## Phthalimide Residue in Coffee: Does It Solely Derive from Folpet?

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# Phthalimide residue in coffee: does it solely derive from Folpet?

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**ABSTRACT**: Folpet, a fungicide used in several crops, easily degrades into phthalimide (PAI) at high temperatures and basic pH. The maximum admitted limit for Folpet in foodstuffs as coffee is defined by the sum of its amount and that of PAI. Noteworthy, PAI can also arise from the reaction between ubiquitous phthalate derivatives and $NH_3$. This work aims to demonstrate that the detection of PAI in roasted coffee is not necessarily diagnostic for Folpet as it can also originate from the reaction between phthalic anhydride (PAA), deriving from phthalates, and amino acids (AAs), as $NH_3$ source. Thermal treatment of AAs with PAA confirmed that PAI generation follows a temperature-dependent path. Experiments with diethyl phthalate (DEP) and AAs have shown that maximum PAI generation via heating occurs at 200°C for 60 min. PAI generation has also been proven for Folpet-free green coffee beans that were heated under laboratory and industrial roasting conditions.

## INTRODUCTION

Folpet is a fungicide that belongs to the thiophthalimide class and is commonly used in agriculture to avoid diseases caused by fungi.[1,2] It degrades into phthalimide (PAI) at high temperatures and at pH values over 9. European regulations on Folpet residues were modified in 2016 to include its degradation product, following the reasoned opinion of the European Food Safety Authority (EFSA). Therefore, the maximum residue limit for this pesticide is now defined as the sum of Folpet and PAI, expressed as Folpet. The European Regulation No 396/2005 established the maximum admitted amount at 0.1 mg/kg for coffee beans. In food samples, the generation of PAI as a by-product of Folpet (**Figure 1**) can occur both during thermal processing and analytical procedures in sample preparation.[3,4,5] Furthermore, the hot injection conditions used for gas chromatography (GC) analyses, which are often used for the detection of Folpet residues, can encourage the degradation of the pesticide into PAI.
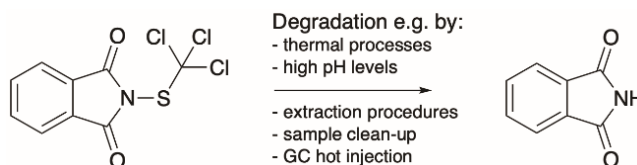


**Figure 1.** Degradation of Folpet into phthalimide.

Modified sample preparation and analytical methods for Folpet determination in tea have been validated in order to overcome the generation of analytical artefacts.[6-9] However, it has recently been reported that PAI may also arise from pathways other than Folpet metabolism/degradation. It was found and reported in Relana® position papers,[10] although in an imprecise and incomplete way, that PAI could be generated *via* the reaction between phthalic anhydride (PAA) and food compounds (e.g. amino acids), when are posed under heating conditions as: food processing (i.e. drying); sample preparation (exothermic reactions), and analytical procedures.[11] Nevertheless, it should be underlined

49 that no toxicity effects for PAI have been reported by the European Chemical Agency (ECHA) for

50 food consumption.[12,13]

51 Phthalates (PAEs) are a group of either dialkyl or alkyl-aryl esters of 1,2-benzendicarboxylic acid

52 (phthalic acid, PA) that are widely used as plasticisers for the production of plastic-based materials.

53 With an estimated annual global production of 11 billion pounds, these compounds are mainly

54 employed to improve polymer characteristics such as flexibility, durability and elasticity.For

55 example, PVC products can contain up to 50 percent of PAEs by weight.[14] There is great economic

56 interest in PAEs as they also find applications in many other fields, such as cosmetics, household and

57 building materials, toys, packaging, medical equipment, pharmaceuticals, pesticides, lubricants,

58 adhesives and printing inks. In particular, some of the most commonly used PAEs are: diethyl

59 phthalate (DEP), di-(2-ethyhexyl)phthalate (DEHP), di-isononylphthalate (DINP),

60 benzylbutylphthalate (BBP) and di-*n*-butylphthalate (DBP).[15] As they are not covalently bound to the

61 plastic polymers, but only dispersed within them, these plasticisers can be released into the

62 environment, *via* leaching for example.[16] In the case of foodstuffs, in addition to migration, another

63 considerable source of these substances is processing, e.g. gloves used to handle food matrices.[17,18]

64 Found in soil, air, natural water and sediments, PAEs are potentially hazardous both for humans and

65 the environment. Due to their environmental persistence and, therefore, bioaccumulation along the

66 food chain, they have been defined as ubiquitous and unavoidable contaminants in food and

67 identifying the contamination source can be challenging.[19-21] It is worth noting that the thermal

68 degradation of PAEs results in monoesters, PA and PAA, from which PAI can be obtained.[22]

69 As the most frequently consumed beverage worldwide, coffee is a common food commodity that

70 canbe polluted by PAEs. It is made from the roasted seeds of *Coffea* plants with two coffee species

71 being of major importance for coffee production: *Coffea arabica* (known as Arabica) and *Coffea*

72 *canephora* (known as Robusta).[23] In addition to caffeine, nitrogen fractions in green coffee beans

73 include trigonelline and protein. Noteworthy, amino acids (AAs) are not only present in proteins, but

74 also in free form, and their content vary between different cultivars (e.g. in a range from traces to

75   0.13% w/w of dry green coffee beans).[24] Coffee roasting is a crucial step that focuses on attaining the

76   required organoleptic characteristics. This procedure is an intense thermal process with variations in

77   time and temperature depending on the desired brewing method, and is usually carried out between

78   120 °C and 240 °C for less than 20 min. The overall roasting treatment ischaracterised by a number

79   of different steps: firstly, the dehydration of the product occurs; next comes the main roasting process

80   itself, which produces the aroma, as well as the typical colour and composition of the beans, mainly

81   through the Maillard and Strecker reactions; finally, the last rapid cooling phase stops the exothermic

82   phase of the roasting operation. During this procedure, about 21% of the proteins content is lost

83   because of their involvement in the above mentioned Maillard reaction and then in the generation of

84   melanoidin compounds. Free AAs (e.g. glycine-Gly, aspartic acid-Asp, and phenylalanine-Phe) are

85   unstable under roasting conditions and therefore a negligible amount of these compounds remains in

86   roasted coffee.[25] **Table 1** shows the main decomposition products of Gly, Asp and Phe as described

87   by Sato *et al.*.[26] The AAs decomposition temperature is reported, but has to be considered that

88   ammonia starts to be released at the lower ones (110-180 °C).[27]

89

90   **Table 1**. Main products of some amino acids thermal decomposition.

| Amino Acid | Main decomposition products | Decomposition temperature |
|---|---|---|
| Glycine (Gly) | ammonia, methyl amine, glycolic acid, formic acid | 290 °C |
| Aspartic acid (Asp) | ammonia, fumaric acid, maleic acid, malic acid, succinic acid, pyruvic acid, lactic acid, acetic acid, formic acid | 271 °C |
| Phenylalanine (Phe) | ammonia, acetic acid, formic acid | 283 °C |

91

92   The aforementioned considerations provide some evidence that the PAI found in food products

93   cannot be exclusively linked to the use and presence of Folpet, thus implying that fungicide false

94   positives can result from analytical artefacts. High temperature coffee roasting treatments may

95   therefore be the optimal conditions under which to evaluate the non-Folpet-related generation of PAI.

96  The importance of process pollutants in food products, together with the possible formation of

97  artefacts, has focused our attention on the study of PAI generation due to the environmental PAEs

98  contamination in Folpet-free coffee samples. To the best of our knowledge, the literature presents

99  significant omissions as regards the mechanisms involved in PAI origin, making it necessary to better

100 clarify the development of this artefact.

101 This work aims to demonstrate that PAI can be formed directly in coffee beans as a result of the

102 reaction between PAA, which is generated from the thermal decomposition of PAEs, and the $NH_3$

103 released by the degradation of AAs during the roasting process.[26-28]

104 Firstly, PAI generation has been monitored through tests performed on several AAs, supplemented

105 with DEP or degradation products of PAEs (i.e. PA, PAA), posed in hermetically sealed systems and

106 heated at different temperatures in a laboratory oven. Subsequently, Folpet free Arabica green coffee

107 bean samples have been subjected to the same thermal treatments in order to evaluate PAI

108 development. Obtained results have been compared with those derived from Folpet-free DEP- or

109 PAA-spiked samples.  Quantitative determinations were performed using GC-FID apparatus. Finally,

110 PAI content in Folpet-free industrially roasted coffee has been considered for discussion.

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112 2. MATERIALS AND METHODS

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114 2.1 Chemicals and coffee samples

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116 Amino acids (aspartic acid, Asp; cysteine, Cys; glycine, Gly; glutamic acid, Glu; lysine, Lys;

117 phenylalanine,Phe), phthalic anhydride (PAA, ≥99.9%), phthalic acid (PA, ≥99.9%), diethyl

118 phthalate (DEP, ≥99.9%), phthalimide (PAI, Pestanal grade) and acetone (HPLC grade, lot:

119 61300645) were purchased from Sigma Aldrich (Sigma–Aldrich, Milan, Italy).

120 Green coffee beans (Arabica, China) certified as Folpet-free (Eurofins Product Testing, Italy S.r.l.,

121 Turin, Italy), were kindly provided by LAVAZZA S.p.A (Settimo Torinese, Turin, Italy).

122 DisQuE QuEChERS kit (150 mg $MgSO_4$, 25 mg PSA, 25 mg C18 and 7 mg GCB, 2 mL dispersive

123 solid phase extraction - d-SPE - tube, 100/pk), employed in coffee sample preparation, was purchased

124 from Waters SpA (Sesto San Giovanni, Milan, Italy; lot:568338291A).

125 Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre was used in green coffee

126 SPME (solid-phase microextraction).

127

128 2.2. Preliminary tests on PAI generation

129 2.2.1. Thermal treatment of AAs in the presence of phthalic anhydride at different temperatures

130

131 Six different AAs have been chosen for trials: Asp, Cys, Gly, Glu, Lys and Phe. 100 mg of each AA

132 and 100 mg of PAA were weighed, mixed with a pestle and mortar, and hermetically sealed into 20

133 mL headspace vials. The samples were then placed in a laboratory oven (G-Therm AG-System Daily,

134 F.lli Galli, Fizzonasco di Pieve Emanuele, Milan, Italy) for 30 minutes at different temperatures: 120

135 °C, 150 °C, 180 °C, 200 °C and 220 °C. After cooling to room temperature, 1 mL of acetone was

136 added to each sample. These mixtures were then filtered through a 0.45 μm filter for analysis by gas

137 chromatography coupled with a flame ionisation detector (GC-FID).

138 Each test was repeated three times to verify method reproducibility.

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140 2.2.2. Thermal treatment of amino acids in presence of phthalic acid

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142 100 mg of Asp, Cys and Gly were respectively mixed with 112 mg (0.67 mmol) of PA with a pestle

143 in a mortar, and then transferred to a 20 mL headspace vial. Hermetically sealed samples were heated

144 in a laboratory oven at 200 °C, for both 30 and 60 min. After cooling to room temperature, 1 mL of

145 acetone was added to each sample, and the mixture was then filtered through a 0.45 μm filter and

146 placed into a vial for GC-FID analyses.

147 Each test was repeated three times to verify the reproducibility of the method.

148    2.2.3. Thermal treatment of amino acids in presence of diethyl phthalate at different temperatures

149

150    100 mg of Asp, Cys and Gly were respectively mixed with 134 µL (0.67 mmol) of DEP and 150 µL

151    (0.0083 mmol) of $H_2O$, which corresponds to 1.24% mol with respect to DEP, in a 20 mL headspace

152    vial and shacked with a Vortex® for 1 min. The hermetically sealed samples were heated in a

153    laboratory oven at 180 °C, 200 °C and 220 °C, in all cases for both 30 and 60 min. After cooling to

154    room temperature, each sample was suspended in 1 mL of acetone, filtered through a 0.45 µm filter

155    and then transferred to a vial for GC-FID analyses.

156    Each test was repeated three times to verify the reproducibility of the method.

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158    2.2.4. Phthalate detection in green coffee beans via SPME

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160    1 g of ground green coffee beans was transfered in a 20 mL headspace vial and submitted to solid-

161    phase microextraction (SPME) employing a Divinylbenzene/Carboxen/Polydimethylsiloxane

162    (DVB/CAR/PDMS) fibre at 80 °C for 12 hours. The sample was then analysed using gas

163    chromatography coupled with mass spectrometry (GC-MS).

164    This test has been repeated three times to verify the reproducibility of the method.

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166    2.3. Folpet free green coffee beans thermal treatment

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168    Tests on green beans were carried out on a 5 g quantity that was milled through a household coffee

169    grinder. Ground coffee samples were transferred to a 20 mL headspace vials and then heated in the

170    laboratory oven for 30 min at different temperatures: 180 °C, 200 °C and 220 °C. The same procedure

171    was used for the PAA and DEP spiked samples achieved by adding either 5 mg (0.037 mmol) of PAA

172    or 7.5 µL (0.037 mmol) of DEP to 5 g of ground green coffee beans before heating.

173    After cooling to room temperature, 6 mL of acetonitrile were added to the samples, and were shacked

174    with a Vortex® for 1 minute. In accordance with the QuEChERS method for multi-residue pesticide

175    analysis,[29,30] 1.5 mL of the obtained extract was transferred into a d-SPE tube for clean-up, and shaken

176    for 1 minute. The d-SPE tube was then centrifuged for 10 minutes at 5000 rpm (Hettich® ROTOFIX

177    32 centrifuge) and 1 ml of the supernatant was evaporated under nitrogen flow. The residue was

178    solubilised in 1 mL of acetone, filtered through a 0.45 μm filter and transferred to a vial for GC-FID

179    analyses.

180    The matrix effect on accuracy was evaluated via recovery experiments that were performed by

181    spiking 5 g of ground coffee, both green and roasted, with PAI at two concentrations (0.5 and 1 mg

182    $g^{-1}$), before the extraction and clean up procedures. Experiments were repeated three times for each

183    concentration, and a range from 86 to 99.9 recovery % was achieved.

184    Moreover, data on PAI content in industrially roasted Folpet-free coffee beans, which were kindly

185    provided by LAVAZZA S.p.A. (certified and analysed by Eurofins), were considered for discussion.

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187    2.3. *Instrumental analyses*

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189    An Agilent Technologies 7820A Network GC System (Santa Clara, California, USA), equipped with

190    an Agilent Technologies GA513A auto sampler and coupled to a flame ionisation detector (FID), was

191    used for the analyses. A HP 5-MS column ((5%-phenyl)-methylpolysiloxane, length 30 m, i.d. 0.25

192    mm, film thickness 0.25μm), with a 1:20 split ratio, 1 μL of injection, a 250 °C injector temperature,

193    and helium as the carrier gas (1.2 mL min$^{-1}$ flow) was employed. The gas chromatography parameters

194    were set as follows: from 70 °C (held 2 min) to 300 °C at 10°C min$^{-1}$ (held for 10 min) with a post-

195    run step at 300°C, which was held for 10 min. Agilent MSD ChemStation software (B.04.03 SP2)

196    was employed for instrument control and data processing. The quantitative analysis of PAI in samples

197    was achieved via a calibration of the GC-FID method. A 1 mg mL$^{-1}$ stock solution was prepared by

198    weighing 50 mg of the PAI standard and dissolving it in 50 mL of acetone. After dilution, 0.75 mg

199     mL$^{-1}$, 0.5 mg mL$^{-1}$, 0.35 mg mL$^{-1}$, 0.25 mg mL$^{-1}$, 0.1 mg mL$^{-1}$ and 0.05 mg mL$^{-1}$ solutions were

200     prepared and injected. A calibration curve was obtained with a linear correlation coefficient ($R^2$) of

201     0.9988 (LOD 0.0025 mg/mL; LOQ 0.005 mg/mL).

202     GC-MS analyses on ground green coffee beans samples that had undergone the SPME were

203     performed on an Agilent Technologies 6850 GC Network GC System equipped with a 7683B

204     Automatic Sampler and coupled with a 5973 Network Mass Selective Detector. Separation was

205     performed using an HP 5-MS column ((5%-phenyl)-methylpolysiloxane, length 30 m, i.d. 0.25 mm,

206     film thickness 0.25μm). Helium was used as the carrier gas with a constant flow of 1.3 mL/min. The

207     split/splitless injector was set in split mode (10:1), and its temperature was maintained at 250 °C. The

208     oven-temperature programme for the separation of the volatile compounds that were adsorbed onto

209     the fibre was as follows: from 50 °C (held 2 min) to 150 °C at 10°C min$^{-1}$; then increased to 260 °C

210     (held 15 min) at 5 °C min$^{-1}$. The main chromatographic peaks were identified by comparing them to

211     the mass spectra of pure standards or using mass-spectra libraries with the match quality index, as

212     calculated by the NIST Similarity and Identity Spectrum Search algorithm (NIST 08 and Wiley MS

213     275).

214     Data analyses refer to measurements performed in triplicate and results are expressed as mean data ±

215     standard deviation (SD).

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217     3. RESULTS AND DISCUSSION

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219     *3.1. Phthalimide formation from amino acids and phthalic anhydride thermal treatment*

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221     As mentioned above, the presence of PAI may represent a false positive for the pesticide Folpet.

222     PAA is an intermediate formed during the thermal degradation of PAEs. As illustrated in **Scheme 1**

223     for DEP, in absence or in trace amount of water the decomposition pathway proceeds *via* the

224   formation of the monoester, an unstable compound in which neighbouring group interactions can lead

225   to the formation of the anhydride compound.[22]

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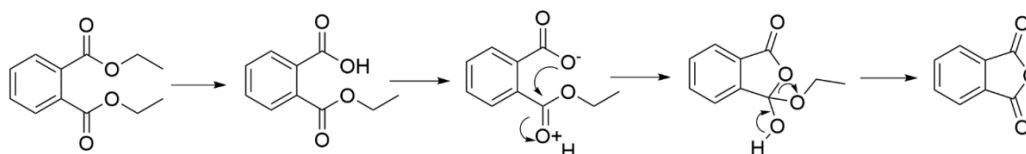


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230   **Scheme 1**. Thermal decomposition of diethyl phthalate to phthalic anhydride in absence or in trace
231   amount of water.

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234   Furthermore, PA is an indicator of the presence of monoester hydrolysis; its generation takes place

235   in presence of at least 10% water, while it is not formed with lower amount of it.

236   Preliminary tests about PAI generation were carried out by adding PAA to Asp, Cys, Gly, Glu, Lys

237   and Phe (**Figure 2**). These AAs have been selected considering some of those that are reported as

238   present in free form in green coffee (Asp, Gly, Glu, Lys, Phe, and Cys),[24] among which Cys and Asp

239   indicated by Sohn and Ho[27] as AAs with higher $NH_3$ release.

240   The thermal treatment was performed in a laboratory oven at a range of temperatures (120°C, 150°C,

241   180°C, 200°C, and 220°C), in order to emulate common coffee roasting conditions.

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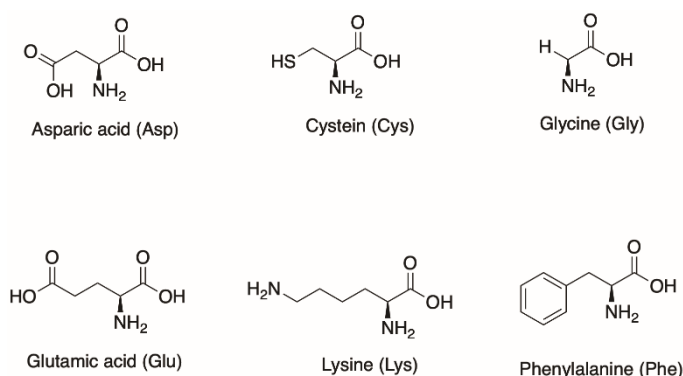243
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255   **Figure 2.** Amino acids chosen for trials.
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259 The quantitative data obtained from the GC-FID analyses (see **Table 2**), show that PAI generation is

260 a temperature-dependent phenomenon, reaching its maximum around 180-200°C for all AAs.

261 The peculiar path of Asp is particularly interesting, since it achieves considerably higher PAI yields

262 than the other AAs tested. Asp is stable up to 150 °C and begins to release $NH_3$ at higher temperatures

263 with a significant increase at 180°C that reaches its maximum at 200°C, thus achieving 32.57 mg of

264 PAI per 100 mg of AA. Moreover, it has been reported that 50% of the α-amino group is decomposed

265 when Asp is heated to 180 °C for 2h.[27]

266 Cys and Gly were also found to lead to better PAI-generation yields, although to a lesser extent than

267 Asp. The increase in PAI formation with temperature for Cys, a sulphur containing AA, may be

268 related to the presence of the reactive thiol group. The attack of the nucleophilic thiol group on the

269 α-carbon of Cys can increase $NH_3$ release even at low temperatures, as seen in **Table 2** (0.69 mg

270 PAI/100 mg Cys *vs* 0.31mg PAI/100 mg Asp).

271 The obtained data show that the $NH_3$ amounts involved are in line with those previously reported by

272 Weiss *et al.*[28], indicating that the thermal treatment of Asp, Cys and Gly can result in at most ½ mol

273 of $NH_3$ per mol of AA.

276 **Table 2.** GC-FID quantitative results from the thermal treatment of tested AAs with phthalic
277 anhydride at different oven temperatures; data indicate phthalimide amount expressed as mg/100mg
278 of AA ± SD.

| | *PAI* mg/100 mg AA | | | | |
|---|---|---|---|---|---|
| | 120°C | 150°C | 180°C | 200°C | 220°C |
| **Asp** | 0.11 ±0.02 | 0.31 ± 0.08 | 10.64 ±0.9 | 32.57 ±2.5 | 32.48 ±1.8 |
| **Cys** | 0.12 ±0.005 | 0.69 ±0.05 | 1.96 ±0.1 | 2.58 ±0.3 | 2.67 ±0.4 |
| **Gly** | 0.11 ±0.03 | 1.22 ± 0.09 | 1.61 ±0.3 | 2.47 ±0.7 | 2.56 ±0.2 |
| **Glu** | 0.07 ±0.002 | 0.30 ± 0.007 | 0.64 ±0.02 | 0.89 ±0.05 | 0.92± 0.004 |
| **Lys** | 0.11 ±0.04 | 0.11 ±0.03 | 0.78 ±0.008 | 1.22± 0.09 | 1.17 ±0.05 |
| **Phe** | 0.12 ±0.06 | 0.71 ±0.005 | 1.20 ±0.09 | 1.49 ±0.1 | 1.42 ±0.08 |

296     *3.2. Phthalimide formation from aspartic acid, cysteine and glycine in presence of phthalic acid*

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298     As PA can dehydrate to PAA at high temperatures, PAI generation has also been evaluated in this

299     study with PA as a reactant (**Figure 3**).  In this case, Asp, Cys and Gly were employed as they were

300     found to be the most responsive AAs in tests described above.

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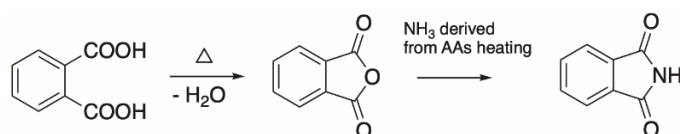


304 **Figure 3.** PAI generation from PA as a result of thermal treatment in the presence of AAs as a $NH_3$
305 source.

309     The thermal decomposition of PA (melting point 207 °C) is a complex process that heavily depends

310     on heating. Chatterjee *et al.*[31] have reported that the onset of PA melting begins at 180 °C and then

311     peaks at 197 °C. This may be due to concomitant dehydration to PAA, small quantities of which

312     depress the melting point, and thus cause the physical process to begin at lower temperatures than

313     expected. As PAA melting point is 130 °C, its generation and evaporation occur simultaneously.

314     200 °C was selected as the temperature to carry out these aiming to maximise the release of $NH_3$ by

315     the AAs, guarantee the formation of PAA from PA and fall within the temperature range used for

316     roasting. Moreover, in the attempt to evaluate the time-of-exposure influence on this phenomenon,

317     each reaction was performed in a sealed vial for both 30 and 60 minutes.

318     From the quantitative GC-FID analyses (see **Graph 1**), it is clear that PAI yield is dramatically higher

319     in the case of Asp, with more than 20 mg/100 mg of AA, while Cys and Gly exhibit lower amounts

320     of PAI.  This confirms the particular behaviour of Asp, which has already been evidenced in the

321     previous trials. Furthermore, data show that an increase in residence time in the oven at 200 °C fails

322     to lead to an improvement in PAI yield for all considered AAs.

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**Graph 1**. Phthalimide formation from AA in the presence of PA at 200°C for 30 and 60 minutes. Data indicate PAI amount expressed as mg/100mg of AA ± SD.



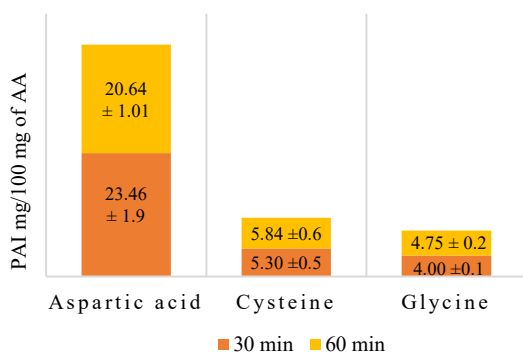*3.3. Phthalimide formation from the thermal treatment of diethyl phthalate with aspartic acid, cysteine and glycine*

Since the presence of PA and PAA in the environment is due to the degradation of ubiquitous PAEs,[21] DEP was chosen as model compound for the tests as it was found to be the only PAE present in detectable quantities in Arabica green coffee beans following the preliminary screening carried out using SPME and GC-MS analyses (data not reported here).

Trials involving the use of DEP and Asp, Cys and Gly were performed at three different temperatures (180 °C, 200 °C and 220 °C), comparing 30 and 60 minutes heating time. Tests carried out in the absence of water were not successful, as the formation of PAI did not occur, whereas the addition of water in minimum amounts (150 µL; 1.24% w/w DEP) allowed its generation. This can be rationalised if we consider the formation of PAA *via* the intermediate monoester (see **Scheme 1**), whose decomposition, we believe, follows the trend reported by Saido *et al.*[22] for DEHP. Moreover, the technical addition of water in these tests also derives from the fact that green coffee beans contain residual moisture, whose value is stated by the International Coffee Organization to be 8 to 12.5%[32], and therefore able to trigger PAE degradation.

Unlike the previous assays that entailed the direct addition of PAA (paragraph 3.1), a significant dependence on higher temperature occurs for DEP. In fact, only at 220 °C it is possible to observe an

358   increase in PAI generation. In particular, the results reported in **Table 3** and **Graph 2** show that, for

359   all temperatures, Asp gave higher PAI yields than Cys and Gly, with the maximum value of 1.176

360   mg PAI/100 mg of AA being observed at 220°C and 60 minutes of reaction time. Cys and Gly gave

361   similar trends with a slight improvement in yields between 180 and 200 °C. Moreover, doubling the

362   residence time of the reagents in the sealed headspace vial proved, in this case, to be effective in

363   increasing the production of PAI for all three AAs tested. These results showed that, despite the lower

364   amounts, PAEs can lead to PAI generation under high-temperature conditions in the presence of a

365   $NH_3$ source.

366

367   **Table 3.** Phthalimide generation from aspartic acid, cysteine and glycine in presence of diethyl
368   phthalate under different heating and temperature conditions. Data indicate PAI amount expressed as
369   mg/100mg of AA ± SD.

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| | PAI mg/100 mg AA | | | | | |
|---|---|---|---|---|---|---|
| | 180°C | | 200°C | | 220°C | |
| | 30 min | 60 min | 30 min | 60 min | 30 min | 60 min |
| **Asp** | 0.066 ±0.002 | 0.157 ±0.04 | 0.262 ±0.05 | 0.450 ±0.01 | 0.855 ±0.03 | 1.676 ±0.08 |
| **Cys** | 0.054 ±0.007 | 0.063 ±0.005 | 0.065 ±0.006 | 0.085 ±0.006 | 0.083 ±0.004 | 0.237 ±0.006 |
| **Gly** | 0.069 ±0.004 | 0.085 ±0.003 | 0.083 ±0.004 | 0.096 ±0.001 | 0.093 ±0.005 | 0.192 ±0.04 |

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**Graph 2**. Phthalimide generation from aspartic acid, cysteine and glycine in presence of diethyl phthalate under heating.
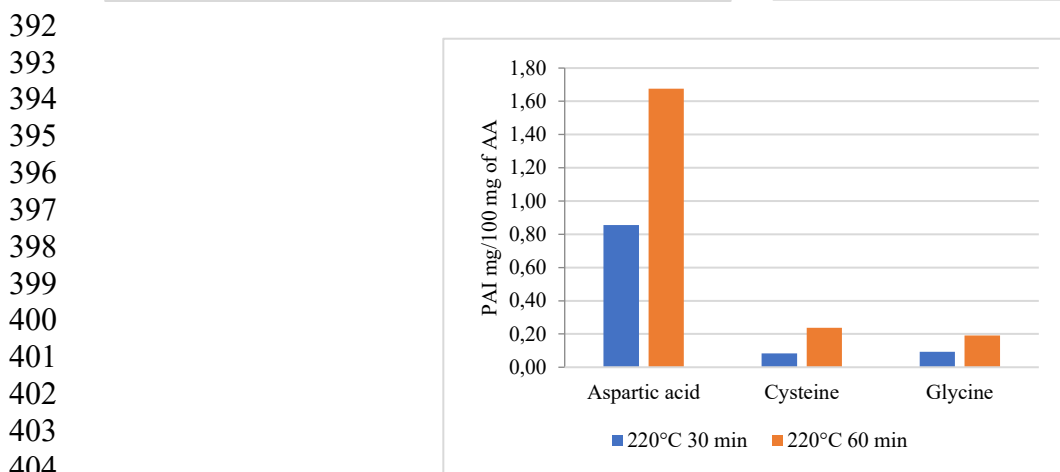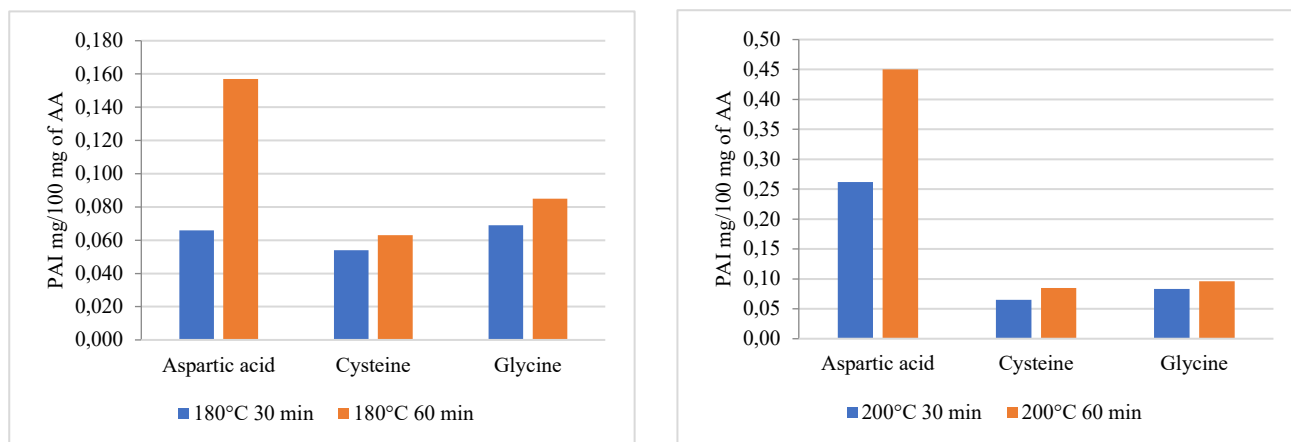


*3.4. Phthalimide formation in folpet free green coffee beans under heating*

Folpet is not listed as an applicable pesticide for coffee as reported by the *International Coffee Organization.*[33] The purpose of this work is to observe and study possible PAI formation during the heating of Folpet free Arabica green coffee. In the case of this complex matrix, the most specific combination of extraction and clean-up procedure should be engaged for quantitative analyses. Therefore, a QuEChERS-based extraction procedure was performed using acetonitrile as the solvent and a d-SPE cartridge for the clean-up step which allows purer extracts with high recovery rates and decreasing matrix effect on the analysis.[30]

No traces of PAI were detected in the GC-FID analysis of green coffee samples that were not submitted to heating. The reliability of both the matrix treatment and the analytical system was

417 evaluated in PAI-spiked coffee samples at two different concentrations, and recovery in the range of

418 86-99.9% was obtained (see paragraph 2.3.). Subsequently, the green coffee samples, either used as

419 such or spiked with PAA and DEP, were submitted to heating at different temperatures (180 °C, 200

420 °C, 220 °C) for 30 minutes in a laboratory oven in order to emulate a variety of roasting degrees. The

421 results obtained from the GC-FID are reported in **Graph 3** and show that PAI was detected in all

422 spiked samples. The formation of PAI follows a temperature-dependant trend with the maximum

423 yield being achieved at 200°C and remaining almost constant at 220°C. This trend seems to reflect

424 the results obtained in tests involving AAs in presence of PAA. In particular, higher PAI yields (0.149

425 mg/g of coffee) were detected in coffee samples with added PAA. For DEP enriched coffee, a

426 maximum amount of 0.038 mg/g was observed. The trend found in tests performed on AAs heated

427 in presence of PAA and DEP was confirmed in this case. Surprisingly, PAI generation was also

428 observed in non-spiked samples, which displayed maximum PAI generation at 200°C (0.0056 mg/g

429 coffee, **Graph 3**). Since the samples were certified Folpet free, it is evident that the heating process

430 leads to PAI generation, the presence of which can only be due to environmental PAEs pollution.

431 Of course, it cannot be assumed that this is the effective PAI concentration in commercial roasted

432 coffee beans as the experimental conditions applied for tests are not comparable to those of a roasting

433 process. For this reason, industrially roasted Folpet-free coffee samples (air roaster) that were

434 obtained at four different roasting degrees (named light, medium light, medium dark, dark) were

435 analysed. PAI was not detected in the lighter samples (light, medium light), while darker samples

436 gave concentrations of 0.034 and 0.038 mg/kg, thus affirming that more intensive thermal conditions

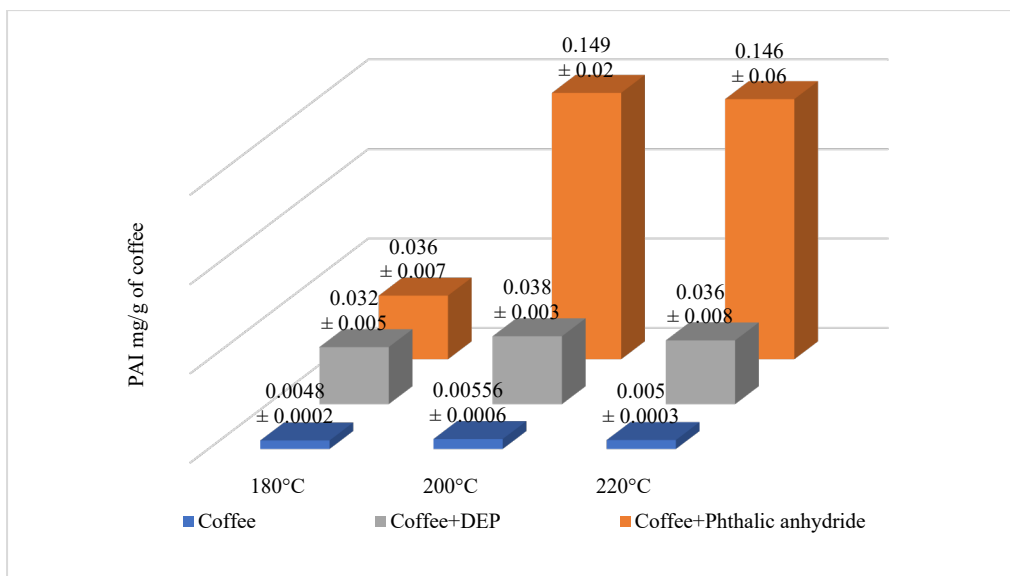437 lead to higher PAI residue.

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440 **Graph 3.** Phthalimide formation (expressed as mg/g of coffee) from Folpet free ground green coffee
441 submitted to heating, either used as such or spiked with diethyl phthalate and phthalic anhydride.
442 Data indicate PAI amount expressed as mg/100mg of AA ± SD.
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Figure data:

| Temperature | Coffee | Coffee+DEP | Coffee+Phthalic anhydride |
|---|---|---|---|
| 180°C | 0.0048 ± 0.0002 | 0.032 ± 0.005 | 0.036 ± 0.007 |
| 200°C | 0.00556 ± 0.0006 | 0.038 ± 0.003 | 0.149 ± 0.02 |
| 220°C | 0.005 ± 0.0003 | 0.036 ± 0.008 | 0.146 ± 0.06 |

PAI mg/g of coffee

PAI, a Folpet by-product, may represent a false positive for the presence of the pesticide, especially in the case of thermally treated foods, such as roasted coffee. In this study, a possible *ex-novo* PAI-generation pathway that considers ubiquitous PAEs to be precursors has been suggested. Some AAs present in their free form in green coffee beans were tested as $NH_3$ sources during thermal treatments. Experiments involving PAA, as a PAE derivative, and AAs under heating conditions that emulate those of roasting process demonstrate a temperature-dependant PAI formation path in which Asp, Cys and Gly exhibit the highest yields.

Ubiquitous DEP was chosen as a model to investigate whether PAEs could represent a source of PAI during thermal processes.

The formation of PAI in moderate amounts provides evidence of how PAEs can degrade and react with $NH_3$. Therefore, although PAEs are potentially harmful for human and environment, the roasting process induces their chemical modification to the non-toxic PAI.

Certified Folpet free Arabica green coffee beans, heated in laboratory oven at 180 °C, 200°C and 220 °C, showed PAI traces.

Despite the experimental conditions of laboratory trials performed in hermetically sealed systems are not comparable with those of industrial roasting processes, it follows that the detection of PAI residue in samples can be ascribed to its generation during thermal processes. Thus, there is a risk of false

464 positives, and overestimation, in the presence of the fungicide Folpet. Lastly, analyses performed on

465 Folpet free coffee samples processed in an industrial air roaster at different roasting degrees only

466 showed PAI traces (0.03-0.04 ppm) for the most intensive thermal conditions (darker roasting).

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ABBREVIATIONS USED: AA, Amino acid; Asp, aspartic acid; Cys, cysteine; Gly, glycine; Glu, glutamic acid; Lys, lysine; DEHP, di-(2-ethyhexyl)phthalate; DEP, diethyl phthalate; d-SPE, dispersive solid phase extraction; EFSA, European Food Safety Authority; GC-FID, gas chromatography coupled to flame ionization detector; GC-MS, gas chromatography coupled to mass spectrometer; LOD, limit of detection; LOQ, limit of quantification; PA, phthalic acid; PAA, phthalic anhydride; PAI, phthalimide; PAEs, phthalates; Phe, phenylalanine; PSA, primary-secondary amine; QuEChERS, Quick, Easy, Cheap, Effective, Rugged and Safe; DVB/CAR/PDMS fibre, Divinylbenzene/Carboxen/Polydimethylsiloxane fibre; SPME, Solid-phase microextraction.

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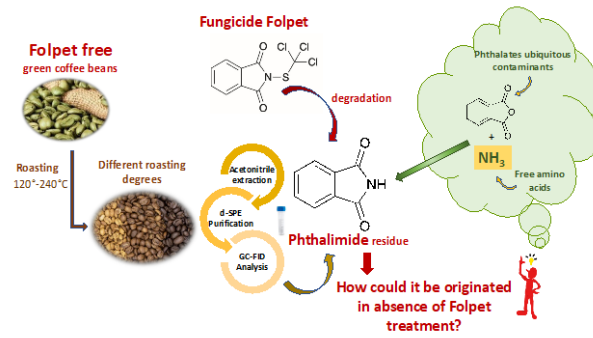630　(assessed October 2020)

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# TOC graphic

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