Ammonia and formaldehyde participate in the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in addition to creati(ni)ne and phenylacetaldehyde

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Abbreviated running title: Ammonia and formaldehyde participate in the formation of PhIP

13 ABSTRACT

14 The formation of formaldehyde from phenylacetaldehyde and phenylalanine, and the
15 contribution of both formaldehyde and ammonia to the production of PhIP from
16 phenylacetaldehyde and creatinine were studied in an attempt to clarify the reaction
17 pathways that produce PhIP. Formaldehyde was produced by thermal degradation of
18 phenylacetaldehyde and, to a lower extent, also by degradation of phenylalanine,
19 phenylethylamine, styrene, and creatinine. When formaldehyde was added to a mixture
20 of phenylacetaldehyde and creatinine, PhIP yield was multiplied by nineteen. When
21 formaldehyde and ammonia were simultaneously present, PhIP yield was multiplied by
22 fifty and the E_a of the reaction decreased by 61%. All these results point out to
23 formaldehyde and ammonia as the two additional reactants required for PhIP formation
24 from both phenylacetaldehyde/creati(ni)ne and phenylalanine/creati(ni)ne mixtures. A
25 general pathway for PhIP formation is proposed. This pathway is suggested to be the
26 main route for PhIP formation in foods.

27

28 *Keywords:* Ammonia; Formaldehyde; Heterocyclic aromatic amines; Maillard reaction;
29 Reactive carbonyls

30

1. Introduction

Heterocyclic aromatic amines (HAAs) are a group of heterocyclic amines containing from 2 to 5 (generally 3) condensed aromatic cycles with one or more nitrogen atoms and, usually, one exocyclic amino group (Alaejos & Afonso, 2011). They are formed in foods during the heating process. HAAs are usually grouped in thermic or pyrolitic HAAs depending on whether they are produced at temperatures lower or higher than 300 ºC, respectively. Among all of them, the thermic 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is one of the most abundant HAAs produced in foods. It is typically found at amounts up to 35 ng/g (Puangsombat, Gadgil, Houser, Hunt, & Smith, 2011), but there are reports of higher levels, especially in fried and barbecued chicken (Solyakov & Skog, 2002). This HAA has been shown to be carcinogenic in animal models and it is suspicious of increased risk in humans for different types of cancer (Barbir, Linseisen, Hermann, Kaas, Teucher, Eichholzer, & Rohrmann, 2012; Tang, Kryvenko, Wang, Trudeau, Rundle, Takahashi, Shirai, & Rybicki, 2013).

The mechanism by which PhIP is produced has been the objective of different studies, and nowadays it has been clearly demonstrated that PhIP is produced by reaction of phenylalanine with creati(ni)ne (Cheng, Wong, Cho, Chu, Sze, Lo, Chen, & Wang, 2008; Felton, Knize, Hatch, Tanga, & Colvin, 1999; Murkovic, 2004; Murkovic, Weber, Geiszler, Fröhlich, & Pfannhauser, 1999; Zöchling, & Murkovic, 2002). In addition, the presence of reactive carbonyls facilitates the reaction by converting phenylalanine into phenylacetaldehyde, which has been shown to be an intermediate in the reaction (Cheng, Wong, Cho, Chu, Sze, Lo, Chen, & Wang, 2008; Zöchling, & Murkovic, 2002). These reactive carbonyls were traditionally considered to proceed from carbohydrates (Murkovic, 2004; Wong, Cheng, & Wang, 2012; Zochling &

Murkovic, 2002), but recent studies have suggested that reactive carbonyls formed from either lipids (Zamora, Alcon, & Hidalgo, 2012; 2013a) or amino acids (Zamora, Alcon, & Hidalgo, 2013b) are also able to contribute to PhIP formation by converting phenylalanine into phenylacetaldehyde.

Differently to these widely accepted steps in PhIP formation, there are others that need further clarification. Thus, PhIP has 13 carbon atoms, but phenylacetaldehyde has 8 carbon atoms and creatinine has 4 carbon atoms. The origin of the additional carbon, and the way of how it is incorporated into the PhIP molecule has not been explained yet, although previous studies have demonstrated that it comes from the phenylalanine molecule but it is not the carboxylic carbon of the amino acid (Murkovic, Weber, Geiszler, Fröhlich, & Pfannhauser, 1999). In addition, PhIP has 4 nitrogen atoms, but phenylacetaldehyde has not nitrogen atoms and creatinine has only 3 nitrogen atoms. Previous studies have suggested that the additional nitrogen atom can proceed from the ammonia formed by either phenylalanine or creatinine thermal decomposition (Murkovic, 2004; Zamora, Alcon, & Hidalgo, 2013a), but the way by which ammonia is incorporated into the PhIP molecule is also unclear at present.

In an attempt to understand the reaction pathways by which PhIP is produced, this study investigated firstly the formation of formaldehyde by thermal decomposition of phenylacetaldehyde and, then, the contribution of both ammonia and formaldehyde to PhIP formation.

2. Materials and methods

2.1. Materials

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was purchased from Toronto Research Chemicals (North York, Ontario, Canada). 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. Formation of formaldehyde and other compounds during phenylacetaldehyde thermal heating

Phenylacetaldehyde (10 μmol) solutions in 500 μL of 0.3 M sodium phosphate buffer, pH 6, (or non-buffered distilled water) were heated at 200 ºC in closed test tubes under air for 1 h. At the end of the heating process, samples were cooled (5 min at room temperature and 10 min at –20 ºC), and the formation of formaldehyde and other compounds was studied by either LC-MS/MS or GC-MS.

For comparison purposes, formaldehyde formation as a consequence of thermal degradation of phenylalanine, phenylethylamine, styrene, benzaldehyde, and creatinine was also studied by LC-MS/MS. Reactions were carried out analogously to the described above but phenylacetaldehyde was substituted by the tested compound.

2.3. Formaldehyde formation by LC-MS/MS

Formaldehyde was identified by LC-MS/MS after derivatization with dansylhydrazine, following a procedure previously described for other carbonyl compounds (Hidalgo, Navarro, Delgado, & Zamora, 2013). The corresponding dansylhydrazone was formed by mixing 150 μL of the cooled solution with 50 μL of trifluoromethanesulfonic acid solution (3% in methanol), and 200 μL of dansylhydrazine solution (2 mg/mL in methanol). The resulting solution was kept at 25 ºC for 1 h and, then, diluted with 200 μL of eluent A (a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate), and analyzed by LC-MS/MS.

The employed equipment was composed by an Agilent liquid chromatography system (1200 Series) consisting of binary pump (G1312A), degasser (G1379B), and

104 autosampler (G1329A), connected to a triple quadrupole API 2000 mass spectrometer
105 (Applied Biosystems) using an electrospray ionization interface in positive ionization
106 mode (ESI^+). Compounds were separated on a Zorbax Eclipse XDB-C18 (150 mm x 4.6
107 mm, 5 μm) column from Agilent. As eluent A, a 30:70 mixture of 0.2% formic acid in
108 acetonitrile and 4 mM ammonium acetate was used. As eluent B, a 0.2% formic acid
109 solution in acetonitrile was employed. The mobile phase was delivered at 0.5 mL/min in
110 isocratic mode using 50% B. 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 40, 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 formaldehyde and mass spectrometric conditions were
119 optimized by using infusion with a syringe pump. Transitions used for formaldehyde
120 identification in addition to its retention time were 278.1→171.1, 278.1→115.2, and
121 278.1→156.1. Operating conditions for detection of these transitions were: DP
122 (declustering potential), 26; FP (focusing potential), 370; EP (entrance potential), 10.5;
123 CEP (collision cell entrance potential), 20; CE (collision energy), 33, 67, and 47,
124 respectively, for the three transitions; and CXP (collision cell exit potential), 6, 4, and 6,
125 respectively, for the three transitions.

126 A semi-quantitative determination of formaldehyde was obtained by using
127 formaldehyde solutions in water as external standard. Thus, solutions of formaldehyde
128 (0–50 nmol) in 500 μL of distilled water were derivatized with dansylhydrazine

following the procedure described above. These samples were injected after the tested samples in the same set of samples. The peak areas for formaldehyde standards were directly proportional to the contents of formaldehyde ($r = 0.99$, $p < 0.01$). These data were employed to estimate formaldehyde content in the tested samples.

2.4. Study of phenylacetaldehyde degradation products by GC-MS

The formation of other products as a consequence of phenylacetaldehyde degradation in addition to formaldehyde was studied by GC-MS. 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 one microliter 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 ºC; oven temperature programmed from 40 ºC (1 min) to 240 ºC at 5 ºC/min and then to 300 ºC at 10 ºC/min; transfer line to MSD, 280 ºC; ionization EI, 70 eV, ion source temperature, 230 ºC, and mass range 28-550 amu.

2.5. PhIP formation in creatinine/phenylacetaldehyde/formaldehyde/ammonia reaction mixtures

A solution of creatinine (10 μmol), phenylacetaldehyde (10 μmol), formaldehyde (0–20 μmol), and/or ammonia (0–20 μmol) in 500 μL of 0.3 M buffer was heated at 180–210 ºC in closed test tubes under air for 0–1 h. The following buffers were employed: sodium citrate for pH 2.15–6, sodium phosphate for pH 6–8 and 11–12, and sodium borate for pH 8–10.

After cooling (5 min at room temperature and 10 min at –20ºC), 100 μL of the reaction mixture was diluted with 50 μL of internal standard solution (1.29 mg of

153 caffeine in 5 mL of a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM
154 ammonium acetate), and 850 μL of a 30:70 mixture of 0.2% formic acid in acetonitrile
155 and 4 mM ammonium acetate. PhIP was determined by LC-MS/MS.

156 *2.6. PhIP determination*

157 Samples were analyzed by using the same equipment and column previously
158 described for formaldehyde determination. As eluent, a 30:70 mixture of 0.2% formic
159 acid in acetonitrile and 4 mM ammonium acetate was used. The mobile phase was
160 delivered at 0.5 mL/min in isocratic mode. Mass spectrometric acquisition was
161 performed by using multiple reaction monitoring (MRM). The nebulizer gas (synthetic
162 air), the curtain gas (nitrogen), and the heater gas (synthetic air) were set at 45, 25, and
163 50 (arbitrary units), respectively. The collision gas (nitrogen) was set at 5 (arbitrary
164 units). The heater gas temperature was set at 500 ºC and the electrospray capillary
165 voltage to 5.5 kV. The fragment ions in MRM mode were produced by collision-
166 activated dissociation of selected precursor ions in the collision cell of the triple
167 quadrupole and analyzed the selected products with the second analyzer of the
168 instrument. Three transitions were acquired for the identification of both PhIP and the
169 IS. To establish the appropriate MRM conditions for the individual compounds, the
170 mass spectrometric conditions were optimized by using infusion with a syringe pump to
171 select the most suitable ion transitions for the target analytes. Precursor and product
172 ions used for confirmation purposes and operating conditions were described previously
173 (Zamora, Alcon, & Hidalgo, 2012). The 225.0 → 210.1 and 195.2 → 138.0 transitions
174 for PhIP and caffeine, respectively, were used for quantification purposes in this study.

175 Quantification of PhIP was carried out by preparing five standard curves of this
176 compound in 500 μL of 0.3M sodium phosphate buffer, pH 8, and following the whole
177 procedure described above. For each curve, seven different concentration levels of PhIP

(0–2 nmol) were used. PhIP content was directly proportional to the PhIP/IS 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 quantificated with a precision less than 15%, was 0.01 nmol.

2.7. Statistical analysis

All data given are mean or 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.7.0220 (OriginLab Corporation, Northampton, MA). The significance level is $p < 0.05$ unless otherwise indicated.

3. Results

3.1. Formation of formaldehyde and other products as a consequence of phenylacetaldehyde thermal heating

When phenylacetaldehyde was heated at 200 ºC for 1 h, a small amount of this aldehyde was decomposed and a number of new products were formed. Among them, the formation of formaldehyde was identified by LC-MS/MS after derivatization with dansylhydrazine. Although the employed water had traces of formaldehyde, when phenylacetaldehyde was heated at 200 ºC for 1 h in water, the formation of formaldehyde could be easily confirmed because the area of this peak increased one order of magnitude. The amount of formaldehyde formed after heating 1 h at 200 ºC in water was 2 nmol per μmol of phenylacetaldehyde (0.2%). This value was higher when

phenylacetaldehyde was heated in 0.3 M sodium phosphate buffer, pH 6. The formaldehyde formed using phosphate buffer in the place of water was 8 nmol per μmol of phenylacetaldehyde (0.8%). Analogously to phenylacetaldehyde, formaldehyde was also produced when heating phenylethylamine, although to a lower extent than when heating phenylacetaldehyde. In addition, formaldehyde was also formed in solutions of phenylalanine and styrene, which were worse formaldehyde producers than either phenylacetaldehyde or phenylethylamine. The heating of creatinine solutions also produced formaldehyde, although to a very low extent. On the other hand benzaldehyde solutions did not produce formaldehyde when heated at 200 ºC for 1 h.

In addition to formaldehyde, other compounds were also formed when heating solutions of phenylacetaldehyde in 0.3 sodium phosphate buffer, pH 6. The compounds detected by GC-MS were benzaldehyde, 1-phenylethanol, phenylacetic acid, and a compound of molecular weight 222 amu, which was tentatively identified as 2,4-diphenylbut-3-enal based on its mass spectra. Identification of other compounds was carried out by both retention indexes and mass spectra.

3.2. PhIP formation in creatinine/phenylacetaldehyde/ammonia/formaldehyde reaction mixtures

When creatinine and phenylacetaldehyde were heated together, PhIP was produced to a significant extent (Fig. 1). However, the presence of either ammonia (A) or formaldehyde (F) increased ($p < 0.05$) the amount of PhIP by three and nineteen times, respectively. Furthermore, when formaldehyde and ammonia were added simultaneously (F+A), the amount of produced PhIP increased fifty times in comparison to the PhIP produced in creatinine/phenylacetaldehyde mixtures.

These values depended on the amount of formaldehyde and ammonia added as well as on the pH of the reaction. Fig. 2A shows the effect of reaction pH on the amount of PhIP produced in creatinine/phenylacetaldehyde/formaldehyde reaction mixtures. As observed in the figure, PhIP was mostly produced at pH 5-8 with a maximum at pH 6-7. A pH value of 6, using sodium phosphate buffer, was used in this study.

When formaldehyde was added to a mixture of 10 μmol of creatinine and 10 μmol of phenylacetaldehyde, the amount of PhIP formed increased very rapidly until 5 μmol of formaldehyde was added, then remain constant until 7.5 μmol of formaldehyde, and decreased afterwards (Fig. 2B). Differently to the addition of formaldehyde, the addition of ammonia increased much more slowly the formation of PhIP and the maximum of PhIP formed was produced with the addition of 20 μmol of ammonia (Fig. 2B). Therefore, 5 μmol of formaldehyde and 20 μmol of ammonia were selected to be added to 10 μmol of creatinine and 10 μmol of phenylacetaldehyde in the different reaction mixtures analyzed in the rest of this study.

PhIP formation also depended on the time and temperature of the reaction. Fig. 3A shows that PhIP increased linearly as a function of time in creatinine/phenylacetaldehyde reaction mixtures between 180 and 210 ºC. However, PhIP was not formed from the initial time, and a lag time was observed. This lag time usually decreased when temperature increased. The lag time observed at 200 ºC for this binary system was 13.4 min. Reaction rates were higher when temperature increased, and they were calculated by using the equation:

$$[\text{PhIP}] = [\text{PhIP}]_0 + kt$$

where $[\text{PhIP}]_0$ represents the intercept, k is the rate constant, and t is the time. These rate constants were used in an Arrhenius plot for the calculation of the activation energy (E_a)

of PhIP formation in creatinine/phenylacetaldehyde mixtures (Fig. 4). The determined E_a was 136.2 kJ/mol.

Analogous behavior was observed when formaldehyde was present in the reaction mixtures, but much higher amounts of PhIP were produced (Fig. 3B). A lag time for the formation of PhIP was also observed in creatinine/phenylacetaldehyde/formaldehyde reaction mixtures, but it was lower than the observed in creatinine/phenylacetaldehyde reaction mixtures. The lag time observed at 200 ºC for the ternary system containing formaldehyde was 9.1 min. By employing an Arrhenius plot (Fig. 4), the E_a of the reaction could be determined and resulted to be 132.2 kJ/mol. This value was very similar to that obtained for creatinine/phenylacetaldehyde reaction mixtures.

The change of formaldehyde by ammonia resulted in a considerable decrease of the PhIP produced (Fig. 3C), but the behavior of this system was analogous to the other above described systems. The lag time observed for creatinine/phenylacetaldehyde/ammonia system heated at 200 ºC was 9.2 min. By employing an Arrhenius plot (Fig. 4), the E_a of the reaction could be determined and resulted to be 95.7 kJ/mol, lower than those values obtained for creatinine/phenylacetaldehyde and creatinine/phenylacetaldehyde/formaldehyde reaction mixtures.

Finally, the simultaneous addition of formaldehyde and ammonia to creatinine and phenylacetaldehyde resulted in the formation of large amounts of PhIP (Fig. 3D), and the reduction of both the lag time (4.1 min at 200 ºC) and the E_a (53.44 kJ/mol).

4. Discussion

A number of studies have been focused to investigate the reaction pathways by which PhIP is produced. Thus, Manabe, Kurihara, Wada, Tohyama, & Aramaki (1992)

found that PhIP was produced by aqueous heating of a mixture of creatinine, phenylalanine, and sugar or aldehyde, which suggested a role for reactive carbonyls in the reaction. Later, Murkovic's group postulated the role of phenylacetaldehyde as an intermediate in the reaction by demonstrating the formation of PhIP from phenylacetaldehyde and creatinine (Murkovic, Weber, Geiszler, Fröhlich, & Pfannhauser, 1999), and by isolating the aldol condensation product between phenylacetaldehyde and creatinine (Zöchling & Murkovic, 2002). However, the later steps needed to complete the PhIP molecule are still unclear.

The results obtained in the present study allow completing the puzzle, firstly by identifying additional compounds that also take part in the reaction and then by explaining how the different compounds can react among them. The most important missing compound was formaldehyde. The results obtained in the present study have shown that formaldehyde is produced from phenylacetaldehyde and also, to a lower extent, by phenylethylamine, phenylalanine, styrene, and creatinine heating. Formaldehyde is suggested to be produced according to the pathway shown in Fig. 5. Although phenylacetaldehyde (**1**) has an α-hydrogen and the aldol condensation (*route B*) is favored over the disproportionation reaction (*route A*), *route A* should also be produced to a certain small extent. Thus, the formation of the condensed aldol product (**11**), phenylacetic acid (**2**) and phenylethanol (**3**) was observed by GC-MS. This last alcohol should be easily dehydrated under the reaction conditions to produce styrene (**4**) (*route A1*). This alkene, which could not be detected in our system, is a well known source of formaldehyde (**7**) by oxidation (see, for example, Fukuzumi, Mizuno, & Ojiri, 2012). As a byproduct of this reaction, benzaldehyde (**6**) would be produced. This last compound was also detected in our reaction mixtures. In addition, this reaction pathway can also explain the observed formation of formaldehyde from phenylalanine,

298 phenylethylamine, and styrene. Thus, both phenylacetaldehyde and styrene are
299 produced by thermal degradation of phenylalanine (Hidalgo & Zamora, 2007) and
300 phenylethylamine elimination produces the corresponding styrene as shown for other
301 amines (Zamora, Delgado, & Hidalgo, 2009). The only data that does not seem to agree
302 with this *route A1* as the unique pathway for formaldehyde production is that styrene
303 produced less formaldehyde than phenylacetaldehyde according to the results obtained
304 in this study.

305 As an alternative route (*route A2*) for formaldehyde production, the degradation of
306 the alcohol (**3**) might be produced in the presence of oxygen to produce small amounts
307 of a hydroperoxide (**8**) with one carbon lesser than the original alcohol, at the same time
308 that formaldehyde is formed. The well known rearrangement of hydroperoxides (see,
309 for example, Pratt, Tallman, & Porter, 2011), would be the responsible for the formation
310 of phenol (**9**) and formaldehyde (**7**) as a major compounds, but the formation of
311 benzaldehyde (**6**) might also be analogously produced. Although phenol (**9**) could not
312 be unequivocally identified in our chromatograms, trace amounts of this compound
313 might be present because some characteristic fragments of this compound at the
314 appropriate retention index were found. In addition, this route would explain the
315 formation of formaldehyde from phenylalanine and phenylethylamine, although to a
316 lower extent than from phenylacetaldehyde because they should be converted first into
317 phenylacetaldehyde (Zamora, Alcón, & Hidalgo, 2013c; Zamora, Delgado, & Hidalgo,
318 2012).

319 Once formaldehyde and ammonia have been produced, the reaction of these
320 compounds with creatinine and phenylacetaldehyde is suggested to be produced as
321 indicated in Fig. 6. According to Zöchling & Murkovic (2002), the first steps of the
322 reaction are the formation of the condensation product **12** between creatinine and

323 phenylacetaldehyde. Thus, phenylacetaldehyde (**1**) and creatinine (**10**) would react to
324 produce the corresponding aldol product (**11**) which lately would loss water to yield the
325 corresponding conjugated olefin (**12**). This adduct would then react with ammonia and
326 formaldehyde according to the indicated scheme (Fig. 6) to produce PhIP. Firstly, the
327 reaction of compound **12** with ammonia would produce the corresponding imine (**13**),
328 which later would evolve the amine (**14**) by tautomerization. This amine (**14**) would
329 then react with formaldehyde to produce the corresponding imine (**15**) which, after
330 electronic rearrangement (**16**), oxidation (**17**), and tautomerization, would be the origin
331 of PhIP (**18**). Ring closures analogous to that suggested in this pathway have been
332 observed, for example, in edible oils heated at high temperatures (Destaillats & Angers,
333 2005).

334 This reaction pathway explains the results obtained in this and in previous studies.
335 Thus, previous assays carried out using ^{13}C-labelled phenylalanine showed that the new
336 carbons in the pyridine ring of PhIP came from phenylalanine (Murkovic, Weber,
337 Geiszler, Fröhlich, & Pfannhauser, 1999). The proposed mechanism is in agreement
338 with this because two of the new carbons belong to phenylacetaldehyde and the third
339 one, which belongs to formaldehyde, is also produced by phenylalanine or
340 phenylacetaldehyde thermal decomposition. In addition, it also agrees that ammonia can
341 proceed from either phenylalanine or creatinine (Murkovic, 2004), because it is
342 ammonia and not the original compound what takes part in the reaction.

343 These reaction pathways are also in agreement with the obtained results showing that
344 reaction yields increased considerably with the addition of formaldehyde and
345 formaldehyde plus ammonia to the creatine/phenylacetaldehyde reaction mixtures, and,
346 to a lesser extent, with the addition of ammonia (Fig. 1). In addition, they also agree
347 with the reduction of lag times and E_a observed when formaldehyde and ammonia were

348 present (Figs. 3 and 4). Thus, the addition of the compounds needed to produce PhIP
349 should increase the reaction yield and reduce the lag time because no previous reactions
350 are required. In addition, reaction yields were higher because formaldehyde and
351 ammonia were added to a higher extent than they are produced in the reaction mixtures
352 by decomposition of phenylacetaldehyde or creatinine. Furthermore, lag times were
353 reduced because reaction was simplified. Thus, the lag time for PhIP formation was
354 reduced from 13.4 min for the creatinine/phenyacetaldehyde reaction mixtures heated at
355 200 ºC to 4.1 min for creatinine/phenylacetaldehyde/ammonia/formaldehyde mixtures
356 heated at the same temperature, and to 2.9 min when this quaternary system was heated
357 at 210 ºC. These data also support that no other reactants are needed. Although a small
358 lag time was always observed, the small lag time observed also in the quaternary system
359 at high temperature is likely a consequence of the time required for the reaction to reach
360 enough temperature so that PhIP can be produced.

361 Although the concentration of formaldehyde seems to be the limiting step of the
362 reaction to produce PhIP to a high extent (Fig. 1), the E_a of the reaction for
363 creatine/phenylacetaldehyde and creatinine/phenylacetaldehyde/formaldehyde systems
364 were very similar. On the contrary, the presence of ammonia decreased E_a by 30% and
365 the simultaneous presence of ammonia and formaldehyde decreased E_a by 61%. Only in
366 the presence of the four reactants, the E_a of the reaction was low enough to suggest that
367 PhIP could be produced to a significant extent without a considerable heating.

368 Differently to the model systems described in this study, formaldehyde and ammonia
369 can be formed in foods by alternative pathways. Therefore, the content of these new
370 reactants, or of the compounds from which they could be produced, should also be
371 considered to understand the amount in which PhIP is produced in food products. To
372 this respect, formaldehyde content ranges from 8–20 mg/kg in meats and 1–98 mg/kg in

fish (World Health Organization, 1989). In addition, all these results also suggest that PhIP content should be reduced if the amount of formaldehyde either present or produced in a food product could be controlled.

Although the existence of other alternative pathways that could also produce PhIP to some extent cannot be discarded, the very big increases (~50 times) observed in the PhIP formed when formaldehyde and ammonia were present and the fact that E_a for the reaction after adding both formaldehyde or ammonia was much reduced, suggest that the pathway described in this study is likely the main route for PhIP formation. Therefore, the formation of PhIP in foods seems to be produced from phenylalanine and creati(ni)ne according to the following general route. The first step is the Strecker degradation of the amino acid to produce phenylacetaldehyde, a reaction that is favored in the presence of reactive carbonyls. Then, phenylacetaldehyde reacts with creati(ni)ne to produce the condensed aldol product **12**. At the same time, the degradation of phenylalanine, phenylacetaldehyde, and creati(ni)ne are responsible for the formation of the ammonia and formaldehyde required to complete the PhIP molecule. Alternatively, and depending on the composition of the food product, ammonia and formaldehyde might also be formed by other routes from other food components. Finally, the reaction between the condensed aldol product **12**, ammonia, and formaldehyde according to the scheme shown in Fig. 6, is the reason by which PhIP is formed.

Abbreviations used

HAAs, heterocyclic aromatic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

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Figure legends

Fig. 1. PhIP formation in equimolecular creatinine/phenylacetaldehyde (Co), creatinine/phenylacetaldehyde/formaldehyde (F), creatinine/phenylacetaldehyde/ammonia (A), and creatinine/phenylacetaldehyde/formaldehyde/ammonia (F+A), reaction mixtures heated in 0.3 M sodium phosphate buffer, pH 6, for 1 h at 200 ºC. Significance: *, $p < 0.05$ *vs.* control; **, $p < 0.01$ *vs.* control.

Fig. 2. Effect of: A, reaction pH, and B, reactant concentration, on the PhIP produced in creatinine/phenylacetaldehyde/formaldehyde (○) or creatinine/phenylacetaldehyde/ammonia (◇) reaction mixtures heated in 0.3 M sodium phosphate buffer, pH 6, for 1 h at 200 ºC. Reaction mixtures contained 10 μmol of creatinine and 10 μmol of phenylacetaldehyde. Abbreviations: A, ammonia; F, formaldehyde.

Fig. 3. Effect of time and temperature on the formation of PhIP in: A, creatinine/phenylacetaldehyde; B, creatinine/phenylacetaldehyde/formaldehyde; C, creatinine/phenylacetaldehyde/ammonia; and D, creatinine/phenylacetaldehyde/formaldehyde/ammonia reaction mixtures. Samples were heated in 0.3 M sodium phosphate buffer, pH 6, for the indicated times at 180 (○), 190 (△), 200 (▽), and 210 (◇) ºC.

Fig. 4. Arrhenius plot for creatinine/phenylacetaldehyde (□), creatinine/phenylacetaldehyde/formaldehyde (○), creatinine/phenylacetaldehyde/ammonia (◇), and creatinine/phenylacetaldehyde/formaldehyde/ammonia (△) reaction mixtures.

Fig. 5. Proposed pathways for formaldehyde formation by phenylacetaldehyde thermal degradation.

Fig. 6. Proposed pathway for PhIP formation from phenylacetaldehyde, creatinine, ammonia, and formaldehyde.

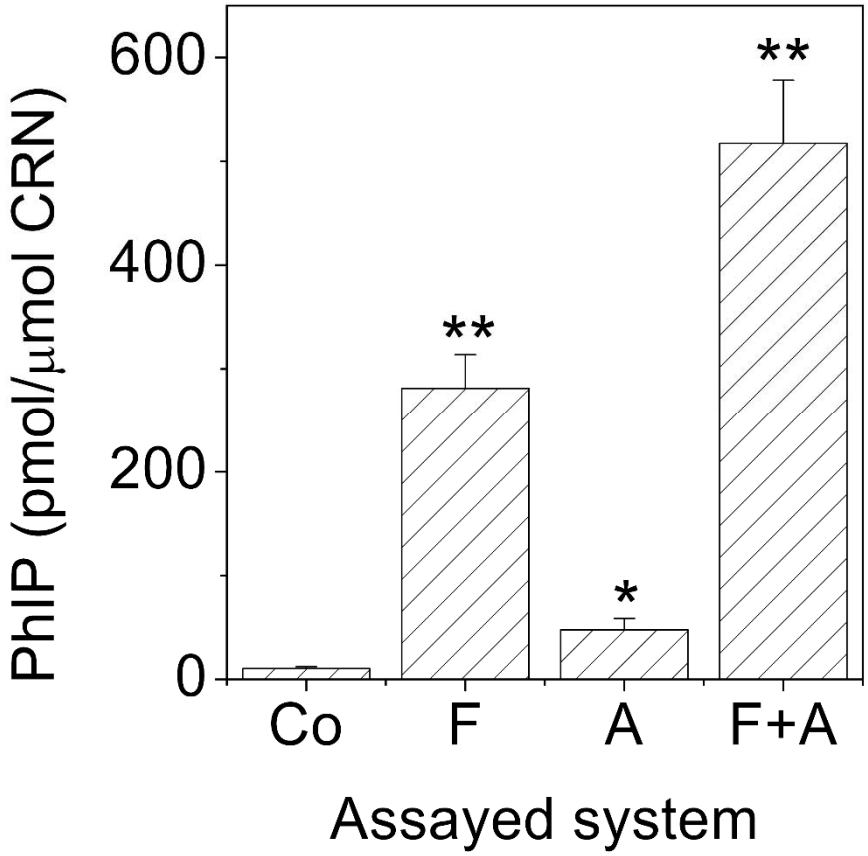

Figure 1

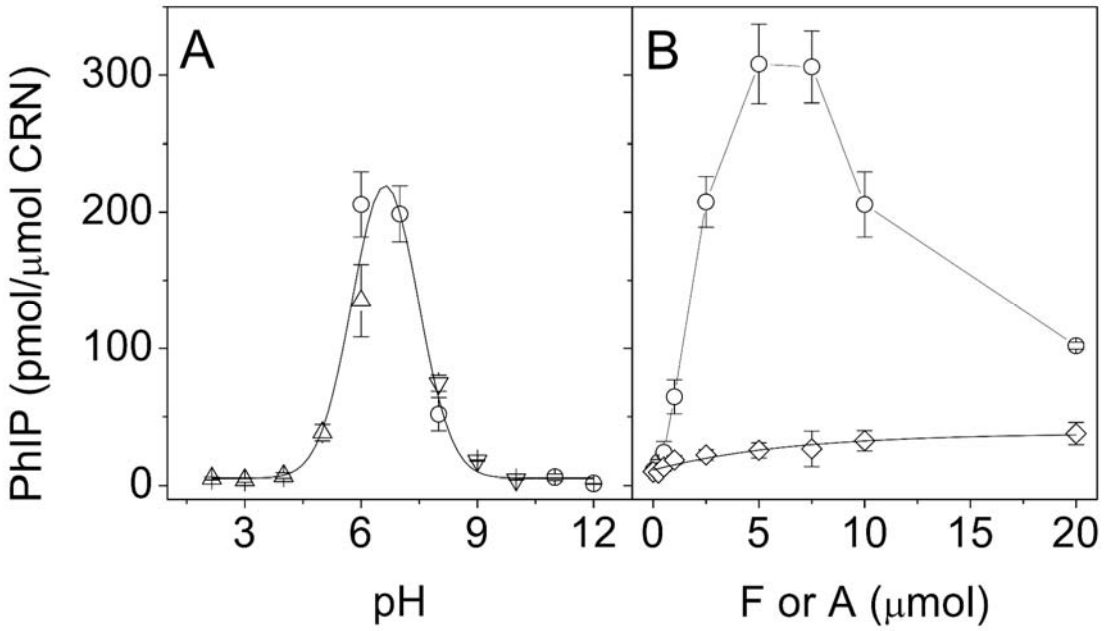
A
B
PhIP (pmol/μmol CRN)
300
200
100
0
3
6
9
12
pH
0
5
10
15
20
F or A (μmol)

Figure 2

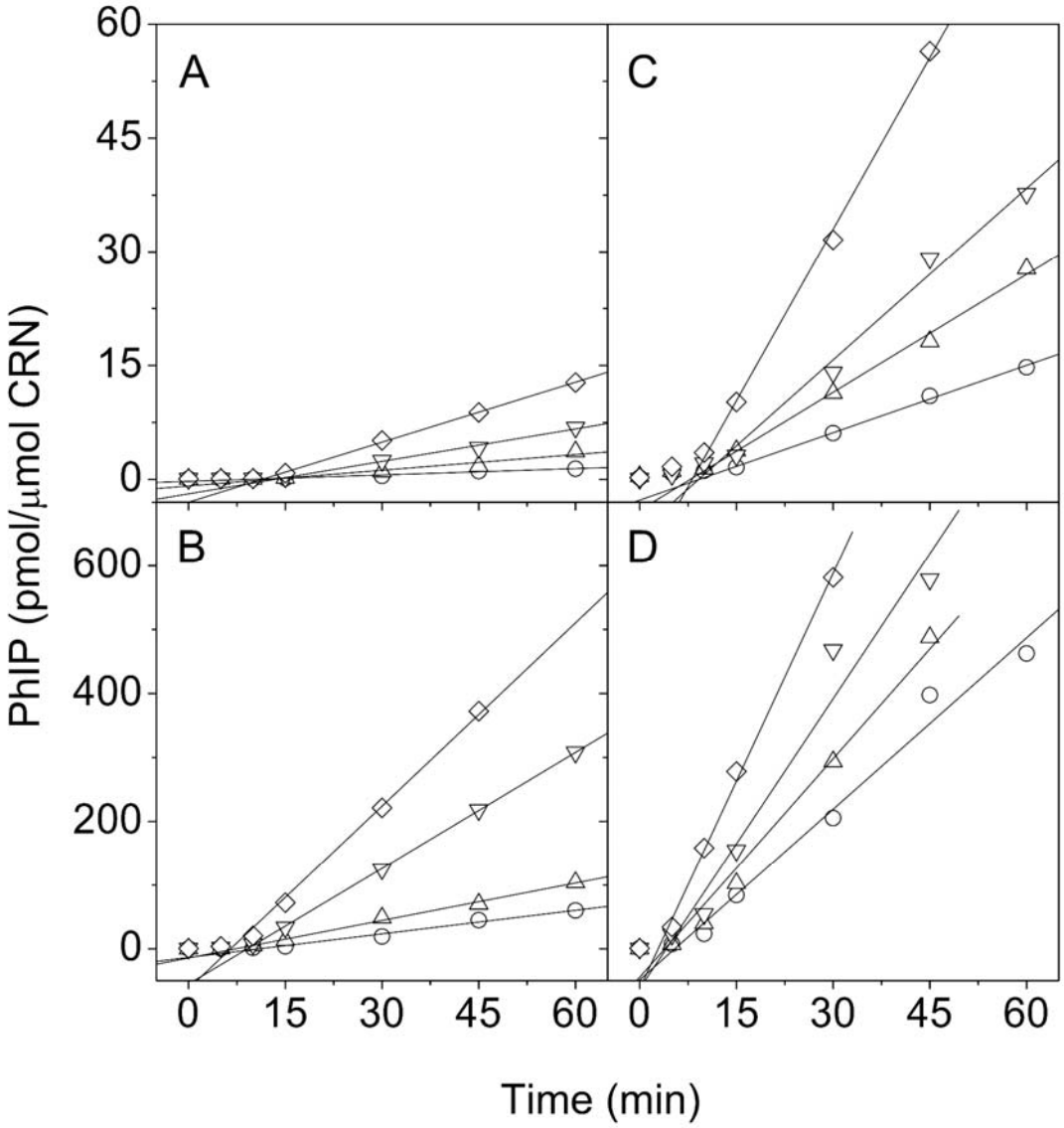

Figure 3

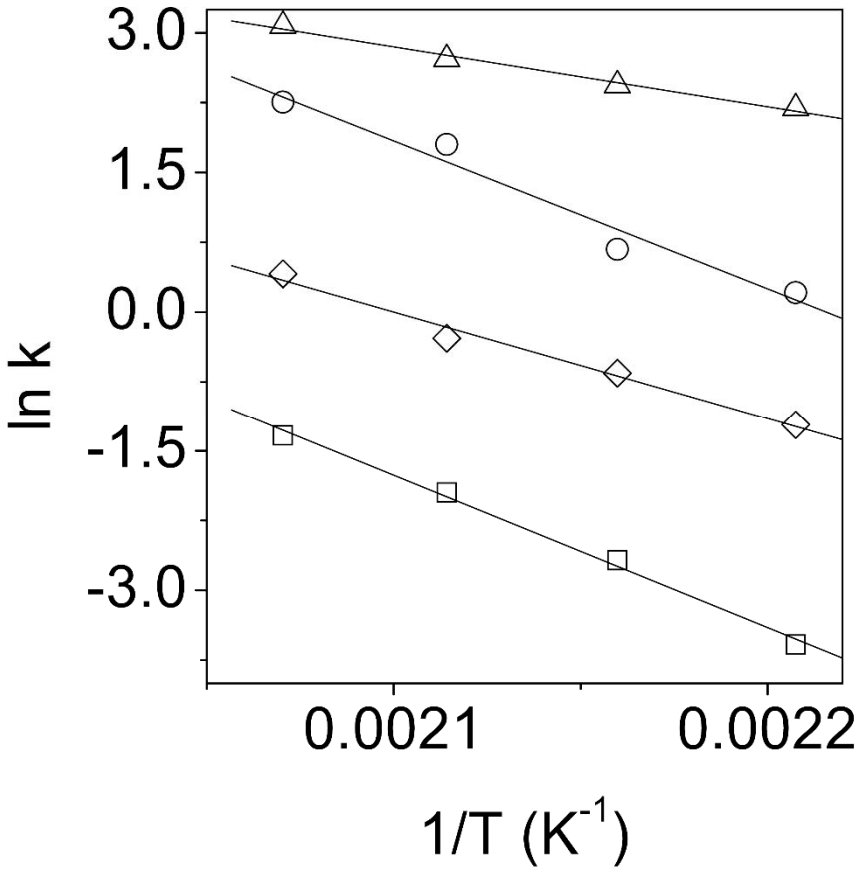

Figure 4

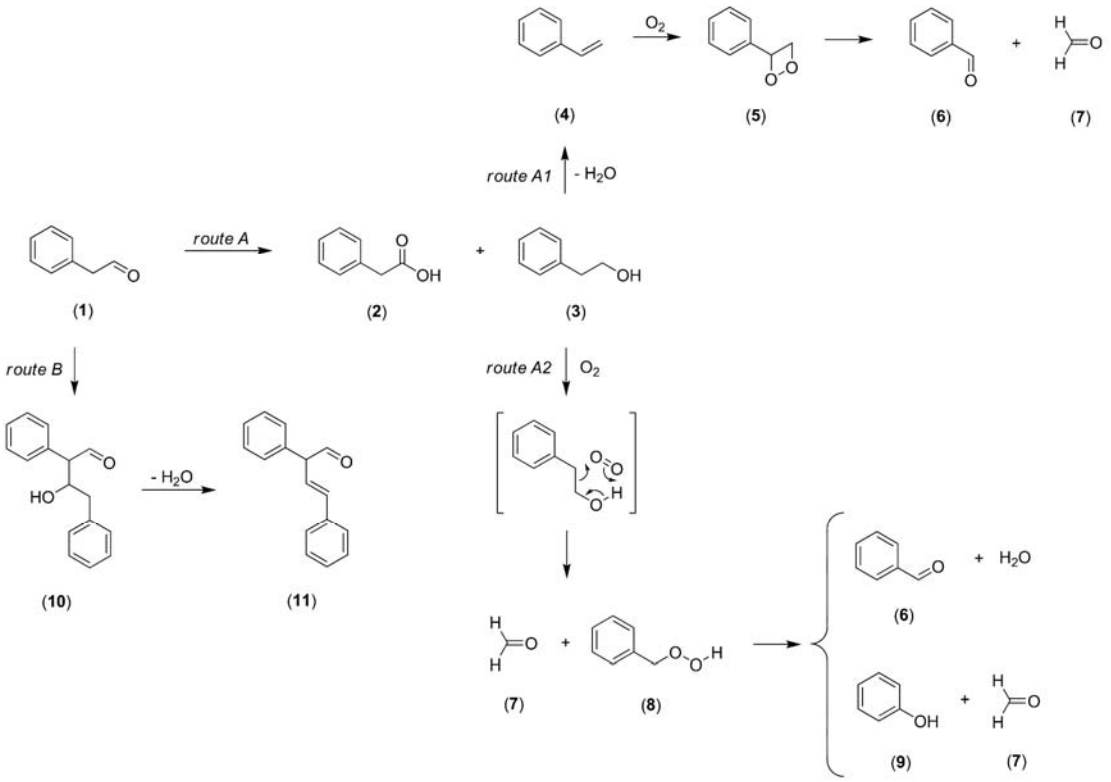
O2
(4)
(5)
(6)
(7)
route A1
- H2O
route A
(1)
(2)
(3)
route B
route A2
O2
- H2O
(10)
(11)
(7)
(8)
+ H2O
(6)
(9)
(7)

Figure 5

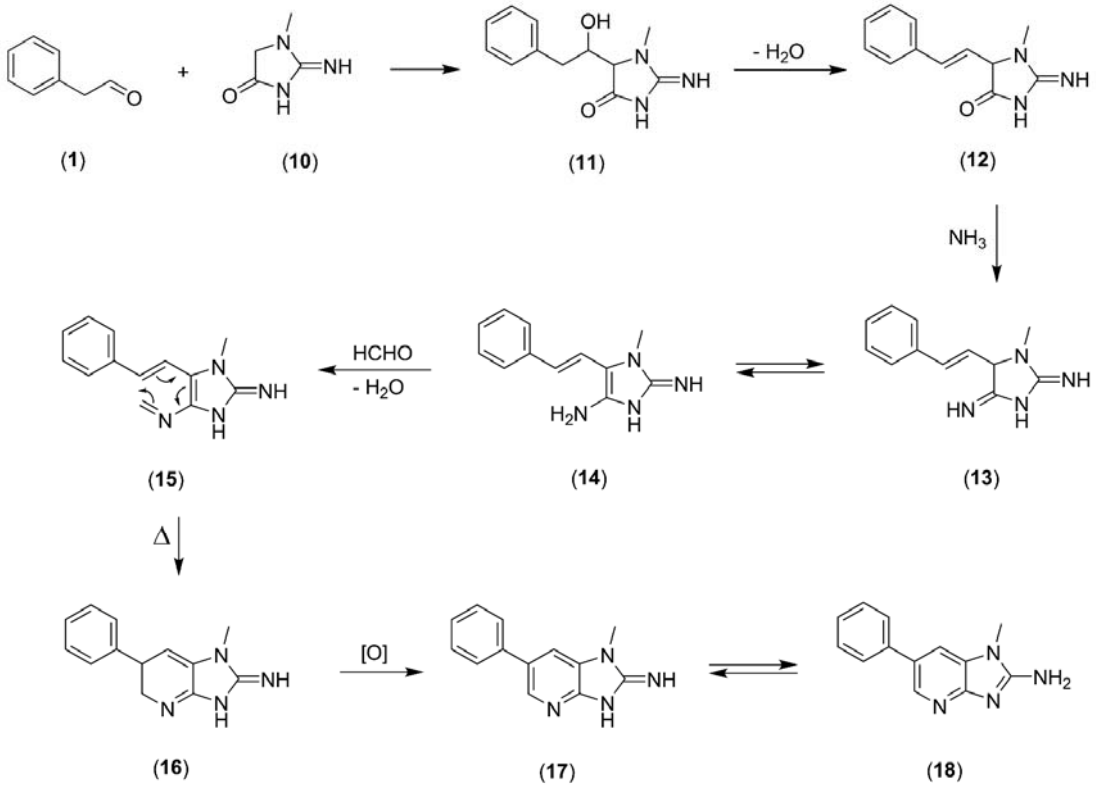

Figure 6