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Protective effect of phenolic compounds on carbonyl-amine reactions produced by lipid-derived reactive carbonyls

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ABSTRACT

The degradation of phenylalanine initiated by 2-pentenal, 2,4-heptadienal, 4-oxo-2-pentenal, 4,5-epoxy-2-heptenal, or 4,5-epoxy-2-decenal in the presence of phenolic compounds was studied to determine the structure-activity relationship of phenolic compounds on the protection of amino compounds against modifications produced by lipid-derived carbonyls. The obtained results showed that flavan-3-ols were the most efficient phenolic compounds followed by single *m*-diphenols. The effectiveness of these compounds was found to be related to their ability to trap rapidly the carbonyl compound, avoiding in this way the reaction of the carbonyl compound with the amino acid. The ability of flavan-3-ols for this reaction is suggested to be related to the high electronic density existing in some of the aromatic carbons of their ring A. This is the first report showing that carbonyl-phenol reactions involving lipid-derived reactive carbonyls can be produced more rapidly than carbonyl-amine reactions, therefore providing a satisfactory protection of amino compounds.

Keywords:

Carbonyl-amine reactions; carbonyl-phenol reactions; lipid oxidation; Maillard reaction; reactive carbonyls

Chemical compounds studied in this article:

Phenylalanine (PubChem ID: 6140); 2-pentenal (PubChem ID: 5364752); 2,4-heptadienal (PubChem ID: 5283321); 4,5-epoxy-2-heptenal (PubChem ID: 6444055); 4,5-epoxy-2-decenal (PubChem ID: 15825667); 4-oxo-2-hexenal (PubChem ID: 6365145); quercetin (PubChem ID: 5280343); morin (PubChem ID: 5281670); catechin (PubChem ID: 73160); epicatechin (PubChem ID: 72276).

1. Introduction

Lipid oxidation is responsible for the deterioration of polyunsaturated fatty acyl chains in food lipids and the later changes produced in flavor, texture, appearance, and nutritional quality of food products (Liu, Gao, McClements, & Decker, 2016; Lv, Lin, Li, Yuan, Gao, & Ma, 2016; Przybylski, Firdaous, Chataigne, Dhulster, & Nedjar, 2016). These changes are a consequence of both the formation of lipid oxidation products with undesirable properties, and the ability of some of these products to modify important macromolecules, including nucleic acids (Kozekov, Turesky, Alas, Harris, Harris, & Rizzo, 2010), aminophospholipids (Zamora & Hidalgo, 2003), amino acids (Hidalgo & Zamora, 2016), and proteins (Zamora & Hidalgo, 2005). Among them, amino acid modifications produced by oxidized lipids during food processing are a recognized source of both desirable beneficial compounds and compounds with deleterious properties, which can also be precursors of processing-related food toxicants such acrylamide (Hidalgo, Leon, & Zamora, 2016; Zamora & Hidalgo, 2008).

Lipid oxidation and its consequences have been traditionally controlled by the use of antioxidants, among which the use of phenolic compounds has received a considerable attention. Thus, these last compounds have been shown both to effectively scavenge free radicals and to chelate transition metals, and, consequently, to stop progressive autoxidative damage and the corresponding production of off-odors and off-flavors (Akhtar, Ismail, Fraternale, & Sestili, 2015; Balboa, Conde, Moure, Falque, & Dominguez, 2013). More recently, some authors have also pointed out to the ability of phenolic compounds to scavenge the carbonyl compounds produced during lipid oxidation, including alkanals, dialdehydes, alkenals, and 4-hydroxyl-2-alkenals, analogously to their better known ability to trap small reactive carbonyls such as glyoxal or methylglyoxal (Delgado, Hidalgo, & Zamora, 2016a; Hidalgo, & Zamora,

2014; Lo, Hsiao, & Chen, 2011; Zhu, Liang, Cheng, Peng, Lo, Shahidi, Chen, Ho, & Wang, 2009; Zhu, Zheng, Cheng, Wu, Zhang, Tang, Sze, Chen, Chen, & Wang, 2009). In fact, carbonyl–phenol adducts have been found to be produced in food products as a consequence of frying (Zamora, Aguilar, Granvogl, & Hidalgo, 2016). These last results have allowed to describe the protection offered by phenolic compounds against lipid oxidation as a triple defensive barrier by, successively, being able of chelating transition metals to inhibit the formation of the first radicals, scavenging lipid radicals to prevent the broadcasting of the initial damage, and, finally, trapping the produced lipid-derived reactive carbonyls to avoid the consequences of carbonyl–amine reactions (Zamora & Hidalgo, 2016).

Because the effectivity of this last defensive barrier is still poorly understood, this manuscript studies the stability of phenylalanine, as model amino acid, in the presence of both lipid-derived carbonyls and phenolic compounds in an attempt to compare the effectiveness of different kinds of phenolic compounds to protect amino acids against carbonyl-amine reactions produced by lipid-derived carbonyl compounds.

2. Materials and methods

2.1. Materials

As model lipid-derived carbonyl compounds, an alkenal (2-pentenal), an alkadienal (2,4-heptadienal), an oxoalkenal (4-oxo-2-hexenal), and two epoxyalkenals (4,5-epoxy-2-heptenal and 4,5-epoxy-2-decenal) were employed. 2-Pentanal and 2,4-decadienal were purchased from Sigma-Aldrich (St. Louis, MO), 4-oxo-2-hexenal was prepared from 2-ethylfuran by ring opening (Zamora, Alcon, & Hidalgo, 2013), and 4,5-epoxy-2-heptenal and 4,5-epoxy-2-decenal were synthetized from their corresponding alkadienals (2,4-heptadienal and 2,4-decadienal) by epoxidation with 3-chloroperoxybenzoic acid (Zamora, Gallardo, & Hidalgo, 2006).

As phenolic compounds a wide array of model compounds as well as flavonoids were employed. They are collected in Fig. 1. They included simple *m*-diphenols: resorcinol (**1**), 2-methylresorcinol (**2**), 2,5-dimethylresorcinol (**3**), and 2,6-dihydroxybenzoic acid (**4**); other simple phenols: 4-methylcatechol (**5**), hydroquinone (**6**), and sesamol (**7**); flavonols: quercetin (**8**), myricetin (**9**), and morin (**10**); flavan-3-ols: catechin (**11**) and epicatechin (**12**); and stilbenoids: resveratrol (**13**). All these compounds, as well as all other chemicals employed in these studies, were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany) and were of the highest available grade.

2.2. Disappearance of phenylalanine and the oxidized lipid, and formation of 3-phenyl-2-(1H-pyrrol-1-yl)propanoic acid in ternary mixtures of phenylalanine, oxidized lipids, and phenolic compounds

Mixtures of phenylalanine (10 μmol), oxidized lipid (20 μmol) and/or the phenolic compound (15 μmol) in 500 μL of 0.3 M sodium phosphate buffer pH 8 were heated at 100 °C for 2 h under nitrogen. After cooling (15 min at room temperature), 50 μL of internal standard solution (10 mg/mL of catechol in water) was added. The obtained reaction mixtures were then treated with methyl chloroformate to obtain the corresponding derivatives to be studied by gas chromatography coupled to mass spectrometry (GC-MS). Briefly, reaction mixtures were treated successively with 100 μL of methanol, 100 μL of 1 M sodium hydroxide, 35 μL of pyridine and 2 × 20 μL of methyl chloroformate (20 s of stirring after each addition). After 5 min, the derivatization reaction was stopped by addition of 0.1 M potassium bicarbonate (400 μL) with stirring and, then, extracted with chloroform (700 μL). The organic layer was separated by centrifugation (2000 × *g* for 7 min) and studied by GC-MS.

The reactions produced between phenylalanine (**14**) and 4,5-epoxy-2-heptenal (**20**), as a model lipid-derived reactive carbonyl assayed in most experiments, and the later derivatization of the compounds present in the reaction mixtures are shown in Fig. 2. The reaction between 4,5-epoxy-2-heptenal and amines and amino acids was described previously and it produces different pyrroles and polymers (Hidalgo & Zamora, 1993). The most stable of the produced compounds with phenylalanine is 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid (**15**).

The different compounds present in the reaction mixtures were derivatizated differently with methyl chloroformate (Fig. 2). Thus, phenylalanine (**14**) was converted into methyl (methoxycarbonyl)phenylalaninate (**16**), the reaction product 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid (**15**) was converted into methyl 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoate (**17**), and the internal standard catechol (**18**) was converted into the corresponding dimethoxycarbonyl derivative (**19**). On the contrary, 4,5-epoxy-2-heptenal (**20**) was not modified as a result of the derivatization reaction.

2.3. GC-MS analyses

GC-MS analyses were conducted with a Hewlett-Packard 6890 GC Plus coupled with an Agilent 5973 MSD (mass selective detector, quadrupole type). A fused-silica HP5-MS capillary column (30 m x 0.25 i.d.; coating thickness, 0.25 μm) was used, and 1 μL of sample was injected in the pulsed splitless mode. Working conditions were as follows: carrier gas, helium (1 mL/min at constant flow); injector, 250 °C; oven temperature programmed from 40 °C (1 min) to 240 °C at 5 °C/min and then to 300°C at 10°C/min; transfer line to MSD, 280 °C; ionization EI, 70 eV; ion source temperature, 230 °C; mass range 28-550 amu.

The ions monitored for the quantitation of the studied analytes were $[C_{10}H_{10}O_2]^+$ (*m/z* 162) for the phenylalanine derivative **16**, $[C_4H_4O]^+$ (*m/z* 68) for the 4,5-epoxy-2-

heptenal **20**, $[C_{14}H_{15}NO_2]^+$ (*m/z* 229) for the derivative **17** of the reaction product 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid (**15**), and $[C_{10}H_{10}O_6]^+$ (*m/z* 226) for the derivative **19** of the internal standard (catechol).

2.4. Determination of phenylalanine, 4,5-epoxy-2-heptenal, and 3-phenyl-2-(1H-pyrrol-1-yl)propanoic acid contents

Quantification of phenylalanine and, in some experiments, also of 4,5-epoxy-2-heptenal, was carried out by preparing standard curves of both compounds and following the derivatization procedure described above. Phenylalanine and 4,5-epoxy-2-heptenal contents were directly proportional to the analyzed compound/internal standard area ratio ($r > 0.999$, $p < 0.0001$). The coefficients of variation were < 10%.

The calibration curve of 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid was prepared in an indirect way because the corresponding standard was not available. Its quantification was carried out by heating at 100 ºC mixtures of phenylalanine methyl ester (20 μmol) and 4,5-epoxy-2-heptenal (40 μmol) in methanol-d_4 (1 mL) and trimethylamine (5 μL). Mixtures heated for different reaction times were studied simultaneously by GC-MS, as described above, and by ^{1}H NMR, as described below. The GC-MS analysis allowed the determination of the concentration of phenylalanine methyl ester and the ^{1}H NMR allowed to determine the ratio of the concentrations between methyl 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoate and phenylalanine methyl ester. With this ratio and the concentration of phenylalanine methyl ester, the concentration of methyl 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoate could be determined. A calibration curve for the area ratios of methyl 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoate/internal standard determined by GC-MS *versus* the corresponding concentrations of the pyrrole calculated from ^{1}H NMR data could be established.

The determination of the ratio between methyl 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoate and phenylalanine methyl ester by ^{1}H NMR could be carried out by integration of the signals corresponding to one of the methylene protons of phenylalanine methyl ester and the pyrrole protons of methyl 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoate which appeared clearly separated in the spectra of the reaction mixture. Thus, the methylene proton of phenylalanine methyl ester used for the integration appeared at δ 3.03 ppm (dd, 1H, J = 13.5 Hz, J = 6.2 Hz) and the pyrrolic protons of methyl 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoate appeared at δ 6.04 ppm (t, 2H, J = 2.1 Hz) and δ 6.73 ppm (t, 2H, J = 2.1 Hz), respectively. ^{1}H NMR spectra were obtained by a Bruker Advance III spectrometer operating at 500 MHz. Acquisition parameters were: spectral width 10000 Hz, relaxation delay 1s, number of scans 16, acquisition time 3.277 s, and pulse width 90°, with a total acquisition time of 1 min 17 s.

2.5. HPLC-HRMS

In order to further characterize the carbonyl-phenol adducts formed, reaction mixtures prepared in Section 2.2 were also fractionated on a Zorbax Eclipse XDB-C18 column (15 cm × 0.46 cm i. d., 5 μm) from Agilent (Santa Clara, CA) and studied on a micrOTOF-QII ultra high resolution time-of-flight (UHR-TOF) mass spectrometer with q-TOF geometry (Bruker Daltonics, Bremen, Germany) as described previously (Zamora, Aguilar, Granvogl, & Hidalgo, 2016).

2.6. Statistical analysis

All data given are mean ± 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. Effect of phenolic compounds on the disappearance of phenylalanine produced by lipid-derived reactive carbonyls

Although amino acids can be degraded upon thermal heating (Zamora, Alcon, & Hidalgo, 2013), amino acid disappearance in the presence of lipid oxidation products is increased considerably because of the carbonyl-amine reactions produced (Zamora & Hidalgo, 2005). However, not all amino acids are modified similarly in the presence of the different lipid oxidation products. The extent of amino acid disappearance depends, in addition to the structure of the involved amino acid, on both the lipid oxidation product responsible for the amino acid modification and the reactions conditions. Thus, under the reaction conditions employed in this study (2 h at 100 ºC under nitrogen and pH 8), only the most reactive lipid oxidation products increased significantly ($p < 0.05$) the disappearance of the amino acid (Table 1). These compounds were the epoxyalkenals 4,5-epoxy-2-heptenal, which decreased the amount of the recovered phenylalanine to about 14%, and 4,5-epoxy-2-decenal, which decreased the amount of the recovered phenylalanine to about 36%. The assayed 2-pentenal, 2,4-heptadienal, and 4-oxo-2-hexenal were not able to produce a disappearance of the amino acid significantly ($p < 0.05$) higher than the control.

When catechin and epicatechin were present as model phenolic compounds, amino acid disappearance was mostly avoided and only the combination of 4,5-epoxy-2-decenal and epicatechin produced a significant ($p < 0.05$) decrease in the phenylalanine recovered. Nevertheless, this decrease was much lower than that produced in the

absence of the phenolic compound. These results suggested that some phenolic compounds can protect amino acids from the modification produced by lipid oxidation products.

3.2. Effect of phenolic structure on the protection exhibited by phenolic compounds against the phenylalanine disappearance produced by 4,5-epoxy-2-heptenal

In order to understand the role of structure-activity relationship of phenolic compounds on their protective effect, the phenolic compounds collected in Fig. 1 were tested to determine their protective effect against the disappearance suffered by phenylalanine in the presence, or not, of 4,5-epoxy-2-heptenal. This lipid oxidation product was selected because it was the lipid oxidation product that produced the highest disappearance of phenylalanine (Table 1). The obtained results are shown in Table 2. Two different behaviors were observed depending on the presence or not of the lipid oxidation product.

When the lipid oxidation product was absent, some phenolic compounds were able to decrease significantly ($p < 0.05$) the amount of phenylalanine recovered, and only one of them (2-methylresorcinol) increased it. Thus, the phenolic compounds that decreased ($p < 0.05$) the amount of phenylalanine recovered were 4-methylcatechol, hydroquinone, and myricetin. These compounds have the common characteristic of having two phenol groups in *ortho* or *para* positions, and they have been shown to be able to degrade amino acids because of their behavior as reactive carbonyls (Delgado, Zamora & Hidalgo, 2016b).

When the lipid oxidation product was present, some phenolic compounds were able to protect phenylalanine and others not. The compounds that protected phenylalanine were epicatechin, catechin, 2-methylresorcinol, resorcinol, 2,5-dimethylresorcinol, morin, resveratrol, and myricetin. On the other hand, 2,6-dihydroxybenzoic acid, 4-

methylcatechol, hydroquinone, sesamol, and quercetin did not show any significant ($p < 0.05$) protective effect.

3.3. Effect of the concentration of the phenolic compounds on the protection exhibited by these compounds against the phenylalanine disappearance produced by 4,5-epoxy-2-heptenal

The protection offered by phenolic compounds against the amino acid disappearance produced by lipid oxidation products depended on the concentration of the phenolic compounds. Thus, for those phenolic compounds that protected the amino acid, the amount of amino acid recovered increased linearly ($r > 0.99$, $p < 0.02$) as a function of the concentration of the phenolic compound in the range 0–15 μmol. Fig. 3 shows that the protective effect of phenolic compounds was observed even at low concentrations of the phenolic compound for the most active phenols. Analogously to that shown in Table 2, the most protective compounds were the flavan-3-ols followed by simple *m*-diphenols. Lower protective effects were exhibited by some flavonols and the stilbenoid analyzed. On the other hand, the protective effect of 2,6-dihydroxybenzoic acid and sesamol was negligible.

3.4. Effect of reaction time on the protection exhibited by phenolic compounds against the phenylalanine disappearance produced by 4,5-epoxy-2-heptenal

In order to understand how the protection of phenylalanine is produced, the disappearance of both phenylalanine (**14**) and 4,5-epoxy-2-heptenal (**20**), and the formation of the adduct 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid (**15**) was studied as a function of reaction time (Fig. 4).

In the absence of phenolic compounds, the disappearance of the amino acid and the aldehyde were parallel and there was a correlation among them ($r = 0.969$, $p = 0.0003$).

This disappearance was also simultaneous to the formation of the oxidized lipid-amino acid adduct 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid. A behavior similar to the control was also observed for quercetin, therefore confirming the null protection of this flavonol. Contrarily to quercetin, the flavan-3-ols catechin and epicatechin completely protected phenylalanine during the studied time. This protection seemed to be a consequence of the ability of these phenols to sequester the 4,5-epoxy-2-heptenal. As observed in Fig. 4B, sequestering of the aldehyde by flavan-3-ols was almost instantaneous and most of the aldehyde had disappeared at $t = 0$ min. This sequestering also avoided the formation of the adduct 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid (Fig. 4C). Other phenols exhibited an intermediate behavior: a slower disappearance of the aldehyde at $t = 0$ min and the appearance of only small amounts of the carbonyl-amine adduct as a function of reaction time. In fact, there was an inverse correlation ($r = -0.91$, $p = 0.0049$) between the amount of 4,5-epoxy-2-heptenal recovered at $t = 0$ min and the amount of phenylalanine recovered at the end of the incubation time. Also, a correlation ($r = 0.95$, $p = 0.0012$) between the amount of 4,5-epoxy-2-heptenal recovered at $t = 0$ min and the amount of 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid produced at the end of the incubation time was observed.

3.5. Formation of carbonyl-phenol adducts

To confirm that the rapid disappearance of 4,5-epoxy-2-heptenal was a consequence of the formation of the corresponding carbonyl-phenol adducts, the reactions between phenylalanine, 4,5-epoxy-2-heptenal, and resorcinol or 2-methylresorcinol were derivatizated with methyl chloroformate and studied by GC-MS to identify the corresponding adducts. These phenolic compounds were selected because they have a small molecular weight and, therefore, the molecular weight of the corresponding adduct should be low enough to be studied by GC-MS. As shown in Figs. S-1 and S-2

279 (Supplementary Material), peaks having the expected molecular weights were identified
280 and tentatively assigned to carbonyl-phenol adducts. The exact molecular masses of
281 these adducts, and therefore, their molecular formulas could be confirmed by HPLC-
282 HRMS, as described below.

283 Both reactions had very similar gas chromatograms, with 6 compounds that
284 corresponded to carbonyl-phenol adducts. The reaction with resorcinol produced 1
285 adduct with a molecular weight of 276 Da (compound **A**) and 5 adducts with a
286 molecular weight of 294 Da (compounds **B-F**; Fig. S-1, Supplementary Material).
287 Compound **A** is likely produced by reaction of 1 molecule of the aldehyde (molecular
288 weight 126 Da) and 1 molecule of resorcinol (molecular weight 110 Da). The formation
289 of this adduct should suffer then the loss of 1 molecule of water and 1 hydroxyl group
290 was derivatizated with methyl chloroformate (126 + 110 – 18 + 58 = 276). The other 5
291 adducts (compounds **B-F**) were produced by reaction of 1 molecule of the aldehyde
292 (molecular weight 126 Da) and 1 molecule of resorcinol (molecular weight 110 Da),
293 and later derivatization of 1 hydroxyl group with methyl chloroformate (126 + 110 + 58
294 = 294). Among compounds **B-F**, adduct **B** had a mass spectrum that was different to the
295 mass spectra of adducts **C-F**. These last compounds had a similar fragmentation pattern.
296 Among all compounds produced, one of them (compound **D**) seemed to be the main
297 reaction product.

298 The exact molecular masses ($M^+ - 1$) of adducts **B-F** were in agreement with the
299 molecular formula of $C_{15}H_{17}O_6$ (error < 3.5 ppm). This formula confirms the
300 participation in these adducts of 1 molecule of phenol, 1 molecule of aldehyde and the
301 derivatization of only 1 hydroxyl group with methyl chloroformate. On the other hand,
302 adduct **A** could not be unambiguously detected by HPLC-HRMS.

303 The reaction with 2-methylresorcinol was very similar (Fig. S-2, Supplementary
304 Material). It produced 1 adduct (compound **G**) with a molecular weight of 290 Da and 5
305 adducts (compounds **H**-**L**) with a molecular weight of 308 Da. Compound **G** is likely
306 produced by reaction of 1 molecule of the aldehyde (molecular weight 126 Da) and 1
307 molecule of 2-methylresorcinol (molecular weight 124 Da). This is a dehydrated adduct
308 with 1 free hydroxyl group that was derivatizated with methyl chloroformate (126 + 124
309 – 18 + 58 = 290). Compounds **H**-**L** were produced by reaction of 1 molecule of the
310 aldehyde (molecular weight 126 Da) and 1 molecule of 2-methylresorcinol (molecular
311 weight 124 Da), and later derivatization of 1 hydroxyl group with methyl chloroformate
312 (126 + 110 + 58 = 294). Analogously to the observed for the reaction with resorcinol,
313 adduct **H** had a mass spectra that was different to the mass spectra of adducts **I**-**L**. These
314 last compounds had a similar fragmentation pattern. Among the different compounds
315 produced, one of them (compound **J**) seemed to be the main reaction product. There
316 was an evident parallelism between both reactions, including the retention times of the
317 corresponding produced adducts and their mass spectra. Thus, structure of adduct **A**
318 should be similar to **G**, structure of adduct **B** to **F**, and so on. The only difference should
319 be the presence of a methyl group in the aromatic ring of the initial phenolic compound.

320 As described above for the reaction involving resorcinol, the exact molecular masses
321 ($M^+ - 1$) of adducts **H**-**L** could be determined and corresponded to the molecular
322 formula $C_{16}H_{19}O_6$ (error < 5 ppm). These results also confirmed the participation in
323 these adducts of 1 molecule of phenol, 1 molecule of aldehyde and the derivatization of
324 only 1 hydroxyl group with methyl chloroformate. Analogously to the above described
325 for adduct **A**, adduct **G** could not be unambiguously detected by HPLC-HRMS.

326 **4. Discussion**

In the presence of lipid oxidation products, amino compounds are rapidly modified to produce a wide range of carbonyl-amine adducts and amine degradation products with mostly deleterious properties in foods (Hidalgo & Zamora, 2016; Zamora & Hidalgo, 2005). To avoid these reactions, the use of antioxidants, including phenolics, has been a constant for food industries during decades. However, at present, only a limited number of phenolics are commercially viable because their prooxidant properties often outweigh their antioxidant properties. This is mostly a consequence of a lack of understanding of the different mechanisms involved in the protective action of phenolics. The results obtained in this study provide new data that can help to better understand their protective action against amino acid damage in the presence of lipid-derived reactive carbonyls. The obtained results confirm that phenols are a really complex mixture of compounds with different functional groups that play different functions and the best way to predict their prooxidant/antioxidant effect is by their classification into different groups according to the number(s) and kind(s) of the function(s) they have (Zamora & Hidalgo, 2016).

The obtained results suggest that the protection of phenolics against the amino acid damage produced by lipid oxidation products is directly related to their ability to sequester the oxidized lipid: the faster they remove the aldehyde, the lower damage is produced in the amino acid. As shown in Fig. 4, when the lipid oxidation product was rapidly removed, it could not react with the amino acid. However, this ability was not randomly observed. In fact, it depended on the phenol structure.

In the studied damage of amino acids produced by epoxyalkenals, the phenols that exhibited the highest protective effect were flavan-3-ols followed by single *m*-diphenols, with the exception of 2,6-dihydroxybenzoic acid. Much lower protective effect was exhibited by the stilbenoid resveratrol and the flavonols morin and myricetin.

352 On the other hand, the simple *o*- or *p*-diphenols assayed as well as sesamol and
353 quercetin, did not exhibited any protection. These results indicated that most of the
354 different phenolic compounds included in the same group had similar protective
355 functions. Only two exceptions were observed to this general rule among the phenolic
356 compounds tested. These were the single *m*-diphenol 2,6-dihydroxybenzoic acid and the
357 flavonol quercetin.

358 The different behavior of quercetin among flavonols was not very relevant because it
359 protected similarly to myricetin, although lower than morin (Table 2). However, the
360 very different protection of 2,6-dihydroxybenzoic acid in relation to that of resorcinol,
361 2-methylresorcinol, and 2,5-dimethylresorcinol, should be related to the presence of the
362 carboxylic group. The protective effect of phenolic compounds against lipid-derived
363 carbonyl compounds is linked to their ability to react with these compounds, and this
364 reaction only occurs when the phenolic compounds have a high electronic density in
365 some of their phenolic carbons, such occurs in *m*-diphenols (Hidalgo & Zamora, 2014;
366 Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014). For that reason resorcinol (**1**), 2-
367 methylresorcinol (**2**), and 2,5-dimethylresorcinol (**3**), but not in 4-methylcatechol (**5**)
368 and hydroquinone (**6**) exhibited this protective effect. The electronic delocalization that
369 favors a high electronic density in *m*-diphenols is shown in Fig. S-3 (Supplementary
370 Material). Although 2,6-dihydroxybenzoic acid has also two hydroxyl groups in *m*-
371 positions, the carboxylic acid blocks the electronic delocalization by forming a strong
372 hydrogen bond (Fig. S-3, Supplementary Material). This is the reason for the strong
373 acidity of this acid which is 800 times more acid than benzoic acid (Ferguson, 1975).

374 Electronic delocalization also explains the behavior within one class of phenolic
375 compounds. The clearest example can be found in the flavonols' group. Morin (**10**) was
376 the flavonol that protected most to phenylalanine. This is consequence of the existence

of *m*-diphenols in rings A and B. On the contrary, quercetin (**8**) only has one *m*-diphenol in ring A and one *o*-diphenol in ring B. Myricetin (**9**) is an intermediate example, it has one *m*-diphenol in ring A and a 1,2,3-triphenol (two *o*-diphenols and one *m*-diphenol) in ring B. For that reason, the protections exhibited were morin $\geq$ myricetin $\geq$ quercetin.

The comparison among phenol classes is more complex, but the different reactivity can also be understood on the basis of both electronic delocalizations and hydrogen bonds, such as that existing between the carbonyl at C4 and the hydroxyl at C5 in flavonols. Analogously to the above described for 2,6-dihydroxybenzoic acid, the existence of this hydrogen bond should decrease electronic delocalization and, therefore, the ability of flavonols to react with epoxyalkenals as compared to that of flavan-3-ols. In addition, flavan-3-ols are more efficient than simple phenols because of a positive effect of ring C in increasing the electronic density in some of the phenolic carbons of ring A.

The obtained results also allow to understand the difficult equilibrium between prooxidant and antioxidant properties of phenolics. These properties can be interpreted on the basis of the relative positions of hydroxyl groups, which determine both electronic densities and the possibility of the phenol to be converted into a quinone. Thus, the existence of an *o*- or *p*-diphenol moiety promotes degradative properties because of the easiness of their conversion into *o*- or *p*-quinones which are highly reactive carbonyl compounds for amino acid disappearance (Delgado, Hidalgo, & Zamora, 2016b). On the other hand, the existence of a *m*-diphenol moiety promotes a protective effect because of their ability of scavenging carbonyl compounds. Both functions can be easily separated in single phenolics, but complex phenolics usually have both kinds of moieties and the fact that one function can outweigh the other function will not only depend on the structure of the phenolic but also on the lipid

oxidation involved, the reaction conditions, and the presence of other groups in the phenol structure. In the reaction analyzed in the present study only myricetin exhibited degradative effects among the complex phenolics analyzed when the oxidized lipid was absent, and, curiously, it also exhibited protective effects, when the oxidized lipid was present (Table 2). Degradative and protective abilities are likely to be a consequence of the existence of the pyrogallol ring with hydroxyl groups in both *o*- and *m*-positions. This potential double action of myricetin has been known for many years (Silva, Gaspar, Rodrigues, daCosta, Laires, & Rueff, 1996).

The obtained results do not allow to propose structures for the produced adducts. However, the obtained mass spectra suggest the formation of a stable core between the phenol and the aldehyde that is dehydrated in a first step (which implies the existence of a hydroxyl group that is not derivatizated with methyl chloroformate) and then suffers the loss of an ethyl group. These data point out to the participation of the three groups of the epoxyalkenal (epoxide, double bond, and carbonyl) in its reaction with the phenol. One hydroxyl group of the phenol seems to be also involved in this reaction.

All these results suggest that phenolic compounds can be employed to control the damage caused by oxidized lipids in amino acids. Not all phenolic compounds have an analogous protective ability because of differences in their structures. The reason seems to be related to the ability of the phenolic compounds to trap the carbonyl compound, an ability which is related to the existence of phenol carbons with a high electronic density. Among the different phenolic compounds assayed in this study, the obtained results point out to flavan-3-ols as the most efficient phenolics that avoid the damage caused by epoxyalkenals in amino acids. Although the structures of the corresponding epoxyalkenal/phenol adducts produced remains to be elucidated, this is the first report showing that carbonyl-phenol reactions involving lipid-derived reactive carbonyls

commonly produced in the course of lipid oxidation can be produced more rapidly than carbonyl-amine reactions, therefore providing a satisfactory protection of amino compounds.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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

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Figure legends

Fig. 1. Chemical structures of the phenolic compounds employed in the study.

Fig. 2. Reaction of phenylalanine (**14**) with 4,5-epoxy-2-heptenal (**20**) to produce 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid (**15**), and later derivatization of reactants and products with methyl chloroformate. Catechol (**18**), added as internal standard, is also derivatizated with methyl chloroformate to produce compound **19**.

Fig. 3. Effect of phenol concentration on phenylalanine disappearance in ternary mixtures of phenylalanine, 4,5-epoxy-2-heptenal, and a phenolic compound. The phenolic compounds assayed were: resorcinol (○), 2-methylresorcinol (△), 2,5-dimethylresorcinol (▲), 2,6-dihydroxybenzoic acid (●), sesamol (■), quercetin (▽), myricetin (▼), morin (◇), catechin (◁), epicatechin (▷), and resveratrol (◆).

Fig. 4. Time course of: (A) phenylalanine (Phe) recovered; (B) 4,5-epoxy-2-heptenal (EH) recovered; and (C) 3-phenyl-2-(1*H*-pyrrol-1-yl)propanoic acid (pyrrole) produced, in ternary mixtures of phenylalanine, 4,5-epoxy-2-heptenal, and a phenolic compound. The phenolic compounds assayed were: resorcinol (○), 2-methylresorcinol (△), quercetin (▽), morin (◇), catechin (◁), and epicatechin (▷). The results obtained in the absence of phenolic compound are also shown (□).

Table 1

Phenylalanine recovered after heating in the presence of lipid-derived reactive carbonyls and either catechin or epicatechin

Lipid derivative	Phenolic compound		
	None	Catechin	Epicatechin
None	81.40 ± 10.05 a	76.90 ± 8.10 a,b,c	81.17 ± 8.52 a
2-Pentenal	86.70 ± 5.87 a	87.67 ± 3.46 a,c	88.57 ± 8.54 a
2,4-Heptadienal	89.47 ± 4.46 a	90.87 ± 4.22 a	85.20 ± 5.38 a
4-Oxo-2-hexenal	79.83 ± 6.43 a	73.87 ± 4.88 c,d	75.90 ± 10.04 a,b
4,5-Epoxy-2-heptenal	14.02 ± 2.85 b	64.90 ± 7.45 b,d	69.40 ± 6.63 a,b
4,5-Epoxy-2-decenal	36.20 ± 3.78 c	63.17 ± 7.16 b,d	60.20 ± 1.87 b

Values correspond to the phenylalanine (in %) recovered at the end of the incubation time (2 h at 100 °C) and are mean ± SD for, at least, three independent experiments. Means in the same column with a different letter are significantly different ($p < 0.05$).

Table 2

Phenylalanine recovered after heating in the presence of 4,5-epoxy-2-heptenal and phenolic compounds

Phenolic compound	Lipid-derived reactive carbonyl	
	None	4,5-Epoxy-2-heptenal
None	81.40 ± 10.05 a,b	14.02 ± 2.85 a
Resorcinol	102.17 ± 6.21 a,c,d	51.17 ± 5.05 b
2-Methylresorcinol	104.90 ± 12.70 c,d	54.57 ± 4.66 b,c
2,5-Dimethylresorcinol	88.83 ± 9.42 a,d,e	46.57 ± 5.08 b
2,6-Dihydroxybenzoic acid	100.87 ± 8.33 a,d	10.00 ± 0.10 a
4-Methylcatechol	46.43 ± 4.92 f,g	9.40 ± 0.46 a
Hydroquinone	42.10 ± 1.57 f,g	9.17 ± 0.06 a
Sesamol	90.03 ± 6.44 a,d,h	15.07 ± 0.50 a,d
Quercetin	67.50 ± 9.11 b,e,g,h	19.00 ± 2.00 a,e
Myricetin	59.60 ± 3.22 f,g,i	26.37 ± 5.06 d,e,f
Morin	79.10 ± 11.31 a,h,i	32.10 ± 5.05 f,g
Catechin	76.90 ± 8.10 a,h,i	64.90 ± 7.45 c,h
Epicatechin	81.17 ± 8.52 a,d,h,i	69.40 ± 6.63 h
Resveratrol	80.80 ± 9.40 a,d,h,i	29.80 ± 4.18 e,g

Values correspond to the phenylalanine (in %) recovered at the end of the incubation time (2 h at 100 °C) and are mean ± SD for, at least, three independent experiments. Means in the same column with a different letter are significantly different ($p < 0.05$).

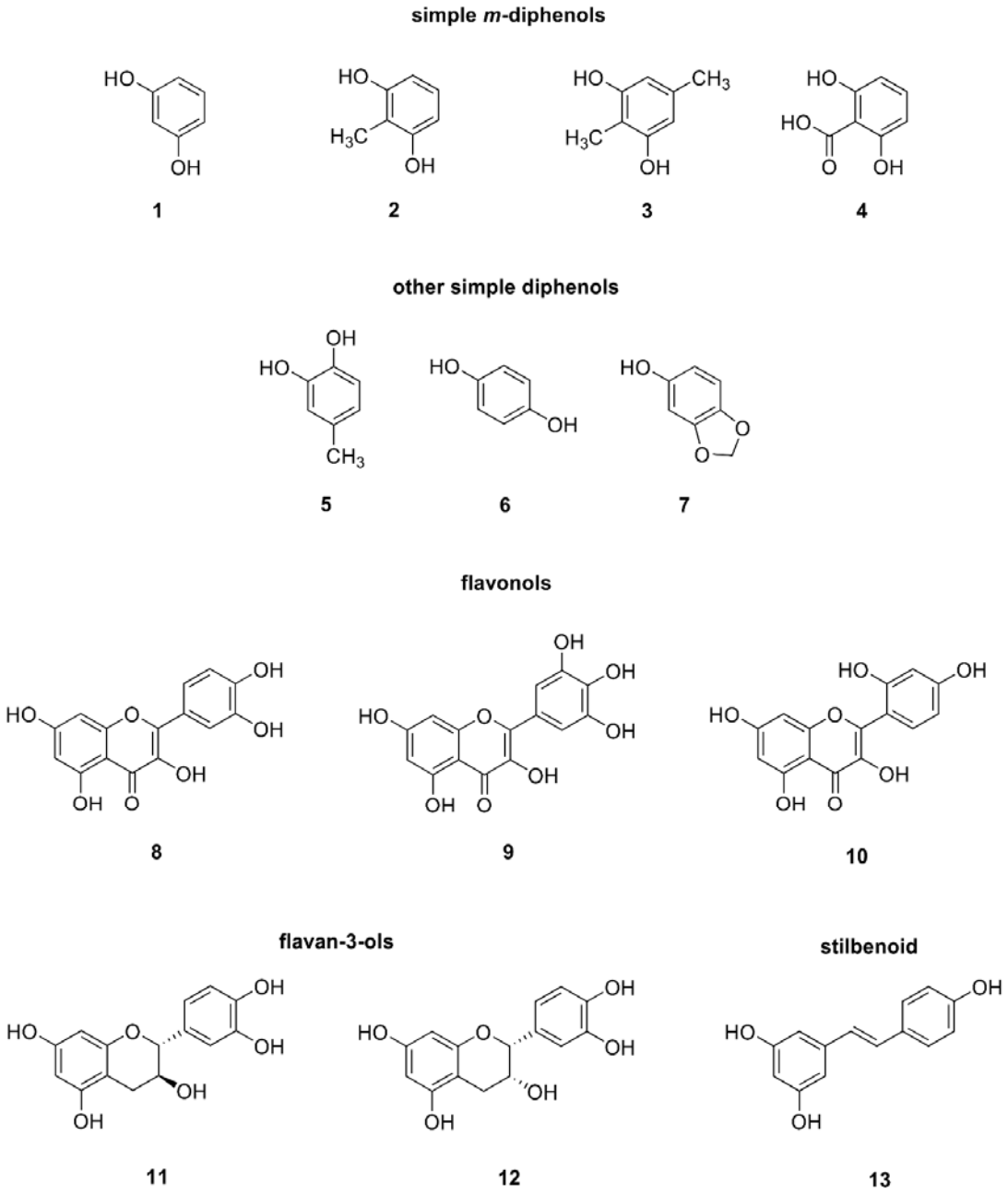
simple *m*-diphenols
1
2
3
4
other simple diphenols
5
6
7
flavonols
8
9
10
flavan-3-ols
11
12
stilbenoid
13

Figure 1

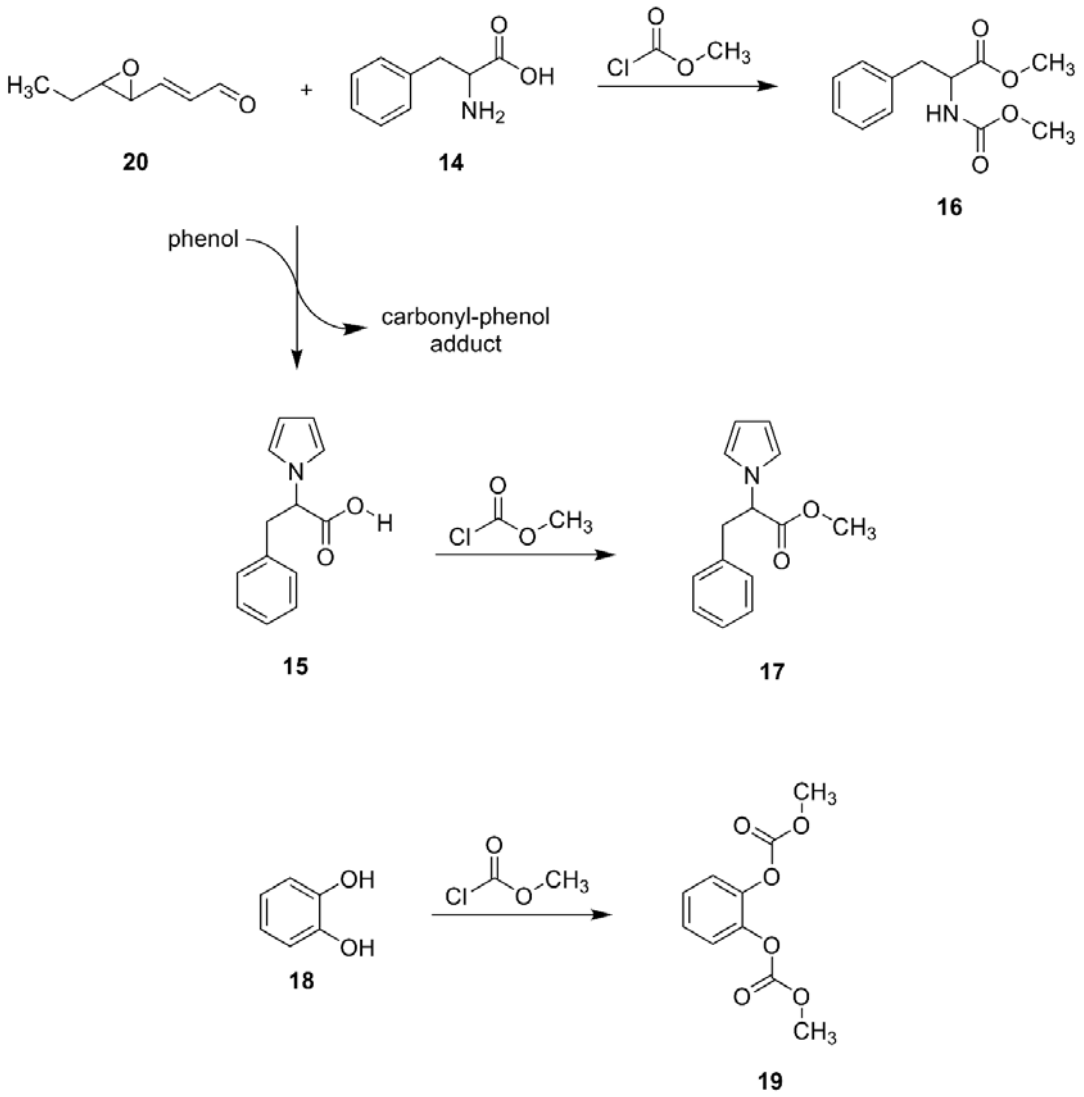

Figure 2

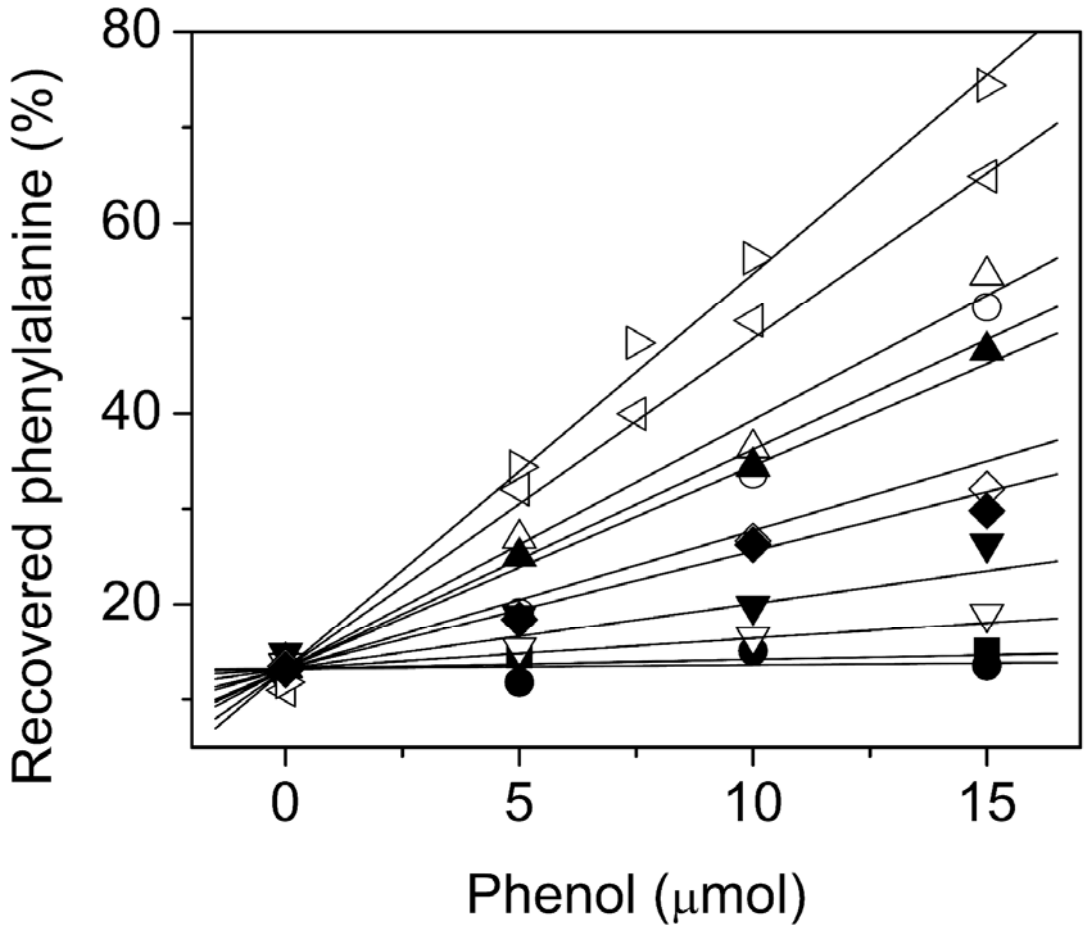

Figure 3

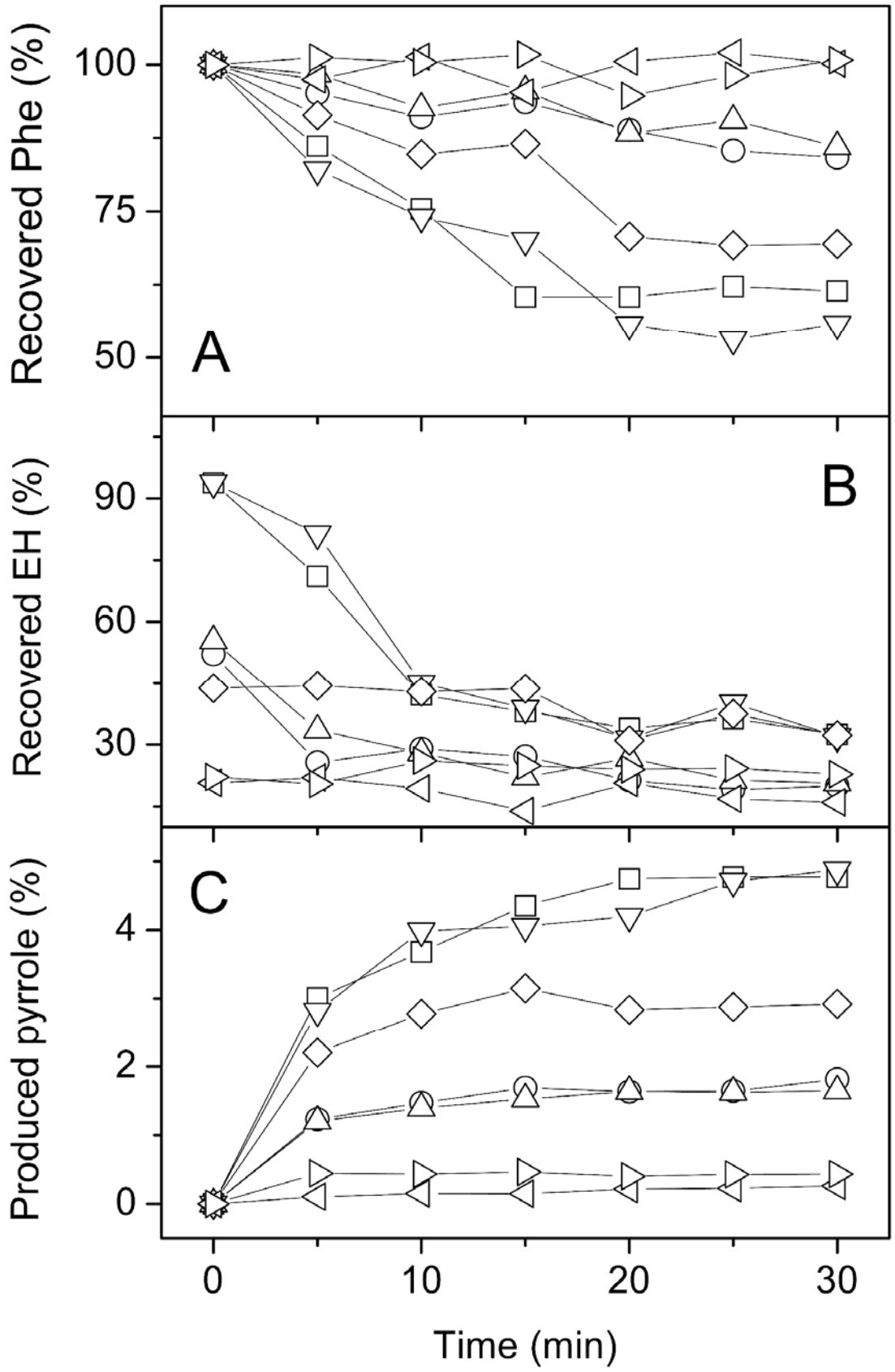
Recovered Phe (%)
100
75
50
A
Recovered EH (%)
90
60
30
B
Produced pyrrole (%)
4
2
0
C
0
10
20
30
Time (min)

Figure 4