Structural characteristics that determine the inhibitory role of phenolic compounds on 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) formation

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ABSTRACT

In an attempt to understand the structural characteristics of phenolic compounds that favor the inhibition of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) formation, this study analyzes the role of twenty-five phenolic compounds on the PhIP produced in phenylalanine/creatinine/oxidized lipid reaction mixtures. The obtained results showed that phenols having two hydroxy groups at *meta* positions of the aromatic ring were the most efficient inhibitors. The presence of alkyl or carboxylic groups as additional substituents in the aromatic ring slightly reduced the inhibitory effect. On the other hand, the introduction of additional hydroxy and amino groups mostly cancelled the inhibitory effect, which was also mostly absent in *ortho* and *para* dihydroxy derivatives. In complex phenols, the presence of several rings with opposite effects produced a reduced inhibitory effect. All these results suggest that it is possible to predict if a phenolic derivative will inhibit the formation of PhIP, or not, based on its structure.

Keywords: Catechin; carbonyl-amine reactions; epicatechin; heterocyclic aromatic amines; Maillard reaction; PhIP; quercetin; resorcinol; resveratrol

1. Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is one of the most abundant heterocyclic aromatic amines (HAAs) formed during thermal processing of proteinaceous foods such as cooked beef, pork, chicken, and fish (Skog, Johansson, & Jägerstad, 1998). This HAA produces colon, prostate, and mammary gland tumors in rodents (Alaejos, Pino, & Afonso, 2008; Cheung, Loy, Li, Liu, & Yang, 2011; Choudhary, Sood, Donnell, & Wang, 2012), and the International Agency for Research on Cancer (IARC) has concluded that there is sufficient evidence in experimental animals for PhIP carcinogenicity (IARC, 1993). In addition, this compound is also considered as possibly carcinogenic to humans (IARC, 1993).

At present, PhIP is believed to be mainly produced from phenylalanine, creati(ni)ne and carbohydrates as a by-product of the Maillard Reaction (Shioya, Wakabayashi, Sato, Nagao, & Sugimura, 1987). The reaction takes place in several steps, among which the formation of phenylacetaldehyde by phenylalanine degradation and the later reaction of the produced phenylacetaldehyde with creati(ni)ne seem to be key steps (Murkovic, Weber, Geiszler, Fröhlich, & Pfannhauser, 1999). Because conversion of phenylalanine into phenylacetaldehyde is not only produced by carbohydrates, but also by other reactive carbonyls such as those formed in the course of lipid oxidation (Hidalgo & Zamora, 2004), recent studies have shown that oxidized lipids are also able to contribute to PhIP formation (Zamora, Alcon, & Hidalgo, 2012; 2013a). Furthermore, the carbonyl compounds produced by thermal decomposition of some amino acids also contribute to PhIP formation (Zamora, Alcon, & Hidalgo, 2013b).

Inhibition of PhIP formation has been the objective of numerous studies, and many authors have shown that the use of phenolic compounds and plants extracts rich in them decreases the PhIP formed (see, for example, Damasius, Venskutonis, Ferracane, &

58 Fogliano, 2011; Gibis, & Weiss, 2012; Janoszka, 2010; Murkovic, Steinberger, &
59 Pfannhauser, 1998; Quelhas, Petisca, Viegas, Melo, Pinho, & Ferreira, 2010). However,
60 the inhibition of PhIP formation has not been well correlated to the antioxidant/free
61 radical scavenging capacity of phenolic compounds and spice extracts (Cheng, Chen, &
62 Wang, 2007; Damasius, Venskutonis, Ferracane, & Fogliano, 2011), which has
63 suggested that an antioxidant-independent mechanism should be playing a role in the
64 inhibition of PhIP by phenolic compounds.

65 In an attempt to understand the structural characteristics of phenolic compounds that
66 favor the inhibition of PhIP formation, this study analyzes the role of twenty-five
67 phenolic compounds on the PhIP produced in phenylalanine/creatinine/oxidized lipid
68 reaction mixtures. This model system was selected because it is an efficient PhIP
69 producer (Zamora, Alcon, & Hidalgo, 2012; 2013a).

70 **2. Materials and methods**

71 *2.1. Materials*

72 Twenty-five phenolic compounds were employed in this study. They are collected in
73 Fig. 1. As can be observed, most of them were simple compounds having two or three
74 hydroxy groups at different positions of the benzene ring. In addition, alkyl, methoxy,
75 amino, and carboxylic groups were also present in some derivatives. Furthermore, some
76 complex phenolic compounds having more than one aromatic ring with hydroxy groups
77 were also studied for comparison purposes. To facilitate the study of the assayed
78 phenols, these compounds have been classified into five groups: *o*-dihydroxy
79 derivatives and analogs, *m*-dihydroxy derivatives and analogs, *p*-dihydroxy derivatives
80 and analogs, trihydroxy derivatives, and complex phenols. All these compounds were
81 purchased from commercial sources (see below).

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). 4-Oxo-2-nonenal was prepared from 2-pentylfuran according to Shimozu, Shibata, Ojika, & Uchida (2009). All other chemicals were purchased from Aldrich (Milwakee, WI, USA), Sigma (St. Louis, MO, USA), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany), and were analytical grade.

2.2. PhIP formation in phenylalanine/creatinine/oxidized lipid/phenolic compound reaction mixtures

A mixture of creatinine (10 μmol), phenylalanine (10 μmol), and the lipid-derived reactive carbonyl (10 μmol) in 500 μL of 0.3 M sodium phosphate, pH 8, was treated, or not (control), with the phenolic compound (0–10 μmol), and heated at 200 ˚C in closed test tubes for 1 h. Three different lipid-derived reactive carbonyls were employed to investigate the effect of the type of lipid on the mitigation of PhIP formation produced by phenolic compounds. These oxidized lipids were: 2-octenal, 2,4-decadienal, and 4-oxo-2-nonenal.

After cooling (20 min at room temperature), 100 μL of the reaction mixture was diluted with 50 μL of internal standard (IS) solution (1.29 mg of caffeine in 5 mL of a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate), and 850 μL of a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate. PhIP content was determined by LC–MS/MS.

2.3. PhIP determination by LC–MS/MS

Samples were analyzed using an Agilent liquid chromatography system (1200 Series) consisting of binary pump (G1312A), degasser (G1379B), and autosampler (G1329A), connected to a triple quadrupole API 2000 mass spectrometer (Applied

106 Biosystems) using an electrospray ionization interface in positive ionization mode
107 (ESI^+). Compounds were separated on a Zorbax Eclipse XDB-C18 (150 mm x 4.6 mm,
108 5 μm) column from Agilent. As eluent, a 30:70 mixture of 0.2% formic acid in
109 acetonitrile and 4 mM ammonium acetate was used. The mobile phase was delivered at
110 0.5 mL/min in isocratic mode. Mass spectrometric acquisition was performed by using
111 multiple reaction monitoring (MRM). The nebulizer gas (synthetic air), the curtain gas
112 (nitrogen), and the heater gas (synthetic air) were set at 45, 25, and 50 (arbitrary units),
113 respectively. The collision gas (nitrogen) was set at 5 (arbitrary units). The heater gas
114 temperature was set at 500 ºC and the electrospray capillary voltage to 5.5 kV. The
115 fragment ions in MRM mode were produced by collision-activated dissociation of
116 selected precursor ions in the collision cell of the triple quadrupole and analyzed the
117 selected products with the second analyzer of the instrument. Three transitions were
118 acquired for the identification of both PhIP and the IS. To establish the appropriate
119 MRM conditions for the individual compounds, the mass spectrometric conditions were
120 optimized by using infusion with a syringe pump to select the most suitable ion
121 transitions for the target analytes. Precursor and product ions used for confirmation
122 purposes and operating conditions were described previously (Zamora, Alcon, &
123 Hidalgo, 2012). The 225.0 → 210.1 and 195.2 → 138.0 transitions for PhIP and
124 caffeine, respectively, were used for quantification purposes in this study.

125 *2.4. PhIP quantification*

126 Quantification of PhIP was carried out by preparing five standard curves of this
127 compound in 500 μL of 0.3 M sodium phosphate buffer, pH 8.0, and following the
128 whole procedure described above. For each curve, seven different concentration levels
129 of PhIP (0–2 nmol) were used. PhIP content was directly proportional to the PhIP/IS
130 area ratio ($r > 0.997$, $p < 0.0001$). The limit of detection (LOD), defined as the lowest

sample concentration that could be detected with a signal-to-noise ratio (S/N) greater than three (Hidalgo, Alaiz, & Zamora, 2001), was 0.005 nmol. The limit of quantitation (LOQ), defined as the lowest concentration that could be quantitated with a precision less than 15%, was 0.01 nmol.

2.5. Formation of phenylacetaldehyde in phenylalanine/oxidized lipid/phenolic compound reaction mixtures

Mixtures of phenylalanine (10 μmol), a lipid-derived reactive carbonyl (10 μmol), and the phenolic compound (0–10 μmol) in 500 μL of 0.3 M sodium phosphate, pH 8, were heated in closed test tubes at 200 ºC for 1 h. The assayed phenolic compounds were 1,2-dihydroxybenzene, resorcinol, and 1,4-dihydroxybenzene as model *o*-, *m*-, and *p*-dihydroxy derivatives, respectively. The lipid-derived reactive carbonyls employed were: 2-octenal, 2,4-decadienal, and 4-oxo-2-nonenal. After cooling (20 min at room temperature), reaction mixtures were diluted with 1 mL of acetonitrile, 50 μL of internal standard solution (54.8 mg of methyl heptanoate in 25 mL of ethanol) was added, and samples were analyzed by GC-MS.

2.6. 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 × 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; and mass range 28−550 amu.

155 *2.7. Determination phenylacetaldehyde content*

156 Quantification of phenylacetaldehyde was carried out, as described previously
157 (Zamora, Gallardo, & Hidalgo, 2007), by preparing standard curves of the aldehyde in
158 the 1.55 mL of solution prepared for GC-MS injection. For each curve, eight different
159 concentration levels of the aldehyde were used. Phenylacetaldehyde content was
160 directly proportional to the aldehyde/internal standard area ratio ($r = 0.999$, $p < 0.0001$).
161 The coefficients of variation were <10%.

162 *2.8. Statistical analysis*

163 All data given are mean or mean ± SD values of, at least, three independent
164 experiments. Statistical comparisons among different groups were made using analysis
165 of variance. When significant *F* values were obtained, group differences were evaluated
166 by the Tukey test (Snedecor & Cochran, 1980). Median effective concentration (EC50)
167 values, which are the concentrations of the phenolic compounds needed to reduce a 50
168 % the PhIP formed, were obtained from the best-fit line plotting concentration of
169 phenolic compound versus PhIP levels. Statistical comparisons and nonlinear curve
170 fitting for EC50 determination were carried out using Origin® v.8.0724 (OriginLab
171 Corporation, Northampton, MA). The significance level is $p < 0.05$ unless otherwise
172 indicated.

173 **3. Results**

174 *3.1. PhIP mitigation produced by o-dihydroxy derivatives and analogs in*
175 *phenylalanine/creatinine/oxidized lipid reaction mixtures.*

176 The curves obtained for PhIP formation as a function of the concentration of the
177 phenolic compound having the two hydroxy groups (or one hydroxy and one methoxy
178 group) at *ortho* positions of the aromatic ring are shown in Fig. 2A. These curves were

similar independently of the lipid involved. As observed in the figure for the 1,2-dihydroxybenzene, the amount of PhIP increased when the phenol was added in the range 0-1 μmol, and decreased afterwards, although this decrease was not very sharp in most cases. For that reason, the EC50 was high in all derivatives assayed (Table 1). Thus, the most simple derivative (1,2-dihydroxybenzene) had a mean EC50 of 7.57 μmol. The introduction of a carboxylic group (3,4-dihydroxybenzoic acid) decreased the effectiveness of the phenolic compound and a mean EC50 >12 μmol was obtained. When the carboxylic group was separated from the aromatic ring by a carbon-carbon double bond and one of the hydroxy groups was substituted by a methoxy group (ferulic acid), the effectiveness of the phenolic compound for PhIP inhibition improved but EC50 was still very high (mean EC50 >10 μmol).

3.2. PhIP mitigation produced by m-dihydroxy derivatives and analogs in phenylalanine/creatinine/oxidized lipid reaction mixtures.

The curves obtained for PhIP formation as a function of the concentration of the phenolic compound having the two hydroxy (or analogue) groups at *meta* positions of the aromatic ring are shown in Fig. 2B. These curves were always similar and independent of the lipid involved. As observed in the figure for the resorcinol, the amount of the produced PhIP decreased exponentially when the concentration of the phenolic compound increased. This rapid decrease of the PhIP produced low mean EC50 values for most *m*-dihydroxy derivatives assayed (Table 1). Thus, the simplest derivative (resorcinol) had a mean EC50 of 1.31 μmol and was the most efficient PhIP inhibitor among the assayed in this study. The introduction of a methyl group in the aromatic ring increased slightly the mean EC50 and this increase depended on the position and the number of methyl groups. Thus, the introduction of the methyl group at the position 2 (2-methylresorcinol) increased the mean EC50 to 1.97 μmol. This value

was very similar to the obtained when the methyl group was introduced at position 5 (orcinol). The mean EC50 was 2.10 μmol for this last compound. When two methyl groups were present at positions 2 and 5 (2,5-dimethylresorcinol), the mean EC50 obtained was 2.67 μmol. The introduction of a carboxylic group (2,6-dihydroxybenzoic acid) increased more the mean EC50. The obtained value was 2.92 μmol. Differently to methyl and carboxylic groups, the introduction of an amino group mostly destroyed the inhibitory effect of resorcinol and the mean EC50 obtained was 8.02 μmol for 2-aminoresorcinol, similar to 1,2-dihydroxybenzene.

The presence of two hydroxy groups at *meta* positions are not needed for the observed effect. In fact, when one hydroxy group was substituted by a methoxy group (3-methoxyphenol), the mean EC50 obtained (2.10 μmol) was higher than the obtained for resorcinol (1.02 μmol), but 3-methoxyphenol was still a very efficient PhIP inhibitor. However, this methoxy group cannot be substituted by a dimethylamino group [3-(dimethylamino)phenol], because the obtained phenol does not inhibit PhIP formation (mean EC50 >12 μmol). Furthermore, the presence of at least one hydroxy group is necessary to observe inhibition. Thus, the mean EC50 for 1,3-dimethoxyphenol was >12 μmol.

3.3. PhIP mitigation produced by p-dihydroxy derivatives and analogs in phenylalanine/creatinine/oxidized lipid reaction mixtures.

The curves obtained for PhIP formation as a function of the concentration of the phenolic compound having the two hydroxy groups (or one hydroxy and one methoxy group) at *para* positions of the aromatic ring are shown in Fig. 2C. These curves were independent of the lipid involved and were very similar to that obtained for *o*-dihydroxy derivatives and analogs. As observed in the figure for the 1,4-dihydroxybenzene, the amount of PhIP increased when the lipid was added in the range 0-1 μmol, and

decreased afterwards, although this decrease was not very rapid for most compounds. For that reason, the EC50 was high in all derivatives assayed (Table 1). In fact, the inhibitory capacity of 1,4-dihydroxybenzene (mean EC50 = 9.74 μmol) was worse than the inhibitory capacity of 1,2-dihydroxybenzene (mean EC50 = 7.57 μmol). However, when its oxidized derivative was studied (benzoquinone), this compound was more efficient (mean EC50 = 6.91 μmol) than the corresponding *p*-dihydroxy derivative (mean EC50 = 9.74 μmol). Introduction of methyl groups (trimethylhydroquinone), one carboxylic group (2,5-dihydroxybenzoic acid), or the substitution of one hydroxy group by a methoxy group (4-methoxyphenol), produced derivatives that did not exhibit PhIP inhibitory effect (EC50 >12 μmol).

3.4. PhIP mitigation produced by trihydroxy derivatives in phenylalanine/creatinine/oxidized lipid reaction mixtures.

Trihydroxy derivatives assayed did not show a homogeneous behavior (Table 1). Thus, pyrogallol had mean EC50 of 9.66 μmol. This value was even higher than that obtained by the analogous compound 2-aminoresorcinol in which the central hydroxy group was substituted by an amino group. The introduction of a carboxylic group in the ring slightly improved the PhIP inhibitory effect but the resulting phenolic compound (gallic acid) was still a poor inhibitor (mean EC50 = 8.98 μmol).

When the three hydroxy groups were not grouped, the PhIP inhibitory effect improved. Thus, 1,2,4-trihydroxybenzene had a mean EC50 of 8.20 μmol and phloroglucinol had a mean EC50 of 3.62 μmol. This last inhibitory effect was worse than the observed for resorcinol (1.02 μmol) in spite of having its three hydroxyl groups at *meta* position from each other.

3.5. PhIP mitigation produced by complex phenols in phenylalanine/creatinine/oxidized lipid reaction mixtures.

254 Resveratrol has two hydroxy groups in *meta* position at the A-ring and one hydroxy
255 group at the B-ring. It is likely that this configuration contributed to this high PhIP
256 inhibitory effect (mean EC50 = 1.37 μmol, Table 1).

257 Quercetin has also two hydroxy groups in *meta* position at the A-ring, but it has also
258 two hydroxy groups in *ortho* position at the B-ring. In addition, it has a conjugated
259 carbonyl and an hydroxy group at the C-ring. This configuration resulted in a worse
260 inhibitory effect of this compound for PhIP formation (mean EC50 = 3.08 μmol) than
261 the inhibitory effect exhibited by resveratrol (mean EC50 = 1.37 μmol).

262 Configuration of epicatechin and catechin was analogous to that of quercentin in
263 rings A and B, but they did not have the conjugated carbonyl group at the C-ring. This
264 change decreased the inhibitory power for PhIP formation of epicathechin and catechin
265 in comparison to that of quercetin. In addition, catechin resulted a better inhibitor (mean
266 EC50 = 3.89 μmol) than epicathechin (mean EC50 = 6.35 μmol) in spite of the
267 similarity among their structures that only differ in the configuration of carbon 2.

268 *3.6. Formation of phenylacetaldehyde in phenylalanine/oxidized lipid/phenolic*
269 *compound reaction mixtures*

270 Phenolic compounds can play a role in PhIP formation either by modification of the
271 conversion of phenylalanine into phenylacetaldehyde or by playing a role in the reaction
272 between phenylacetaldehyde and creatinite to produce PhIP. To determine the role of
273 phenolic compounds in the first step of the reaction, these compounds, together with
274 phenylalanine and the oxidized lipids were heated and the amount of
275 phenylacetaldehyde formed was determined. Creatinine was not added to the reaction
276 mixture to avoid the disappearance of the formed phenylacetaldehyde. The obtained
277 results for 1,2-dihydroxybenzene, resorcinol, and 1,4-dihydroxybenzene, as model *o*-,
278 *m*-, and *p*-dihydroxy derivatives, respectively, are shown in Fig. 3. As can be observed,

279 and differently to PhIP formation, all assayed phenolic compounds decreased
280 exponentially the amount of phenylacetaldehyde produced when the amount of the
281 phenolic compound increased.

282 **4. Discussion**

283 Many studies have analyzed the antioxidant capacity of phenolic compounds,
284 concluding that it is strongly related to the number and position of their hydroxy groups
285 as well as their aromatic structure (Rigo et al., 2000). The results obtained in this study
286 suggest that this conclusion is also valid for the inhibition of PhIP formation, although
287 the structural characteristics that promote antioxidative properties do not seem to be the
288 same as that those that favor the inhibition of PhIP formation. Thus, PhIP formation
289 seems to be mainly inhibited by phenolic compounds having two hydroxy groups at
290 *meta* positions in the aromatic ring. For that reason, resorcinol, its derivatives having
291 methyl, methoxy, or carboxylic groups as additional substituents in the aromatic ring,
292 and resveratrol were the best inhibitors for PhIP formation found in this study. The
293 introduction of hydroxy, amino, or alkylamino groups at the aromatic ring decreased
294 significantly the PhIP inhibitory potential of the phenolic compounds, most likely as a
295 consequence of the electronic effects of these groups. If these groups are introduced at
296 *ortho* or *para* positions in relation to the other two hydroxy groups, the PhIP inhibitory
297 activity is much reduced such as in pyrogallol and 1,2,4-trihydroxybenzene. Only when
298 the new group is introduced at the *meta* position in relation to the other two hydroxy
299 groups, as occurs in phloroglucinol, the inhibitory activity is lower than the exhibited by
300 resorcinol but the resulting phenol is still a good inhibitor of PhIP formation. The same
301 occurs when the phenol has more than one ring with opposing effects, such as in
302 quercetin, catechin and epicatechin. On one hand, the resorcinol-like A-ring present in
303 these complex phenols promotes the PhIP inhibitory effect. On the other, the B-ring

304 containing two hydroxy groups at *ortho* positions reduces the inhibitory effect of the A-
305 ring. Nevertheless, although reduced with respect to resorcinol and its simple
306 derivatives, the assayed complex phenols still exhibited a significant PhIP inhibitory
307 effect. For that reason, many studies have shown that black and green tea as well as
308 wine marinades efficiently reduce PhIP formation in both foods and model systems
309 (see, for example, Busquets, Puignou, Galcerán & Skog, 2006; Quelhas, Petisca,
310 Viegas, Melo, & Ferreira, 2010; Melo, Viegas, Petisa & Ferreira, 2008; Weisburger,
311 Nagao, Wakabayashi, & Oguri,1994).

312 The mechanism by which resorcinol-like phenols inhibit PhIP formation is unclear at
313 present and should be further investigated. Nevertheless, the results obtained by
314 studying the effect of phenols in phenylacetaldehyde formation showed that the assayed
315 *o*-, *m*-, and *p*-dihydroxy derivatives decreased phenylacetaldehyde formation, which
316 might be a consequence of either the reaction between phenylacetaldehyde and phenolic
317 compounds shown by Cheng, Wong, Cho, Chu, Sze, Lo, Chen, & Wang (2008), or
318 between the phenolic compounds and the carbonyl compounds that promote
319 phenylalanine degradation. However, to explain the behavior observed for PhIP
320 formation in the presence of *o*- and *p*-dihydroxy derivatives, these compounds should
321 promote the reaction of phenylacetaldehyde with creatinine. This would explain the
322 curve obtained in Fig. 2 for *ortho* and *para* derivarives. Thus, at low phenol
323 concentration, phenylacetaldehyde formation is only slightly reduced and the promoted
324 reaction between phenylacetaldehyde and creatinine should produce more PhIP. When
325 the amount of phenolic compound increased, the formation of phenylacetaldehyde was
326 so inhibited that PhIP was decreased also for *ortho* and *para* derivatives. On the
327 contrary, *meta* dihydroxy derivatives should not influence the reaction between
328 phenylacetaldehyde and creatinine, and a correlation ($r > 0.973$, $p < 0.0001$) between

329 the effect of these phenolic compounds on phenylacetaldehyde disappearance and PhIP
330 formation was observed. The reason by which *ortho* and *para* dihydroxy derivatives
331 seem to promote the formation of PhIP at low concentration of *o*- and *p*-dihydroxy
332 derivatives will be the objective of future investigations.

333 The results obtained in this study provide the basis to understand the role of phenolic
334 compounds in PhIP formation and suggest that it is possible to predict if a phenolic
335 derivative will inhibit or not the formation of PhIP based on its structure. Although the
336 validity of these conclusions should be further confirmed in real foods, the above results
337 suggest that to decrease PhIP formation, the use of *meta* dihydroxy derivatives should
338 be employed. Thus, the use of *meta* dihydroxy derivatives in marinating and meat
339 processing industries may be an attractive way for adding value to these products in
340 terms of minimizing the risk of exposure to PhIP and to improve nutritional
341 characteristics of foods in which antioxidants may be used in their formulation.
342 Nevertheless, this conclusion is only valid for PhIP and not for other HAAs that are
343 produced by other reaction pathways. Therefore, additional studies are needed to
344 investigate the role, if any, of *meta* dihydroxy derivatives on the formation of other
345 HAAs. A different role of phenolic compounds on the formation of the several HAAs
346 might be expected because the several HAAs are produced by different pathways.
347 Therefore, the use of a specific phenolic derivative might decrease one HAA and either
348 not produce any result or even increase the formation of other HAAs, as it have been
349 observed in many studies (see, for example, Damasius, Venskutonis, Ferracane, &
350 Fogliano, 2011).

351 **Abbreviations used**

352 EC50, median effective concentration; HAAs, heterocyclic aromatic amines; IARC,
353 International Agency for Research on Cancer; PhIP, 2-amino-1-methyl-6-
354 phenylimidazo[4,5-*b*]pyridine.

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Table 1

EC50 values determined for phenolic compounds in phenylalanine/creatinine/oxidized lipid reaction mixtures

	Oxidized lipid			
Compound	2-Octenal	2,4-Decadienal	4-Oxo-2-nonenal	Mean EC50
	***o*-dihydroxy derivatives and analogs**			
1,2 Dihydroxybenzene	8.22 ± 0.98 ^{f,g,h}	8.55 ± 1.59 ^{c,d,e}	5.93 ± 1.21 ^{c,d}	7.57
3,4-Dihydroxybenzoic acid	> 12	> 12	> 12	> 12
Ferulic acid	11.22 ± 1.05 ^{i}	> 12	7.87± 0.36 ^{d,e}	> 10
	***m*-dihydroxy derivatives and analogs**			
Resorcinol	1.09 ± 0.37 ^{a}	1.82 ± 0.38 ^{a,b}	1.02 ± 0.26 ^{a}	1.31
2-Methylresorcinol	2.62 ± 0.52 ^{a,b,c}	1.19 ± 0.14 ^{a}	2.09 ± 0.66 ^{a,b}	1.97
Orcinol	1.98 ± 0.66 ^{a,b}	2.53 ± 0.98 ^{a,b}	1.80 ± 0.20 ^{a}	2.10
2,5-Dimethylresorcinol	2.77 ± 0.89 ^{a,b,c}	2.62 ± 0.99 ^{a,b}	2.63 ± 0.79 ^{a,b}	2.67
2-Aminoresorcinol	7.72 ± 0.36 ^{f,g,h}	8.83 ± 1.03 ^{c,d,e}	7.50 ± 1.49 ^{d,e}	8.02
2,6-Dihydroxybenzoic acid	3.03 ± 0.10 ^{a,b,c}	4.31 ± 1.43 ^{b}	1.42 ± 0.73 ^{a}	2.92
3-Methoxyphenol	1.08 ± 0.60 ^{a}	3.61 ± 0.83 ^{a,b}	1.62 ± 0.38 ^{a}	2.10
3-(Dimethylamino)phenol	> 12	> 12	> 12	> 12
1,3-Dimethoxybenzene	> 12	> 12	> 12	> 12
	***p*-dihydroxy derivatives and analogs**			
1,4-Dihydroxybenzene	11.50 ± 2.39 ^{i}	10.96 ± 0.60 ^{e}	6.77 ± 1.48 ^{d,e}	9.74
1,4-Benzoquinone	6.79 ± 0.39 ^{e,f,g}	7.72 ± 0.67 ^{c}	6.26 ± 0.60 ^{c,d,e}	6.91
Trimethylhydroquinone	> 12	> 12	> 12	> 12
2,5-Dihydroxybenzoic acid	> 12	> 12	> 12	> 12
4-Methoxyphenol	> 12	> 12	> 12	> 12
	trihydroxy derivatives			
Pyrogallol	9.51 ± 1.24 ^{h,i}	10.50 ± 0.74 ^{d,e}	8.97 ± 1.23 ^{e}	9.66
1,2,4-Trihydroxybenzene	9.38 ± 0.61 ^{g,h,i}	9.18 ± 0.55 ^{c,d,e}	6.05 ± 0.95 ^{c,d,e}	8.20
Phloroglucinol	3.95 ± 0.03 ^{b,c,d}	4.19 ± 0.40 ^{b}	2.72 ± 1.00 ^{a,b}	3.62
Gallic acid	11.26 ± 0.53 ^{i}	8.18 ± 1.02 ^{c,d}	7.49 ± 0.84 ^{d,e}	8.98
	complex phenols			
Resveratrol	1.41 ± 0.34 ^{a,b}	1.25 ± 0.66 ^{a}	1.46 ± 0.61 ^{a}	1.37
Quercetin	2.69 ± 0.84 ^{a,b,c}	3.69 ± 0.62 ^{a,b}	2.87 ± 0.32 ^{a,b}	3.08
(+)-Catechin	4.91 ± 1.23 ^{c,d,e}	3.33 ± 0.76 ^{a,b}	3.44 ± 0.14 ^{a,b,c}	3.89
(-)-Epicatechin	6.56 ± 0.71 ^{d,e,f}	7.56 ± 1.22 ^{c}	4.92 ± 0.64 ^{b,c,d}	6.35

Values are mean ± SD (in µmol) for, at least, three independent experiments. Means in the same column with different superscripts are significantly different ($p < 0.05$).

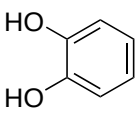
Figure legends

Fig. 1. Chemical structures of the phenolic compounds employed in this study. They have been classified according to the number and position of hydroxy groups in the benzene ring.

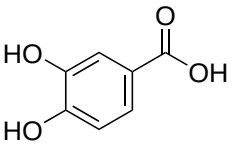
Fig. 2. Effect of the concentration of the phenolic compound on the PhIP produced in phenylalanine/creatinine/oxidized lipid mixtures heated for 1 h at 200 ºC. The phenolic compounds assayed were: A, 1,2-dihydroxybenzene; B, resorcinol; and C, 1,4-dimethylbenzene. The oxidized lipids assayed were: 2-octenal (○), 2,4-decadienal (△), and 4-oxo-2-nonenal (▽).

Fig. 3. Effect of the concentration of the phenolic compound on the phenylacetaldehyde produced in phenylalanine/oxidized lipid mixtures heated for 1 h at 200 ºC. The phenolic compounds assayed were: A, 1,2-dihydroxybenzene; B, resorcinol; and C, 1,4-dimethylbenzene. The oxidized lipids assayed were: 2-octenal (○), 2,4-decadienal (△), and 4-oxo-2-nonenal (▽).

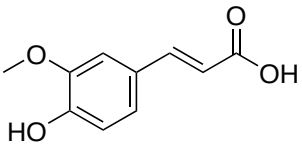
o-dihydroxy derivatives and analogs



1,2-Dihydroxybenzene

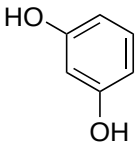


3,4-Dihydroxybenzoic acid

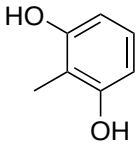


Ferulic acid

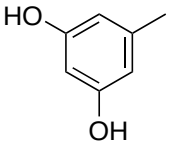
m-dihydroxy derivatives and analogs



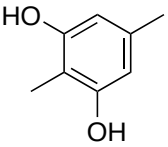
Resorcinol



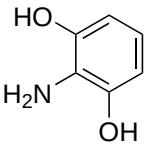
2-Methylresorcinol



Orcinol



2,5-Dimethylresorcinol



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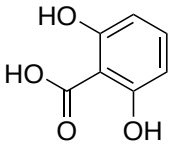
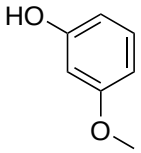
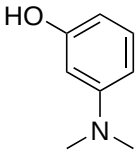


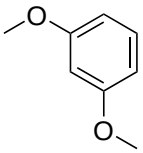
2,6-Dihydroxybenzoic acid



3-Methoxyphenol

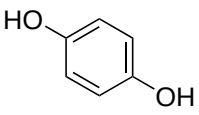


3-(Dimethylamino)phenol

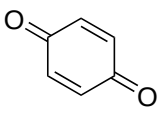


1,3-Dimethoxybenzene

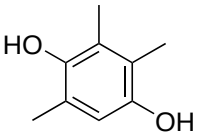
p-dihydroxy derivatives and analogs



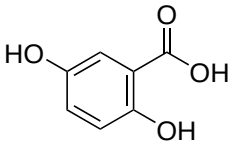
1,4-Dihydroxybenzene



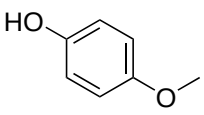
1,4-Benzoquinone



Trimethylhydroquinone

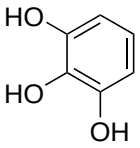


2,5-Dihydroxybenzoic acid

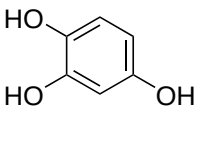


4-Methoxyphenol

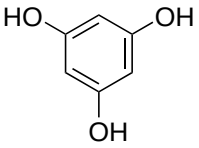
trihydroxy derivatives



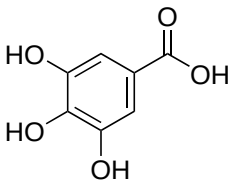
Pyrogallol



1,2,4-Trihydroxybenzene

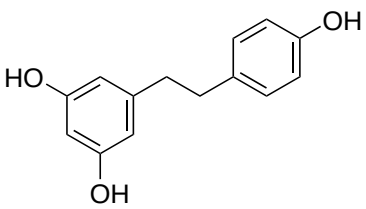


Phloroglucinol

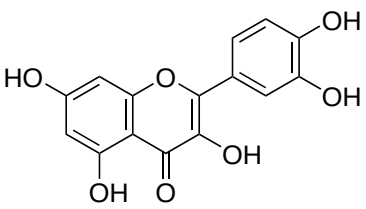


Gallic acid

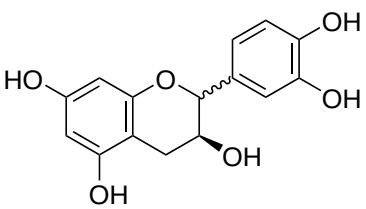
complex phenols



Resveratrol



Quercetin



(-)-Epicatechin & (+)-catechin

Fig. 1

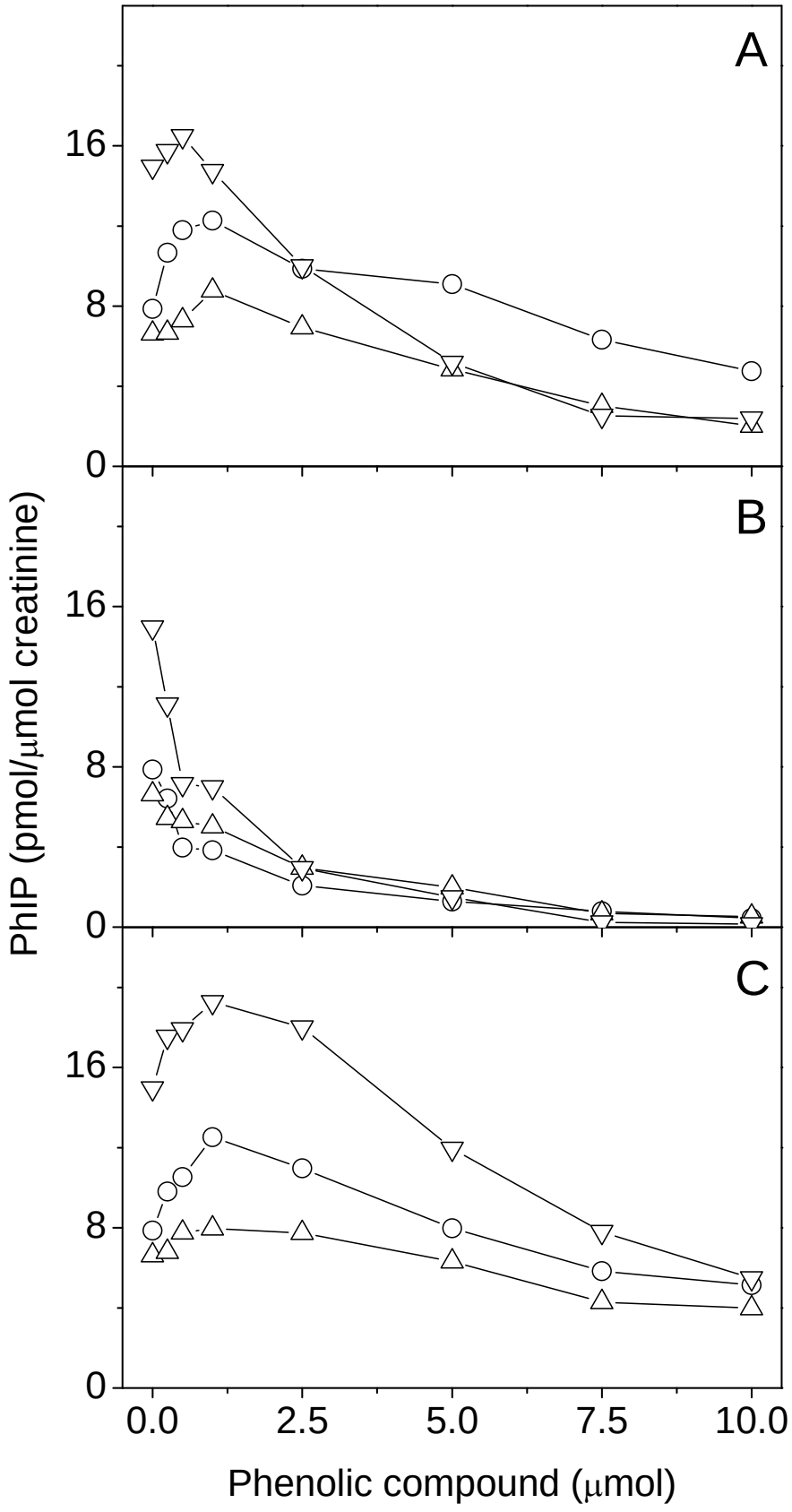

A
B
C
16
8
0
0.0
2.5
5.0
7.5
10.0
PhIP (pmol/μmol creatinine)
Phenolic compound (μmol)

Fig. 2

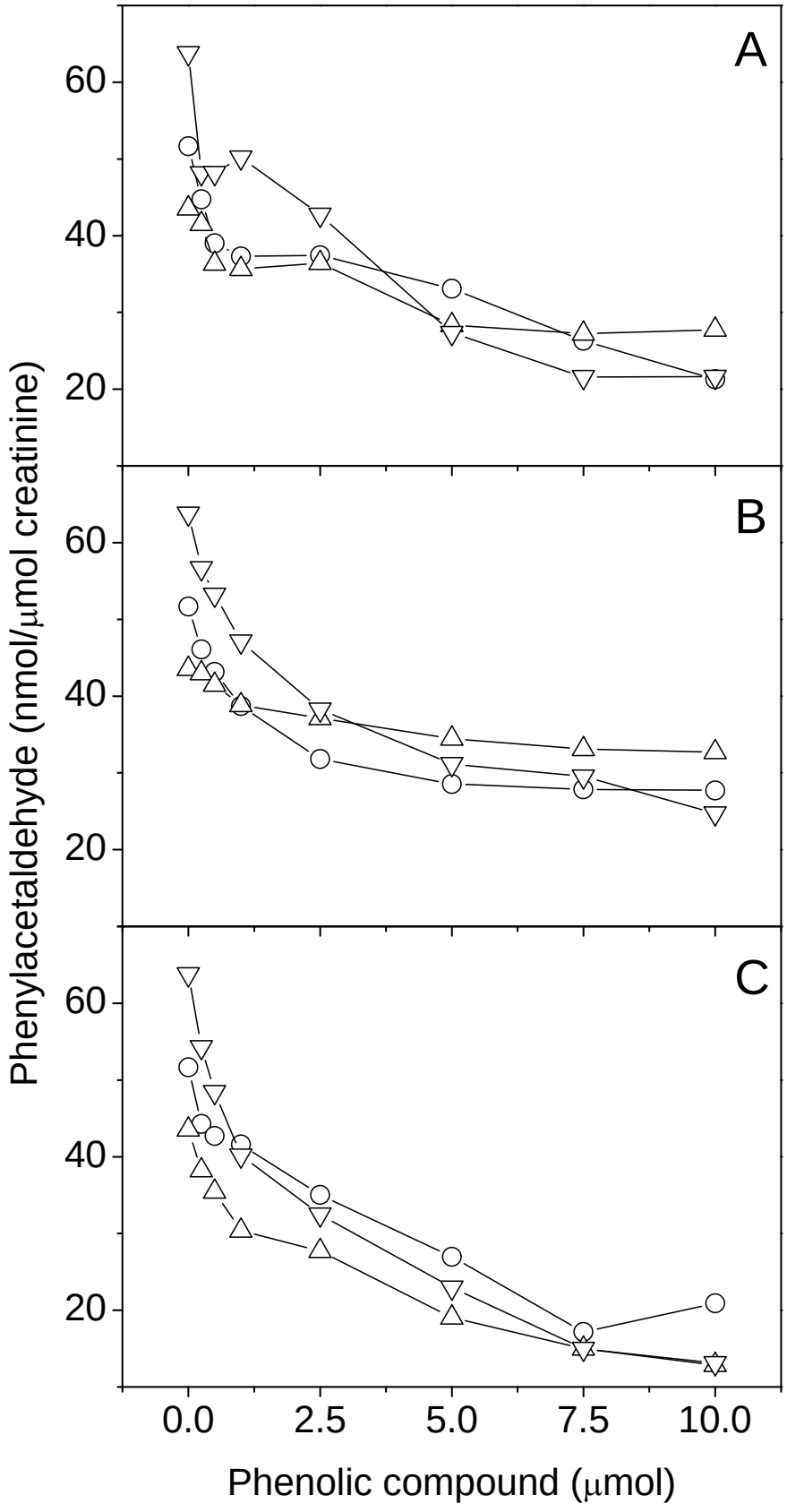

A
B
C
60
40
20
0.0
2.5
5.0
7.5
10.0
Phenylacetaldehyde (nmol/μmol creatinine)
Phenolic compound (μmol)

Fig. 3