Maillard reaction initiated by carbohydrates, although a few studies have proposed the contribution of lipids via the formation of acrolein (*17*). In addition, other studies have shown that oxidized lipids compete very efficiently with carbohydrates for carbonyl−amine reactions, and the same products are frequently produced from either carbohydrates or lipids by identical or very similar reaction pathways (*18*). Thus, among amino acid degradation studies, different investigations have shown that suitable amino acids may be converted into their corresponding Strecker aldehydes by different tertiary lipid oxidation products at low or moderate temperatures (*19–23*). In addition, certain secondary lipid oxidation products may convert amino acids either into their corresponding Strecker aldehydes or into vinylogous derivatives depending on the amount of oxygen present in the reaction (*24, 25*), therefore suggesting a potential route for the contribution of these secondary lipid oxidation products to acrylamide formation in thermally treated foods.

As a continuation of these last studies, the present investigation was undertaken to determine the different lipid oxidation products that can contribute to the formation of acrylamide from asparagine. Model reactions were carried out with both methyl linoleate (**1**) and methyl stearate as well as with primary, secondary, and tertiary products of the oxidation of compound **1**. In addition, different mixtures of lipids and carbohydrates were also studied to understand the reactions that take place in real foodstuffs in which amino acids, lipids, and carbohydrates are in close contact.

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10.1021/jf073047d CCC: $40.75 © 2008 American Chemical Society
Published on Web 07/15/2008

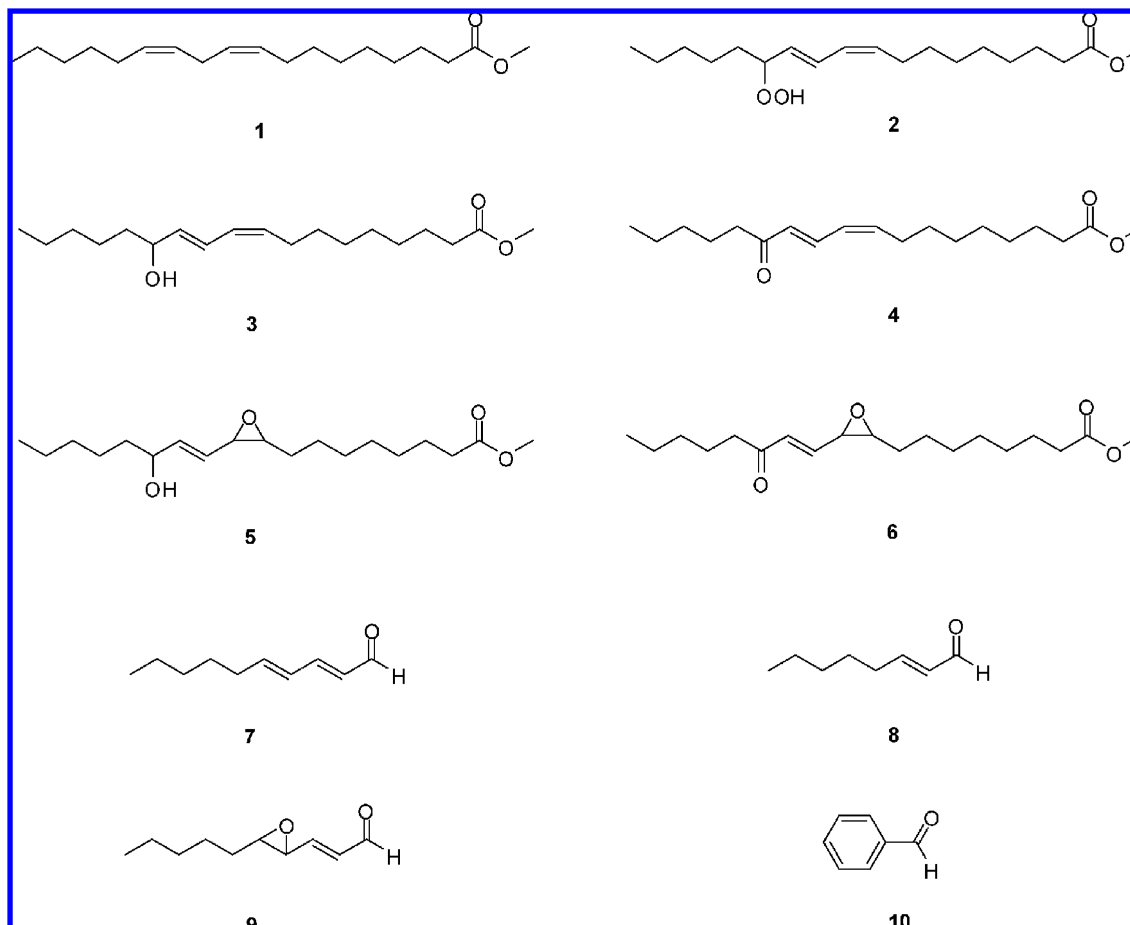

Figure 1. Chemical structures of the fatty acid derivatives employed in this study. They were derived from the ω6 linoleic acid. **1**, Methyl linoleate; **2**, methyl 13-hydroperoxyoctadeca-9,11-dienoate; **3**, methyl 13-hydroxyoctadeca-9,11-dienoate; **4**, methyl 13-oxooctadeca-9,11-dienoate; **5**, methyl 9,10-epoxy-13-hydroxy-11-octadecenoate; **6**, methyl 9,10-epoxy-13-oxo-11-octadecenoate; **7**, 2,4-decadienal; **8**, 2-octenal; **9**, 4,5-epoxy-2-decenal. Also included is the chemical structure of benzaldehyde (**10**), which was employed for comparison purposes.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Aldrich (Milwakee, WI), Sigma (St. Louis, MO), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany) and were of analytical grade. Labeled [1,2,3-$^{13}C_3$]acrylamide was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

Lipids and Lipid Oxidation Products. Unoxidized lipids as well as primary, secondary, and tertiary lipid oxidation products derived from ω6 linoleic acid were employed in this study. The chemical structures of the lipid derivatives employed in this study are given in **Figure 1**. This figure also includes the chemical structure of benzaldehyde, which was employed for comparison purposes. On the contrary, the chemical structure of methyl stearate has not been included. It is identical to that of compound **1**, but the fatty chain is saturated.

Methyl linoleate (**1**) (>98.5%) and methyl stearate (>99%) were obtained from Fluka and were used without further purification.

Methyl 13-hydroperoxyoctadeca-9,11-dienoate (**2**) was prepared by oxidation of the corresponding fatty acid with lipoxygenase following a previously described procedure (*26*). The obtained hydroperoxide was esterified with diazomethane and purified by column chromatography on silica gel using hexane/diethyl ether (7:3) as the eluent. Compound **2** was obtained chromatographically pure. Additional confirmations of identity and purity were obtained by 1D and 2D NMR. ^{13}C NMR ($CDCl_3$, 75.4 MHz) of compound **2**: δ 174.49 (C1), 133.96 (C9), 131.23 (C12), 130.11 (C11), 127.51 (C10), 86.85 (C13), 51.54 (OCH_3), 34.06 (C2), 32.49 (C14), 31.72 (C16), 29.35, 29.03, 29.00, 28.87 (C4−C7), 27.69 (C8), 24.98 (C15), 24.85 (C3), 22.51 (C17), and 14.04 (C18). This spectrum was identical to that previously described by Dussault et al. (*27*).

Methyl 13-hydroxyoctadeca-9,11-dienoate (**3**) was prepared by reducing the 13-hydroperoxide of linoleic acid with sodium borohydride and later esterification with diazomethane (*26*). Compound **3** was purified by column chromatography on silica gel using hexane/diethyl ether (7:3) as the eluent. This compound was obtained chromatographically pure. Additional confirmations of identity and purity were obtained by 1D and 2D NMR. ^{13}C NMR ($CDCl_3$, 75.4 MHz) of compound **3**: δ 174.36 (C1), 135.84 (C12), 132.80 (C9), 127.71 (C10), 125.69 (C11), 72.87 (C13), 51.47 (OCH_3), 37.21 (C14), 34.00 (C2), 31.72 (C16), 29.40 (C6), 28.99, 28.99, 28.89 (C4−C6), 27.61 (C8), 25.08 (C15), 24.82 (C3), 22.56 (C17), and 14.03 (C18). This spectrum was identical, for example, to that previously collected by Hämäläinen and Kamal-Eldin (*28*).

Methyl 13-oxooctadeca-9,11-dienoate (**4**) was prepared by oxidation of 13-hydroxyoctadeca-9,11-dienoic acid with chromium trioxide and later esterification with diazomethane (*26*). Compound **4** was purified by column chromatography on silica gel using hexane/diethyl ether (4:1) as the eluent. This compound was obtained chromatographically pure and exhibited the previously described ^{1}H and ^{13}C NMR spectra (*26*).

Methyl 9,10-epoxy-13-oxo-11-octadecenoate (**6**) was prepared by epoxidation of **4** with 3-chloroperoxybenzoic acid (*29*). Compound **6** was purified by column chromatography on silica gel using hexane/diethyl ether (4:1) as the eluent. This compound was obtained chromatographically pure and exhibited the previously described ^{1}H and ^{13}C NMR spectra (*26*).

Methyl 9,10-epoxy-13-hydroxy-11-octadecenoate (**5**) was prepared by reduction of **6** with sodium borohydride (*26*). Compound **5** was purified by column chromatography on silica gel using hexane/diethyl

ether (3:2) as the eluent. This compound was obtained chromatographically pure and exhibited the previously described ^{1}H and ^{13}C NMR spectra (*26*).

2,4-Decadienal (**7**) (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.

4,5-Epoxy-2-decenal (**9**) was prepared by epoxidation of **7** with 3-chloroperoxybenzoic acid (*23*). Compound **9** was purified by column chromatography on silica gel using hexane/diethyl ether (95:5) as the eluent. This compound was obtained chromatographically pure. Additional confirmations of identity and purity were obtained by ^{1}H and ^{13}C NMR and GC-MS.

2-Octenal (**8**) and benzaldehyde (**10**) were used without further purification.

All of these lipid derivatives, among other compounds, are produced during oxidation of compound **1**. Thus, the oxidation of **1** produces **2**, which is later decomposed to form **3**, **4**, **7**, and **8**, among other compounds. Later oxidation of **3**, **4**, and **7** is the origin of **5**, **6**, and **9**, respectively. These pathways have been broadly studied (*30, 31*). Additional pathways for the formation of some of these compounds have also been described (*30, 31*), for example, the formation of **9** by the degradation of an epoxyhydroperoxy fatty acid. These additional pathways are also in agreement with the conclusions obtained in the present study.

Asparagine/Lipid Reaction Mixtures. Model reactions were carried out analogously to that used by Granvogl and Schieberle (*8*), with the modifications described by Hidalgo and Zamora (*25*). Briefly, mixtures of asparagine and the lipid derivative (75 μmol each) were singly homogenized with 0.063−0.200 mm silica gel 60 (600 mg) (Macherey-Nagel, Düren, Germany), 60 μL of 0.3 M sodium phosphate, pH 6, and 360 μL (31%) of water. Samples were heated under nitrogen at 180 °C in closed test tubes for 10 min. After cooling (15 min at −20 °C), 60 μL of internal standard solution (0.1 mg/mL of labeled [1,2,3-$^{13}C_3$]acrylamide in water) 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 was determined.

Asparagine/Glucose/Lipid Reaction Mixtures. Model reactions were carried out, and acrylamide was determined, analogously to the above-described method for asparagine/lipid reaction mixtures, but 75 μmol of asparagine, 37.5 μmol of the glucose, and 37.5 μmol of the lipid were employed. In addition, some mixtures also included 9.4 μmol of BHT.

Analysis of Acrylamide in Model Systems. Acrylamide was analyzed as the stable 2-bromopropenamide by gas chromatography−mass spectrometry (GC-MS) using the method of Castle et al. (*32*) with the modifications of Andrawes et al. (*33*). 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 the addition of 1 M sodium thiosulfate until the solution became colorless, and the solution was extracted with 1 mL of ethyl acetate/hexane (4:1). The organic layer was finally dried with sodium sulfate, evaporated to a volume of ~50 μL, treated with 50 μL of triethylamine, and analyzed 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 quantitation. 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 quantitation. Mass spectra of both compounds are collected, for example, by Pittet et al. (*34*). 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 temperature, 250 °C; oven temperature, raised from 70 °C (1 min) to 240 at 5 °C/min and then to 325 °C at 10 °C/min; transfer line to MSD, 280 °C; and ionization EI, 70 eV.

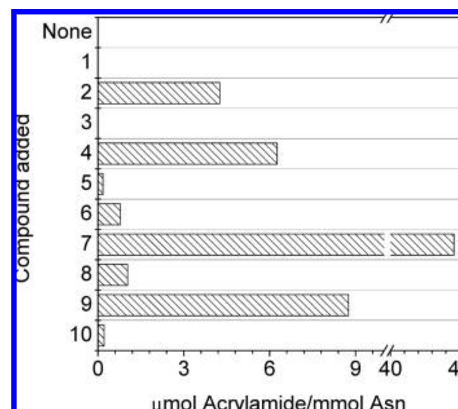

Figure 2. Acrylamide produced in asparagine/lipid (1:1) model systems heated at 180 °C for 10 min under nitrogen. Chemical structures for the assayed lipids are given in **Figure 1**.

Quantification of acrylamide was carried out by preparing standard curves of this compound in the 600 mg of silica gel and following the whole procedure described above. For each curve, 15 different concentration levels of acrylamide (0−200 μg) were used. Acrylamide content was directly proportional to the acrylamide/internal standard area ratio ($r = 0.999$, $p < 0.0001$). The coefficients of variation at the different concentrations were <10%. All data given are mean values of, at least, two independent experiments.

RESULTS

Acrylamide Formation in Asparagine/Lipid Reaction Mixtures. When heated with asparagine, different lipid oxidation products converted this amino acid into acrylamide to some extent (**Figure 2**). **Figure 2** also includes the data obtained with **10**, which is not a lipid oxidation product, for comparison purposes.

The assayed unoxidized lipid (**1**) did not produce acrylamide analogously to the asparagine heated in the absence of lipid (control). Analogous results were also obtained with the secondary and tertiary lipid oxidation products, which did not have a carbonyl group. Thus, the hydroxydiene **3** was not able to convert asparagine into acrylamide, and its epoxy derivative **5** produced <0.02% of acrylamide. On the other hand, the hydroperoxide **2** and all of the lipid derivatives assayed having a carbonyl group in their structures were able to convert asparagine into acrylamine. The yield of acrylamide formation depended on the lipid oxidation product employed and ranged from the 4.7% obtained for compound **7** to the 0.08% obtained for compound **6**.

Among the long-chain and short-chain derivatives studied, the highest reaction yields were obtained for the corresponding $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl compounds (**4** and **7**, respectively). In addition, the epoxidation of one of the double bonds in both **4** and **7** to produce **6** and **9**, respectively, decreased considerably the reaction yield (between 5 and 8 times).

The yield of the hydroperoxide **2** was relatively high for a long-chain lipid oxidation product (0.4%) and was only lower than that obtained for the long-chain derivative **4** (0.6%).

Although short-chain aldehydes were more reactive than the analogous long-chain ketone derivatives, the reaction yield for short-chain aldehydes was low if the structure was not appropriate. Thus, the yield of compound **8** was only 0.1%, despite the difference between compounds **8** and **7** being only one double bond. Furthermore, the reaction yield for **10**, an aromatic aldehyde, was 0.02%.

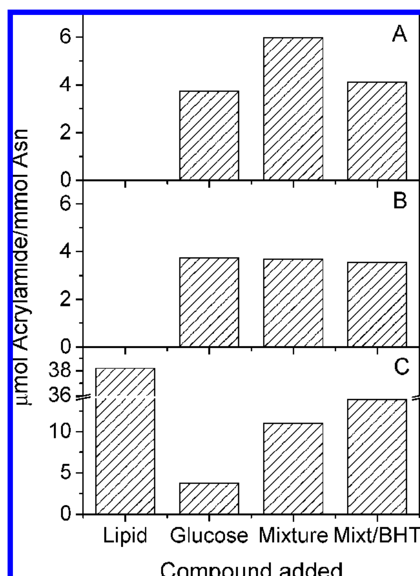

Figure 3. Acrylamide produced in asparagine/glucose/lipid (2:1:1) and asparagine/glucose/lipid/BHT (2:1:1:0.25) model systems heated at 180 °C for 10 min under nitrogen (mixture and mixt/BHT, respectively). Acrylamide produced in binary mixtures (2:1) of asparagine with either the lipid or glucose is also given for comparison. The lipids added were **A**, methyl linoleate (**1**); **B**, methyl stearate; and **C**, 2,4-decadienal (**7**).

Acrylamide Formation in Asparagine/Glucose/Lipid Reaction Mixtures. In foods, lipids are not the unique compounds in contact with asparagine. Therefore, lipid/carbohydrate interactions (see, for example, ref *18* for a recent review) can play a role in the amount of acrylamide produced. **Figure 3A** shows the acrylamide produced when asparagine was heated in the presence of the unsaturated fatty ester **1**, glucose, the mixture of both compounds, and the mixture of compound **1**, glucose, and BHT. As described above, compound **1** did not produce acrylamide. On the other hand, the reaction yield for glucose was 0.4%. However, when asparagine was incubated in the presence of both compound **1** and glucose, a positive synergism was observed, and acrylamide was produced with a reaction yield of 0.6%. The synergism factor for this mixture, which is defined as the ratio between the experimental value and the value calculated considering the effect offered by both components when tested individually, was 1.6. This synergism was much reduced when the compound **1**/glucose/asparagine mixture was incubated in the presence of BHT (synergism factor = 1.1).

To obtain further confirmation that the oxidation of the lipid was the responsible for the acrylamide produced, the same mixtures were analyzed but methyl stearate was employed in place of compound **1** (**Figure 3B**). Neither the methyl stearate/glucose/asparagine mixture (synergism factor = 1.0) nor the methyl stearate/glucose/asparagine/BHT mixture (synergism factor = 0.9) exhibited any increase in the acrylamide determined.

Different from the behavior exhibited by compound **1** or by methyl stearate, when compound **7** was heated with asparagine in the presence of glucose, the amount as acrylamide decreased and a negative synergism was observed for these two compounds (**Figure 3C**). Thus, the acrylamide yield obtained for **7** alone (3.8%) decreased to 1.1% in the presence of glucose (synergism factor = 0.26). This decrease was lower in the presence of BHT (synergism factor = 0.36).

DISCUSSION

The results obtained in this study indicate that, in addition to carbohydrates, different lipid oxidation products are able to convert asparagine into acrylamide. This conversion is favored by oxidized lipids having an $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl group. The obtained results suggest that the diunsaturated aldehyde **7** is more reactive than the diunsaturated ketone **4**. This is likely a consequence of both a higher reactivity of the aldehyde group in comparison to the ketone group for this reaction and the different solubilities of short-chain and long-chain lipid oxidation products. Short-chain aldehydes are more hydrophilic and, therefore, they should be much more easily in close contact with asparagine. However, the structure of the compound is much more important than the solubility, and short-chain aldehydes not having the $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl group produced very reduced amounts of acrylamide. Thus, for example, an aromatic aldehyde, such as benzaldehyde, produced only very small amounts of acrylamide.

When the $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl group suffered a further oxidation step (such as in the formation of compounds **9** and **6**), its reactivity for this reaction was reduced considerably in comparison to their corresponding secondary lipid oxidation products (compounds **7** and **4**, respectively). However, the reactivity of **9** for this reaction was still very high. Additional studies are needed to investigate the reactivity of this aldehyde, which might be related to a reversion of the epoxidation reaction at high temperature. These studies are being developed at present in this laboratory.

In addition to $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl compounds, the diunsaturated hydroperoxide **2** was also very reactive for this reaction. This may be a consequence of the easy decomposition of hydroperoxides to produce $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl compounds, among other compounds (*30, 31*). This result is very important because oxidized lipids are usually present in foods at lower concentrations than employed in this study. However, the obtained results suggest that the presence of $\alpha,\beta,\gamma,\delta$-diunsaturated carbonyl compounds is not necessary to convert asparagine into acrylamide. The presence of the primary lipid oxidation products, or even unoxidized lipids under conditions that can be oxidized, is the only requisite needed to observe a positive contribution of the lipids to acrylamide formation during food heating.

These conclusions were confirmed when mixtures of lipids and glucose were heated in the presence of asparagine. The diunsaturated lipid **1** alone was found to be unable to convert asparagine into acrylamide. However, the Maillard reaction between asparagine and glucose is likely to produce free radicals (*35–37*) that should oxidize compound **1** to **2**. The formation of this hydroperoxide **2** should be responsible for the positive synergism observed in compound **1**/glucose/asparagine mixtures. On the contrary, methyl stearate, which cannot be oxidized, did not exhibit any positive synergism. Furthermore, the addition of an antioxidant to compound **1**/glucose/asparagine mixtures inhibited the oxidation of the unsaturated fatty ester and, therefore, mostly reduced the synergism in those samples. Moreover, the production of free radicals in compound **7**/glucose/asparagine mixtures should convert the $\alpha,\beta,\gamma,\delta$-diunsaturated aldehyde **7** into a much less reactive derivative for this reaction, which is in agreement with both the negative synergism observed in these mixtures and the reduction of this negative synergism in the presence of antioxidants.

The effect of antioxidants on acrylamide formation in heated foodstuffs is not completely clarified yet, and it may be related

to the type of both food and antioxidant employed. Thus, although early studies indicated that antioxidants did not have any significant effect in the formation of acrylamide (*38*), more recent studies have suggested a positive role of antioxidants for food protection (*39, 40*). This protection offered by antioxidants might be related to the inhibition of the contribution of lipid oxidation products to the formation of acrylamide described in the present study.

All of these results suggest that both unoxidized and oxidized lipids are able to contribute to the conversion of asparagine into acrylamide, but unoxidized lipids need to be oxidized as a preliminary step. This oxidation, which occurs, for example, when a concurrent Maillard reaction is taking place, can be avoided with the use of antioxidants.

ACKNOWLEDGMENT

We are indebted to José L. Navarro for technical assistance.

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Received for review October 16, 2007. Revised manuscript received January 10, 2008. Accepted May 18, 2008. This study was supported in part by the European Union (FEDER funds), the Junta de Andalucía (Project P07-AGR-2846), and the Plan Nacional de I + D of the Ministerio de Educación y Ciencia of Spain (Project AGL2006-01092).

JF073047D