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Structure-Activity Relationship (SAR) of Phenolics for 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) Formation in Phenylalanine/Creatinine Reaction Mixtures Including (or not) Oxygen and Lipid Hydroperoxides

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ABSTRACT

Phenolics can act as either promoters or inhibitors in 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) formation. In an attempt to clarify the structure-activity relationship (SAR) of phenolics for this reaction, the formation of PhIP in mixtures of phenylalanine, creatinine, 13-hydroperoxide of linoleic acid (LOOH) or 4-oxo-2-nonenal, and a wide array of phenolics was studied in the presence and in the absence of oxygen. The obtained results suggested that those phenolics having a high carbonyl scavenging ability inhibited the formation of PhIP. On the other hand, those phenolics that mainly acted as free radical scavengers and, therefore, were easily converted into quinones, promoted the formation of PhIP. Phenolics of the first type were *m*-diphenols and 1,3,5-triphenols. Phenolics of the second type were *o*- and *p*-diphenols. Other phenolics, like 1,2,3- and 1,2,4-triphenols, exhibited a behavior either as carbonyl scavengers or as free radical scavengers depending on ring substitutions. Among the studied derivatives, the presence of a carboxylic or a methoxyl group at certain positions inhibited their behavior as carbonyl scavengers and, therefore, promoted the formation of PhIP. A procedure to classify phenolics as either carbonyl or free radical scavengers is proposed.

KEYWORDS: *Heterocyclic aromatic amines; Lipid oxidation; Maillard reaction; Phenols; PhIP; Reactive carbonyls*

INTRODUCTION

In 2015, the Working Group of the International Agency for Research on Cancer (IARC) classified the consumption of processed red meat as "carcinogenic to humans" based on the evidences for colorectal cancer.[1] This carcinogenicity is thought to be a consequence of the chemical substances produced in meat during processing, preservation, and cooking.[2] Among these substances, the formation of carcinogenic and mutagenic heterocyclic aromatic amines (HAAs) has been known for the last three decades,[3,4] and, consequently, significant efforts have been dedicated to mitigate their formation.[5,6]

At present, numerous mitigation strategies have been described, and many of them include the addition of a number of additives. Among them, the addition of phenolic-rich extracts has been repeatedly suggested.[7] Nevertheless, these additives do not always produce the desired results, and an increase in the formation of HAAs is sometimes observed.[8] Therefore, nowadays the only way to propose a mitigation strategy based on phenolics is its testing to confirm whether the desired results are produced or not.

One of the reasons for this lack of understanding of the dual role of phenolics as promoters or inhibitors of this reaction is that the reaction pathways responsible for the formation of most HAAs are still very poorly understood.[9] In fact, the complete formation pathway of only one of these HAAs has been described at present.[10] This HAA is 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP). It is one of the HAAs produced to a highest extent,[11] and its formation pathway is shown in Figure 1. The reaction is initiated by the Strecker degradation of phenylalanine to produce phenylacetaldehyde. This degradation is promoted by a wide variety of reactive carbonyls, included those produced as a consequence of lipid oxidation.[12] The later reaction of phenylacetaldehyde with creatinine forms the corresponding carbonyl-creatinine adduct: 2-amino-2-(1-

hydroxy-2-phenylethyl)-1-methyl-1*H*-imidazol-4(5*H*)-one in a first step, and, then, after dehydration, 2-amino-1-methyl-5-(2-phenylethylidene)-1*H*-imidazol-4(5*H*)-one.[13] Finally, the reaction of this last adduct with the *in situ* formed ammonia and formaldehyde is the responsible for finishing PhIP pyridine ring formation.[14]

In an attempt to clarify the dual role of phenolics in this reaction and, therefore to determine their structure-activity relationship (SAR), this study describes the formation of PhIP in the presence of a wide array of phenolic compounds. Some of the studied model systems also included the 13-hydroperoxide of linoleic acid (LOOH) because lipids are common components in foods where PhIP formation has been observed and their contribution to PhIP formation has been described.[12] Although it has not been yet clarified, this contribution might be related to either the free radicals or the reactive carbonyls that are produced as a consequence of LOOH decomposition.[15]

MATERIALS AND METHODS

Chemicals. LOOH was prepared by oxidation of linoleic acid with lipoxygenase following a previously described procedure.[16] 4-Oxo-2-nonenal (ON) was prepared by ring opening of the corresponding 2-pentylfuran.[17] Twenty-three phenolic compounds and three quinones were employed in this study. They included *o*-, *m*-, and *p*-diphenols, *p*-quinones, and 1,2,3-, 1,2,4-, and 1,3,5-triphenols. The structures of all of them are given in Figure 2. Each line in the figure corresponds to one type of derivative, although some groups also include compounds with a slightly different structure that were included in these groups for comparison purposes. All these compounds as well as all other chemicals employed in this study were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany), with the exception of PhIP, which was purchased from Toronto Research Chemicals (North York, Ontario, Canada).

Formation of PhIP in phenylalanine/creatinine/LOOH/phenolic model systems under air. A solution of creatinine (20 μmol) and phenylalanine (20 μmol) in 400 μL of 0.3 M sodium phosphate buffer, pH 8.0), was treated with LOOH (10 μmol) and/or the phenolic compound (10 μmol) and heated at 200 ºC in closed Pyrex® test tubes for 1 h in a heating block. After cooling (5 min at room temperature and 10 min at –20 ºC), 20 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) was added, and the samples were filtered and PhIP content determined by LC-MS/MS.

Formation of PhIP in phenylalanine/creatinine/formaldehyde/ammonia/LOOH/phenolic model systems under nitrogen. The reaction was carried out under the same reaction conditions than under air, but formaldehyde and ammonia were added because these compounds are mainly produced from phenylalanine and creatinine under air. Thus, a solution of creatinine (20 μmol), phenylalanine (20 μmol), formaldehyde (10 μmol), and ammonia (10 μmol) in 400 μL of 0.3 M sodium phosphate buffer, pH 8.0), was treated with LOOH (10 μmol) and/or the phenolic compound (10 μmol) and heated at 200 ºC in closed Pyrex® test tubes for 1 h in a heating block. Similarly to that described above, after cooling, the internal standard was added, and the samples were filtered and studied by LC-MS/MS.

PhIP determination. PhIP was determined as described previously.[18] Briefly, reaction mixtures were fractionated on a Zorbax Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) column from Agilent. As the eluent, a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate was used. The mobile phase was delivered at a rate of 0.5 mL/min in isocratic mode. Mass spectrometric detection was performed by

using multiple reaction monitoring (MRM). Mass detector conditions were described previously.[18] Precursor and product ions used for confirmation purposes were also described previously.[12] The *m/z* 225.0 → *m/z* 210.1 and *m/z* 195.2 → *m/z* 138.0 transitions for PhIP and caffeine, respectively, were used for quantitation purposes in this study.

PhIP quantitation was conducted by preparing five standard curves of this compound in 400 μL of 0.3 M sodium phosphate buffer (pH 8.0) and following the whole procedure described above. For each curve, seven different concentration levels of PhIP (0–2 nmol) were used. The PhIP content was directly proportional to the PhIP/IS area ratio ($r > 0.997$; $p < 0.0001$). The limits of detection (LOD) and quantitation (LOQ) were 0.005 and 0.01 nmol, respectively.[18]

Statistical analysis. All data given are mean ± standard deviation (SD) of at least three independent experiments. Statistical comparisons with the corresponding controls were made using Student *t*-test.[19] 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.[19] These studies were conducted using Origin version 7.0 (OrigenLab Corp., Northampton, MA). The significance level is $p < 0.05$ unless otherwise indicated.

RESULTS AND DISCUSSION

The study of antioxidant properties of phenolics and their consequent health benefits have been the objective of numerous investigations during the last decades.[20–22] However, at present, only a limited number of phenolics are commercially available because their prooxidant properties often outweigh their antioxidant properties.[23] This is mostly a consequence of both the competence among the different mechanisms taking place under oxidative conditions and the different abilities of phenolics against the oxidation-induced

damage as a function of their structures.[24] The different mechanisms that can play a role in the formation of PhIP have been collected in Figure 3.

In the absence of either LOOH or phenolics, the formation of PhIP should occur to a low extent because the conversion of phenylalanine into phenylacetaldehyde is not favored. However, the thermal degradation of hydroperoxides produce both lipid radicals and reactive carbonyls that should promote the conversion of phenylalanine into phenylacetaldehyde. Thus, free radicals degrade phenylalanine[25] and oxidize *o*- and *p*-diphenols,[26] and both reactions should promote the formation of phenylacetaldehyde and, therefore, of PhIP. In particular, the free radical scavenging ability of *o*- and *p*-diphenols promote the formation of *o*- and *p*-quinones, which are reactive carbonyls and favor PhIP formation.[27] Furthermore, the conversion of phenolics into quinones implies the formation of phenoxyl radicals,[28] which should also favor the formation of PhIP. Moreover, this conversion of *o*- and *p*-diphenols into *o*- and *p*-quinones also takes place in the presence of oxygen, although lipid radicals are not present,[26] and, therefore, PhIP formation should also be promoted. Thermal degradation of LOOH also produces lipid-derived reactive carbonyls and these compounds also contribute to the formation of phenylacetaldehyde[29] and, therefore, of PhIP. However, this reaction is inhibited in the presence of *m*-diphenols because of the ability of these compounds to trap carbonyls[30] and to form the corresponding carbonyl-phenol adducts.[31] Furthermore, the ability of *m*-diphenols to trap phenylacetaldehyde and quinones should also be considered.[32] All these processes should be competing among them and the formation of PhIP in the different reaction mixtures and conditions should be a consequence of this competence.

Effect of phenolics on PhIP formation in a mixture of phenylalanine and creatinine heated under air. Heating a mixture of phenylalanine and creatinine under air produced minute amounts of PhIP (Table 1). However, when this heating was carried

out in the presence of phenolics, significant differences were observed, and these differences depended on the structure of the involved phenolics.

When the heating was carried out in the presence of *o*-diphenols, the amount of PhIP increased considerably (Table 1). This behavior is likely related to the conversion of *o*-diphenols into *o*-quinones. As described previously,[26] quinones behave as reactive carbonyls and promote the conversion of phenylalanine into phenylacetaldehyde, the first step in the formation of PhIP.

Differently to *o*-diphenols, when the reaction mixture contained *m*-diphenols, the amount of PhIP did not increase with the exception of compound **11** (Table 1). This behavior is likely related to the carbonyl-scavenging ability described for these derivatives,[31] which is consequence of the high nucleophilicity of certain aromatic carbons.[14] The electronic delocalization responsible for this high nucleophilicity is absent in compound **11** because of the existence of a strong intramolecular hydrogen bond between the carboxylic group and one of the hydroxyl groups,[23] and this compound had a behavior similar to that of *o*-diphenols.

An analogous behavior to that of *o*-diphenols, and different to that of *m*-diphenols, was also observed for *p*-diphenols (Table 1). Surprisingly, when *p*-diphenols were substituted by *p*-quinones, the amount of PhIP formed was lower than that produced by *p*-diphenols. These results suggested that heating *p*-diphenols (and also *o*-diphenols) in the presence of air did not only produce the corresponding quinones, but something else that promoted PhIP formation. According to the obtained results for hydroquinone (compound **12**) and benzoquinone (compound **16**), benzoquinone produced about one half of the PhIP produced by hydroquinone. This means that about 50% of the PhIP was produced as a consequence of the conversion of hydroquinone into benzoquinone. The other 50%

should be likely produced by the free radicals (phenoxyl radicals) involved in this conversion.[33]

When three hydroxyl groups were present in the phenolic compound, the simultaneous existence of *ortho*, *meta*, and *para* effects played their corresponding roles and its behavior was intermediate between that of *o*- and *p*-diphenols, on one hand, and *m*-diphenols on the other (Table 1). Thus, in 1,2,3-triphenols (compounds **19**–**21**), there are two *ortho* and one *meta* substitutions. For that reason, the increase in PhIP was usually lower than the observed in *o*- and *p*-diphenols. 1,2,4-Triphenols and analogues (compounds **22**–**24**) have one *ortho*, one *meta*, and one *para* substitutions. For all assayed compounds PhIP increased 2-3 times and the PhIP produced was lower than that in *o*- and *p*-derivatives. Finally, 1,3,5-triphenols (compounds **25**–**26**) have three *meta* substitutions. Therefore, they exhibited a behavior similar to that of *m*-diphenols and did not contribute to PhIP formation.

In relation to the effect of additional substituents in the phenolic ring, the presence of a carboxylic group, methyl groups, or an alkyl chain increased considerably the amount of PhIP produced in *o*- and *p*-diphenols. This effect was smaller in *m*-diphenols and triphenols.

Effect of phenolics and LOOH on PhIP formation in mixtures of phenylalanine and creatinine heated under air. In the absence of phenolics, LOOH increased the formation of PhIP by six times (Table 1). This is likely related to the thermal decomposition of LOOH that produces both lipid radicals and lipid-derived reactive carbonyls. As described above, both reactive carbonyls and free radicals promote PhIP formation.

Simultaneous addition of LOOH and *o*-diphenols did not increase the amount of PhIP produced in the absence of LOOH, and an analogous effect was observed for *p*-diphenols (Table 1). These results suggested that, in the presence of *o*- or *p*-diphenols, the contribution of LOOH to this system was negligible, most likely because different reactions with contrary effects were simultaneously produced as described in Figure 3.

Analogously to *p*-diphenols, the simultaneous addition of LOOH and *p*-quinones did not increase the amount of PhIP produced. In fact, quinones produced less PhIP than *p*-diphenols, most likely because they do not introduce new free radicals in the reaction mixture.

Differently to *o*- or *p*-diphenols, the simultaneous addition of *m*-diphenols and LOOH produced more PhIP than when reaction mixtures were heated in the absence of LOOH. Nevertheless, the amount of PhIP produced was usually lower than that produced by LOOH in the absence of *m*-diphenols (Table 1). As indicated in Figure 3, this behavior is likely related to the carbonyl-scavenging ability of *m*-diphenols, which do not have a free radical scavenging ability. As discussed previously, compound **11** exhibited an anomalous behavior because this compound did not have a carbonyl-scavenging ability.

Analogous effects were observed in triphenols and analogues (Table 1). Because all of them had, at least, one *m*-diphenol in its structure, the amount of PhIP formed usually increased when LOOH and phenolics were present in relation to the PhIP produced when LOOH was absent. However, this increase was usually lower than that observed in *m*-diphenols. Considering that phloroglucinol (**25**) should be an efficient carbonyl scavenger, the PhIP produced in LOOH/phloroglucinol mixtures should be a consequence of the free radicals produced by LOOH decomposition. The amount of PhIP produced in the presence of LOOH/phloroglucinol was about 46% of the PhIP produced in the absence

of this phenol derivative. Therefore, about 50% of the PhIP produced by LOOH should be a consequence of the free radicals and the other 50% should be a consequence of the reactive carbonyls produced as a consequence of LOOH decomposition.

As discussed previously, the introduction of LOOH in the system increased the number of reactions taking place and the behavior of phenolics was different in the presence or in the absence of LOOH. The best way to study the structure-activity relationship (SAR) of phenolics for this reaction is to plot the ratio between the PhIP obtained in the presence of LOOH and the PhIP obtained in its absence as a function of the kind of phenol derivative (Figure 4). As can be observed, diphenols could be divided into two groups that were significantly ($p < 0.05$) different (Figure 4B). Thus, this ratio was close to 1 for phenolics having mainly a free radical scavenging function (*o*- and *p*-diphenols) and higher than 2 for phenolics having mainly a carbonyl scavenging function (*m*-diphenols). By using this ratio it is possible to distinguish whether, in complex phenolics, the free radical or the carbonyl scavenger function is the one that plays a main role. Thus, triphenols having a behavior similar to that of *m*-diphenols were compounds **19**, **22**, **25** and **26**. On the other hand, compounds **20**, **21**, and **24** had a behavior close to that of *o*- and *p*-diphenols. Finally, compound **23** had an intermediate behavior. Although the number of triphenols assayed was low, some preliminary conclusions can be obtained. Thus, the presence of a carboxylic group in *meta* or *para* with respect to the hydroxyl groups converted a carbonyl scavenging phenol (compound **19**) into a phenol that exhibited a behavior similar to *o*- and *p*-derivatives (compounds **20** or **21**). Similarly, the change of a hydroxyl group by a methoxyl group in *meta* or *para* in relation to two other hydroxyl groups converted a carbonyl scavenger phenol (compound **22**) into a phenol with the characteristics of *o*- and *p*-diphenols (compound **24**).

Effect of phenolics on PhIP formation in mixtures of phenylalanine, creatinine, ammonia and formaldehyde heated under nitrogen. As described above, oxygen played a role in all these reactions by converting *o*-and *p*-diphenols into the corresponding quinones at the same time that phenoxyl radicals were generated. To avoid the interference of this reaction, the same model system discussed above was heated under nitrogen. Nevertheless, because PhIP formation requires the presence of ammonia and formaldehyde and these compounds are better produced under oxidative conditions, both compounds were added to the assayed reaction mixtures.

When phenylalanine, creatinine, formaldehyde, and ammonia were heated under nitrogen, PhIP was produced (Table 2), and the amount of PhIP formed was twice higher than that produced when phenylalanine and creatinine were heated under air (Table 1). This is likely a consequence of the addition of formaldehyde and ammonia to the reaction mixture heated under nitrogen.

When *o*-diphenols were also added to the reaction mixture, the values obtained for PhIP (Table 2) were lower than those obtained under air (Table 1). This behavior is likely a consequence of phenolic oxidation not being favored under nitrogen. Therefore, less quinones should have been formed and less PhIP was produced. The only exception was compound **5**, which unexpectedly produced a similar amount of PhIP under air and under nitrogen. In addition, most assayed *o*-diphenols did not increase the amount of PhIP produced by the control (reaction mixture without phenol). The only exceptions were compounds **3** and **5**.

Analogous effects were observed for *p*-diphenols, although *p*-diphenols increased the amount of PhIP produced by the control (Table 2). Again, the values obtained under nitrogen (Table 2) were lower than those obtained under air (Table 1), more likely because

of the absence of phenoxyl radicals. On the contrary, when quinones were assayed, they produced more PhIP under nitrogen (Table 2) than under air (Table 1), more likely because formaldehyde and ammonia were already present in the reaction mixture.

Differently to *o*- and *p*-diphenols, and analogously to *p*-quinones, the PhIP produced in the presence of *m*-diphenols under nitrogen (Table 2) was usually higher than that produced under air (Table 1).

Triphenols and analogues had a behavior that was intermediate between that of *o*- and *p*-diphenols, on one hand, and that of *m*-diphenols, on the other. For most of them the PhIP produced when they were added was similar to the PhIP produced by the control. The only exceptions were compound **24**, which increased the PhIP produced, and compound **26**, which decreased the PhIP produced.

Figure 5 shows the plot of the ratio between the PhIP formed under nitrogen and the PhIP formed under air as a function of the kind of phenol derivative studied. As observed in Figure 5B, *o*-, *m*-, and *p*-diphenols were not significantly ($p < 0.05$) different and it was not possible to classify phenolics as either free radical or carbonyl scavengers according to this figure. In addition, *p*-diphenols had a behavior significantly ($p < 0.05$) different to that of *p*-quinones because, in the absence of oxygen and the free radicals produced by LOOH decomposition, *p*-diphenols could not be converted into *p*-quinones.

Effect of phenolics and LOOH on PhIP formation in mixtures of phenylalanine, creatinine, ammonia and formaldehyde heated under nitrogen. The addition of LOOH increased the PhIP of the phenylalanine/creatinine/ammonia/formaldehyde reaction mixtures (Table 2) because of the presence of the free radicals and reactive carbonyls produced as a consequence of the thermal decomposition of the LOOH. Furthermore, and differently to the behavior observed when reaction mixtures were

286 incubated under air (Table 1), the addition of LOOH increased the amount of PhIP
287 produced by most phenolics (Table 2). Thus, the mixture of *o*-diphenols and LOOH
288 produced an amount of PhIP similar to that of control LOOH, and higher than the PhIP
289 produced in reaction mixtures heated in the presence of phenolics but without LOOH.
290 These results confirmed that *o*-diphenols did not contribute to PhIP formation unless
291 oxygen was present. The only exceptions were compounds **4** and **5**. These compounds
292 increased the amount of PhIP produced by control LOOH. This behavior might be related
293 to a certain oxidability of these phenolics in the presence of lipid hydroperoxides.

294 A similar behavior was also observed for *p*-diphenols, but not for *p*-quinones (Table
295 2). Thus, the amount of PhIP produced by mixtures of *p*-diphenols and LOOH produced
296 the same amount of PhIP as compared to a reaction mixture without *p*-diphenols, but the
297 effect of LOOH and phenolics was superior to that of phenolics alone. Differently to *p*-
298 diphenols, the mixture of LOOH and *p*-quinones increased the amount of PhIP produced
299 by LOOH, most likely as a consequence of the quinone behavior as reactive carbonyls.[26]

300 Mixtures of *m*-diphenols and LOOH also produced an amount of PhIP similar to that
301 produced by control LOOH and this amount of PhIP was higher than that produced by
302 the phenolics in the absence of LOOH (Table 2). Exceptions were compounds **7** and **10**.
303 As described above, these compounds are strong carbonyl scavengers and mixtures of
304 them with LOOH produced less PhIP than LOOH alone.

305 Mixtures of triphenols and LOOH usually produced less PhIP than control LOOH
306 (Table 2). In addition, 1,2,3- and 1,3,5-triphenols, but not 1,2,4-triphenols and analogues,
307 produced more PhIP in the presence of LOOH than in its absence (Table 2). The amount
308 of PhIP produced by all triphenols was mostly similar to that of *m*-diphenols and lower

than that produced by *o*- and *p*-diphenols. Therefore, under these reaction conditions, these phenolics had a behavior mostly similar to that of *m*-diphenols.

Because most phenolics produced similar amounts of PhIP (Table 2), when the ratio between the PhIP produced with LOOH and the PhIP produced without LOOH was plotted as a function of the kind of phenols studied (Figure 6), most phenolics had a similar ratio. In fact, no significant ($p < 0.05$) differences were observed among the different types of diphenols (Figure 6B).

Effect of phenolics and ON on PhIP formation in mixtures of phenylalanine, creatinine, ammonia and formaldehyde heated under nitrogen. To try to distinguish among phenolics as a function of their structure when reactions were carried out in the absence of oxygen, additional reactions involving ON (a reactive carbonyl compound derived from LOOH)[34] were studied. This change of LOOH by ON should suppress all free radical reactions (the reactions showed on the left half of Figure 3) and differences among phenolics as a function of their structures should increase.

The addition of ON to the model system increased the formation of PhIP to a similar extent that LOOH (Table 2). The increase in the PhIP produced is likely a consequence of being ON a reactive carbonyl that promotes the degradation of phenylalanine into phenylacetaldehyde, the first step of PhIP formation.[14]

When *o*- or *p*-diphenols were also present, the amount of PhIP formed mostly increased in relation to the PhIP produced in the absence of lipid, although there was not much difference in relation to the PhIP produced in the presence of ON but in the absence of phenolics (Table 2). On the contrary, when *m*-diphenols were present, the amount of PhIP formed usually decreased in relation to the samples without the lipid because of the carbonyl-scavenging ability of these phenolics.[30] As discussed previously, compound **11**

was an exception because the electronic delocalization needed to exhibit a carbonyl scavenger activity is absent in this compound.[23]

Triphenols and analogues again exhibited an intermediate behavior between that of *o*- and *p*-diphenols, on one hand, and that of *m*-diphenols, on the other (Table 2). Thus, compounds **19**, **22**, and **25** exhibited a behavior closer to that of *m*-diphenols by decreasing the amount of PhIP produced when ON was present, compound **21** exhibited a behavior similar to *o*- and *p*-diphenols by increasing the amount of PhIP produced when ON was present, and the other assayed compounds (**20**, **23**, **24**, and **26**) produced similar amounts to PhIP when ON was present or not.

When the ratio between the PhIP produced with ON and the PhIP produced without ON was plotted as a function of the kind of phenols studied (Figure 7), some differences appeared. Except for the anomalous behavior of compound **11**, which is not typical of *m*-diphenols as discussed above, diphenols began to be separated as a function of their structures. Thus, *m*-diphenols were significantly ($p < 0.05$) different to *o*-diphenols although not to *p*-diphenols.

A complete separation of diphenols as a function of their structures was obtained when the ratio between the PhIP produced with LOOH and the PhIP produced with ON was plotted as a function of the kind of phenol studied (Figure 8). As observed in Figure 8B, *m*-diphenols, except for anomalous compound **11**, were significantly ($p < 0.05$) different to *o*- and *p*-diphenols and, therefore, it was possible to distinguish between free radical and carbonyl scavenger functions also when reactions were carried out under nitrogen. Thus, phenolics with a ratio higher than 3 are good carbonyl scavengers and they produced an inhibition of PhIP formation. On the other hand, most phenolics that are poor carbonyl scavengers had a ratio close to 1. Figure 8 is quite similar to Figure 4, which

was obtained with the data of reactions carried out under air. Thus, among the assayed triphenols, only compounds **19**, **25**, and **26** exhibited a behavior similar to that of *m*-diphenols. These compounds exhibited the same effect under air. The only difference between both figures is the behavior of compound **22** that behave as a carbonyl scavenger under air and as a free radical scavenger under nitrogen. Additional experiments are needed to confirm and explain this potential dual behavior of some phenolics as a function of oxygen accessibility.

All these results show that the role of phenolics in the formation of PhIP is complex because the required phenylacetaldehyde is formed, as shown in Figure 3, by degradation of phenylalanine in the presence of both free radicals and reactive carbonyls, and the thermal degradation of lipid hydroperoxides produces both of them. Therefore, the main role of phenolics as free radical or carbonyl scavengers will determine if they will promote or inhibit the formation of PhIP. In the presence of oxygen, the ratio between PhIP formed in the presence and in the absence of LOOH will determine the main role of phenolics as free radical or carbonyl scavengers (< 1.5 or > 2, respectively). In the absence of oxygen, the ratio of PhIP formed in the presence of LOOH versus the PhIP formed in the presence of ON will determine their main behavior as free radical or carbonyl scavengers (< 3 or > 3, respectively). The use of these ratios is suggested to classify complex phenolics. The study of other reactions are needed to know if the behavior of phenolics as free radical or carbonyl scavengers is always the same or it can change as a function of the involved reaction.

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Notes

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FIGURE CAPTIONS

Figure 1. PhIP formation pathway.

Figure 2. Phenolic compounds (or analogues) employed in this study. Each row corresponds to one kind of studied phenolics (or analogues): *o*-diphenols, *m*-diphenols, *p*-diphenols, *p*-quinones, 1,2,3-triphenols, 1,2,4-triphenols, and 1,3,5-triphenols.

Figure 3. Reactions (free radical on the left and carbonyl-amine on the right) involved in the formation of PhIP in the presence of LOOH and phenolics. Abbreviations: LOOH, 13-hydroperoxide of linoleic acid; PAC, phenylacetaldehyde; Phe, phenylalanine.

Figure 4. Ratio of PhIP produced under air in the presence of LOOH and that produced in its absence as a function of the kind of phenolic compound studied. The numbers correspond to compound numbering shown in Figure 2. Individual ratios are collected in panel A. In panel B, the values obtained for the first four types of compounds have been substituted by a box plot. Boxes with different letters are significantly ($p < 0.05$) different. Abbreviations: 1,2, *o*-diphenols; 1,3, *m*-diphenols; 1,4, *p*-diphenols; 1,4q, *p*-quinones; 1,2,3, 1,2,3-triphenols; 1,2,4, 1,2,4-triphenols; and 1,3,5, 1,3,5-triphenols.

Figure 5. Ratio of PhIP produced under nitrogen and that produced under air as a function of the kind of phenolic compound studied in the absence of LOOH. The numbers correspond to compound numbering shown in Figure 2. Individual ratios are collected in panel A. In panel B, the values obtained for the first four types of compounds have been substituted by a box plot. Boxes with different letters are significantly ($p < 0.05$) different. Abbreviations are shown in Figure 4 legend.

Figure 6. Ratio of PhIP produced under nitrogen in the presence of LOOH and that produced in its absence as a function of the kind of phenolic compound studied. The

numbers correspond to compound numbering shown in Figure 2. Individual ratios are collected in panel A. In panel B, the values obtained for the first four types of compounds have been substituted by a box plot. Boxes with different letters are significantly ($p < 0.05$) different. Abbreviations are shown in Figure 4 legend.

Figure 7. Ratio of PhIP produced under nitrogen in the presence of 4-oxo-2-nonenal (ON) and that produced in its absence as a function of the kind of phenolic compound studied. The numbers correspond to compound numbering shown in Figure 2. Individual ratios are collected in panel A. In panel B, the values obtained for the first four types of compounds have been substituted by a box plot. Boxes with different letters are significantly ($p < 0.05$) different. Abbreviations are shown in Figure 4 legend.

Figure 8. Ratio of PhIP produced under nitrogen in the presence of LOOH and that produced in the presence of ON as a function of the kind of phenolic compound studied. The numbers correspond to compound numbering shown in Figure 2. Individual ratios are collected in panel A. In panel B, the values obtained for the first four types of compounds have been substituted by a box plot. Boxes with different letters are significantly ($p < 0.05$) different. Abbreviations are shown in Figure 4 legend.

Table 1. Effect of phenolics and LOOH on PhIP formation in a mixture of phenylalanine and creatinine heated under air[a]

	No LOOH added		LOOH added		
Phenolic	PhIP (pmol/μmol creatinine)	Effect of phenolic (*p* value obtained by comparison with control)	PhIP (pmol/μmol creatinine)	Effect of phenolic (*p* value obtained by comparison with control)	Effect of LOOH (*p* value obtained by comparing the same phenol with and without LOOH)
None (control)	0.87 ± 0.57		5.62 ± 0.67		0.001
o-Diphenols					
Catechol (**1**)	3.11 ± 0.70	0.013	3.40 ± 0.48	0.009	n.s.[a]
4-Methylcatechol (**2**)	4.95 ± 1.14	0.005	4.65 ± 0.68	n.s.[a]	n.s.[a]
3-(3,4-dihydroxyphenyl)propanoic acid (**3**)	11.42 ± 2.75	0.003	12.15 ± 2.65	0.014	n.s.[a]
3,4-Dihydroxybenzoic acid (**4**)	10.60 ± 0.42	< 0.001	11.09 ± 1.44	0.004	n.s.[a]
Hydroxytyrosol (**5**)	11.30 ± 2.71	0.003	12.66 ± 0.96	< 0.001	n.s.[a]
Tyrosol[b] (**6**)	3.87 ± 0.73	0.005	3.30 ± 0.17	0.004	n.s.[a]
m-Diphenols					
Resorcinol (**7**)	0.36 ± 0.14	n.s.[a]	1.08 ± 0.11	0.000	0.002
2-Methylresorcinol (**8**)	0.81 ± 0.11	n.s.[a]	2.02 ± 0.06	0.001	< 0.001
2,5-Dimethylresorcinol (**9**)	0.87 ± 0.06	n.s.[a]	3.47 ± 0.75	0.020	0.004
Orcinol (**10**)	0.76 ± 0.27	n.s.[a]	1.89 ± 0.22	0.001	0.005
2,6-Dihydroxybenzoic acid (**11**)	2.36 ± 0.39	0.020	10.53 ± 1.19	0.003	< 0.001
p-Diphenols					

Hydroquinone (**12**)	6.31 ± 0.89	0.001	6.74 ± 1.64	n.s.[a]	n.s.[a]
2,3,5-Trimethylbenzene-1,4-diol (**13**)	10.73 ± 0.38	< 0.001	10.20 ± 1.89	0.017	n.s.[a]
tert-Butylhydroquinone (**14**)	11.92 ± 2.95	0.003	11.77 ± 0.67	< 0.001	n.s.[a]
2,5-Dihydroxybenzoic acid (**15**)	10.26 ± 0.62	0.001	11.94 ± 0.85	0.001	n.s.[a]
p-Quinones					
Benzoquinone (**16**)	2.88 ± 1.02	0.040	3.85 ± 0.89	n.s.[a]	n.s.[a]
Methylbenzoquinone (**17**)	2.41 ± 0.69	0.040	2.51 ± 0.30	0.002	n.s.[a]
2,5-Dimethylbenzoquinone (**18**)	5.74 ± 0.57	< 0.001	3.91 ± 0.23	0.014	0.007
1,2,3-Triphenols					
Pyrogalol (**19**)	1.50 ± 0.37	n.s.[a]	3.43 ± 0.19	0.005	0.001
Methyl gallate (**20**)	4.85 ± 1.16	0.006	6.55 ± 0.94	n.s.[a]	n.s.[a]
Propyl gallate (**21**)	2.60 ± 0.41	0.013	3.71 ± 0.37	0.012	0.026
1,2,4-Triphenols and analogues					
1,2,4-Trihydroxybenzene (**22**)	2.06 ± 0.44	0.046	5.56 ± 0.55	n.s.[a]	0.001
Methoxyhydroquinone[c] (**23**)	2.83 ± 0.82	0.027	5.44 ± 0.79	n.s.[a]	0.016
4-Methoxycatechol[c] (**24**)	2.65 ± 0.68	0.025	3.61 ± 0.90	0.035	n.s.[a]
1,3,5-Triphenols					
Phloroglucinol (**25**)	1.09 ± 0.32	n.s.[a]	3.18 ± 0.33	0.005	0.001
2-Phenyl-1-(2,4,6-trihydroxyphenyl)ethan-1-one (**26**)	0.49 ± 0.06	n.s.[a]	1.77 ± 0.22	0.001	< 0.001

[a]Abbreviations: LOOH, 13-hydroperoxide of linoleic acid; n.s., not significant. [b]This compound is not a *o*-diphenol. It was included in this group for comparison purposes. [c]This compound is not a 1,2,4-triphenol. It was included in this group for comparison purposes.

Table 2. Effect of phenolics, LOOH, and ON on PhIP formation in a mixture of phenylalanine, creatinine, ammonia, and formaldehyde heated under nitrogen[a]

	Neither LOOH nor ON added		LOOH added			ON added		
Phenolic	PhIP (pmol/μmol creatinine)	Effect of phenolic (*p* value obtained by comparison with control)	PhIP (pmol/μmol creatinine)	Effect of phenolic (*p* value obtained by comparison with control)	Effect of LOOH (*p* value obtained by comparing the same phenol with and without LOOH)	PhIP (pmol/μmol creatinine)	Effect of phenolic (*p* value obtained by comparison with control)	Effect of ON (*p* value obtained by comparing the same phenol with and without ON)
None (control)	1.73 ± 0.49		5.28 ± 1.38		0.014	5.88 ± 1.14		0.004
o-Diphenols								
Catechol (**1**)	1.31 ± 0.31	n.s.[a]	4.17 ± 0.35	n.s.[a]	< 0.001	3.58 ± 0.19	0.001	< 0.001
4-Methylcatechol (**2**)	1.62 ± 0.13	n.s.[a]	5.64 ± 0.13	n.s.[a]	< 0.001	2.17 ± 0.16	0.005	0.002
3-(3,4-dihydroxyphenyl)propanoic acid (**3**)	3.89 ± 0.39	< 0.001	6.97 ± 1.40	n.s.[a]	< 0.001	4.07 ± 0.86	0.041	n.s.[a]
3,4-Dihydroxybenzoic acid (**4**)	2.26 ± 0.64	n.s.[a]	8.46 ± 1.20	0.014	< 0.001	7.88 ± 1.05	n.s.[a]	< 0.001
Hydroxytyrosol (**5**)	10.90 ± 1.80	< 0.001	16.22 ± 2.82	0.004	0.010	10.50 ± 0.80	0.000	n.s.[a]
Tyrosol[b] (**6**)	2.32 ± 0.43	n.s.[a]	4.80 ± 1.01	n.s.[a]	< 0.001	7.12 ± 0.96	n.s.[a]	< 0.001
m-Diphenols								
Resorcinol (**7**)	0.59 ± 0.27	< 0.001	1.03 ± 0.46	< 0.001	0.005	0.28 ± 0.10	< 0.001	0.005
2-Methylresorcinol (**8**)	3.08 ± 1.11	n.s.[a]	4.23 ± 1.28	n.s.[a]	0.041	0.76 ± 0.23	< 0.001	< 0.001
2,5-Dimethylresorcinol (**9**)	2.76 ± 0.70	0.041	4.60 ± 0.70	n.s.[a]	0.001	0.93 ± 0.18	0.001	0.001
Orcinol (**10**)	1.13 ± 0.29	0.017	2.58 ± 0.72	0.003	< 0.001	0.73 ± 0.23	< 0.001	0.017
2,6-Dihydroxybenzoic acid (**11**)	6.26 ± 0.43	< 0.001	6.83 ± 0.24	n.s.[a]	n.s.[a]	10.56 ± 1.01	0.001	< 0.001
p-Diphenols								

Hydroquinone (**12**)	3.84 ± 0.67	< 0.001	7.37 ± 0.45	n.s.[a]	< 0.001	6.99 ± 0.46	n.s.[a]	< 0.001
2,3,5-Trimethylbenzene-1,4-diol (**13**)	4.31 ± 0.43	< 0.001	8.67 ± 0.55	n.s.[a]	< 0.001	8.16 ± 0.30	0.0238	< 0.001
tert-Butylhydroquinone (**14**)	3.03 ± 0.48	0.007	6.43 ± 0.59	n.s.[a]	< 0.001	4.38 ± 0.61	n.s.[a]	0.008
2,5-Dihydroxybenzoic acid (**15**)	4.44 ± 0.66	< 0.001	7.51 ± 0.09	n.s.[a]	0.001	6.76 ± 0.23	n.s.[a]	0.001
p-Quinones								
Benzoquinone (**16**)	11.41 ± 2.39	< 0.001	13.68 ± 1.34	0.000	n.s.[a]	14.28 ± 1.96	0.003	n.s.[a]
Methylbenzoquinone (**17**)	18.84 ± 1.57	< 0.001	21.54 ± 1.38	0.000	0.014	17.93 ± 2.85	0.002	n.s.[a]
2,5-Dimethylbenzoquinone (**18**)	15.04 ± 0.94	< 0.001	16.08 ± 1.71	0.001	n.s.[a]	15.91 ± 1.32	0.002	n.s.[a]
1,2,3-Triphenols								
Pyrogalol (**19**)	1.89 ± 0.79	n.s.[a]	3.28 ± 1.02	0.042	0.011	0.46 ± 0.26	0.000	0.001
Methyl gallate (**20**)	1.86 ± 0.38	n.s.[a]	2.75 ± 0.89	0.012	0.019	2.01 ± 0.43	0.000	n.s.[a]
Propyl gallate (**21**)	2.06 ± 0.28	n.s.[a]	3.33 ± 0.35	0.019	< 0.001	2.65 ± 0.72	0.001	0.043
1,2,4-Triphenols and analogues								
1,2,4-Trihydroxybenzene (**22**)	2.46 ± 0.50	n.s.[a]	2.58 ± 0.54	0.034	n.s.[a]	1.11 ± 0.44	0.000	< 0.001
Methoxyhydroquinone[c] (**23**)	2.37 ± 0.43	n.s.[a]	2.39 ± 0.09	0.022	n.s.[a]	1.88 ± 0.28	0.004	n.s.[a]
4-Methoxycatechol[c] (**24**)	3.06 ± 0.34	0.018	3.44 ± 1.02	0.034	n.s.[a]	2.68 ± 0.48	0.000	n.s.[a]
1,3,5-Triphenols								
Phloroglucinol (**25**)	1.76 ± 0.53	n.s.[a]	4.12 ± 0.04	n.s.[a]	< 0.001	0.93 ± 0.01	0.002	0.034
2-Phenyl-1-(2,4,6-trihydroxyphenyl)ethan-1-one (**26**)	0.28 ± 0.07	< 0.001	1.37 ± 0.08	0.031	< 0.001	0.28 ± 0.07	0.001	n.s.[a]

[a]Abbreviations: LOOH, 13-hydroperoxide of linoleic acid; n.s., not significant; ON, 4-oxo-2-nonenal. [b]This compound is not a *o*-diphenol. It was included in this group for comparison purposes. [c]This compound is not a 1,2,4-triphenol. It was included in this group for comparison purposes.

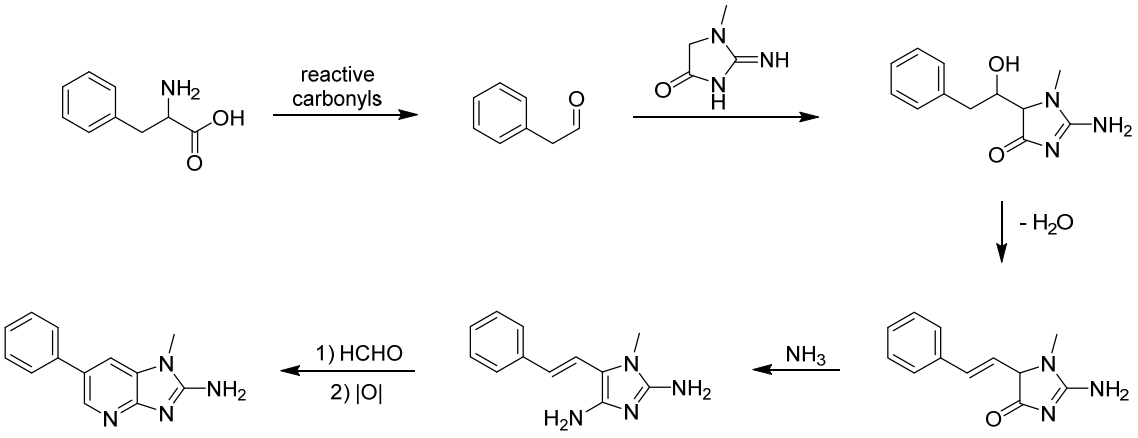


Figure 1

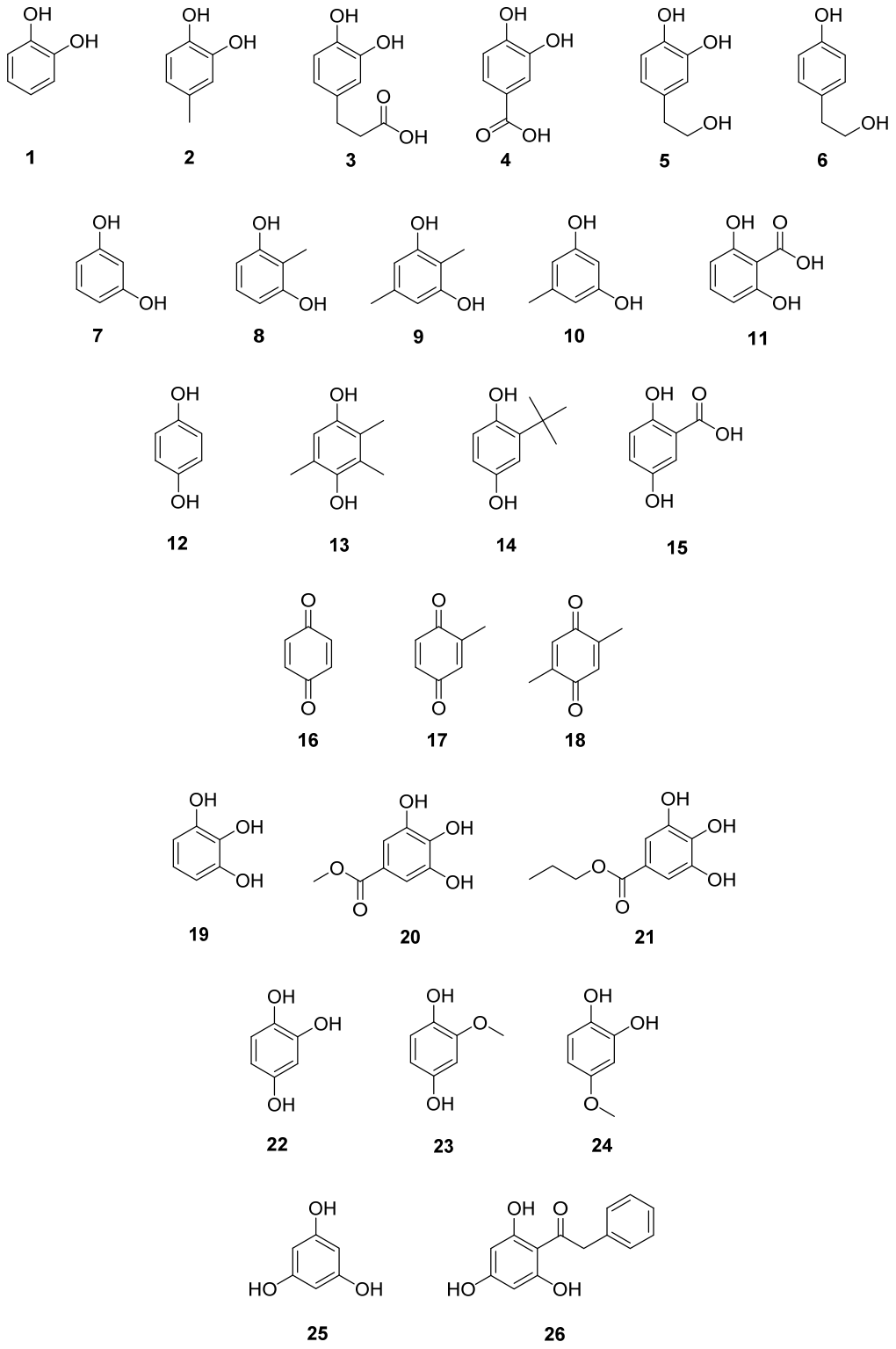
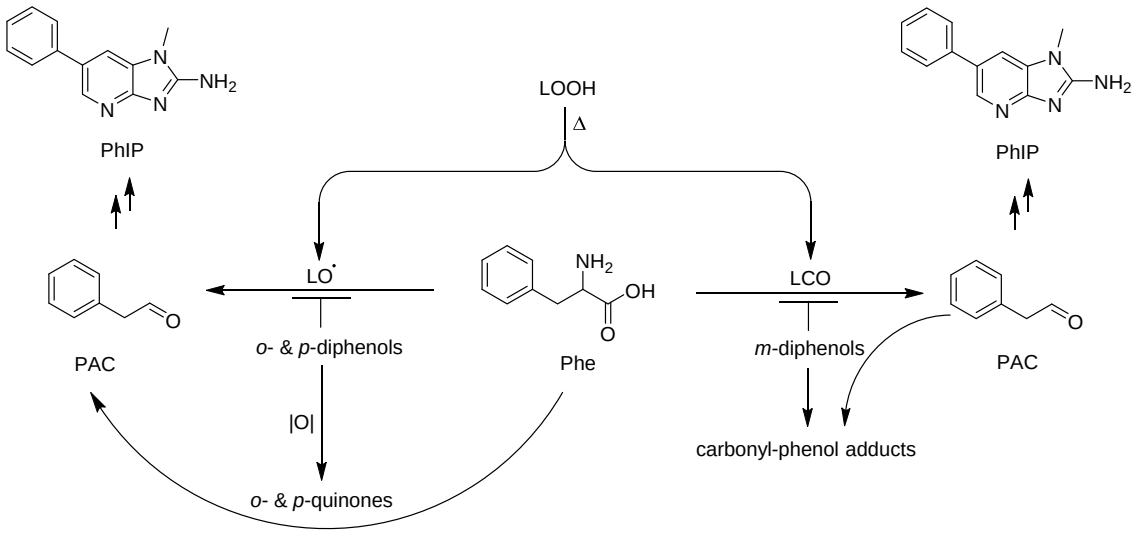


Figure 2


PhIP
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Δ
PhIP
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o- & p-diphenols
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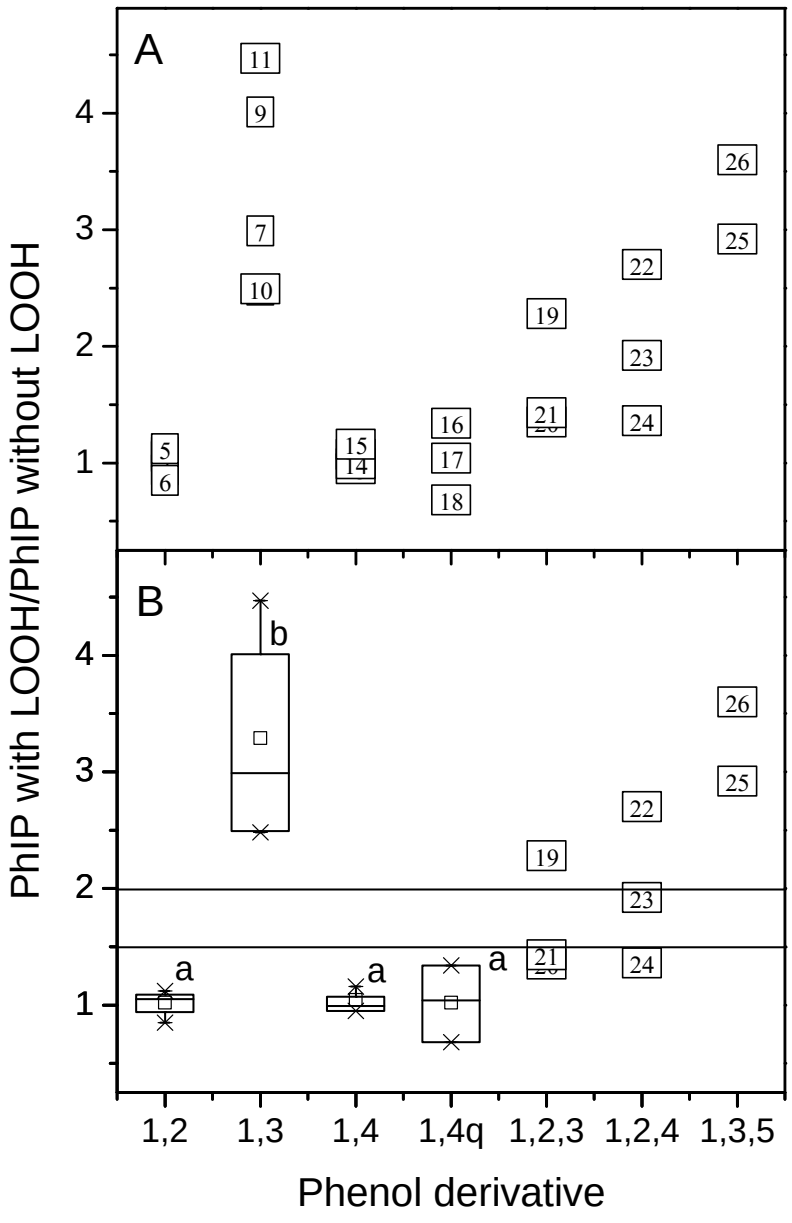
carbonyl-phenol adducts
o- & p-quinones

Figure 3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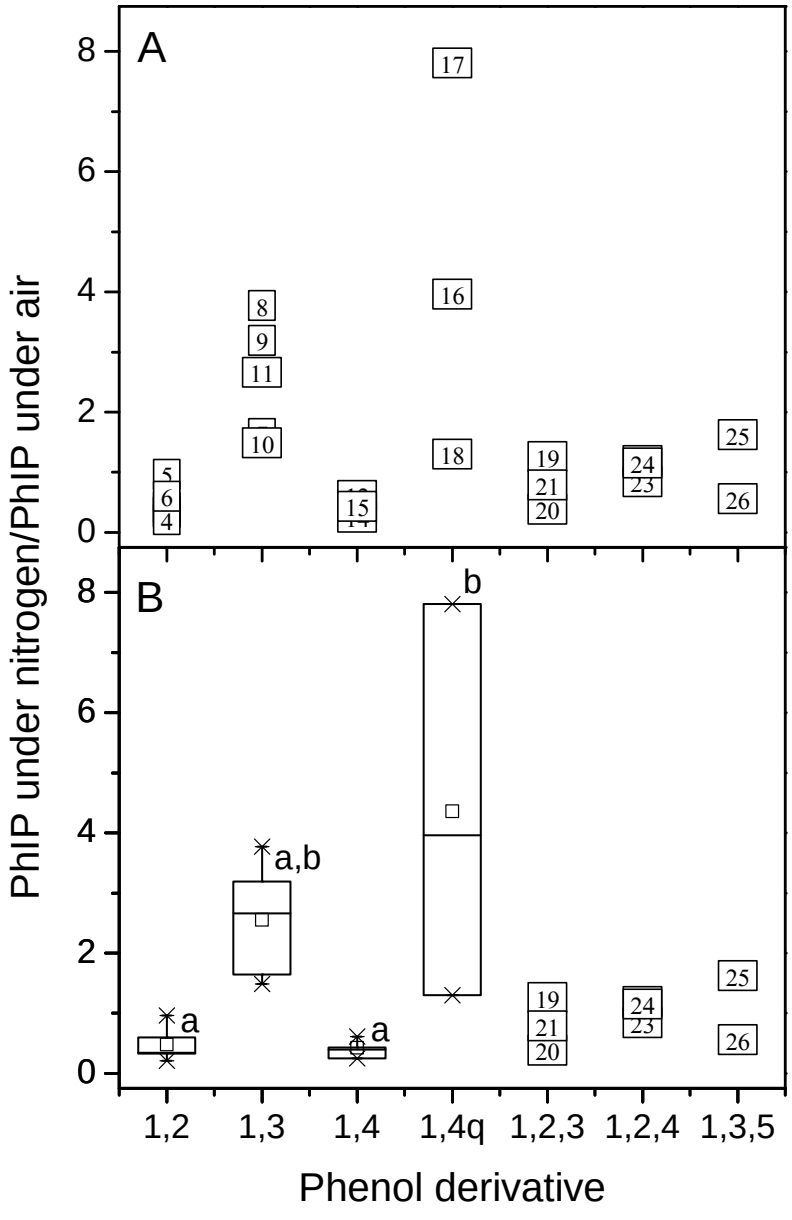
Phenol derivative

Figure 4


A
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PhIP under nitrogen/PhIP under air
Phenol derivative
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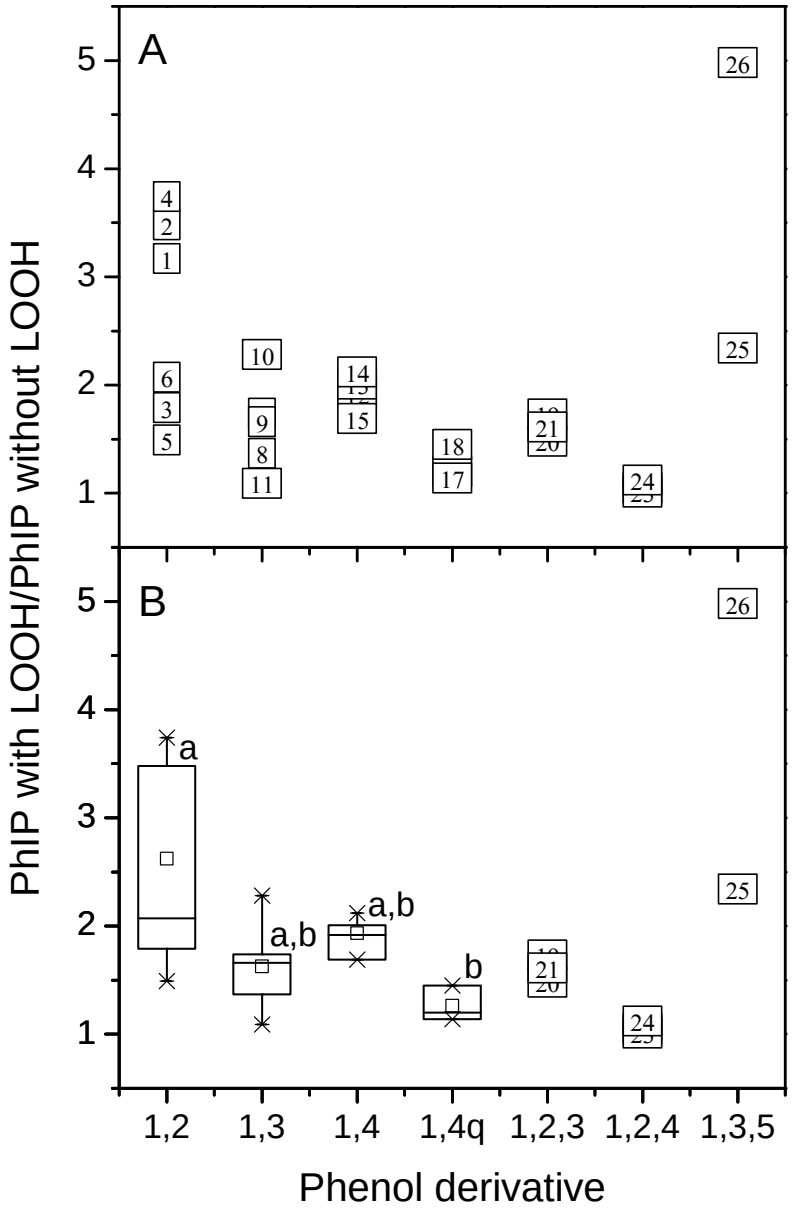
Figure 5


A
B
PhIP with LOOH/PhIP without LOOH
Phenol derivative
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a,b
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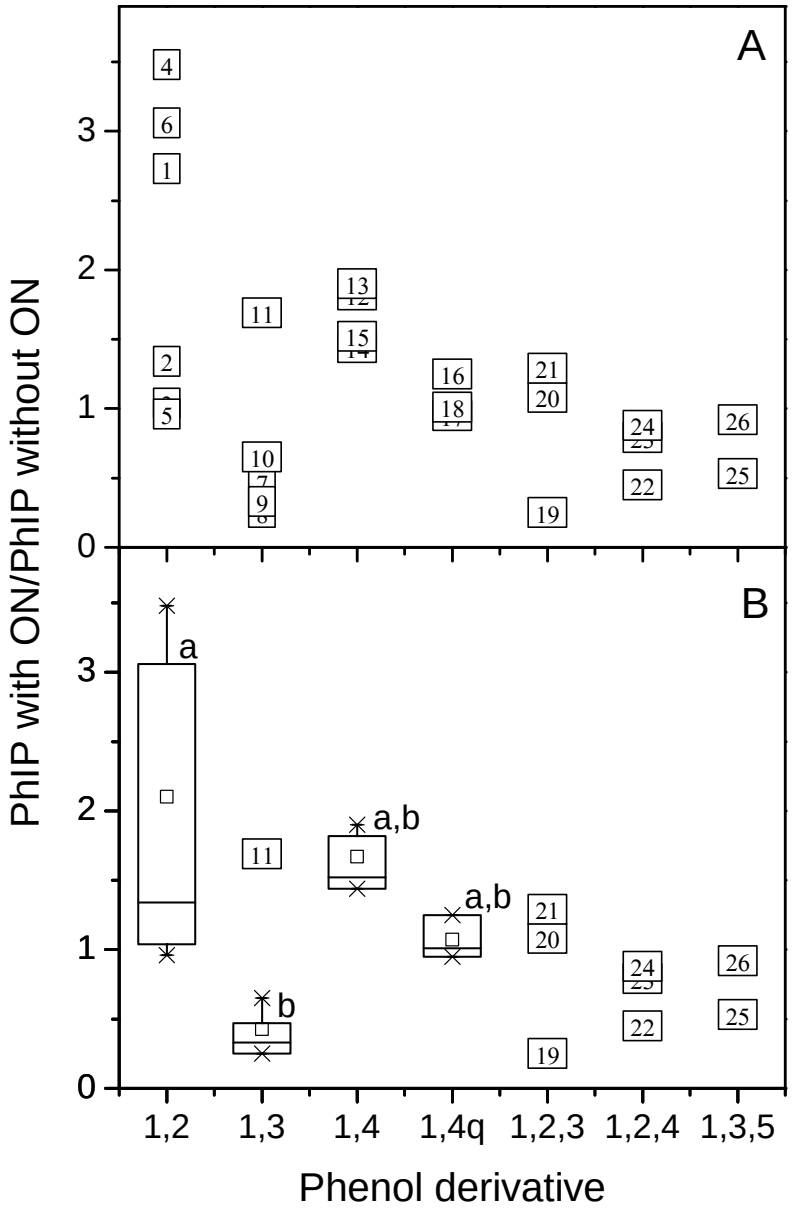
Figure 6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PhIP with ON/PhIP without ON
Phenol derivative
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1,4q
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1,3,5
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b
a,b
a,b

Figure 7

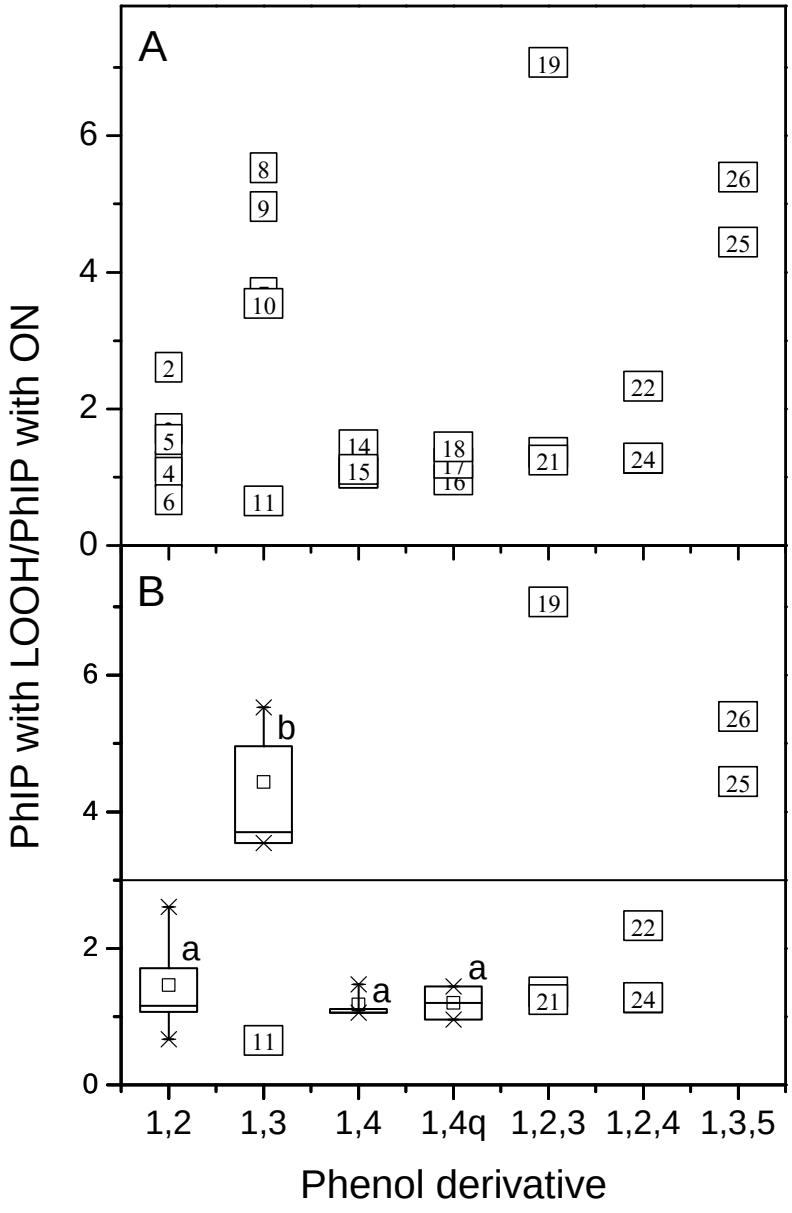
A
B
PhIP with LOOH/PhIP with ON
Phenol derivative
1,2
1,3
1,4
1,4q
1,2,3
1,2,4
1,3,5
a
b
a
a

Figure 8

GRAPHIC FOR TABLE OF CONTENTS