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(Article begins on next page)

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

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KEYWORDS: Phthalimide; amino acids; phthalates; coffee beans; roasting; pesticides degradation.

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.

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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.

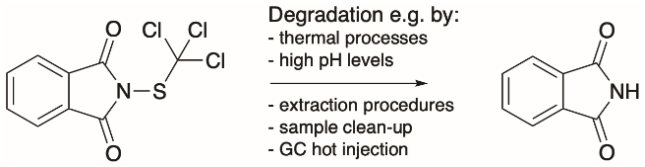


Figure 1. Degradation of Folpet into phthalimide.

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

0.13% w/w of dry green coffee beans).[24] Coffee roasting is a crucial step that focuses on attaining the required organoleptic characteristics. This procedure is an intense thermal process with variations in time and temperature depending on the desired brewing method, and is usually carried out between 120 °C and 240 °C for less than 20 min. The overall roasting treatment ischaracterised by a number of different steps: firstly, the dehydration of the product occurs; next comes the main roasting process itself, which produces the aroma, as well as the typical colour and composition of the beans, mainly through the Maillard and Strecker reactions; finally, the last rapid cooling phase stops the exothermic phase of the roasting operation. During this procedure, about 21% of the proteins content is lost because of their involvement in the above mentioned Maillard reaction and then in the generation of melanoidin compounds. Free AAs (e.g. glycine-Gly, aspartic acid-Asp, and phenylalanine-Phe) are unstable under roasting conditions and therefore a negligible amount of these compounds remains in roasted coffee.[25] **Table 1** shows the main decomposition products of Gly, Asp and Phe as described by Sato *et al.*.[26] The AAs decomposition temperature is reported, but has to be considered that ammonia starts to be released at the lower ones (110-180 °C).[27]

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

The aforementioned considerations provide some evidence that the PAI found in food products cannot be exclusively linked to the use and presence of Folpet, thus implying that fungicide false positives can result from analytical artefacts. High temperature coffee roasting treatments may 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.
111
112 ## 2. MATERIALS AND METHODS
113
114 ### 2.1 Chemicals and coffee samples
115
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).

DisQuE QuEChERS kit (150 mg $MgSO_4$, 25 mg PSA, 25 mg C18 and 7 mg GCB, 2 mL dispersive solid phase extraction - d-SPE - tube, 100/pk), employed in coffee sample preparation, was purchased from Waters SpA (Sesto San Giovanni, Milan, Italy; lot:568338291A).
Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre was used in green coffee SPME (solid-phase microextraction).

2.2. Preliminary tests on PAI generation

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

Six different AAs have been chosen for trials: Asp, Cys, Gly, Glu, Lys and Phe. 100 mg of each AA and 100 mg of PAA were weighed, mixed with a pestle and mortar, and hermetically sealed into 20 mL headspace vials. The samples were then placed in a laboratory oven (G-Therm AG-System Daily, F.lli Galli, Fizzonasco di Pieve Emanuele, Milan, Italy) for 30 minutes at different temperatures: 120 °C, 150 °C, 180 °C, 200 °C and 220 °C. After cooling to room temperature, 1 mL of acetone was added to each sample. These mixtures were then filtered through a 0.45 μm filter for analysis by gas chromatography coupled with a flame ionisation detector (GC-FID).
Each test was repeated three times to verify method reproducibility.

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

100 mg of Asp, Cys and Gly were respectively mixed with 112 mg (0.67 mmol) of PA with a pestle in a mortar, and then transferred to a 20 mL headspace vial. Hermetically sealed samples were heated in a laboratory oven at 200 °C, for both 30 and 60 min. After cooling to room temperature, 1 mL of acetone was added to each sample, and the mixture was then filtered through a 0.45 μm filter and placed into a vial for GC-FID analyses.
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.

157

158 2.2.4. Phthalate detection in green coffee beans via SPME

159

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.

165

166 2.3. Folpet free green coffee beans thermal treatment

167

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.

After cooling to room temperature, 6 mL of acetonitrile were added to the samples, and were shacked with a Vortex® for 1 minute. In accordance with the QuEChERS method for multi-residue pesticide analysis,[29,30] 1.5 mL of the obtained extract was transferred into a d-SPE tube for clean-up, and shaken for 1 minute. The d-SPE tube was then centrifuged for 10 minutes at 5000 rpm (Hettich® ROTOFIX 32 centrifuge) and 1 ml of the supernatant was evaporated under nitrogen flow. The residue was solubilised in 1 mL of acetone, filtered through a 0.45 μm filter and transferred to a vial for GC-FID analyses.

The matrix effect on accuracy was evaluated via recovery experiments that were performed by spiking 5 g of ground coffee, both green and roasted, with PAI at two concentrations (0.5 and 1 mg g^{-1}), before the extraction and clean up procedures. Experiments were repeated three times for each concentration, and a range from 86 to 99.9 recovery % was achieved.

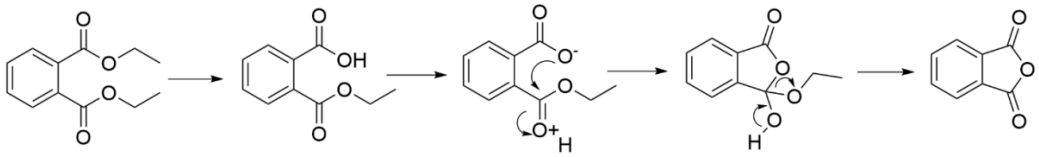
Moreover, data on PAI content in industrially roasted Folpet-free coffee beans, which were kindly provided by LAVAZZA S.p.A. (certified and analysed by Eurofins), were considered for discussion.

2.3. *Instrumental analyses*

An Agilent Technologies 7820A Network GC System (Santa Clara, California, USA), equipped with an Agilent Technologies GA513A auto sampler and coupled to a flame ionisation detector (FID), was used for the analyses. A HP 5-MS column ((5%-phenyl)-methylpolysiloxane, length 30 m, i.d. 0.25 mm, film thickness 0.25μm), with a 1:20 split ratio, 1 μL of injection, a 250 °C injector temperature, and helium as the carrier gas (1.2 mL min^{-1} flow) was employed. The gas chromatography parameters 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-run step at 300°C, which was held for 10 min. Agilent MSD ChemStation software (B.04.03 SP2) was employed for instrument control and data processing. The quantitative analysis of PAI in samples was achieved via a calibration of the GC-FID method. A 1 mg mL^{-1} stock solution was prepared by 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).
216
217 3. RESULTS AND DISCUSSION
218
219 *3.1. Phthalimide formation from amino acids and phthalic anhydride thermal treatment*
220
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

formation of the monoester, an unstable compound in which neighbouring group interactions can lead to the formation of the anhydride compound.[22]



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

Furthermore, PA is an indicator of the presence of monoester hydrolysis; its generation takes place in presence of at least 10% water, while it is not formed with lower amount of it.

Preliminary tests about PAI generation were carried out by adding PAA to Asp, Cys, Gly, Glu, Lys and Phe (**Figure 2**). These AAs have been selected considering some of those that are reported as present in free form in green coffee (Asp, Gly, Glu, Lys, Phe, and Cys),[24] among which Cys and Asp indicated by Sohn and Ho[27] as AAs with higher NH_3 release.

The thermal treatment was performed in a laboratory oven at a range of temperatures (120°C, 150°C, 180°C, 200°C, and 220°C), in order to emulate common coffee roasting conditions.

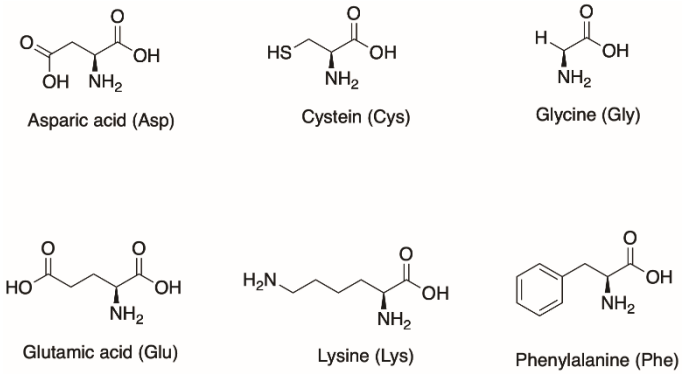


Figure 2. Amino acids chosen for trials.

The quantitative data obtained from the GC-FID analyses (see **Table 2**), show that PAI generation is a temperature-dependent phenomenon, reaching its maximum around 180-200°C for all AAs.

The peculiar path of Asp is particularly interesting, since it achieves considerably higher PAI yields than the other AAs tested. Asp is stable up to 150 °C and begins to release NH_3 at higher temperatures with a significant increase at 180°C that reaches its maximum at 200°C, thus achieving 32.57 mg of PAI per 100 mg of AA. Moreover, it has been reported that 50% of the α-amino group is decomposed when Asp is heated to 180 °C for 2h.[27]

Cys and Gly were also found to lead to better PAI-generation yields, although to a lesser extent than Asp. The increase in PAI formation with temperature for Cys, a sulphur containing AA, may be related to the presence of the reactive thiol group. The attack of the nucleophilic thiol group on the α-carbon of Cys can increase NH_3 release even at low temperatures, as seen in **Table 2** (0.69 mg PAI/100 mg Cys *vs* 0.31mg PAI/100 mg Asp).

The obtained data show that the NH_3 amounts involved are in line with those previously reported by Weiss *et al.*[28], indicating that the thermal treatment of Asp, Cys and Gly can result in at most ½ mol of NH_3 per mol of AA.

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

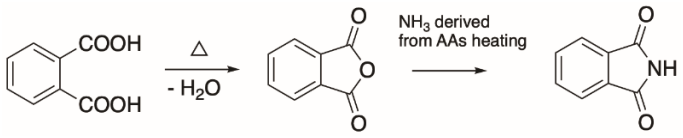
	PAI* mg*/100 mg AA				
	120°C	**150°C**	**180°C**	**200°C**	**220°C**
Asp	0.11 ±0.02	0.31 ± 0.08	10.64 ±0.9	32.57 ±2.5	32.48 ±1.8
Cys	0.12 ±0.005	0.69 ±0.05	1.96 ±0.1	2.58 ±0.3	2.67 ±0.4
Gly	0.11 ±0.03	1.22 ± 0.09	1.61 ±0.3	2.47 ±0.7	2.56 ±0.2
Glu	0.07 ±0.002	0.30 ± 0.007	0.64 ±0.02	0.89 ±0.05	0.92± 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*

297

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.

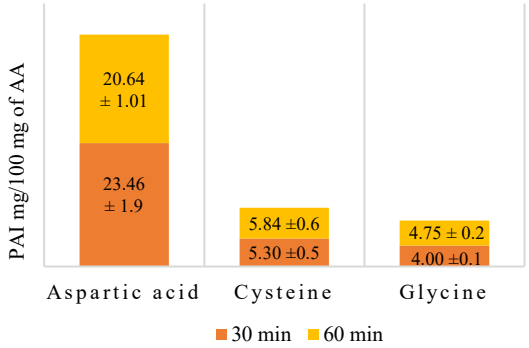
301



302
303
304 **Figure 3.** PAI generation from PA as a result of thermal treatment in the presence of AAs as a NH_3
305 source.
306
307
308
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.

323

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.

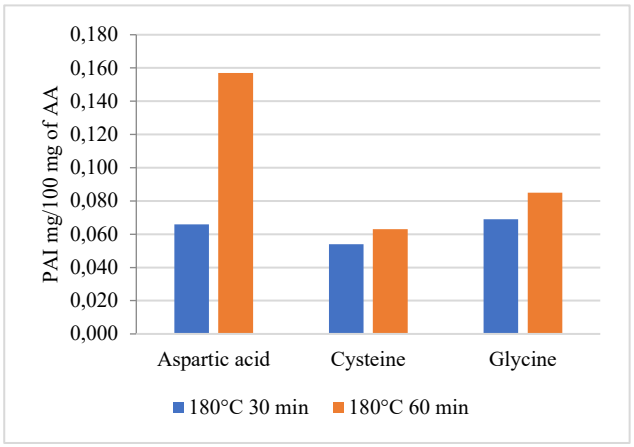
Unlike the previous assays that entailed the direct addition of PAA (paragraph 3.1), a significant dependence on higher temperature occurs for DEP. In fact, only at 220 °C it is possible to observe an

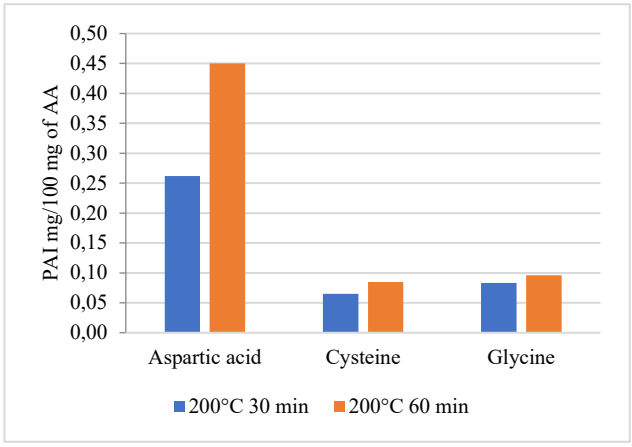
increase in PAI generation. In particular, the results reported in **Table 3** and **Graph 2** show that, for all temperatures, Asp gave higher PAI yields than Cys and Gly, with the maximum value of 1.176 mg PAI/100 mg of AA being observed at 220°C and 60 minutes of reaction time. Cys and Gly gave similar trends with a slight improvement in yields between 180 and 200 °C. Moreover, doubling the residence time of the reagents in the sealed headspace vial proved, in this case, to be effective in increasing the production of PAI for all three AAs tested. These results showed that, despite the lower amounts, PAEs can lead to PAI generation under high-temperature conditions in the presence of a NH_3 source.

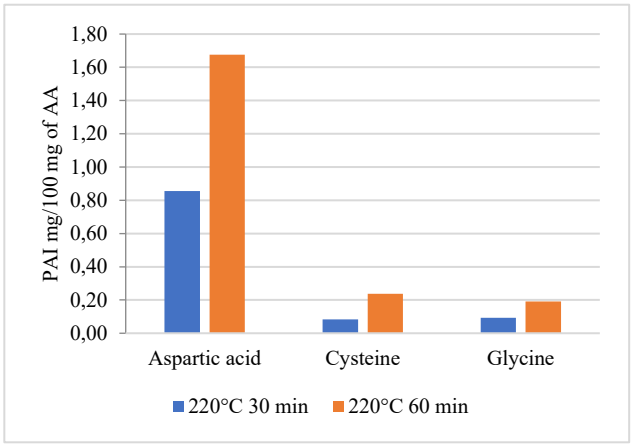
Table 3. Phthalimide generation from aspartic acid, cysteine and glycine in presence of diethyl phthalate under different heating and temperature conditions. Data indicate PAI amount expressed as mg/100mg of AA ± SD.

	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

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.
438
439
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.
443
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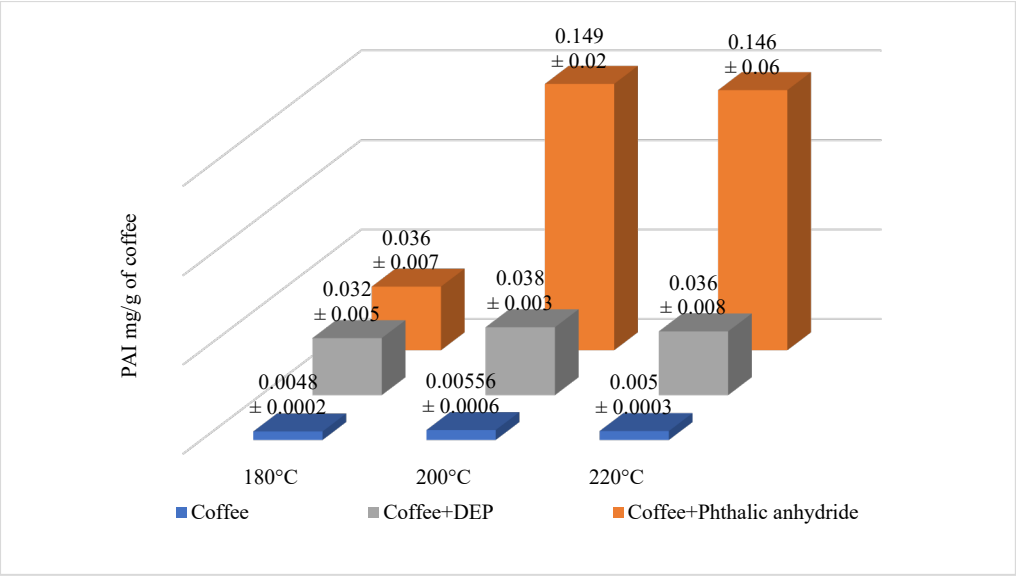

445
446

447 PAI, a Folpet by-product, may represent a false positive for the presence of the pesticide, especially
448 in the case of thermally treated foods, such as roasted coffee. In this study, a possible *ex-novo* PAI-
449 generation pathway that considers ubiquitous PAEs to be precursors has been suggested. Some AAs
450 present in their free form in green coffee beans were tested as NH_3 sources during thermal treatments.
451 Experiments involving PAA, as a PAE derivative, and AAs under heating conditions that emulate
452 those of roasting process demonstrate a temperature-dependant PAI formation path in which Asp,
453 Cys and Gly exhibit the highest yields.
454 Ubiquitous DEP was chosen as a model to investigate whether PAEs could represent a source of PAI
455 during thermal processes.
456 The formation of PAI in moderate amounts provides evidence of how PAEs can degrade and react
457 with NH_3. Therefore, although PAEs are potentially harmful for human and environment, the roasting
458 process induces their chemical modification to the non-toxic PAI.
459 Certified Folpet free Arabica green coffee beans, heated in laboratory oven at 180 °C, 200°C and 220
460 °C, showed PAI traces.
461 Despite the experimental conditions of laboratory trials performed in hermetically sealed systems are
462 not comparable with those of industrial roasting processes, it follows that the detection of PAI residue
463 in samples can be ascribed to its generation during thermal processes. Thus, there is a risk of false

positives, and overestimation, in the presence of the fungicide Folpet. Lastly, analyses performed on Folpet free coffee samples processed in an industrial air roaster at different roasting degrees only 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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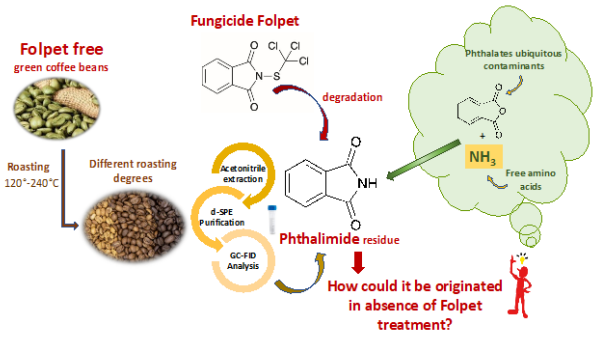
TOC graphic


Fungicide Folpet
Folpet free
green coffee beans
degradation
Phthalates ubiquitous contaminants
Roasting
120°-240°C
Different roasting degrees
Acetonitrile extraction
d-SPE Purification
GC-FID Analysis
NH3
Free amino acids
Phthalimide residue
How could it be originated in absence of Folpet treatment?