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Characterization of Carbonyl-Phenol Adducts Produced by Food Phenolic Trapping of 4-Hydroxy-2-hexenal (HHE) and 4-Hydroxy-2-nonenal (HNE)

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ABSTRACT

4-Hydroxy-2-alkenals disappear in the presence of food phenolics (i.e. cathechin or quercetin) and the corresponding carbonyl-phenol adducts are produced. In an attempt to identify structure(s) of formed adducts, the reactions between model phenolics (resorcinol, 2-methylresorcinol, orcinol, and 2,5-dimethylresorcinol) and hydroxyalkenals (4-hydroxy-2-hexenal and 4-hydroxy-2-nonenal) were studied and the produced adducts were isolated by column chromatography and unambiguously characterized by 1D and 2D NMR and MS as dihydrobenzofuranols (**1**), chromane-2,7-diols (**2**), and 2*H*-chromen-7-ols (**3**). These compounds were mainly produced at slightly basic pH values and moderate temperatures. Their activation energies (E_a) of formation were ~25 kJ·mol^{-1} for adducts **1**, ~32 kJ·mol^{-1} for adducts **2**, and ~38 kJ·mol^{-1} for adducts **3**. A reaction pathway that explains their formation is proposed. All these results confirm that, analogously to other lipid-derived carbonyl compounds, phenolics can trap 4-hydroxy-2-alkenals in an efficient way. Obtained results provide the basis for the potential detection of carbonyl-phenol adducts derived from hydroxyalkenals in food products.

KEYWORDS:

Carbonyl-phenol reactions; 4-hydroxy-2-alkenals; lipid oxidation; Maillard reaction; phenolics; reactive carbonyls

INTRODUCTION

Food lipid profiles change during processing and storage because of the oxidation of fatty acyl chains. This process is responsible for both changes in food quality and formation of undesirable compounds.[1,2] Among the produced compounds, the formation of short-chain carbonyl compounds has long attracted the attention of researchers because of their role in the formation of food flavors and off-flavors[3,4] and their potential toxicity.[5,6] In particular, 4-hydroxy-2-alkenals have been specially studied in this sense. They have been shown to be produced both during food processing[7] and food digestion,[8] to contribute to the generation of food flavors,[9] and to play a major role in food safety and body health and disease.[10]

Toxicity of all these compounds has been related to their ability to react with the nucleophiles (mostly amino compounds) present in the nearby and to produce the corresponding carbonyl-amine adducts. Consequently, the modification of important biomolecules, such as nucleic acids, proteins and amino phospholipids, by short-chain aldehydes and other lipid-derived reactive carbonyls has been described in many studies.[11–13]

A possibility for mitigating these reactions, and avoiding the destruction of important biomolecules, is the use of alternative nucleophiles that can trap the produced carbonyl compounds. To this respect, recent studies have shown that certain phenolic compounds[14] can effectively trap a wide range of lipid-derived reactive carbonyls and protect, in that way, amino compounds from degradation.[15] This carbonyl-trapping occurs under common cooking conditions,[16] and this ability constitutes an additional protective function of phenolics for avoiding lipid oxidation consequences.[17] Thus, the reactions of phenolics with alkanals, 2-alkenals, 2,4-alkadienals, 4-oxo-2-alkenals, and 4,5-epoxy-2-alkenals have been studied so far, and the corresponding carbonyl-phenol

adducts produced have been isolated and characterized.[18] In addition, formation of these carbonyl-phenol adducts has been related to the protective function of some phenolics on the formation of heterocyclic aromatic amines and biogenic amines by carbonyl-amine reactions,[19,20] and the beneficial properties of polyphenolic-rich foods such as tea or coffee.[21]

Although carbonyl-phenol reactions with different lipid-derived reactive carbonyls have been described,[22–27] the ability of phenolic compounds to trap and produce carbonyl-phenol adducts with 4-hydroxyalkenals has not been described. To the best of our knowledge, although several studies have shown that 4-hydroxy-2-nonenal (HNE) seems to be effectively trapped by phenolics,[28,29] neither the produced reaction has been described in detail nor the structure(s) of the formed adduct(s) has been unequivocally characterized. In addition, the formation of carbonyl-phenol adducts between 4-hydroxy-2-hexenal (HHE) and phenolics has not been investigated so far.

In an attempt to fill this gap, this manuscript describes the carbonyl-trapping of HNE by catechin and quercetin. In addition, it unambiguously characterizes the formed adducts by studying the reactions of HNE and HHE with simple phenolics. For these characterization studies, resorcinol, 2-methylresorcinol, orcinol, and 2,5-dimethylresorcinol were employed as model phenolics because of both their high trapping potential,[14] similar to that of food phenolics,[15] and their low molecular weight that facilitated the isolation and characterization of the produced adducts.

MATERIALS AND METHODS

Materials. HNE was prepared according to the procedure described by Gardner et al.[30] (1992), who epoxidated 3(*Z*)-nonenol with 3-chloroperoxybenzoic acid and later oxidized the 3,4-epoxynonanol obtained with periodinane. This procedure was also

applied to the synthesis of HHE, although it had to be modified. Briefly, a solution 3(*Z*)-hexenol (50 mmol) in 140 mL of dichloromethane were treated slowly with 3-chloroperoxybenzoic acid (9.3 g) and the obtained mixture was stirred for 1 h at room temperature. After this time, 10% potassium bicarbonate (140 mL) was added, and the mixture was stirred for 2 min. The organic layer was then collected and dried over sodium sulfate. The obtained solution, which contained the produced 3,4-epoxyhexanol, was added slowly to a solution of periodinane (16 g in 150 mL of dichloromethane) and the obtained mixture was stirred under dark for 30 min at room temperature. Then, 500 mL of diethyl ether and 230 mL of 1.3 M NaOH were added. The solution was vigorously stirred for 2 min and the organic layer was collected, dried over sodium sulfate, and taken to dryness. The residue was fractionated by column chromatography on silica gel using mixtures of hexane and diethyl ether as eluent. Identity and purity of obtained HHE were confirmed by means of 1D and 2D NMR and GC-MS. Spectroscopic and spectrometric data of this compound are collected in the Supplementary Material. Synthesized hydroxyalkenals were stored at –30 ºC under nitrogen. They were stable for long time periods under these conditions.

Periodinane, unsaturated alcohols, phenolics, and all other chemicals employed in these studies were purchased from Sigma-Aldrich (St. Louis, MO), Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland), and were of the highest available grade.

Disappearance of the Hydroxyalkenal and Formation of Carbonyl-Phenol Adducts in the Reaction of HNE with Catechin or Quercetin. A mixture of the phenolic (30 μmol in 170 μL of methanol), HNE (30 μmol in 30 μL of methanol) and 0.3 M sodium phosphate buffer (300 μL), pH 8.0, were heated under nitrogen for different times at 80 ºC. At the end of the heating, samples were cooled at room temperature and 50 μL of internal standard (54.8 mg of methyl heptanoate in 25 mL of

methanol) was added. One hundred microliters of the obtained solution was diluted with acetonitrile (100 μL), centrifuged at 2000 *g* for 5 min, and, finally, studied for HNE disappearance by GC-MS and for carbonyl-phenol adduct formation by MS.

Determination of HNE. HNE disappearance was determined directly by GC-MS. The ions monitored for quantitation of HNE and the internal standard were *m/z* 86 and 74, respectively. GC-MS analyses were conducted with an Agilent 7820 gas chromatograph coupled with an Agilent 5977B mass selective detector (MSD), quadrupole type. Separations were carried out on a fused-silica DB-5MS UI capillary column (30 m × 0.25 mm i.d; coating thickness, 0.25 μm), and 1 μL of sample was injected in the pulsed splitless mode. Working conditions were: carried gas, helium (0.8 mL/min); injector, 250 ºC; oven temperature programmed from 40 ºC (3 min) to 200 ºC at 12 ºC/min and then 1 min at 200 ºC; transfer line to MSD, 280 ºC; ionization EI, 70 eV; ion source temperature, 230 ºC; mass range 50-550 amu.

The quantitation of HNE was carried out by preparing standard curves of this compound and using the same procedure described above. HNE concentration was directly proportional to the adduct/internal standard area ratio ($r > 0.99$, $p < 0.001$). RSD was < 10%.

Detection of Carbonyl-Phenol Adducts in the Reaction Between HNE and Catechin or Quercetin. Detection of carbonyl-phenol adducts was carried out by direct injection on a triple quadrupole API 2000 mass spectrometer (Applied Biosystems, Foster City, CA) using an electrospray ionization interface in the negative ionization mode (ESI^-). The nebulizer gas and the curtain gas were set at 19 and 10 (arbitrary units), respectively. The electrospray capillary voltage was set to –4.5 kV, the declustering potential was –50 V, the focusing potential was –400 V, and the entrance potential was –10 V.

Formation of Carbonyl-Phenol Adducts in the Reaction of 4-Hydroxy-2-Alkenals and Simple Phenolic Compounds. Determination of conditions that favored the formation of carbonyl-phenol adducts and the characterization of structures of the produced adducts was carried out with simple phenolic compounds and by using two procedures: one for analytical purposes and another for preparative purposes. Both procedures included the acetylation of the formed adducts to get their stabilization.

For analytical purposes, a mixture of the phenolic (30 μmol in 170 μL of water), the hydroxyalkenal (30 μmol in 30 μL of methanol) and 0.3 M buffer (300 μL) were heated under nitrogen at the indicated times and temperatures. At the end of the incubation time, samples were cooled at room temperature, treated with ethanol (1.2 mL) and taken to dryness under nitrogen. Then, 30 μL of internal standard (36.64 mg of 3(*Z*)-nonenol in 5 mL of dried pyridine), 1 mL of dry pyridine, and 500 μL of acetic anhydride were added, and the mixture was allowed to react under dark for 20 h at room temperature. After that time, 2 mL of dichlorometane and 2 mL of water were added. The mixture was stirred for 1 min and the organic layer was collected, and washed 4 times with 2 mL of 5% HCl and once with 2 mL of water. Layers were separated by centrifugation (2000 *g* for 5 min) and the organic layer was studied by GC-MS.

For preparative purposes, reactions were carried out analogously but the phenolic (2.4 mmol in 13.6 mL of water) and the hydroxyalkenal (2.4 mmol in 2.4 mL of methanol) were dissolved in 24 mL of 0.3 M sodium phosphate and heated under nitrogen at 100 ºC for 18 h. After cooling, 120 mL of ethanol were added and the reaction mixture was taken to dryness. The acetylation was carried out by adding 80 mL of pyridine and 40 mL of acetic anhydride. After 20 h, 160 mL of dichloromethane and 160 mL of water were added, and the solution was washed four times with 160 mL of 5% HCl and once with 160 mL of water. The organic layer was taken to dryness and the

residue fractionated by column chromatography on silica gel using mixtures of hexane and diethyl ether as eluent. Separation was controlled by GC-MS using the conditions described below.

Characterization of the Carbonyl-Phenol Adducts Formed in the Reaction of 4-Hydroxy-2-alkenals and Phenolic Compounds. The reaction between hydroxyalkenals and phenolics is complex and different carbonyl-phenol adducts were produced. Figure 1 shows the portion of the total ion chromatogram where carbonyl-phenol adducts appeared. This figure shows the chromatograms obtained in the reactions of HHE with resorcinol (Figure 1A), 2-methylresorcinol (Figure 1C), orcinol (Figure 1E), and 2,4-dimethylresorcinol (Figure 1G), and the chromatograms obtained in the reactions of HNE with the same phenolics (Figs. 1B, 1D, 1F, and 1H, respectively). As discussed below, and independently of the phenolic involved, three types of adducts were always produced (compounds **1**–**3**). Structures for these adducts are collected in Figure 2. Adducts **1** were isolated and characterized from the reaction of HHE and 2-methylresorcinol, and adducts **2** and **3** were isolated and characterized from the reaction of HNE and 2-methylresorcinol. The adducts isolated and characterized were (*Z*)-(6-acetoxy-2-(1-acetoxybutyl)-7-methylbenzofuran-3(2*H*)-ylidene)methyl acetate (as the pair of diastereomers **1e** and **1f**), 2-hydroxy-4-(1-hydroxyhexyl)-8-methylchroman-7-yl acetate (as the pair of diastereomers **2g** and **2h**) and 1-(7-acetoxy-8-methyl-2*H*-chromen-2-yl)hexyl acetate (**3g**). Spectroscopic and spectrometric data of all these compounds, as well as of other adducts identified in the assayed reactions, are collected in Figures S-1, S-2 and S-3, and in Tables S-1, S-2, and S-3 of the Supplementary Material.

Phenolic-Hydroxyalkenal Adduct Determination. To study the formation of carbonyl-phenol adducts and the effect of reaction conditions, formed adducts were

determined by GC-MS. GC-MS were conducted using the equipment described above and chromatographic and spectrometric conditions were the same with the exception of the oven temperature program. Oven temperature was programmed from 100 ºC (1 min) to 300 ºC at 15 ºC/min and then 5 min at 300 ºC. Carbonyl-phenol adducts were quantified by preparing standard curves of the isolated adducts (**1e** and **1f** as models of adducts **1**, **2g** and **2h** as models of adducts **2**, and **3g** as model of adduct **3**). Six concentration levels were used. Adduct content was directly proportional to adduct/internal standard area ratio ($r > 0.99$, $p < 0.001$). RSD was always < 10%.

NMR Spectroscopy. 1D and 2D NMR spectra were obtained in a Bruker Advance III spectrometer operating at 500 MHz for protons. Experiments were performed at 24 ºC and acquisition parameters were described previously.[16]

Statistical Analysis. All quantitative data are mean ± SD values of, at least, three independent experiments. Analysis of variance was employed to compare different groups. When *F* values were significantly different, group differences were evaluated by the Tukey test.[31] Statistical comparisons were carried out using Origin® v. 7.0 (OriginLab Corporation, Northampton, MA). The significance level is $p < 0.05$ unless otherwise indicated.

RESULTS

Disappearance of HNE and Formation of Carbonyl-Phenol Adducts in the Reaction of HNE with Catechin or Quercetin. Analogously to other lipid oxidation products,[32] HNE is relatively unstable upon heating. Therefore, it disappeared slowly when heated in sodium phosphate buffer, pH 8 (Figure 3). This loss was linear ($r = -0.975$, $p = 0.005$) during the first 10 h. Nevertheless, when catechin or quercetin were also present, the hydroxyalkenal disappeared more rapidly (Figure 3). This difference

195 was a consequence of the carbonyl-trapping ability of phenolic compounds. Thus, when
196 reaction mixtures were studied by MS, the appearance of the molecular ions
197 corresponding to carbonyl-phenol adducts were observed (Figure S-4, Supplementary
198 material). Thus, the mixture of HNE and catechin exhibited the $M^+ - 1$ molecular ion of
199 the adducts at *m/z* 445 and the mixture of HNE and quercetin exhibited the $M^+ - 1$
200 molecular ion of the adduct at *m/z* 457. However, these adducts resulted unstable and
201 they could not be isolated and characterized. To carry out this characterization, the
202 corresponding adducts with simpler phenolics were prepared.

203 **Characterization of the Adducts Produced in the Reaction Between**
204 **Hydroxyalkenals and Phenolic Compounds**. When the reaction between
205 hydroxyalkenals and simple phenolics was studied, the formation of different carbonyl-
206 phenol adducts was observed by GC-MS after acetylation (Figure 1). Thus, when the
207 reaction was carried out with HHE, two main adducts (adducts **1**) were always observed
208 in the different reactions assayed (Figs. 1A, 1C, 1E, and 1G). This kind of adducts were
209 also produced in the reaction of HNE and phenolics (Figs. 1B, 1D, 1F, and 1H). They
210 had a molecular weight that corresponded to the addition of the molecular weights of
211 the carbonyl compound, the phenolic, and three acetyl groups, and the loss of one
212 molecule of hydrogen. All of them had identical fragmentation pattern (Table S-1,
213 Supplementary Material), and they were identified as 2,3-dihydrobenzofuranols on the
214 basis of the 1D and 2D NMR spectra of adducts **1e** and **1f**, which were isolated and
215 characterized. Adducts **1e** and **1f** were produced in the reaction of HHE and 2-
216 methylresorcinol, but analogous adducts were produced in all assayed reactions.
217 Spectroscopic and spectrometric data of adducts **1** are collected in the Supplementary
218 Material.

Adducts **1e** and **1f** had very similar NMR spectra. The only difference was the coupling constants of protons H2 and H1". This suggested that both adducts were a pair of diastereomers and carbons C2 and C1" were chiral carbons with different configuration for both isomers. 1D NMR showed the presence of one isolated olefinic proton, the existence of two saturated carbons bonded to oxygen, and the occurrence of the unchanged ethyl group. In addition, most of the initial phenolic molecule remained unchanged with the exception of one of the hydroxyl groups and its contiguous aromatic carbon. The study of HMBC and HSQC spectra allowed to determine a structure of 2,3-dihydrobenzofuranol for the produced adducts (complete chemical structure is shown in Figure 2). This compound has two chiral carbons (C2 and C1") and one double bond between carbons C3 and C1'. The two distereomers that appeared in the chromatograms are likely the pairs of stereoisomers *R*,*R* and *S*,*S* on one hand and *R*,*S* and *S*,*R*, on the other.

Differently to adducts **1**, adducts **2** and **3** only appeared to a certain extent in reactions involving HNE. For that reason, they were isolated and characterized from the reaction of HNE and 2-methylresorcinol. Adducts **2** always appeared to a higher extent than adducts **3** under the assayed reaction conditions, and, analogously to adducts **1**, they were always produced as a mixture of two diastereomers.

The molecular ion of adducts **2** was not observed in the mass spectra. This is not strange because of the presence of free hydroxylic groups observed by NMR. Thus, the ion with the highest *m/z* ratio observed by MS was a dehydrated ion (Table S-2, Supplementary Material). This ion suffered a new dehydration, which suggested the existence of two free hydroxylic groups in the molecule. Having this into account, the molecular weight of these adducts corresponded to the addition of the molecular weights of the carbonyl compound, the phenolic, and only one acetyl group. All adducts

2 had identical fragmentation pattern, although fragment intensities were different for the two diastereomers of each adduct (Table S-2, Supplementary Material). They were identified as chromane-2,7-diols on the basis of the 1D and 2D NMR spectra of adducts **2g** and **2h**, which were isolated and characterized. Adducts **2g** and **2h** were produced in the reaction of HNE and 2-methylresorcinol, but analogous adducts were produced in all assayed reactions involving HNE. Spectroscopic and spectrometric data of adducts **2** are collected in the Supplementary Material.

Adducts **2g** and **2h** had very similar NMR spectra. However, multiplicities and coupling constants of protons H2, H3, H4, and H1' were different for both of them. This suggested that both adducts were a pair of diastereomers. 1D NMR showed the presence of two carbons bonded to non-acetylated hydroxyl groups, and the disappearance of both the carbon-carbon double bond and the carbonyl group initially present in the hydroxyalkenal. On the contrary, the hydroxyl and pentyl groups initially present in the hydroxyalkenal remained unchanged. In addition, most of the initial phenolic molecule remained unchanged with the exception of one of the hydroxyl groups and its contiguous aromatic carbon. The study of the HMBC and HSQC spectra of both diastereomers allowed to identify their structure as chromane-2,7-diol derivatives (chemical structure is shown in Figure 2). This compound has three chiral carbons (C2, C4, and C1'). However, only two of them are fixed (C4 and C1'). This is likely the reason for the isolation and characterization of only two diastereomers.

Finally, a third kind of adducts were also produced. These adducts **3** were the adducts produced to a lower extent under the assayed conditions. They were only produced to a certain extent in reactions involving HNE and, differently to adducts **1** or **2**, only one adduct could be detected for each phenolic.

Analogously to adducts **2**, the molecular ion of adducts **3** was not observed in the mass spectra. The ions with highest *m/z* ratio observed corresponded to the loss of either ketene or acetic acid (Table S-3, Supplementary Material). Having this into account, the molecular weight of these adducts corresponded to the addition of the molecular weights of the carbonyl compound, the phenolic, the loss of one molecule of water, and the incorporation of two acetyl groups. No free hydroxyl groups could be detected in these adducts, analogously to adducts **1** and differently to adducts **2**. All adducts **3** had identical fragmentation pattern (Table S-3, Supplementary Material). They were identified as 2*H*-chromen-7-ols on the basis of the 1D and 2D NMR spectra of adduct **3g**, which was isolated and characterized from the reaction between HNE and 2-methylresorcinol. Spectroscopic and spectrometric data of adducts **3** are collected in the Supplementary Material.

Adduct **3g** had NMR spectra that were, to a certain extent, similar to NMR spectra of adducts **2g** and **2h**, although significant differences were observed. Thus, the spectra showed that adduct **3g** had one carbon-carbon double bond and only two hydroxyl groups (both of which were acetylated). On the other hand, and analogously to adducts **2**, the occurrence of the unchanged hydroxyl and pentyl groups initially present in the hydroxyalkenal was observed. In addition, most of the initial phenolic molecule remained unchanged with the exception of one of the hydroxyl groups and its contiguous aromatic carbon. The study of their HMBC and HSQC spectra allowed to identify adducts **3** as 2*H*-chromen-7-ol derivatives (chemical structure is shown in Figure 2). This compound has two chiral carbons (C2 and C1'). However, only one diastereomer could be identified and isolated in studied reactions.

Effect of pH on the Formation of Carbonyl-Phenol Adducts in the Reaction of HNE and 2-Methylresorcinol. Three adducts were produced in reactions involving

293 HNE and only adducts **1** were produced to a certain extent in reactions involving HHE.
294 In addition, the yields of all of them depended on the reaction conditions. The study of
295 the effect of reaction conditions on the disappearance of the phenolic and the formation
296 of carbonyl adducts **1**–**3** was carried out by employing HNE as model carbonyl
297 compound and 2-methylresorcinol as model phenolic because their reaction produced
298 the three adducts. Figure 4 shows the effect of reaction pH on the disappearance of 2-
299 methylresorcinol (Figure 4A) and the formation of adducts **1g** and **1h** (Figure 4B),
300 adducts **2g** and **2h** (Figure 4C), and adduct **3g** (Figure 4D). As observed in Figure 4A,
301 the remaining 2-methylresorcinol recovered at the end of the heating time (20 h at 100
302 ºC) decreased as a function of reaction pH. This decrease was produced because of both
303 the formation of the carbonyl-phenol adducts and the instability of phenolic compounds
304 at basic pH values.[33]

305 Adducts **1** were mostly produced at pH ~8 (Figure 4B). At this pH, the reaction yield
306 was about 2% for both isomers **1g** and **1h**. As shown in Figure 4B, the concentration of
307 both adducts **1g** and **1h** increased from pH 6 to pH 8 and then decreased significantly (p
308 < 0.05) at pH 10.

309 Adducts **2** were also mostly produced at pH ~8 (Figure 4C). Thus, the concentration
310 of both adducts **2** increased significantly ($p < 0.05$) from pH 6 to pH 8 and, then,
311 decreased to higher pH values. Both diastereomers exhibited a similar behavior and
312 adduct **2h** was produced to a slightly higher yield than adduct **2g** (5% vs. 4% after 20 h
313 at 100 ºC).

314 Differently to adducts **1** and **2**, formation of adduct **3g** was not so clearly pH-
315 dependent and was produced approximately with the same yield (~3% after 20 h at 100
316 ºC) between pH 6 and pH 9 (Figure 4D). However, its concentration decreased
317 significantly at pH 10.

Effect of Phenolic and Hydroxyalkenal Concentrations on the Formation of Carbonyl-Phenol Adducts in the Reaction of HNE and 2-Methylresorcinol. Adduct formation depended on the concentration of both the phenolic and the hydroxyalkenal present. Figure 5 shows the remaining 2-methylresorcinol (Figure 5A) and the formed adducts **1** (Figure 5B), **2** (Figure 5C), and **3** (Figure 5D), as a function of the concentration of the phenolic (0–50 μmol) in the reaction between 2-methylresorcinol and 30 μmol of HNE after 20 h at 100 ºC. Because increasing amounts of 2-methylresorcinol were added, the amount of remaining 2-methylresorcinol increased as a function of the amount of 2-methylresorcinol added (Figure 5A). However, this increase achieved a maximum when 30 μmol of 2-methylresorcinol was added, although only 30 μmol of HNE was available for the reaction with the phenolic. Formation of polymeric structures at high concentrations of the phenolic might be hypothesized.

The effect of phenolic concentration on adduct **1** formation was also quite surprising (Figure 5B). These adducts were produced to a high extent at low concentrations of the phenolic and the presence of higher amounts of 2-methylresorcinol decreased linearly the concentration of adducts **1**. Thus, reaction yield of adduct **1g** was 3% when 10 μmol of 2-methylresorcinol was added, and this yield decreased to 1.7% when 50 μmol of 2-methylresorcinol was added. Something similar occurred with adduct **1h**, and its formation yield decreased from 2.9% to 1.4%.

Differently to adducts **1**, adducts **2** exhibited a much more expectable behavior and a linear increase in their concentrations was observed when the amount of 2-methylresorcinol increased from 0 to 30 μmol (Figure 5C). Thus, yield of adduct **2g** increased from 0 to 3.4% when 2-methylresorcinol concentration increased from 0 to 30

342 μmol and then remained approximately constant. Analogously, adduct **2h** increased
343 from 0 to 4.2% when 2-methylresorcinol increased from 0 to 30 μmol and then
344 increased slightly to 5.3% when 50 μmol of 2-methylresorcinol was added.

345 The behavior of adduct **3g** was analogous to that of adducts **2**, and its yield increased
346 from 0 to 2.2% when 2-methylresorcinol increased from 0 to 30 μmol and, then,
347 decreased slightly at higher concentrations of 2-methylresorcinol.

348 Figure 6 shows the remaining 2-methylresorcinol (Figure 6A) and the formed
349 adducts **1** (Figure 6B), **2** (Figure 6C), and **3** (Figure 6D), as a function of the
350 concentration of the hydroxyalkenal (0–50 μmol) in the reaction between 30 μmol of 2-
351 methylresorcinol and HNE after 20 h at 100 ºC. As expected, increasing amounts of
352 hydroxyalkenal produced a decrease in the remaining 2-methylresorcinol (Figure 6A).
353 This decrease was linear between 0 and 30 μmol of HNE and higher amounts of the
354 aldehyde did not produce a higher decrease in the remaining 2-methylresorcinol.

355 Differently to that observed for increasing amounts of 2-methylresorcinol, the yield
356 of adducts **1** increased linearly between 0 and 40 μmol of HNE (Figure 6B). Thus, the
357 yield of adduct **1g** increased to 3.3% when 40 μmol of HNE were added and the yield of
358 adduct **1h** was 2.6% when the same amount of HNE was added.

359 A similar behavior was observed for adducts **2**, although adduct **2g** only increased
360 linearly between 0 and 20 μmol of HNE, and adduct **2h** increased linearly between 0
361 and 50 μmol of HNE (Figure 6C).

362 Finally, adduct **3g** also increased linearly between 0 and 40 μmol of HNE (Figure
363 6D). However, and differently to other adducts, the yield obtained with 50 μmol of
364 HNE was significantly much higher than that expected for a linear increase.

Effect of Time and Temperature on the Formation of Carbonyl-Phenol Adducts in the Reaction of HNE and 2-Methylresorcinol. Time courses of adducts formation in the reaction between 2-methylresorcinol and HNE are collected in Figures 7, 8, and 9 for adducts **1**, **2**, and **3**, respectively. Concentration of all adducts increased linearly as a function of time at all assayed temperatures (from 60 to 140 ºC). Formation rates were determined for all adducts at the different assayed temperatures by using the equation:

$$[\text{adduct}] = k \cdot t$$

where k is the rate constant and t is the time. Determined rate constants were then used in an Arrhenius plot to calculate the activation energies (E_a) of formation of the different adducts (Figure 10). The E_a were determined from the slopes of the lines of best fit. The E_a determined for the formation of the different adducts were: 25.0 ± 2.1 kJ·mol^{-1} for adduct **1g**, 25.8 ± 2.1 kJ·mol^{-1} for adduct **1h**, 33.2 ± 1.7 kJ·mol^{-1} for adduct **2g**, 31.9 ±1.2 kJ·mol^{-1} for adduct **2h**, and 38.4 ± 3.8 kJ·mol^{-1} for adduct **3g**.

DISCUSSION

Previous studies have shown that the lipid-derived carbonyl trapping by phenolic compounds is a complex reaction in which the different nucleophilic groups of the phenolics (the hydroxyl groups and their contiguous aromatic carbons in *m*-diphenols) react with the reactive carbons of the carbonyl compound.[18] Because there is a large variety of lipid-derived carbonyl structures and because some of these carbonyls are easily degraded to produce new lipid carbonyls,[32] the variety of structures of the initially produced carbonyl-phenol adducts is also considerable.[18] Furthermore, many of these adducts still have reactive groups and further reactions, including polymerizations, are usually produced.

388 Nevertheless, the number of mechanisms by which the initial carbonyl-phenol
389 adducts are produced is relatively limited. Thus, when only an isolated carbonyl group
390 is present, such as in alkanals, the reaction is an addition of the aromatic carbon of the
391 phenolic to the carbonyl carbon of the alkanal.[22] However, the reaction is more complex
392 when a conjugated carbon-carbon double bond is present in the carbonyl compound. In
393 this case, the reaction is always initiated by the addition of either the hydroxyl group or
394 its contiguous aromatic carbon in the phenolic to the α,β carbon-carbon double bond of
395 the lipid carbonyl. After this initial addition, the molecule is then stabilized by blocking
396 the carbonyl group, usually by formation of a hemiacetalic structure. This occurs in 2-
397 alkenals,[23,24] 2,4-alkadienals,[25] and 4-oxo-2-alkenals.[26] As an exception, in 4,5-epoxy-2-
398 alkenals, the reaction is initiated by the attack of the hydroxyl group of the phenolic to
399 the epoxide ring of the lipid carbonyl because of the high reactivity of this three
400 membered ring.[27]

401 4-Hydroxy-2-alkenals are basically α,β-unsaturated carbonyl compounds. Therefore,
402 its behavior, at least at the initial steps, should be similar to other unsaturated carbonyl
403 compounds. In addition, and because of the presence of an additional hydroxylic group
404 in the carbonyl compound, the involvement of this group in the stabilization of the
405 formed structure might be expected. This is what has been observed in this study,
406 although the later stabilization of adducts **1** was different to that previously observed for
407 other lipid carbonyls.

408 Figure 11 collects a possible reaction pathway that explains the formation of the
409 different produced adducts. Analogously to other unsaturated carbonyl compounds, the
410 reaction is initiated by addition of either the hydroxyl group or its contiguous aromatic
411 carbon in the phenol to the carbon-carbon double bond of the aldehyde. This would
412 produce adducts **5** and **4**, respectively.

Stabilization of adduct **4** occurs simply by forming the corresponding hemiacetal (**6**), analogously to that observed in reactions involving 2-alkenals[24] or 2,4-alkadienals.[25] The later acetylation of adduct **6** stabilizes the structure of the adduct and produce the adducts **2** isolated and characterized in this study. Curiously, acetylation only occurs in the phenolic hydroxyl group. An interaction between the other two hydroxyl groups might be hypothesized to explain that they were not acetylated under standard acetylation conditions (20 h at 25 ºC).

Stabilization of adduct **5** is more complex. A possibility of stabilization is the addition of the aromatic carbon contiguous to the hydroxyl group to the carbonyl group and the formation of the chromane-4,7-diol (**7**). However, this compound is not stable and, after dehydration, the most stable 2*H*-chromen-7-ol (**8**) is produced. An analogous dehydration was observed in the reaction between phenolics and 2-alkenals.[24] After acetylation, the corresponding adducts **3** were produced.

An additional possibility, not observed previously with other lipid carbonyls, is the oxidation of adduct **5** to produce the corresponding α,β-unsaturated carbonyl compound **9**, which would be latter stabilized by addition of the aromatic carbon of the phenol. A stabilization of this kind was previously observed in 4,5-epoxy-2-alkenals.[26] A later tautomerization would be responsible of the existence of adduct **10** and, after acetylation, of adduct **1**.

The effect of studied reaction conditions is in agreement with the proposed reaction pathways. Thus, a slight basicity would increase the nucleophilicity of phenolic active groups and would favor the reaction as observed in Figure 4 for most adducts. In addition, the reaction involves equimolecular amounts of the phenolic and the carbonyl compound. Therefore, the concentration of most adducts increased linearly when the concentration of either 2-methylresorcinol or HNE increased from 0 to 30 μmol, which

438 is the amount present of the other reactant (Figures 5 and 6). The most surprising result
439 in relation to this conclusion was that observed for adducts **1**, because their
440 concentration decreased when the concentration of 2-methylresorcinol increased (Figure
441 4). This behavior might be likely related to the required oxidation step from adduct **5** to
442 adduct **9** (Figure 11) that can be hypothesized to be inhibited because of the antioxidant
443 activity of the phenolic compound. Thus, the higher amount of phenolic present, the
444 higher inhibition of the oxidative reaction and the lower yield of adduct **1**. Other
445 possibility is a higher reactivity of adduct **10** in comparison to that of compounds **2** and
446 **8**. Thus, compound **10** might react away in the presence of excess of phenolics more
447 easily than compounds **2** or **8**. Finally, adduct concentrations increased linearly as a
448 function of incubation times and E_a increased in the order adducts **1** < adducts **2** <
449 adducts **3**. For that reason, adducts **1** were the main adducts produced at low
450 temperature and were produced for the two hydroxyalkenals assayed. However, adduct
451 stability also played a major role and, at high temperatures and long incubation times,
452 the main adducts present in hydroxyalkenal/phenolic reaction mixtures were always
453 adducts **2**. This kind of hemiacetalic adducts, although derived from acrolein, has been
454 detected in commercially crispy fried onions,[16] which suggest a certain thermostability.

455 All these results confirm that, analogously to other lipid-derived carbonyl
456 compounds, phenolics are also able to trap 4-hydroxy-2-alkenals, which is likely
457 contributing to the mitigation of some adverse consequences described for these
458 undesirable products of lipid oxidation.[34–35] Furthermore, they provide the basis for the
459 potential determination of carbonyl-phenol adducts derived from hydroxyalkenals in
460 food products. This determination may help to evaluate the oxidative stress to which
461 such food has been exposed during processing or storage.

462 **ASSOCIATED CONTENT**

463 **Supporting Information**

464 The Supporting Information is available free of charge on the ACS Publications website
465 at DOI:

466 Spectroscopic and spectrometric data of compounds isolated and characterized in this
467 study. Proposed fragmentation, retention indexes and mass spectra of 2,3-
468 dihydrobenzofuranols (**1**), chromane-2,7-diols (**2**), and 2H-chromen-7-ols (3). Mass
469 spectra of catechin/HNE and quercetin/HNE reaction mixtures (PDF)

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480 **Notes**

481 The authors declare no competing financial interest.

482

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FIGURE CAPTIONS

Figure 1. Total ion chromatograms obtained for the reactions between: A, 4-hydroxy-2-hexenal (HHE) and resorcinol; B, 4-hydroxy-2-nonenal (HNE) and resorcinol; C, HHE and 2-methylresorcinol; D, HNE and 2-methylresorcinol; E, HHE and orcinol; F, HNE and orcinol; G, HHE and 2,5-dimethylresorcinol; and H, HNE and 2,5-dimethylresorcinol, in sodium phosphate buffer, pH 8, after 20 h at 100 ºC under nitrogen, and later acetylation.

Figure 2. Adducts identified in this study. Compounds **1e** (R = ethyl; R' = methyl; R" = H), **1f** (R = ethyl; R' = methyl; R" = H), **2g** (R = pentyl; R' = methyl; R" = H), **2h** (R = pentyl; R' = methyl; R" = H), and **3g** (R = pentyl; R' = methyl; R" = H) were isolated and characterized by NMR and MS. Carbon numbering corresponds to that employed in NMR spectra (spectroscopic and spectrometric data are collected in the Supplementary Material).

Figure 3. Time-course of 4-hydroxy-2-nonenal (HNE) disappearance at 100 ºC in sodium phosphate buffer, pH 8, and in the presence of catechin (○) and quercetin (△). HNE disappearance in the absence of phenolics is also shown for comparison (□).

Figure 4. Effect of pH on: A, remaining 2-methylresorcinol (Rem. MeRes); B, adduct **1** formation; C, adduct **2** formation; and D, adduct **3** formation, in the reaction of 2-methylresorcinol (30 μmol) and 4-hydroxy-2-nonenal (30 μmol) after 20 h at 100 ºC under nitrogen. The compounds determined were: 2-methylresorcinol (□,■), adduct **1g** (◇,◆), adduct **1h** (◁,◀), adduct **2g** (○,●), adduct **2h** (△,▲), and adduct **3g** (▽,▼). Open symbols correspond to sodium phosphate buffers and closed symbols correspond to sodium borate buffers.

Figure 5. Effect of 2-methylresorcinol (MeRes) concentration on: A, remaining 2-methylresorcinol (Rem. MeRes); B, adduct **1** formation; C, adduct **2** formation; and D, adduct **3** formation, in the reaction of 2-methylresorcinol and 4-hydroxy-2-nonenal (30 μmol) in sodium phosphate buffer, pH 8, after 20 h at 100 ºC under nitrogen. The compounds determined were: 2-methylresorcinol (□), adduct **1g** (◇), adduct **1h** (◁), adduct **2g** (○), adduct **2h** (△), and adduct **3g** (▽).

Figure 6. Effect of 4-hydroxy-2-nonenal (HNE) concentration on: A, remaining 2-methylresorcinol (Rem. MeRes); B, adduct **1** formation; C, adduct **2** formation; and D, adduct **3** formation, in the reaction of 2-methylresorcinol (30 μmol) and HNE in sodium phosphate buffer, pH 8, after 20 h at 100 ºC under nitrogen. The compounds determined were: 2-methylresorcinol (□), adduct **1g** (◇), adduct **1h** (◁), adduct **2g** (○), adduct **2h** (△), and adduct **3g** (▽).

Figure 7. Time courses of formation of adducts: A, **1g**, and B, **1h**, at 60 (□), 80 (○), 100 (△), 120 (▽), and 140 ºC (◇). Structures of adducts **1g** and **1h** are given in Figure 2.

Figure 8. Time courses of formation of adducts: A, **2g**, and B, **2h**, at 60 (□), 80 (○), 100 (△), 120 (▽), and 140 ºC (◇). Structures of adducts **2g** and **2h** are given in Figure 2.

Figure 9. Time courses of formation of adduct **3g** at 60 (□), 80 (○), 100 (△), 120 (▽), and 140 ºC (◇). Structure of adduct **3g** is given in Figure 2.

Figure 10. Arrhenius plot of adducts **1g** (◇), **1h** (◁), **2g** (○), **2h** (△), and **3g** (▽). Structures of adducts **1g**, **1h**, **2g**, **2h**, and **3g** are given in Figure 2.

Figure 11. Proposed reaction pathways for the formation of adducts **1**–**3** in the reaction of phenolics and 4-hydroxy-2-alkenals. R can be either ethyl or pentyl groups. R' and

R" can be either hydrogen or a methyl group. Ac_2O is acetic anhydride and Pyr is pyridine.

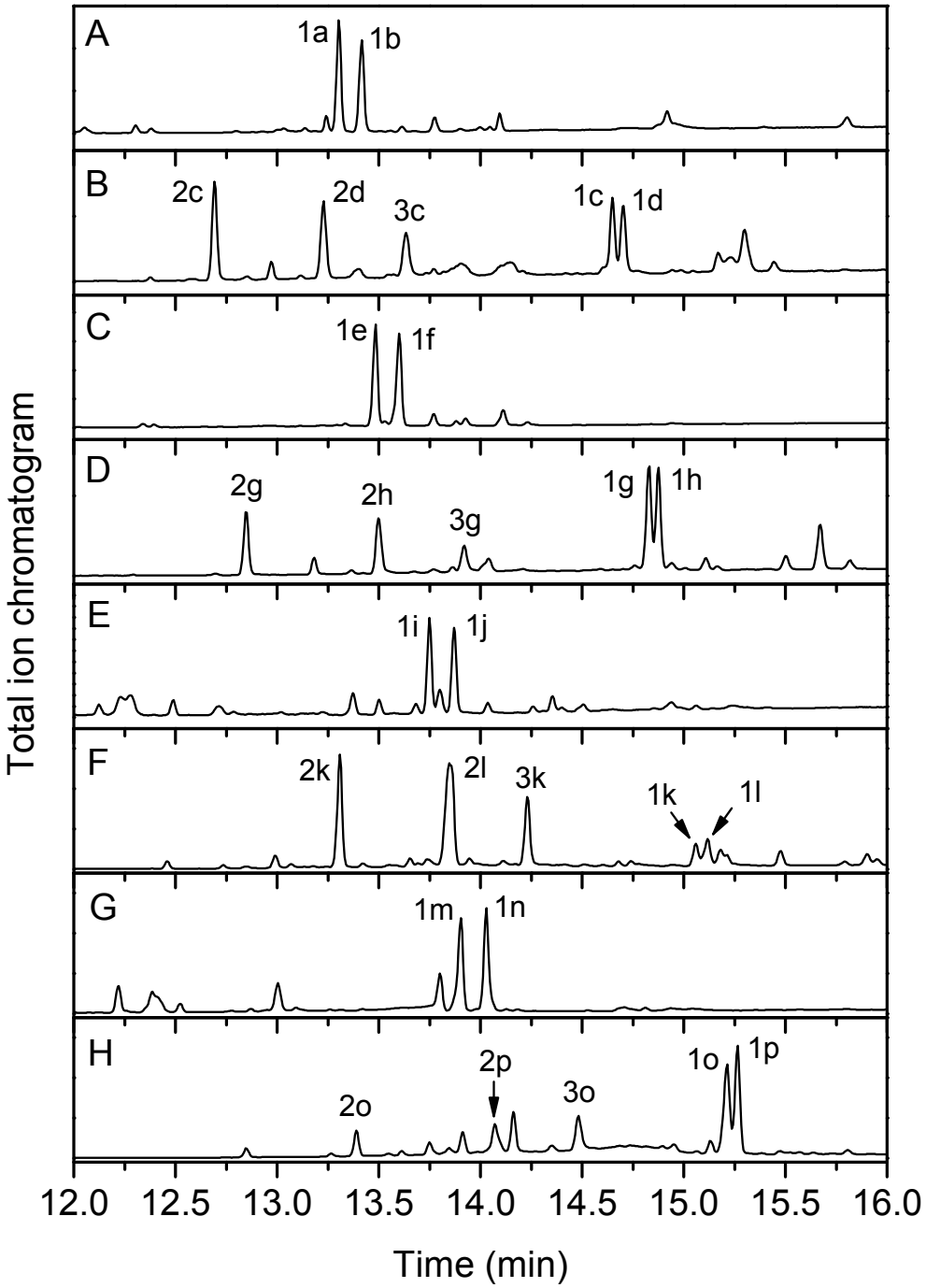
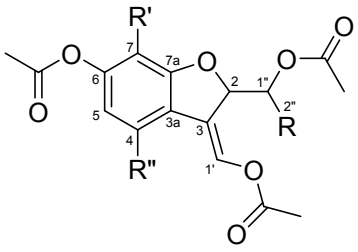
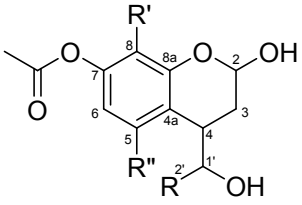

A
1a
1b
B
2c
2d
3c
1c
1d
C
1e
1f
D
2g
2h
3g
1g
1h
E
1i
1j
F
2k
2l
3k
1k
1l
G
1m
1n
H
2o
2p
3o
1o
1p
Total ion chromatogram
12.0
12.5
13.0
13.5
14.0
14.5
15.0
15.5
16.0
Time (min)

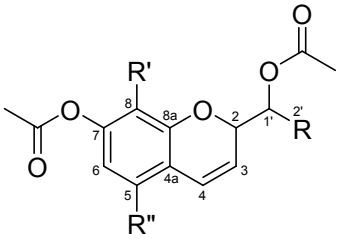
Figure 1


R'
O
O
7
7a
O
2
1"
O
O
6
2"
5
3a
3
R
4
R"
1'
O
O

1


R'
8
O
8a
O
2
OH
O
7
6
4a
3
5
4
R"
2'
1'
R
OH

2


O
R'
O
8
8a
O
2
2'
O
7
1'
R
6
4a
3
5
4
R"

3

Figure 2

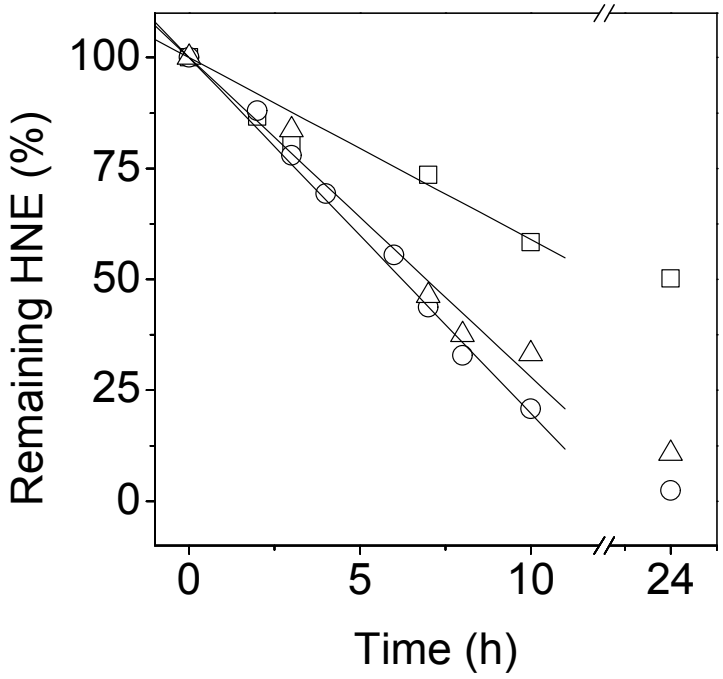


Figure 3

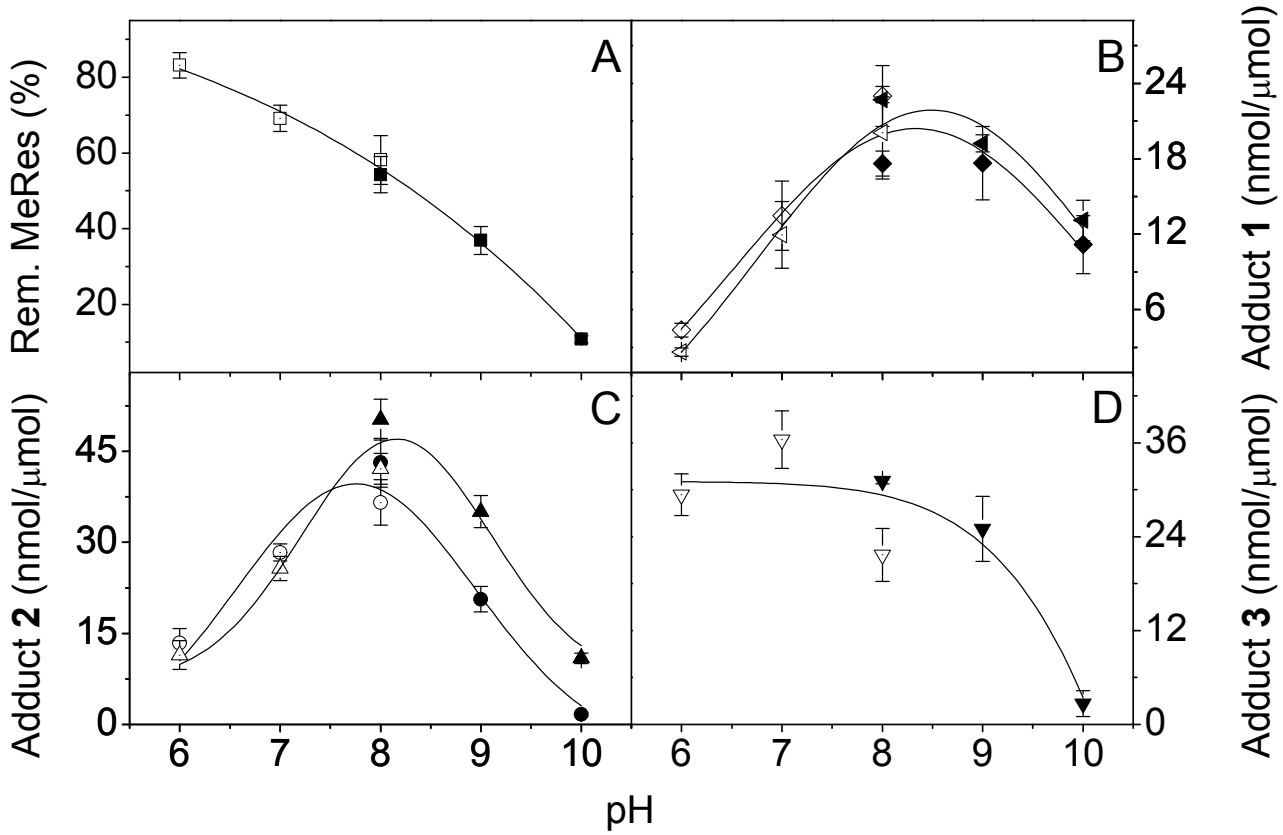

A
B
C
D
Rem. MeRes (%)
Adduct 2 (nmol/μmol)
Adduct 1 (nmol/μmol)
Adduct 3 (nmol/μmol)
pH
80
60
40
20
24
18
12
6
45
30
15
0
36
24
12
0
6
7
8
9
10

Figure 4

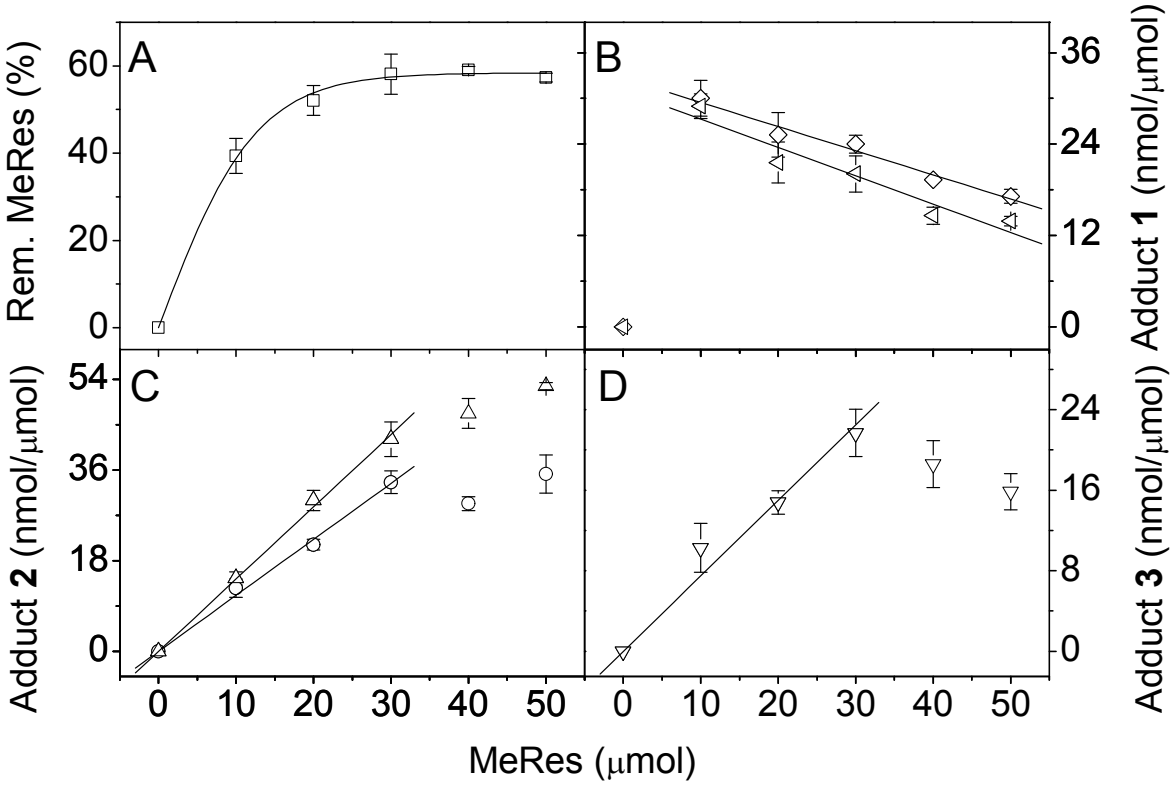

A
B
C
D
Rem. MeRes (%)
Adduct 2 (nmol/μmol)
Adduct 3 (nmol/μmol)
Adduct 1 (nmol/μmol)
MeRes (μmol)
0
20
40
60
0
12
24
36
0
18
36
54
0
8
16
24
0
10
20
30
40
50

Figure 5

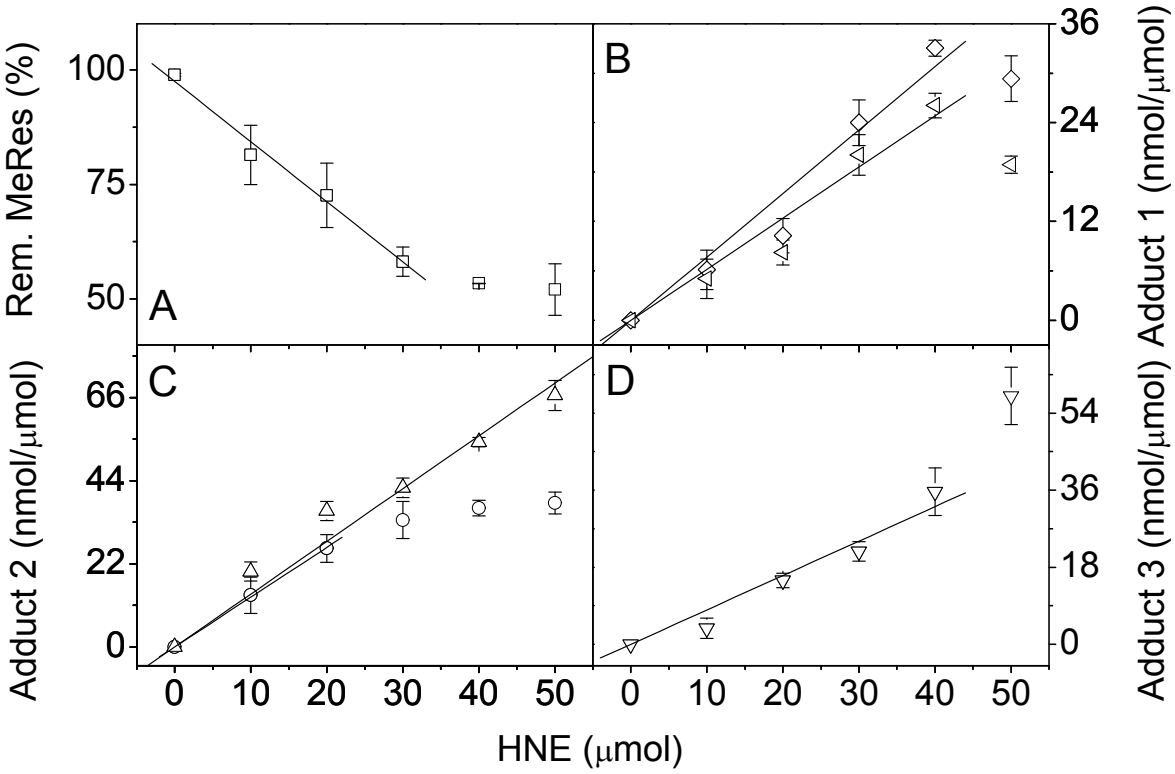

Rem. MeRes (%)
Adduct 2 (nmol/μmol)
Adduct 3 (nmol/μmol)
Adduct 1 (nmol/μmol)
HNE (μmol)
A
B
C
D
100
75
50
66
44
22
0
36
24
12
54
18
0
10
20
30
40

Figure 6

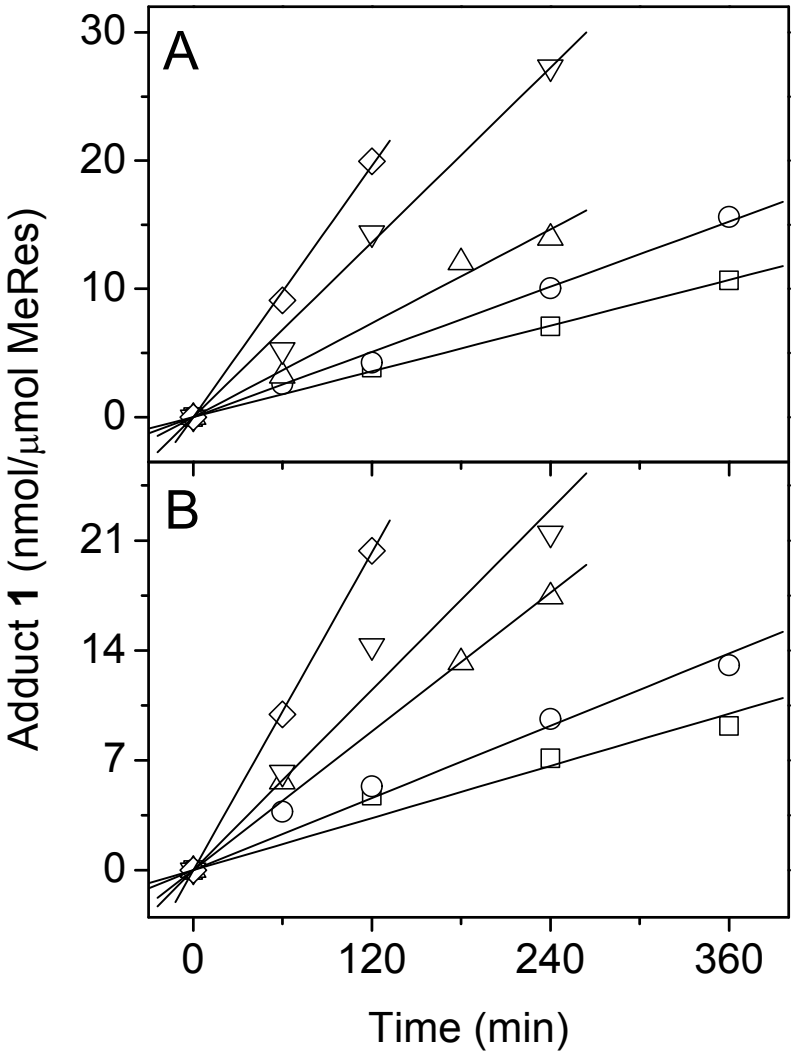

A
B
30
20
10
0
21
14
7
0
0
120
240
360
Time (min)
Adduct 1 (nmol/μmol MeRes)

Figure 7

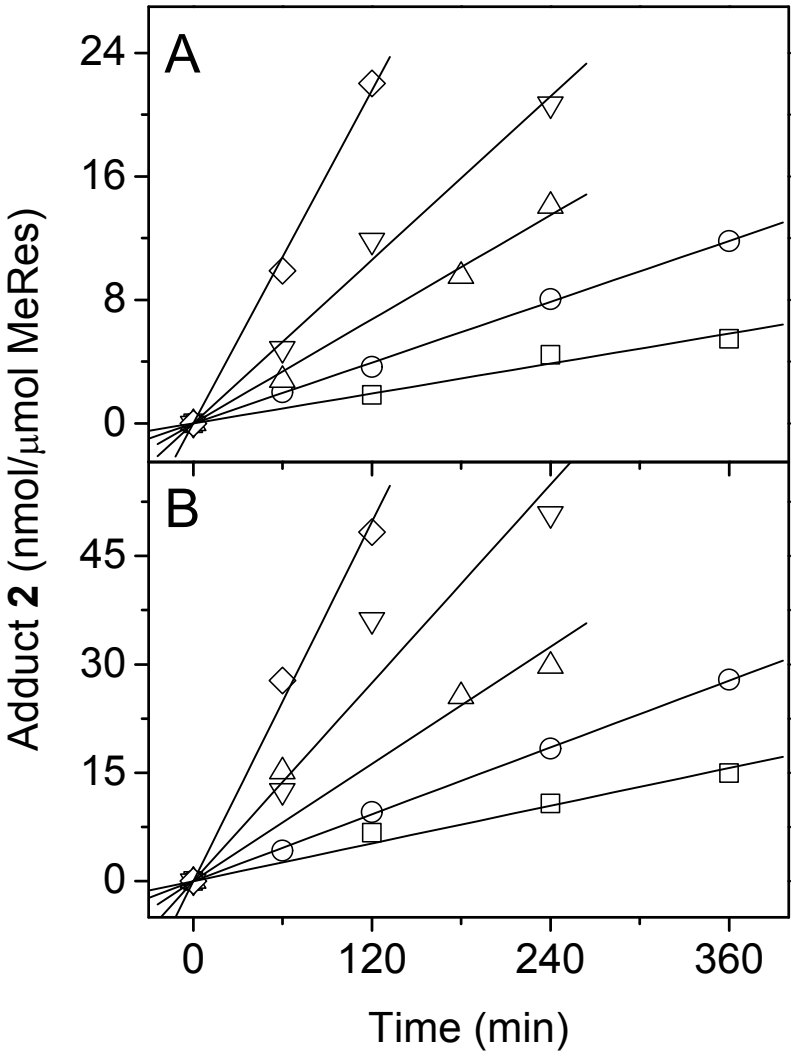

A
B
24
16
8
0
45
30
15
0
0
120
240
360
Time (min)
Adduct 2 (nmol/μmol MeRes)

Figure 8

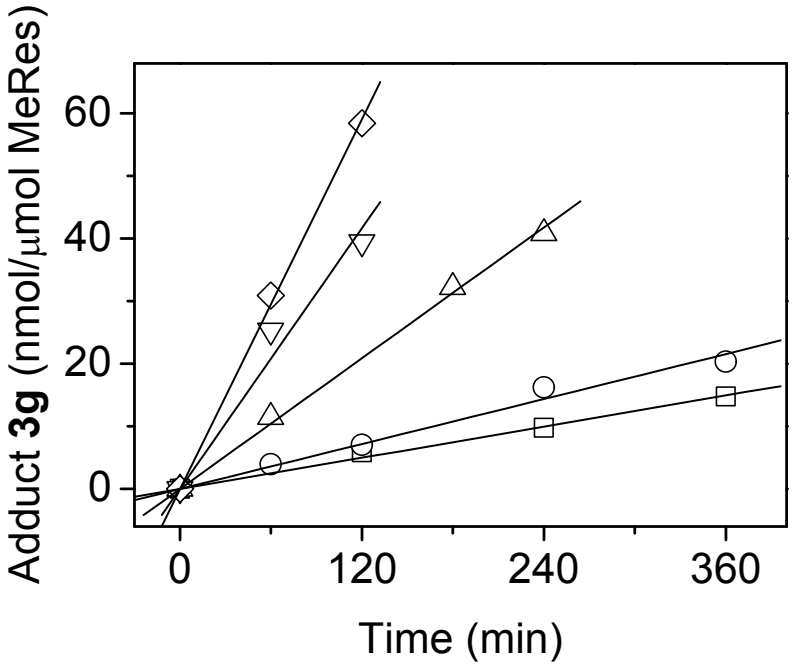


Figure 9

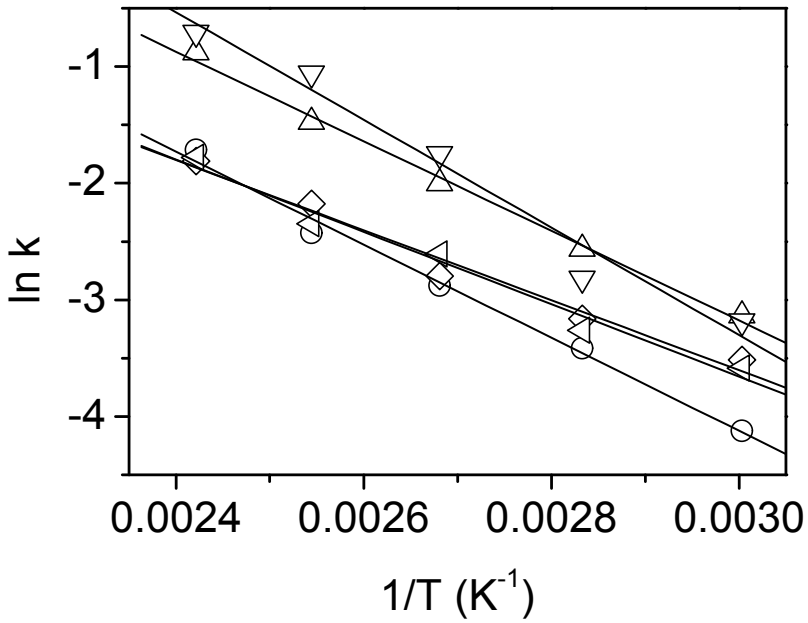


Figure 10

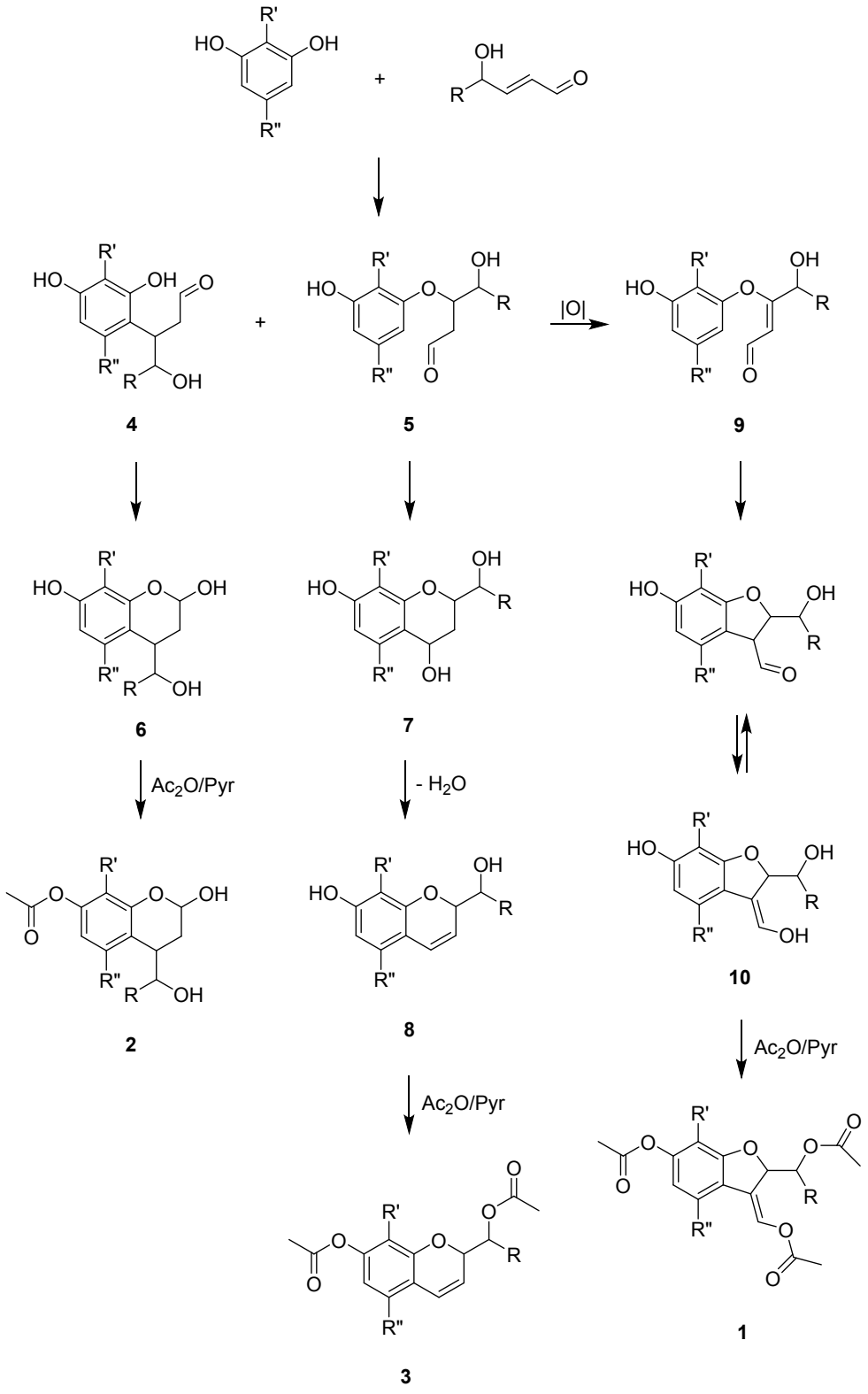


Figure 11

GRAPHIC FOR TABLE OF CONTENTS