1 **Epoxyalkenal-trapping ability of phenolic compounds**

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9 ABSTRACT

10 Lipid oxidation products have been shown to produce changes in food quality and
11 safety as a consequence of carbonyl-amine reactions. Some of these reactions can be
12 prevented by the use of phenolics, although the lipid-derived carbonyl trapping ability
13 of phenolics is still poorly understood. In an attempt to fill this gap, the reactions of 4,5-
14 epoxy-2-hexenal, 4,5-epoxy-2-heptenal, and 4,5-epoxy-2-decenal, with 2-
15 methylresorcinol and 2,5-dimethylresorcinol were studied. These reactions produced
16 diverse 1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromene-2,7-diols and 3,4,4a,9a-
17 tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diols, which were isolated and
18 characterized by 1D and 2D nuclear magnetic resonance (NMR) and mass spectrometry
19 (MS). The produced epoxyalkenal-phenol reaction was characterized and carbonyl-
20 phenol adducts were produced firstly by epoxide-ring opening initiated by the attack of
21 one phenolic hydroxyl group and, then, by addition of one aromatic phenol carbon to
22 the carbon-carbon double bond of the epoxyalkenal. This reaction rapidly deactivated
23 the most important reactive groups of epoxyalkenals, decreasing in this way their ability
24 to modify amino compounds.

25 *Keywords:*

26 4,5-Epoxy-2-alkenals; Carbonyl-amine reactions; Carbonyl-phenol reactions; Lipid
27 oxidation; Maillard reaction; Reactive carbonyls

28 *Chemical compounds studied in this article:*

29 2,5-Dimethylresorcinol (PubChem ID: 24863551); 4,5-epoxy-2-heptenal (PubChem ID:
30 6444055); 4,5-epoxy-2-decenal (PubChem ID: 5352429); 2-methylresorcinol
31 (PubChem ID: 11843).

1. Introduction

The oxidation of unsaturated fatty acyl chains is a major concern during food processing or storage because of its consequences on quality and safety of foods, including both changes in flavor, texture, appearance, and nutritional quality, and the formation of potentially toxic compounds (Gramza-Michalowska, Kobus-Cisowska, Kmiecik, Korczak, Helak, Dziedzic, et al., 2016; Liu, Liu, Xue, & Gao, 2016; Sainsbury, Grypa, Ellingworth, Duodu, & De Kock, 2016). Among the myriad of compounds produced as a consequence of the lipid oxidation in complex food systems, the formation toxic oxygenated unsaturated aldehydes has been the objective of numerous studies (Guillen & Goicoechea, 2008; Guillen & Uriarte, 2012). These compounds are highly reactive, and their ability to modify important food components has been thoroughly studied (Zamora & Hidalgo, 2005; Hidalgo & Zamora, 2016). Nevertheless, some studies have suggested that these highly reactive lipid oxidation products can be trapped by phenolic compounds (Delgado, Hidalgo, & Zamora, 2016; Hidalgo & Zamora, 2014; Zhu, Zheng, Cheng, Wu, Zhang, Tang, et al, 2009), a reaction that would constitute an additional protective mechanism of phenolic compounds in food products (Zamora & Hidalgo, 2016). Thus, some of these lipid-derived carbonyl compounds have been shown to be trapped by phenolic compounds in food products at the same time that they are being produced (Zamora, Aguilar, Granvogl, & Hidalgo, 2016). In addition, other highly reactive lipid oxidation products, namely 4,5-epoxy-2-alkenals, have been shown to be trapped by phenolic compounds with preference to other lipid oxidation products and this reaction is fast enough to prevent the production of the competitive carbonyl-amine reactions (Hidalgo, Delgado, & Zamora, 2017).

In spite of the important role that carbonyl-phenol reactions might be playing in the prevention of the consequences produced in food by carbonyl-amine reactions initiated

by epoxyalkenals, epoxyalkenal-phenol reactions remains to be characterized. In an attempt to filling this gap, this study analyzes the reaction of 4,5-epoxy-2-alkenals and phenolic compounds by both isolating and identifying the produced compounds, and studying the reaction conditions that favors the formation of the corresponding carbonyl-phenol adducts.

2. Materials and methods

2.1. Materials

Three 4,5-epoxy-2-alkenals were employed in this study. They were 4,5-epoxy-2-hexenal, 4,5-epoxy-2-heptenal, and 4,5-epoxy-2-dexenal, which were synthesized from the corresponding 2,4-alkadienals (2,4-hexadienal, 2,4-heptadienal, and 2,4-decadienal, respectively) by epoxidation with 3-chloroperoxybenzoic acid (Zamora, Gallardo, & Hidalgo, 2006).

As model phenolic compounds, only simple *m*-diphenols were employed, namely 2-methylresorcinol and 2,5-dimethylresorcinol. These phenolic compounds were selected because carbonyl-phenol adducts are formed mainly with phenolic compounds having two hydroxyl groups at *meta* positions (Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014), and simple *m*-diphenols are efficient epoxyalkenal-trapping agents (Hidalgo, Delgado, & Zamora, 2017).

Alkadienals, phenolic compounds and all other chemicals employed in this study were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany) and were either analytical grade or the highest available grade.

2.2. Formation of carbonyl-phenol adducts in the reaction of epoxyalkenals and phenolic compounds

For analytical studies, a mixture of 30 μ mol of the phenolic compound (170 μ L of a 0.176 mM solution in water), 40 μ mol of the epoxyalkenal (40 μ L of a 1 M solution in methanol), and 300 μ L of 0.3 M buffer was heated at 100 $^{\circ}$ C for 30 min under nitrogen, unless other conditions are indicated. At the end of the heating process, samples were cooled at room temperature (22 $^{\circ}$ C) for 15 min, 1.2 mL of ethanol added, and taken to dryness using a flow of nitrogen.

Dried samples were acetylated by adding 30 μ L of internal standard (a solution of 36.64 mg of *cis*-3-nonen-1-ol in 5 mL of anhydrous pyridine), 1 mL of anhydrous pyridine, and 500 μ L of acetic anhydride. Mixtures were left 20 h in the dark at room temperature.

Acetylated samples were treated with 2 mL of water and 2 mL of chloroform, and stirred for 10 s. After phase separation, the aqueous layer was removed and discarded. The organic layer was washed several times (usually three) with 2 mL of 5% HCl until the pyridine was removed. Finally, the organic layer was washed with 2 mL of water to remove the HCl. The resulting organic extracts were treated with sodium sulfate to eliminate the remaining humidity and then studied by GC-MS.

For preparative studies, the reaction conditions were the same but the reaction was carried out using 2.4 mmol of phenolic compound, 3.4 mmol of epoxyalkenal, and 24 mL of 0.3 M sodium phosphate buffer, pH 8. Acetylation of the heated and dried mixtures was carried out with 80 mL of anhydrous pyridine and 40 mL of acetic anhydride. Acetylated compounds were extracted with chloroform, and the pyridine was removed as described above. The mixtures of acetylated compounds obtained were fractionated by column chromatography on silica gel 60 (230-400 mesh; Macherey-Nagel, Düren, Germany) using hexane:diethyl ether mixtures as eluent and the

separation was controlled by gas chromatography coupled to mass spectrometry (GC-MS). The following compounds were isolated and characterized (Fig. 1):

4-Ethyl-2-hydroxy-6-methyl-1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromen-7-yl acetate (**9a**) was isolated in the reaction between 2-methylresorcinol (**1a**) and 4,5-epoxy-2-heptenal (**2b**). ¹H NMR (CDCl₃): δ (ppm) 1.04t (3H, *J* = 7.4 Hz, CH₃CH₂), 1.65qu (2H, *J* = 7.4 Hz, CH₃CH₂), 2.02s (3H, CH₃C6), 2.21m and 2.29m (2H, H1), 2.33s (3H, CH₃CO), 3.52m (1H, H4), 3.89q,br (1H, *J* = 7.5 Hz, H9b), 4.65t (1H, *J* = 9.3 Hz, H3a), 6.07dd (1H, *J* = 2.7 Hz and 5.2 Hz, H2), 6.59d (1H, *J* = 8.0 Hz, H8), and 6.97d (1H, *J* = 8.0 Hz, H9). ¹³C NMR (CDCl₃): δ (ppm) 9.19 (CH₃C6), 9.45 (CH₃CH₂C4), 20.76 (CH₃CO), 26.22 (CH₃CH₂C4), 29.58 (C1), 36.56 (C9b), 74.33 (C4), 82.31 (C3a), 92.17 (C2), 113.51 (C6), 114.39 (C8), 120.90 (C9), 125.77 (C9a), 149.71 (C7), 158.60 (C5a), 169.39 (CH₃CO). This compound suffered a dehydration when studied by GC-MS and was converted into 2-ethyl-8-methyl-4-(2-oxoethyl)-2*H*-chromen-7-yl acetate (**10a**). MS, *m/z* (% ion structure): 274 (19, M⁺), 232 (33, M⁺ – CH₂CO), 204 (15, 232 – ethene), 203 (100, 232 – CH₃CH₂), 189 (11, 204 – CH₃), 175 (19, 204 – CHO), 174 (31, 204 – HCHO) and 161 (13, 204 – CH₂CHO).

4-Ethyl-2-hydroxy-6-methyl-1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromen-7-yl acetate (**9b**) was also isolated in the reaction between 2-methylresorcinol (**1a**) and 4,5-epoxy-2-heptenal (**2b**). This compound is a stereoisomer of compound **9a**. ¹H NMR (CDCl₃): δ (ppm) 1.06t (3H, *J* = 7.4 Hz, CH₃CH₂), 1.64qu (2H, *J* = 7.4 Hz, CH₃CH₂), 2.02s (3H, CH₃C6), 1.95m and 2.30m (2H, H1), 2.32s (3H, CH₃CO), 3.51m (1H, H9b), 3.84dt (1H, *J* = 3.9 Hz, *J* = 8.7 Hz, *J* = 8.7 Hz, H4), 4.58t (1H, *J* = 9.3 Hz, H3a), 6.15dd (1H, *J* = 5.9 Hz and 8.5 Hz, H2), 6.55d (1H, *J* = 8.0 Hz, H8), and 6.96d (1H, *J* = 8.0 Hz, H9). ¹³C NMR (CDCl₃): δ (ppm) 9.24 (CH₃C6), 9.55 (CH₃CH₂C4), 20.74 (CH₃CO), 26.36 (CH₃CH₂C4), 30.56 (C1), 37.42 (C9b), 71.81 (C4), 84.16 (C3a), 91.92 (C2),

113.39 (C6), 114.13 (C8), 121.30 (C9), 126.16 (C9a), 149.65 (C7), 159.16 (C5a),
 169.35 (CH₃C=O). This compound also suffered a dehydration when studied by GC-MS
 and was converted into 2-ethyl-8-methyl-4-(2-oxoethyl)-2*H*-chromen-7-yl acetate
 (**10a**). This last compound was identical to the compound produced in the dehydration
 of compound **9a**. MS, m/z (% ion structure): 274 (19, M⁺), 232 (33, M⁺ – CH₂CO), 204
 (15, 232 – ethene), 203 (100, 232 – CH₃CH₂), 189 (11, 204 – CH₃), 175 (19, 204 –
 CHO), 174 (31, 204 – HCHO), and 161 (13, 204 – CH₂CHO).

2-Hydroxy-6,9-dimethyl-4-pentyl-1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromen-7-yl
 acetate (**9c**) was isolated in the reaction between 2,5-dimethylresorcinol (**1b**) and 4,5-
 epoxy-2-decenal (**2c**). ¹H NMR (CDCl₃): δ (ppm) 0.89t (3H, *J* = 6.8 Hz, CH₃CH₂), 1.30
 m (6H, CH₃CH₂CH₂CH₂), 1.58m (2H, CH₂C4), 1.85m and 2.36m (2H, H1), 1.98s (3H,
 CH₃C6), 2.23s (3H, CH₃C9), 2.31s (3H, CH₃CO), 3.42m (1H, H9b), 3.95ddd (1H, *J* =
 1.3 Hz, *J* = 4.4 Hz, *J* = 5.6 Hz, H4), 4.54t (1H, *J* = 8.9 Hz, H3a), 6.16dd (1H, *J* = 6.1 Hz
 and 9.1 Hz, H2), and 6.37s (1H, H8). ¹³C NMR (CDCl₃): δ (ppm) 9.04 (CH₃C6), 13.97
 (CH₃CH₂), 18.15 (CH₃C9), 20.95 (CH₃CO), 22.48 (CH₃CH₂), 25.12 (CH₃CH₂CH₂CH₂),
 29.00 (C1), 29.74 (CH₂C4), 31.61 (CH₃CH₂CH₂), 37.04 (C9b), 71.85 (C4), 84.61 (C3a),
 92.03 (C2), 110.49 (C6), 115.18 (C8), 124.63 (C9), 132.17 (C9a), 149.52 (C7), 159.03
 (C5a), 169.45 (CH₃C=O). This compound suffered a dehydration when studied by GC-
 MS and was converted into 8-methyl-4-(2-oxoethyl)-2-pentyl-2*H*-chromen-7-yl acetate
 (**10c**). MS, m/z (% ion structure): 330 (27, M⁺), 288 (59, M⁺ – CH₂CO), 273 (5, 288 –
 CH₃), 259 (10, 288 – CH₃CH₂), 245 (12, 288 – CH₃CH₂CH₂), 218 (15, 288 – pentene),
 217 (100, 288 – CH₃CH₂CH₂CH₂CH₂), 189 (41, 218 – CHO), 188 (67, 218 – HCHO),
 175 (69, 218 – CH₂CHO), and 151 (84).

1,8-Dimethyl-3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diyl diacetate
 (**11a**) was isolated in the reaction between 2-methylresorcinol (**1a**) and 4,5-epoxy-2-

155 hexenal (**2a**). ^1H NMR (CDCl_3): δ (ppm) 1.26d (3H, $J = 6.6$ Hz, $\text{CH}_3\text{C1}$), 1.99s (3H,
 156 $\text{CH}_3\text{C8}$), 2.08s (3H, CH_3CO), 2.28m (2H, H4), 2.32s (3H, CH_3CO), 3.12d (1H, $J = 3.9$
 157 Hz, H4a), 4.24d (1H, $J = 7.8$ Hz, H9a), 4.79dt (1H, $J = 7.8$ Hz, $J = 6.6$ Hz, H1), 5.84d
 158 (1H, $J = 3.2$ Hz, H3), 6.57d (1H, $J = 8.1$ Hz, H6), and 6.85d (1H, $J = 8.1$ Hz, H5). ^{13}C
 159 NMR (CDCl_3): δ (ppm) 8.99 ($\text{CH}_3\text{C8}$), 16.81 ($\text{CH}_3\text{C1}$), 20.82 (CH_3CO), 21.31
 160 (CH_3CO), 30.21 (C4), 37.57 (C4a), 69.77 (C1), 91.39 (C9a), 99.35 (C3), 113.77 (C6),
 161 118.64 (C8), 123.50 (C5), 126.17 (C4b), 149.18 (C7), 150.59 (C8a), 169.35 (CH_3CO),
 162 and 170.31 (CH_3CO). MS, m/z (%), ion structure): 320 (8, M^+), 278 (1, $\text{M}^+ - \text{CH}_2\text{CO}$),
 163 260 (8, 278 – H_2O), 245 (43, 260 – CH_3), 232 (5, 260 – CO), 218 (38, 260 – CH_2CO),
 164 203 (100, 218 – CH_3), 190 (50, 218 – CH_3CH), 176 (9, 218 – CH_2CO), 175 (43, 218 –
 165 CH_3CO), and 161 (66, 176 – CH_3).

 166 1-Ethyl-8-methyl-3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diyl
 167 diacetate (**11b**) was isolated in the reaction between 2-methylresorcinol (**1a**) and 4,5-
 168 epoxy-2-heptenal (**2b**). ^1H NMR (CDCl_3): δ (ppm) 0.91t (3H, $J = 7.4$ Hz, CH_3CH_2),
 169 1.57m and 1.78m (2H, $\text{CH}_2\text{C1}$), 1.99s (3H, $\text{CH}_3\text{C8}$), 2.15s (3H, CH_3CO), 2.29m (2H,
 170 H4), 2.32s (3H, CH_3CO), 3.05d (1H, $J = 3.9$ Hz, H4a), 4.29d (1H, $J = 8.3$ Hz, H9a),
 171 4.75dt (1H, $J = 3.8$ Hz, $J = 8.1$ Hz, H1), 5.83d (1H, $J = 3.3$ Hz, H3), 6.56d (1H, $J = 8.1$
 172 Hz, H6), and 6.83d (1H, $J = 8.1$ Hz, H5). ^{13}C NMR (CDCl_3): δ (ppm) 8.99 ($\text{CH}_3\text{C8}$),
 173 9.78 (CH_3CH_2), 20.77 (CH_3CO), 21.11 (CH_3CO), 23.93 ($\text{CH}_2\text{C1}$), 30.19 (C4), 37.72
 174 (C4a), 73.76 (C1), 89.85 (C9a), 99.34 (C3), 113.74 (C6), 118.61 (C8), 123.52 (C5),
 175 126.15 (C4b), 149.18 (C7), 150.59 (C8a), 169.31 (CH_3CO), and 170.62 (CH_3CO). MS,
 176 m/z (%), ion structure): 334 (4, M^+), 292 (1, $\text{M}^+ - \text{CH}_2\text{CO}$), 274 (2, 278 – H_2O), 245 (55,
 177 274 – CHO), 232 (16, 274 – CH_2CO), 217 (2, 232 – CH_3), 203 (100, 232 – CH_3CH_2),
 178 191 (18, 232 – C_3H_5), 190 (5, 232 – CH_2CO), 189 (15, 232 – CH_2CHO), 175 (8, 190 –
 179 CH_3), and 161 (39, 190 – CH_3CH_2).

180 1-Ethyl-8-methyl-3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diyl
 181 diacetate (**11c**) was also isolated in the reaction between 2-methylresorcinol (**1a**) and
 182 4,5-epoxy-2-heptenal (**2b**). This compound is a stereoisomer of compound **11b**. ¹H
 183 NMR (CDCl₃): δ (ppm) 0.85t (3H, *J* = 7.5 Hz, CH₃CH₂), 1.65m and 1.76m (2H,
 184 CH₂C1), 1.98s (3H, CH₃C8), 2.03s (3H, CH₃CO), 2.25dd (2H, *J* = 2.4 Hz, *J* = 3.2 Hz,
 185 H4), 2.31s (3H, CH₃CO), 3.20dd (1H, *J* = 3.0 Hz, *J* = 5.1 Hz, H4a), 4.22dd (1H, *J* = 3.2
 186 Hz, *J* = 8.5 Hz, H9a), 4.47ddd (1H, *J* = 4.1 Hz, *J* = 6.5 Hz, *J* = 8.5 Hz, H1), 5.80br (1H,
 187 H3), 6.55d (1H, *J* = 8.1 Hz, H6), and 6.68d (1H, *J* = 8.1 Hz, H5). ¹³C NMR (CDCl₃): δ
 188 (ppm) 8.62 (CH₃CH₂), 9.07 (CH₃C8), 20.78 (CH₃CO), 21.00 (CH₃CO), 24.37 (CH₂C1),
 189 32.87 (C4), 38.40 (C4a), 73.94 (C1), 87.95 (C9a), 99.13 (C3), 113.75 (C6), 118.24
 190 (C8), 122.00 (C4b), 125.11 (C5), 149.52 (C7), 150.15 (C8a), 169.30 (CH₃CO), and
 191 169.61 (CH₃CO). MS, m/z (% ion structure): 334 (4, M⁺), 292 (2, M⁺ – CH₂CO), 274
 192 (3, 278 – H₂O), 245 (18, 274 – CHO), 232 (28, 274 – CH₂CO), 217 (1, 232 – CH₃), 203
 193 (100, 232 – CH₃CH₂), 191 (10, 232 – C₃H₅), 190 (7, 232 – CH₂CO), 189 (43, 232 –
 194 CH₂CHO), 175 (11, 190 – CH₃), and 161 (17, 190 – CH₃CH₂).

195 1-Ethyl-5,8-dimethyl-3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diyl
 196 diacetate (**11d**) was isolated in the reaction between 2,5-dimethylresorcinol (**1b**) and
 197 4,5-epoxy-2-heptenal (**2b**). ¹H NMR (DMSO-*d*₆): δ (ppm) 0.83t (3H, *J* = 7.4 Hz,
 198 CH₃CH₂), 1.48m and 1.69m (2H, CH₂C1), 1.82s (3H, CH₃C8), 2.11s (3H, CH₃CO),
 199 2.19s (3H, CH₃C5), 2.26m (2H, H4), 2.27s (3H, CH₃CO), 3.31m (1H, H4a), 4.07d (1H,
 200 *J* = 8.0 Hz, H9a), 4.68dt (1H, *J* = 3.5 Hz, *J* = 8.2 Hz, H1), 5.88d (1H, *J* = 3.2 Hz, H3),
 201 and 6.47s (1H, H6). ¹³C NMR (CDCl₃): δ (ppm) 9.22 (CH₃C8), 9.62 (CH₃CH₂), 18.22
 202 (CH₃C5), 20.93 (CH₃CO), 21.23 (CH₃CO), 23.88 (CH₂C1), 30.07 (C4), 33.34 (C4a),
 203 73.53 (C1), 89.92 (C9a), 99.27 (C3), 115.35 (C8), 115.74 (C6), 125.42 (C5), 131.76
 204 (C4b), 148.48 (C7), 150.42 (C8a), 169.40 (CH₃CO), and 170.57 (CH₃CO). MS, m/z (% ion structure): 334 (4, M⁺), 292 (2, M⁺ – CH₂CO), 274 (3, 278 – H₂O), 245 (18, 274 – CHO), 232 (28, 274 – CH₂CO), 217 (1, 232 – CH₃), 203 (100, 232 – CH₃CH₂), 191 (10, 232 – C₃H₅), 190 (7, 232 – CH₂CO), 189 (43, 232 – CH₂CHO), 175 (11, 190 – CH₃), and 161 (17, 190 – CH₃CH₂).

ion structure): 348 (28, M^+), 306 (10, $M^+ - CH_2CO$), 288 (1, 292 – H_2O), 259 (53, 288 – CHO), 246 (49, 288 – CH_2CO), 231 (3, 246 – CH_3), 217 (100, 246 – CH_3CH_2), 205 (35, 246 – C_3H_5), 204 (14, 246 – CH_2CO), 203 (75, 246 – CH_2CHO), 189 (9, 204 – CH_3), 176 (96, 204 – ethene), 175 (80, 204 – CH_3CH_2), and 161 (20, C_3H_5).

2.3. GC-MS analyses

GC-MS analyses were conducted with an Agilent 7820 gas chromatograph coupled with an Agilent 5977B mass selective detector (MSD), quadrupole type. The employed column was a fused-silica DB-5MS UI capillary column (30 m $\times$ 0.25 i.d.; coating thickness, 0.25 μ m), and 1 μ L of sample was injected in the pulsed splitless mode. The following working conditions were employed: carried gas, helium (0.8 mL/min); injector, 250 $^{\circ}C$; oven temperature programmed from 100 $^{\circ}C$ (1 min) to 300 $^{\circ}C$ at 15 $^{\circ}C$ /min and then 5 min at 300 $^{\circ}C$; transfer line to MSD, 280 $^{\circ}C$; ionization EI, 70 eV; ion source temperature, 230 $^{\circ}C$; mass range 50–550 amu.

2.4. Epoxyalkenal-phenol adduct determination

The quantification of aldehyde-phenol adducts was carried out by preparing standard curves of the isolated adducts (**9a**, **9b**, **11b**, **11c**) in the reaction between 4,5-epoxy-2-heptenal and 2-methylresorcinol. For each curve, six different concentration levels of the adducts were used. Adduct content was directly proportional to the adduct/internal standard area ratio ($r > 0.99$, $p < 0.001$). The coefficients of variation were $<10\%$. Because the reaction between 4,5-epoxy-2-heptenal and 2-methylresorcinol produced four isomers (Fig. S-1, Supplementary data) of 1-ethyl-8-methyl-3,4,4a,9a-tetrahydro-1H-pyrano[3,4-*b*]benzofuran-3,7-diyl diacetates (**11**) and only two of them could be isolated and employed for preparing the corresponding standard curves (**11b** and **11c**), the quantification of the other two isomers (**11e**, **11f**) was carried out by employing a

calibration curve that was the media of the other two. This approximation should be considered satisfactory because the calibration curves of the adducts **11b** and **11c** were very similar between them.

2.5. NMR Spectroscopy

All NMR spectra were obtained using a Bruker Advance III spectrometer operating at 500 MHz for protons. For ^1H and ^{13}C NMR spectra, acquisition parameters were described previously (Zamora, Aguilar, Granvogl, & Hidalgo, 2016). All experiments were performed at 24 °C and COSY, HMQC and HMBC experiments were carried out to determine the chemical structures of the isolated compounds. Main HMBC couplings exhibited by compounds **9a** and **11b** are shown in Fig. S-2 (Supplementary data). HMBC couplings of compounds **9b** and **9c** were identical to those of compound **9a**. HMBC couplings of compounds **11a**, **11c**, and **11d** were identical to those of compound **11b**.

2.6. Statistical analysis

All data given are mean $\pm$ SD values of, at least, three independent experiments. Statistical comparisons among different groups were made using analysis of variance. When significant F values were obtained, group differences were evaluated by the Tukey test (Snedecor & Cochran, 1980). Statistical comparisons were carried out using Origin® v. 7.0 (OriginLab Corporation, Northampton, MA). The significance level is $p < 0.05$ unless otherwise indicated.

3. Results

3.1. Characterization of the adducts produced in the reaction between epoxyalkenals and phenolic compounds

The reaction between epoxyalkenals and phenolic compounds is very fast and produces a complex mixture of compounds (Hidalgo, Delgado, & Zamora, 2017). Different attempts were carried out to isolate the produced compounds, but all of them were unsuccessful because of the high reactivity of the formed adducts, which spontaneously suffered further reactions, including polymerizations. Only after their stabilization, they could be isolated and characterized. This stabilization was achieved by acetylation of the hydroxyl groups present in the formed molecules.

Two different kinds of adducts were isolated by column chromatography and characterized by 1D and 2D NMR and MS (Fig. 1). The first type of adducts were 1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromen-7-yl acetates (**9**). This cyclic form was the only observed in solution by NMR. However, because the hemiacetalic hydroxyl group was not acetylated, when studied by GC-MS, the cyclic hemiacetal was opened and the free hydroxyl group suffered a dehydration to produce the corresponding 4-(2-oxoethyl)-2*H*-chromen-7-yl acetates (**10**).

Compounds **9** had only one acetyl group and two phenolic protons (or one in the case of compound **9c**), indicating that one phenolic hydroxyl group and one of the phenolic protons of the original phenol were involved in the reaction with the epoxyalkenal. The epoxyalkenal chain could be easily identified in the adduct by means of a COSY experiment. The study of its chain showed that its epoxy, double bond and carbonyl groups were also involved in the reaction. The final characterization of their structures was carried out by using HMQC and HMBC experiments. In particular, HMBC showed long distance couplings, which were employed for the unequivocal identification of the formed structures.

Two isomers (**9a,9b**) were isolated in the reaction between 4,5-epoxy-2-heptenal and 2-methylresorcinol. This is consequence of the different chiral centers present in the

molecule (C4, C3a, and C9b). Two of them (C3a and C4) corresponded to the epoxy carbons of the epoxyalkenal (C4 and C5) and were already present in the aldehyde. Because the initial alkadienal (2,4-heptadienal) was *trans, trans* and the epoxidation was syn, the obtained epoxyalkenal should be a mixture of the isomer *4R,5R* and its enantiomer. Supposing that the formation of the adduct did not imply a racemization, the creation of the new chiral center at C9b would explain the existence of the two isomers **9a** and **9b**. In addition, C2 is also chiral, although it is not fixed and the interconversion between the two absolute configurations of this carbon might be produced. The ¹H NMR spectra of both isomers were quite similar. The most significant differences, as expected, corresponded to the protons belonging to chiral carbons C4, C3a, C9b, and C2. One difference was the chemical shift of protons H4 and H9b, which were interchanged in compounds **9a** and **9b** as confirmed by HMQC experiments. Other differences were the coupling constants, which were particularly different for H2. After dehydration, both compounds produced the same compound **10a**.

Differently to the reaction between 4,5-epoxy-2-heptenal and 2-methylresorcinol, in the reaction between 4,5-epoxy-2-decenal and 2,5-dimethylresorcinol only one isomer (**9c**) could be isolated and characterized. According to the chemical shifts of protons H4 and H9b, this compound was similar to compound **9b**. However, isomer **9c** differed from isomer **9b** in the coupling constants of H4, which were lower in compound **9c**. On the other hand, the mass spectrum of the dehydrated compound **10c** was similar to the mass spectra of compound **10a**.

The second type of adducts produced in these reactions were 3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diyl diacetates (**11**). Compounds **11** had two acetyl groups and two phenolic protons (or one in the case of compound **11d**), indicating that, at least, one of the phenolic protons of the original phenol was involved in the reaction

with the epoxyalkenal. The epoxyalkenal chain could also be easily identified in the adduct by means of a COSY experiment. The study of its chain showed that epoxy, double bond and carbonyl groups of the epoxyalkenal were also involved in the reaction. The final characterization of the structures was carried out by using HMQC and HMBC experiments.

Analogously to the above described for compounds **9a** and **9b**, the reaction between 4,5-epoxy-2-heptenal and 2-methylresorcinol produced several compounds **11**. In this case, four isomers were detected by GC-MS (Fig. S-2, Supplementary data), two of which (**11b** and **11c**) could be isolated and characterized. The existence of these 4 isomers is a consequence of the presence in the molecule of 4 chiral carbons: C1, C9a, C4a, and C3. Two of them (C1 and C9a) were already present in the initial epoxyalkenal, but C4a and C3 were created in the reaction with the phenol and C3 was fixed in the later acetylation.

The ¹H NMR spectra of stereoisomers **11b** and **11c** were similar between them and the main differences were again the chemical shifts and coupling constants of protons close to chiral carbons. Thus, for example, the acetyl group of C3 was different for the two isomers. On the other hand, the acetyl group of C7, which is quite far from the chiral centers was identical for the two isomers. These differences were also observed in the mass spectra. Thus, although the fragmentation pattern was identical for the two isomers, the intensities of the produced fragments were different.

When the reaction was carried out between 4,5-epoxy-2-hexenal and 2-methylresorcinol, only one isomer (**11a**) could be isolated and characterized. This isomer was similar to compound **11b** because it had similar coupling constants. Something comparable occurred with compound **11d**, which was isolated from the

reaction between 4,5-epoxy-2-heptenal and 2,5-dimethylresorcinol. In addition, all compounds **11** had very similar fragmentation patterns by MS.

Although only a limited number of compounds could be isolated and characterized in the studied reactions, no other compounds having different mass fragmentation patterns were formed in the studied reactions to a significant extent. Therefore, it can be concluded that the reaction between epoxyalkenals and phenolic compounds mainly produces in a first step 1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromene-2,7-diols (**7**) and 3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diols (**8**). These compounds have reactive hydroxyl and carbonyl groups that can be involved in further reactions, which is in agreement with the reactivity observed for the formed adducts.

3.2. Effect of reaction conditions on the formation of epoxyalkenal-phenol adducts in the reaction of 4,5-epoxy-2-heptenal and 2-methylresorcinol

The effect of reaction conditions on reaction between 4,5-epoxy-2-heptenal and 2-methylresorcinol was studied to understand how this reaction is produced. A total ion chromatogram of the acetylated compounds formed in the reaction is shown in Fig. S-2 (Supplementary data). This reaction produced 1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromene-2,7-diols (**7**) (which would produce compounds **9a** or **9b** after acetylation and compound **10a** after dehydration by GC-MS) and 3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diols (**8**) (which would produce compounds **11b**, **11c**, **11e**, **11f** after acetylation). Compounds **11e** and **11f** could not be isolated, but their mass spectra were very similar to those of compounds **11b** and **11c**, therefore confirming that they were isomers of them. The 1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromene-2,7-diol and the four 3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diols were the primary reaction products. However, they were unstable and spontaneously polymerized (the reaction developed brown color as a function of time,

data not shown). To avoid further reactions of these compounds, reactions were stopped by acetylation and studied by GC-MS.

Fig. 2 shows the effect of increasing amounts of 4,5-epoxy-2-heptenal on the disappearance of the phenolic compounds and the formation of epoxyalkenal-phenol adducts. The addition of increasing amounts of aldehyde produced the linear ($r = -0.996$, $p = 0.0003$) disappearance of the phenolic compound (Fig. 2A). The highest amount of epoxyalkenal tested (40 μmol) produced the disappearance of the 26% of the phenolic compound present ($7.89 \pm 1.38 \mu\text{mol}$ of the phenol disappeared). This disappearance was parallel to the appearance of the adducts **10a** and **11b**, **11c**, **11e**, **11f**. The amount of all of them increased linearly as a function of the amount of epoxyalkenal added and were inversely correlated ($r > 0.98$, $p < 0.003$) to the disappearance of the phenol.

An analogous behavior was observed when increasing amounts of phenol were added (Fig. 3). Thus, the addition of increasing amounts of phenol produced the linear increase ($r > 0.98$, $p < 0.002$) of the concentration of formed adducts **10a** and **11b**, **11c**, **11e**, **11f** (Fig. 3B), therefore confirming that formation of adducts required the participation of both epoxyalkenal and phenolic compound.

Phenol disappearance and adduct formation were also pH-dependent (Fig. 4). Adducts were not formed at $\text{pH} < 6$. However, the formation of some adducts (**10a**, **11b**, and **11c**) was already significant at $\text{pH} 7$, and the concentration of most of them increased at higher pH values. For most adducts, there were not significant differences between the amount formed at $\text{pH} 8$ in the presence of either phosphate or borate, therefore confirming that it was the pH and not the employed buffer the responsible for the formation of these compounds.

Phenol disappearance and adduct formation also depended on reaction times and temperatures (Fig. 5). In general, phenol disappearance and adduct formation increased with time and temperature, although there were differences among the different adducts. Thus, adduct **10a** was produced similarly at 60–100 °C but its concentration increased considerably at 120 °C (Fig. 5B). At this temperature, its maximum concentration was achieved after only 20 min heating.

The concentration of adduct **11e** exhibited a great dependence on the assayed temperature. Thus, the maximum concentration of this compound was obtained at low temperature because at temperatures higher than 80 °C it suffered a significant decomposition (Fig. 5C).

Adducts **11b** and **11c** (Figs. 5E and 5D, respectively), exhibited a similar behavior among them as a function of time and temperature. Thus, they were produced to a similar extent at 80 and 100 °C, but the amount at which they were produced decreased significantly ($p < 0.5$) at 60 °C and increased significantly ($p < 0.5$) at 120 °C.

Finally, the adduct **11f** was the most temperature-dependent. It was almost not produced at 60 °C and its concentration increased considerably when heating temperature increased. Thus, adducts **10a**, **11b**, and **11f** were the main products at 120 °C. On the other and, adduct **11f** was the adduct produced to a lower extent at 60 °C.

4. Discussion

Epoxyalkenals are minor lipid oxidation products, but they react rapidly with amino acids, aminophospholipids and proteins producing their rapid degradation at the same time that non-enzymatic browning is produced by means of carbonyl-amine reactions (Hidalgo & Zamora, 1993). However, previous studies have pointed out that they are

rapidly scavenged in the presence of certain phenolic compounds, which included single *m*-diphenols and flavan-3-ols (Hidalgo, Delgado, & Zamora, 2017).

The above described results confirm that the reaction between phenolic compounds and epoxyalkenals takes place very rapidly and involves the three functional groups of the epoxyalkenal, which is an essential requirement to protect the amino compounds from degradation. However, although this reaction preserves amino compounds from degradations, the formed adducts still have some reactive groups that can produce further reactions. Therefore, the stabilization of these first reaction products was required for their isolation and characterization. This characterization showed that the first reaction products between epoxyalkenals and phenolics were 1,3a,4,9b-tetrahydro-2*H*-furo[2,3-*c*]chromene-2,7-diols (**7**) and 3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diols (**8**).

Fig. 6 proposes a reaction pathway to explain the first steps of the reaction between phenols and epoxyalkenals and the formation of epoxyalkenal-phenol adducts. The reaction is initiated by the opening of the epoxy ring of the aldehyde (**2**) by one of the hydroxyl groups of the phenol (**1**). Two different compounds (**3** and **4**) are produced. Because of the disposition of the groups in the phenol, the aromatic carbon at the α -position of the hydroxyl group has enough nucleophilia to be added to the carbon-carbon double bond of compounds **3** and **4**. These compounds are α,β -unsaturated carbonyl compounds. Therefore, the addition of the aromatic carbon to the carbon-carbon double bond occurs at the β -carbon. The result is the formation of a six-membered ring in compound **5** when starting from compound **3**, and a five-membered ring in compound **6** when starting from compound **4**. Both adducts still have a carbonyl group and a hydroxyl group to an adequate distance to produce cyclic hemiacetals. Therefore, the predominant forms of these adducts are 1,3a,4,9b-tetrahydro-2*H*-

furo[2,3-*c*]chromene-2,7-diol (**7**) and 3,4,4a,9a-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diol (**8**), respectively. Upon acetylation under soft conditions, compound **7** only suffers the acetylation of the phenolic hydroxyl group to produce adduct **9**. However, the hydroxyl group of the six-membered ring of the hemiacetal **8** is acetylated at the same time that the phenolic hydroxyl group to produce adduct **11**. This explains the concordance between NMR spectra and GC-MS spectra for adducts **11**. On the other hand, when the acetylated compound **9** is submitted to GC-MS, the hemiacetal ring is opened and a dehydration is produced to form the corresponding 4-(2-oxoethyl)-2*H*-chromen-7-yl acetate (**10**). This last compound was that observed by GC-MS.

Although only two products are formed in the first steps of these reactions, the reaction mixtures are quite complex because of the existence of different isomers (Fig. S-2, Supplementary data). According to the reaction pathway proposed in Fig. 6, the initial epoxyalkenal **2** is a mixture of 4*R*,5*R* plus its enantiomer. These two carbons have different numberings depending on the produced compounds. Thus, they are 3a*R*,4*R* plus its enantiomer if the numbering of compound **7** is employed or 9a*R*,1*R* plus its enantiomer if the numbering of compound **8** is used. The opening of the epoxide ring in compounds **3** and **4** implies the inversion of the stereochemistry of the carbon that suffers the attack of the phenolic hydroxyl group. As a result, compound **3** should be a mixture of 3a*S*,4*R* plus its enantiomer and compound **4** should be a mixture of 9a*R*,1*S* plus its enantiomer. The addition of the phenolic carbon to the carbon-carbon double bond creates a new chiral center. This means that compound **5** should be a mixture of 3a*S*,4*R*,9b*R*, 3a*S*,4*R*,9b*S* and their corresponding enantiomers. Something similar should be happening in compound **6**, which should be a mixture of 1*S*,4a*R*,9a*R*, 1*S*,4a*S*,9a*R*, and their corresponding enantiomers.

Acetylation does not change the stereochemistry of compound **7**, in which the stereochemistry of C2 is not fixed, and the later dehydration implies the loss of two chiral centers (C3a and C9b), and the formation of a carbon-carbon double bond with a fixed stereochemistry because of the six-membered ring. For that reason, compound **10** has only one chiral center, and only one dehydrated compound is observed in the gas chromatogram of Fig. S-2 (Supplementary data).

On the other hand, acetylation of compound **8** fixes a new chiral center (C3). This means that compounds **11** should be a mixture of 1*S*,3*R*,4*aR*,9*aR*, 1*S*,3*S*,4*aR*,9*aR*, 1*S*,3*R*,4*aS*,9*aR*, 1*S*,3*S*,4*aS*,9*aR*, and their corresponding enantiomers. For that reason, the gas chromatogram of Fig. S-2 (Supplementary data) shows the existence of four peaks with the same mass spectra that correspond to compounds **11b**, **11c**, **11e**, and **11f**. Obviously, not all isomers have to be formed to the same extent, and those having less steric hindrance would be preferred. Thus, compound **11b** was the 3,4,4*a*,9*a*-tetrahydro-1*H*-pyrano[3,4-*b*]benzofuran-3,7-diol formed to a higher extent in the reaction between 4,5-epoxy-2-alkenal and 2-methylresorcinol at the different assayed temperatures (Fig. 5).

The presence of hydroxyl and carbonyl groups in compounds **5** and **6** also explain the reactivity of these molecules and, therefore, the difficulty for their study. Saturated carbonyl compounds have been shown to react with phenolic compounds (see, for example, Totlani & Peterson, 2005) and also with amino compounds (Kitryte, Adams, Venskutonis, & De Kimpe, 2012), but their reactivity is much reduced in comparison to that of epoxyalkenals (see, for example, Hidalgo, Delgado, Navarro, & Zamora, 2010). Therefore, phenolic compounds are able to reduce rapidly the high reactivity of epoxyalkenals, although the produced adducts still have functional groups having a potential reactivity with both phenolics and amino compounds.

Reaction pathway proposed in Fig. 6 is also in agreement with the effects of reaction conditions on adduct formation described previously. Thus, both epoxyalkenal (Fig. 2) and phenol (Fig. 3) take part in the reaction; the basic pH (Fig. 4) is needed for the addition of the aromatic carbon to the carbon-carbon double bond, as observed previously for 2-alkenals (Hidalgo & Zamora, 2014); and reaction times and temperatures are expected to favor the formation of adducts. However, many steps in Fig. 6 are reversible and the relative proportions among final products will change as a function of reaction conditions.

All obtained results contribute to a better understanding of the carbonyl-trapping ability of phenolic compounds in which both hydroxyl groups and phenolic carbons are involved. Thus, for saturated aldehydes, only the addition of phenolic aromatic carbons to the aldehydic carbonyl carbon has been described (Shao, Bai, He, Ho, Yang, & Sang, 2008). However, in the case of 2-alkenals, the reaction can be initiated by addition of either the phenolic hydroxyl group or the phenolic aromatic carbon to the carbon-carbon double bond of the aldehyde (Hidalgo & Zamora, 2014). Nevertheless, in the case of epoxyalkenals, the reaction is always initiated by the epoxide-ring opening produced by the phenolic hydroxyl group and then the later addition of the phenolic aromatic carbon to the carbon-carbon double bond of the initial aldehyde. These results suggest a versatility of phenolic compounds, which are able to react differently with the diverse carbonyl compounds as a function of their structure. This provides an additional explanation of the differences observed in the scavenging of carbonyl compounds by phenolic compounds, which depends on the structures of both the lipid-derived carbonyl compounds and the phenolic compounds involved (Hidalgo, Delgado, & Zamora, 2017).

Conflict of interest

497 The authors declare no conflicts of interest.

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503 **Appendix A. Supplementary data**

504 Supplementary data associated with this article can be found, in the online version, at

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567

Figure legends

Fig. 1. Compounds isolated and characterized in the reaction of epoxyalkenals and simple phenolics.

Fig. 2. Effect of epoxyalkenal concentration on: A, phenol disappearance; and B, formation of carbonyl-phenol adducts. Reactions were carried out by heating a mixture of 4,5-epoxy-2-heptenal and 2-methylresorcinol (30 μ mol, $\triangleright$) for 30 min at 100 °C in the presence of 0.3 mM sodium phosphate buffer. The carbonyl-phenol adducts produced were compounds **10a** ($\square$), **11b** (∇), **11c** ($\triangle$), **11e** ($\circ$), and **11f** ($\diamond$).

Fig. 3. Effect of phenol concentration on: A, remaining phenol; and B, formation of carbonyl-phenol adducts. Reactions were carried out heating a mixture of 4,5-epoxy-2-heptenal (40 μ mol) and 2-methylresorcinol ($\triangleright$) for 30 min at 100 °C in the presence of 0.3 mM sodium phosphate buffer. The carbonyl-phenol adducts produced were compounds **10a** ($\square$), **11b** (∇), **11c** ($\triangle$), **11e** ($\circ$), and **11f** ($\diamond$).

Fig. 4. Effect of reaction pH on: A, phenol disappearance; and B, formation of carbonyl-phenol adducts. Reactions were carried out heating a mixture of 4,5-epoxy-2-heptenal (40 μ mol) and 2-methylresorcinol (30 μ mol, $\triangleright$) for 30 min at 100 °C. The carbonyl-phenol adducts produced were compounds **10a** ($\square$), **11b** (∇), **11c** ($\triangle$), **11e** ($\circ$), and **11f** ($\diamond$). Two buffers were employed: 0.3 M sodium phosphate buffer (open symbols) and 0.3 M sodium borate buffer (closed symbols).

Fig. 5. Effect of reaction time and temperature on: A, phenol disappearance; B, formation of compound **10a**; C, formation of compound **11e**; D, formation of compound **11c**; E, formation of compound **11b**; and F, formation of compound **11f**. Reactions were carried out heating a mixture of 4,5-epoxy-2-heptenal (40 μ mol) and 2-methylresorcinol

(30 μmol) in 0.3 mM sodium phosphate buffer at the indicated times and temperatures.

The assayed temperatures were: 120 ($\square$), 100 ($\circ$), 80 ($\triangle$), and 60 $^{\circ}\text{C}$ (∇).

Fig. 6. Proposed reaction pathway for the reaction between epoxyalkenals and phenolic compounds. R and R' are H or alkyl groups. The assayed epoxyalkenals and phenolic compounds were: 2-methylresorcinol (**1a**, R = H), 2,5-dimethylresorcinol (**1b**, R = CH_3), 4,5-epoxy-2-hexenal (**2a**, R' = CH_3), 4,5-epoxy-2-heptenal (**2b**, R' = CH_2CH_3), and 4,5-epoxy-2-decenal (**2c**, R' = $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5