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"Development of new extraction, purification and analytical methods in the coffee production chain"

TESI PRESENTATA DA: Janet Menzio

TUTOR(S): Dott.ssa Arianna Binello

COORDINATORE DEL DOTTORATO: Prof.ssa Roberta Cavalli

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Sommario

Abbreviations

AAs, amino acids; Asp, asparagine; ATSDR, Agency for Toxic Substances and Disease Registry; BaA, Benzo[a]anthracene; BaP, Benzo[a]pyrene; BbF, Benzo[b]fluoranthene; CHR, Chrysene; CF-SPME, cold fibre solid phase microextraction; Cys, cysteine; DEP, diethyl phthalate; DLLME, dispersive liquid–liquid microextraction; d-SPE, dispersive solid phase extraction; EPA, Environmental Protection Agency; EFSA, European Food Safety Authority; EU, European Union; FLD, fluorescence detection; GC, gas chromatography; GC-FID, gas chromatography with flame ionization detection; GPC, gel permeation chromatography; Gly, glycine; Glu, glutamine; HACCP, Hazard Analysis and Critical Control Points; HPLC, high performance liquid chromatography; 5-HMF, 5-Hydroxymethylfurfural; IARC, International Agency for Research on Cancer; ICO, International Coffee Organization; ISO, International Organization for Standardization; JECFA, Joint FAO/WHO Expert Committee on Food Additives; MR, Maillard reaction; ME, matrix effect; MRL, maximum residue limit; MAE, microwave assisted extraction; NADES, natural deep eutectic solvents; LOD, limit of detection; LOQ, limit of quantification; LC, liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; LE–CO_2, liquid CO_2 extraction; LLE, liquid–liquid extraction; LLME, liquid–liquid microextraction; Lys, lysine; OTA, ochratoxin A; OPP, *o*-phenylphenol; Phe, phenylalanine; PAEs, phthalates; PAA, phthalic anhydride; PA, phthalic acid; PAI, phthalimide; PAHs, Polycyclic aromatic hydrocarbons; PSA sorbent, primary and secondary amine sorbent; PUFAs, polyunsaturated fatty acids; QuEChERS, Quick, Easy, Cheap, Effective, Rugged and Safe; RP, reversed-phase; SOPP, sodium *o*-phenylphenate; SPE, solid phase extraction; SPME, solid phase microextraction; SD, standard deviation; SFE–CO_2, supercritical CO_2 extraction; UPLC-ESI-MS/MS, ultra-performance liquid chromatography-electrospray tandem mass spectrometry; US, ultrasound; UAE, ultrasound-assisted extraction; US-SFE-CO_2, ultrasound assisted supercritical CO_2 extraction.
For the complete list of 16 PAHs abbreviations see Table 1 section 6.

General summary of PhD project

Coffee is a principal food commodity as it is the most frequently consumed beverage worldwide. It is made from the roasted seeds of the Coffea plant, and mainly two coffee species are successfully employed in commercial cultivation: *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta).

The procedure used in the manufacturing is to transform the raw fruit of the coffee plant into the finished product. The first phase of the coffee production chain is the harvesting of the fruits which can be performed using manual and/or mechanical techniques, and, since the fruits on the plant ripen at different time, the final quality of the product depends a lot on the accuracy of this phase. The post-harvest operations include pulping, processing, drying, hulling, cleaning, sorting, grading, storage, roasting, grinding, and cupping. The post-harvest processing contributes about 60% of the quality of green coffee beans.

Roasting is clearly a crucial step consisting in an intense thermal process (120-240°C) with variations in time and temperature depending on the desired organoleptic features. It leads to the development of colour, aroma and flavour involving a number of chemical reactions, as the well-known Maillard reaction, responsible of the overall beans' composition change. A lot of scientific research has been done to examine the different components of this valuable vegetal matrix.

Public health issues relating to food have increased the focus of the relevant authorities on the regulation of pollutants, adulterants, process or environmental contaminants in recent years. At the same time, the industry has been compelled to concentrate on the quality of products and raw materials.

The need for in-depth analysis of high-consumption food matrices, including coffee - whose safety and quality are functions of the entire production line - has been prompted by the growing interest in a more protected diet and the evolving regulatory framework.

Chemical residues and pollutants can enter the food supply chain through food processing or environmental variables like air, soil, and water.

The work carried out in this thesis fits into this context; the main objective was to analyse the presence of some pollutants in green and roasted coffee beans. The various projects have focused both on the optimization of analytical methods and on sample preparation of such complex matrix responsible of analytical interferences, and on the formation of artifacts during the phases of the processing that can be incorrectly correlated to the presence of the pollutants. In particular, phthalimide residue in roasted coffee samples was investigated as it is considered a degradation product and marker of Folpet, a pesticide that International Coffee Organization does not list as being applicable in coffee cultivation. Concerning *o*-phenylphenol (OPP), which was claimed to only be detected in roasted beans and not in green beans, the goal was to examine the potential for false negative results in unroasted samples by using the typical multiresidue analytical methodologies for pesticides. Finally, among the process pollutants, acrylamide has been studied. Due to the limited technological advancements available, the mitigation of acrylamide poses a significant problem for the coffee business making crucial the choice of raw material. Thus, it has been explored how green coffee parameters, such as ripening and post-harvest treatments, affect the production of acrylamide in both Arabica and Robusta samples.

In addition, another part of the coffee manufacturing chain was considered during this project by evaluating the use of improved green decaffeination techniques on green beans. With concerns regarding extraction efficiency and time savings, acoustic cavitation was chosen to enhance supercritical CO_2 extraction of caffeine.

The Covid-19 lockdown in 2020 has been dedicated to the drafting and publication of a review article on polycyclic aromatic hydrocarbons (PAHs) considered to be potentially genotoxic and carcinogenic to humans. Since the main cause of coffee PAHs contamination is thought to be their generation during processing stages, the focus of this work was that to summarise and discuss the knowledge and recent advances in PAHs formation during roasting and the residue in different coffee samples including brews, as well as the evaluation of analytical techniques.

I. Phthalimide Residue in Coffee

Folpet is a fungicide belonging to the class of thiophthalimides and is commonly used in agriculture to prevent diseases caused by fungi. In food samples, generation of phthalimide (PAI) as a byproduct of Folpet can occur either during heat treatment or during analytical procedures in sample preparation. Thus, the maximum residue limit for this pesticide is defined as the sum of Folpet and PAI. It has recently been reported for the parsley (Relana position paper No. 16-03; Relana position paper No. 17-01) that PAI can also result from pathways other than Folpet metabolism/degradation (phthalic anhydride (PAA) + NH_3 from amino acids (AAs) pyrolysis), however without defining the source of PAA.
Since Folpet is not listed as an applicable pesticide for coffee, this work aimed to observe and study possible PAI formation during the heating of Folpet-free Arabica green coffee, indeed, the high temperatures reached during roasting result in optimal conditions for the PAI generation not correlated to the pesticide. It follows that the presence of PAI in coffee cannot be solely related to the use of Folpet.
We aimed to demonstrate that PAI can form during the roasting of coffee beans following the thermal decomposition of phthalates (PAE), ubiquitous and unavoidable contaminants in foods, and subsequent reaction of the degradation products (phthalic acid, PA, and PAA) with the released NH_3 from the pyrolysis of free AAs. Various experiments were performed to monitor the generation of PAI employing GC-FID for quantitative determinations. Therefore, the work was structured as follows:

1) Execution of preliminary tests on different AAs, supplemented with diethyl phthalate (DEP) or PAE degradation products (i.e., PA, PAA), placed in hermetic systems and heated to different temperatures in a laboratory oven to verify and optimize the experimental conditions.
2) Tests on samples of Folpet-free Arabica green coffee beans subjected to the same heat treatments to evaluate the development of PAI.
3) Tests on Folpet-free green coffee samples added with DEP or PAA.
4) Tests on Folpet-free industrially roasted coffee samples.

Preliminary tests on PAI generation were carried out by adding PAA to Asp, Cys, Gly, Glu, Lys, and Phe. The thermal treatment was performed in a laboratory oven at a range of temperatures (120, 150, 180, 200, and 220 °C) in order to emulate common coffee roasting conditions.
The quantitative data obtained from the GC-FID analyses (see **Table 1**) 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 and reaches its maximum at 200 °C, thus achieving 32.57 mg of PAI per 100 mg of AA (molar ratio 1:3.4).

Table 1. 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	32.48 ± 2 1.8
Cys	0.12 ±0.01	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 ±0.01	0.30 ± 0.01	0.64 ±0.02	0.89 ±0.05	0.92± 0.01
Lys	0.11 ±0.04	0.11 ±0.03	0.78 ±0.01	1.22 ±0.09	1.17 ±0.05
Phe	0.12 ±0.06	0.71 ±0.01	1.20 ±0.09	1.49 ±0.1	1.42 ±0.08

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 sulfur 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 1** (0.69 mg of PAI/100 mg of Cys vs 0.31 mg of PAI/100 mg of Asp).

As phthalic acid (PA) can dehydrate to PAA at high temperatures, PAI generation has also been evaluated in this study with PA as a reactant. In this case, Asp, Cys, and Gly were employed as they were found to be the most responsive AAs in the tests described above. 200°C was selected as the temperature to carry out these experiments aiming to maximize AAs NH_3 release to guarantee the formation of PAA from PA, and as it is the temperature used for roasting. Moreover, in the attempt to evaluate the time-of-exposure influence on this phenomenon, each reaction was performed in a sealed vial for both 30 and 60 min. Also in this case PAI generation was confirmed with higher levels for Asp but no differences with respect to time employed.

Since the presence of PA and PAA in the environment is due to the degradation of ubiquitous PAEs, DEP was chosen as the model compound for the testing PAI generation, 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 (solid phase micro extraction) and GC-MS analyses.

Trials involving the use of DEP and Asp, Cys, and Gly were performed at three different temperatures (180, 200, and 220 °C), and 30 and 60 min of heating time were compared.

PAI generation was observed only in the tests carried out with the addition of water in minimal quantities (1.24% w/w DEP). This can be rationalized by considering the formation of PAA through the intermediate monoester (see **Figure 1**), whose decomposition, in our opinion, follows the trend reported by Saido et al. (1979) for DEHP. Furthermore, the addition of water in these tests also derives from the fact that green coffee beans contain residual moisture of about 8% and 12.5% as indicated by the International Coffee Organization which allows triggering the degradation of PAE.

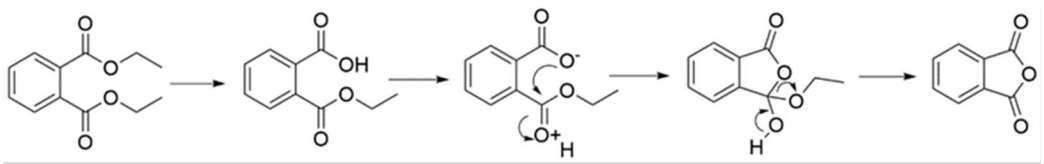

Figure 1. Thermal decomposition of diethyl phthalate to phthalic anhydride in absence or in trace amount of water.

Unlike the previous assays that entailed the direct addition of PAA, a significant dependence on higher temperature occurs for DEP. In fact, only at 220 °C is it possible to observe an increase in PAI generation. In particular, the results reported in **Table 2** show that, for all temperatures, Asp gave higher PAI yields than Cys and Gly with the maximum value of 1.68 mg of PAI/100 mg of AA being observed at 220°C and 60 min of reaction time (molar ratio 1:66). 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 2. 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	0.16 ±0.04	0.26 ±0.05	0.45 ±0.01	0.86 ±0.03	1.68 ±0.08
Cys	0.054 ±0.007	0.063 ±0.005	0.065 ±0.006	0.085 ±0.006	0.083 ±0.004	0.24 ±0.01
Gly	0.069 ±0.004	0.085 ±0.003	0.083 ±0.004	0.096 ±0.001	0.093 ±0.005	0.19 ±0.04

As regards the tests carried out on green coffee, the samples used as such or with the addition of PAA and DEP were subjected to heating at various temperatures (180, 200, 220 °C) for 30 minutes in a laboratory oven in order to emulate different roasting degrees. The results obtained by GC-FID are shown in **Figure 2** and show that PAI was detected in all spiked samples. It is important to underline that the coffee bean, being a complex matrix, requires analysis pre-treatments that are able to best purify the extract and at the same time to enrich the sample. Therefore, an extraction procedure based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) was performed on all the coffee bean samples subjected to the tests, using acetonitrile as solvent and a d-SPE cartridge for the cleaning phase, resulting in purer extracts with high recovery rates (Anastassiades, Lehotay, Stajnbaher & Schenck, 2003) The formation of PAI follows a temperature-dependent trend with the maximum yield being achieved at 200 °C and remaining almost constant at 220 °C. This trend seems to reflect the results obtained in tests involving AAs in the presence of PAA. In particular, higher PAI yields (0.15 mg/g coffee) were detected in coffee samples with added PAA. For DEP enriched coffee, a maximum amount of 0.038 mg/g was observed. The trend found in tests performed on AAs heated in the presence of PAA and DEP was confirmed in this case. Surprisingly, PAI generation was also observed in non-spiked samples, which displayed a maximum PAI generation at 200 °C (0.0056 mg/g coffee, **Figure 2**). Since the samples were certified Folpet-free, it is evident

that the heating process leads to PAI generation, the presence of which can only be due to environmental PAE pollution.

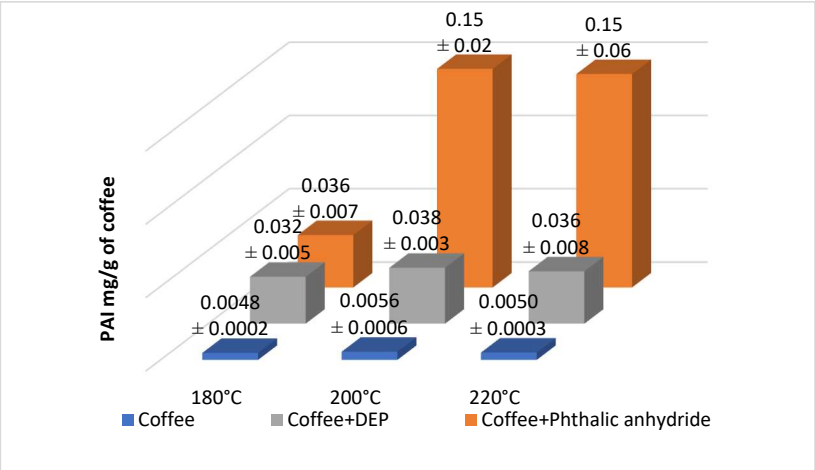

Figure 2. Phthalimide formation (expressed as mg/g of coffee) from Folpet free ground green coffee submitted to heating, either used as such or spiked with diethyl phthalate and phthalic anhydride. Data indicate PAI amount expressed as mg/100mg of AA ± SD.

Of course, it cannot be assumed that this is the effective PAI concentration in commercial roasted coffee beans as the experimental conditions applied for tests are not comparable to those of a roasting process. For this reason, industrially roasted Folpet-free coffee samples (air roaster) that were obtained at four different roasting degrees (named light, medium light, medium dark, dark) were analysed. PAI was not detected in the lighter samples (light, medium light), while darker samples gave concentrations of 0.034 and 0.038 mg/kg, thus affirming that more intensive thermal conditions lead to a higher PAI residue.
It can be concluded that 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.
The results of this study have been published on the Journal of Agricultural and Food Chemistry in 2021.

II. *o*-phenylphenol detection in coffee

Biphenyl-2-ol or *o*-phenylphenol (2-phenylphenol, OPP) and its water-soluble salts (i.e., sodium *o*-phenylphenate, SOPP) are widely employed in the post-harvest treatment of diseases in fresh fruits and vegetables thanks to their antimicrobial activity. OPP and SOPP have not only been used in agriculture to control fungal and bacterial growth on stored crops but are still widely used as general surface disinfectants and in packaging materials.
EFSA sets the maximum residue limit (MRL) for OPP at 0.05 mg/kg for coffee beans. However, OPP and SOPP are not listed among the pesticides used on coffee beans, as regulated by the EU. (European Commission MRLs Coffee beans,https://ec.europa.eu/food/plant/pesticides/eu-pesticides-data-base/products/?event=details&p=244).
A recent paper published by Theurillat et al. (2022) reports that OPP is detected by common pesticide residue analysis methods only after coffee roasting and never in green beans. The authors excluded the possibility that the traces of OPP found in the roasted coffee are due to the possible use of the fungicide on green coffee and to packaging contamination, while they attributed the presence of OPP in the coffee to its generation during roasting. They report

higher OPP levels with increasing roasting degrees highlighting a correlation with the species as Arabica samples have slightly higher amounts than Robusta.
As far as we know, OPP cannot be considered a natural phenol because it is not a component of any food; furthermore, although coffee is considered a rich source of phenolic compounds, the possible chemical pathway of OPP formation during roasting from naturally occurring precursors in the beans is difficult to define.
Therefore, the occurrence of OPP residues in green and roasted coffee beans has been investigated in this work considering its potential as a foodstuff contaminant.
The first step of this work was to evaluate OPP residues in green and industrially roasted coffee samples. Arabica (Brazil) and Robusta (Vietnam) green coffee beans and related roasted samples were analysed with different roasting degrees defined by the colour and identified with a number on a scale between 45 (darker) and 60 (lighter). Due to the very low concentrations of OPP, samples were prepared from 200 g of green and roasted coffee. Sample preparation for OPP determination was performed with a QuEChERS-based method and a triple quadrupole system equipped with a collision cell integrated in a UPLC-ESI-MS/MS instrument was used for analysis, due to its high sensitivity and selectivity.
As reported in **Table 3**, the data show higher biocide content in samples subjected to lighter roasting, while no OPP was detected in the green samples. Moreover, a comparison of the two species showed that higher levels are found in Robusta samples than in Arabica, especially at the degrees of roasting identified, 55 and 60 (32.1 µg/kg vs 16.4 µg/kg and 34.2 µg/kg vs 17.1 µg/kg, respectively).
The results were in complete agreement with those of Theurillat et al. (2022), who found OPP concentrations ranging from < 10 µg/kg to 46 µg/kg in commercial roasted coffee. However, some differences were noted, as they reported an OPP increase with roasting degree and higher values for the Arabica species.

Table 3. OPP concentration in ground green and industrially roasted coffee analyzed via the QuEChERS-method and UPLC-MS/MS.

Roasting degree	Arabica µg/kg (±SD)	Robusta µg/kg (±SD)
Dark (45)	11.3 (±0.3)	traces
Medium dark (50)	11.8 (±0.2)	12.5 (±0.2)
Medium light (55)	16.4 (±0.5)	32.1 (±0.6)
Light (60)	17.1 (±0.1)	34.2 (±0.8)
Green coffee	-	-

To better understand the correlation between OPP detection and thermal treatment, Arabica and Robusta green coffees were subjected to isothermal heating in a laboratory oven. Using industrial roasting temperatures, tests were carried out for 1 h at 120°C, 150°C, 180°C and 220°C.
After heating, the beans were grinded in order to maximize the extractable surface and were then analysed and the graph reported in **Figure 3** shows the mean values of OPP amount found at every thermal condition.

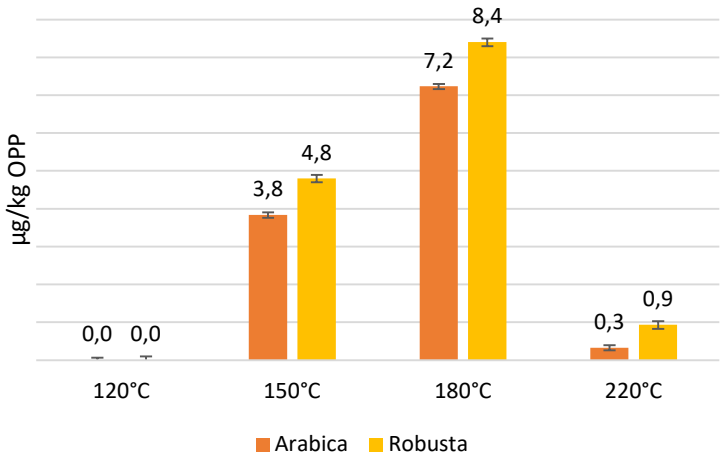

Figure 3. OPP concentration detected via the QuEChERS-method and UPLC-MS/MS in thermally treated Arabica (Brazil) and Robusta (Vietnam) green coffee samples. The treatment was carried out in a laboratory oven for 1 hour.

The obtained results show a significant increase in the OPP values in both species at 150 °C (Robusta 4.8 µg/kg, Arabica 3.8 µg/kg) and especially at 180 °C (Robusta 8.4 µg/kg, Arabica 7.2 µg/kg). The lower levels of fungicide found in these trials, compared to industrially roasted samples, can be attributed to the different levels of thermal shock suffered by the beans in the roaster. As regards the comparison between the two species Arabica and Robusta, the trend previously established in the industrially roasted coffees is confirmed here.

A EURL-SRM (EU reference Laboratories for Residues of Pesticides) report assessed that pesticides bearing reactive chemical groups, such as carboxy-, amino- and phenol groups, tend to form covalent bonds with primary or secondary plant metabolites, generating compounds commonly defined as “conjugates” or “conjugate residues”.

It follows that the presence of conjugates such as ester compounds must be taken into consideration when defining pesticide residues and that any analytical procedure must foresee their breakdown. These observations may provide a rationale for the non-detection of OPP in green coffee, although this matrix was not specifically reported in the EURL-SRM report.

The surface of the coffee bean is covered with a thin layer of wax which makes up 0.2-0.3% of its total weight. Assuming that OPP interacts with components of this outermost layer, we performed the extraction of green coffee beans without grinding at room temperature for 30 min with hexane in order to obtain possible "OPP conjugates", then extracts were submitted to heat treatment in a laboratory oven. To evaluate the sensitivity and efficiency of the method, tests were performed on Arabica coffee, which has a lower OPP content in roasted samples. It was possible to detect the OPP (5.9 µg/kg), only when water was added to the dry hexane extract (10% w/w) before its heat treatment (180°C) in order to reconstruct the humidity of the green coffee. Analysis of thermally treated polar solvent extracts resulted negative in the presence of OPP confirming the hypothesis that the fungicidal conjugates are localized in the lipophilic layer of the coffee.

The exhaustiveness of the extraction method described above was confirmed by an analysis with a negative OPP result, which was obtained by heating (1 h, 180°C) green coffee beans that had previously been treated with hexane.

Since the extracts in methanol are rich in phenolic compounds, it is interesting to note that the presence of OPP was not observed in these trials, thus leading to the conclusion that its

origin may not be linked to its generation from precursors that are naturally present in green coffee beans.

Based on the EURL-SRM report, The preliminary hydrolytic procedure at pH 12 in methanol (70°C, 1 h) on the dry hexane extract and the analysis of the neutralized sample treated with the QuEChERS method allowed to detect OPP at a concentration of 34.8 µg/kg.

This result confirms that OPP is present in green coffee as lipophilic conjugates that undergo partial degradation during roasting. As the OPP levels previously detected on the corresponding industrially roasted coffee samples (17.1 µg/kg for roasting degree 60, **Table 3**) can now be considered underestimated, the hexane extraction and subsequent hydrolysis in methanol were carried out on the same sample, giving a concentration of 32.2 µg/kg. This result may demonstrate that, for full OPP quantification, it is necessary to proceed with the breakup of its "conjugates". It is worth noting that in the case of a complex matrix such as coffee, preliminary sample treatment with hexane is a key step in extracting lipophilic OPP-conjugates. In fact, the direct hydrolysis of roasted coffee in methanol provided OPP detection of 24.8 µg/kg. Finally, the same increase in OPP concentration (64.8 ± 0.4 µg/ kg vs 34.2 ± 0.6 µg/kg) was detected when the modified sample preparation procedure was applied, for the sake of comparison between the two species, on the Robusta sample with a roasting degree 60.

In **Figure 4**, it is possible to observe the influence of sample preparation on the detected OPP levels in both green and roasted samples, and that the optimized procedure (extraction with hexane, basic hydrolysis in methanol, neutralization, QuEChERS and UPLC-MS/MS) allows comparable results to be obtained for Arabica samples both before and after the roasting process. It is also evident that roasting only causes partial hydrolysis in the OPP-conjugates, with the quantity that is detected using the simple QuEChERS method only corresponding to ~50 % of the total amount.

The possibility that OPP-conjugates may be ester compounds formed from the fungicide and free fatty acids present in green coffee beans was investigated by synthesizing *o*-phenylphenyl stearate and adding this compound to a sample of Arabica ground green coffee, at a concentration of 124 µg/kg (i.e. 48 µg/kg of OPP), before subjecting the sample to the above-described procedure. The UPLC-MS/MS analysis of the obtained sample revealed an increase in OPP amount, at 41.5 µg/kg (86% of *o*-phenylphenyl stearate conversion), thus confirming that the conditions for the extraction in hexane and the basic hydrolysis are sufficient to achieve a reasonable release of the fungicide from this kind of conjugate.

Moreover, in order to estimate the possible transfer of OPP into the coffee brew, the beverage was prepared by percolation using the same sample of Arabica roasted coffee (roasting degree 60) and was subjected to freeze- drying. The residue was then solubilized in methanol and hydrolyzed at pH 12 before the QuEChERS procedure, and the analysis observed no OPP, thus demonstrating that there is no risk of OPP exposure for coffee beverage consumers.

For comparison, a commercial sample of soluble coffee was also tested with the direct hydrolysis procedure, and the previous observation of an absence of OPP was therefore corroborated. Furthermore, since there is no specific evidence for the use of this biocide in coffee, its detection may be due to accidental or technically unavoidable contamination during green coffee production.

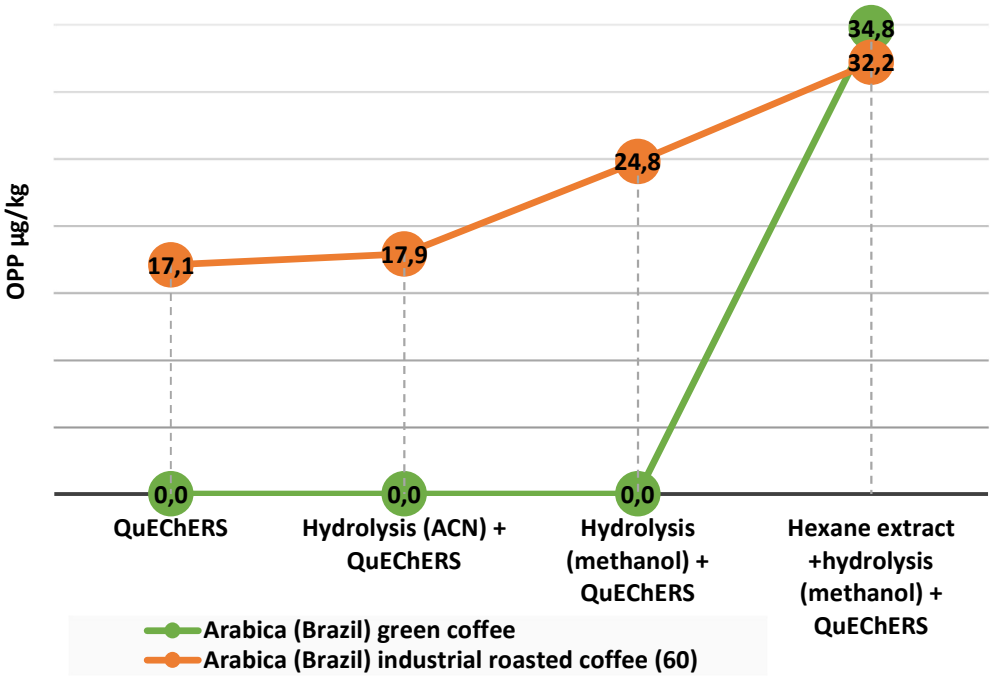

Figure 4. OPP amount (µg/kg) in green vs roasted Arabica coffee according to sample preparation method and UPLC-MS/MS analysis.

III. Acrylamide in roasted coffee

Owing to the growing concern for this contaminant, and with the aim of meeting the recommended levels imposed by regulatory agencies, several studies have been reported on the formation of acrylamide in coffee often pursuing the challenge of finding appropriate conditions able to mitigate its generation.

Even if the Maillard reaction is considered the main route of acrylamide formation, other possible pathways are also contemplated including asparagine degradation, reaction between ammonia and acrylic acid deriving from aspartic acid degradation or fatty acids/glycerol oxidation, and interaction between hydroxymethylfurfural and asparagine.

The characteristic chemical properties of related to each coffee species (mainly Arabica e Robusta) can be influenced by many parameters as variety, cultivation climatic conditions and post-harvest treatments (dry or natural, semi-dry or semi-wet, and wet methods). It follows that all these variables can affect the levels of acrylamide precursors in green beans, making the choice of the raw material of essential importance as reduction strategy for the contaminant.

The number of defective green beans, especially the unripe ones, has been reported as related to an increase of acrylamide content in the roosted product, probably as a consequence to their significantly higher amounts of free asparagine and reducing sugars whit respect to the ripe ones (Mazzafera, 1999). Moreover, it has also been observed that asparagine is present at more significant levels in unripe beans submitted to dry method as post-harvest protocol (Dias, Borém, Pereira and Guerreiro, 2012).

Despite the use of very sensitive analytical methods, the accuracy of acrylamide detection at low levels remains still difficult, not only as regards the sample preparation, but also for the so called “matrix effect”, namely the interference caused by the coffee extract components when tandem mass spectrometry is employed for quantitative determination. This phenomenon, that occurs when co-eluting molecules with the compound of interest alter the

ionization efficiency of the electrospray interface, is commonly observed as suppression or enhancement of analyte signal that implies the need for its assessment during the development and validation of LC-MS/MS methods, and the adoption of compensation strategies as the use of internal standard isotopically labelled.

In this work it has been evaluated how green beans ripening and different post-harvest treatments can affect acrylamide levels in roasted coffee by considering both Arabica and Robusta samples. To quantify the levels of this process contaminant through the UPLC–MS/MS technique, an analytical method based on a single extraction step with water, followed by a two-stage solid phase extraction clean-up was optimized. Deuterium labelled acrylamide (acrylamide-d_3) has been employed to compensate, through the estimation of the recovery %, both the analyte losses and matrix effect during sample preparation and quali/quantitative analysis. The UPLC–MS/MS reliability was evaluated analysing standard solutions of acrylamide and acrylamide-d_3 at different concentration levels (0.05, 0.1, 0.3, 0.5, 0.7, 1 μg/mL), and good linearity was found, with the determination coefficients being >0.99. The deviations of the obtained concentrations from the nominal values were examined for all calibration points and in all cases were <10%. The limit of detection (LOD) and the limit of quantification (LOQ) were determined in 0.02 μg/mL and 0.05 μg/mL, respectively.

As concerns sample preparation, in this study the QuEChERS procedure did not result a performing purification method, evidencing a consistent analyte loss (~60%) during the extraction and partitioning steps, with final recovery results that are quite in accordance with De Paola et al. (2017).

A water extraction of ground coffee samples, followed by an effective SPE clean-up step, has been pursued to avoid the interference of co-extracted components (e.g., polysaccharides, proteins, acids, caffeine, trigonelline, dark coloured colloidal particles) on the quali-/quantitative analysis outcome.

In detail, silica-based C18 bonded phase, anion exchange (QMA), strong cation exchange (SCX), weak cation-exchange (CM) and amino (DSC-NH_2) sorbents were selected for this purpose.

After checking the recovery rate of the analyte (aqueous standard solution of both acrylamide and acrylamide-d_3 at 0.5 and 1 μg/mL) through each SPE cartridge elution, which was found to be greater than 99% on average, the same procedure was applied to evaluate how coffee matrix could affect the contaminant detection. Given the impossibility of having acrylamide-free roasted coffee samples, acrylamide-d_3 aqueous standard solution was employed to spike two samples of water coffee extract (1 ml each), previously obtained from 2 g of grinded roasted coffee, in order to reach final concentrations equal to 0.5 and 1 μg/mL. Following elution through the selected cartridges, the average analyte recovery indicated that acrylamide-d_3 amount significantly decreased after SPE, with best recovery data for C18 (65.2 ±1) and QMA (63.8 ±2) sorbents (**Figure 5**)

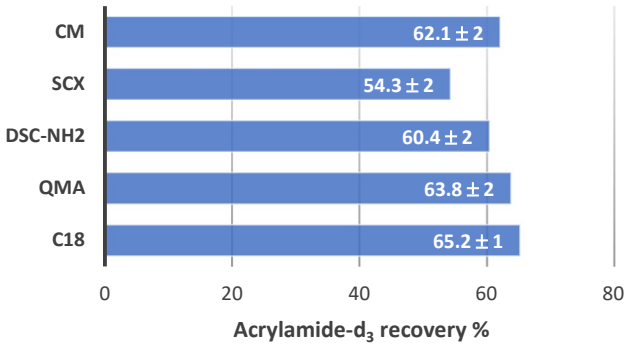

Figure 5. Average acrylamide-d_3 recovery % from elution through different SPE cartridges (silica-based C18 bonded phase, anion exchange QMA, strong cation exchange SCX, weak cation-exchange CM and amino DSC-NH_2) of water coffee extract spiked at 0.5 and 1 μg/mL. Data obtained from 6 replicates.

In order to obtain a cleaner sample and limit the matrix effect, the two most performing stationary phases (C18 and QMA) were then employed in a two-step SPE clean-up procedure with improved results in terms of extract clearing and analyte recovery (77.3 ±1%).
The effectiveness of the two-step clean-up method was then evaluated by directly spiking the ground-roasted coffee with acrylamide-d_3 before water extraction at two different concentration levels (250 and 500 μg/kg). Each obtained sample was submitted to freeze-drying and then solubilized in 500 μL of water to obtain final solutions as concentrate as possible to be submitted at UPLC-MS/MS analysis. Both Arabica and Robusta coffee samples were analysed six times for method performance evaluation and the results, indicating an internal standard average recovery % of 69.3 ±4 %, evidenced the negative coffee matrix effect on acrylamide detection.
The stability of the analyte in the final extract provides information about the storage requirements for the samples until further analysis. The need to perform the quantitative analysis of acrylamide immediately after sample preparation has been demonstrated by a set of experiments carried out within the sample preparation day, after 24h and after 48h (**Table 4**). The extracts were stored at 4°C and the mean percentage reduction in acrylamide-d_3 recovery was found always less than 5% within the first day, of about 17% on average after 24 hours and of about 50% on average after 48 hours for both spiking levels (250 and 500 μg/kg).

Table 4. Acrylamide-d_3 recovery % at two spiking levels after different time from sample preparation.

AC-d_3 recovery % at two spiking levels after sample preparation*		
	250 μg/kg	**500 μg/kg**
Within sample preparation day	67.5 ±2%	69.7 ±2%
24h after sample preparation	57.8 ±2%	56.8 ±1%
48h after sample preparation	35.8 ±1%	34.7 ±2%

* SD calculated over 6 replicates of each sample.

Moreover, since it was not possible to go beyond 69.3 ±4% of acrylamide-d_3 recovery with the optimized cleaning conditions, the entity of analyte signal suppression via UPLC-MS/MS analysis was investigated by comparing the MS/MS response of the spiked coffee extract with the MS/MS response of analyte aqueous standard solution at various concentration levels. To this aim, freeze-dried samples, obtained from extraction and purification of not spiked coffee powders, were redissolved in 500 μL of distilled water and added with acrylamide-d_3 water

standard solution only before the UPLC-MS/MS analysis to obtain the following final concentrations: 0.05, 0.1, 0.3, 0.5, 0.7, 1 μg/mL. As showed by the calibration curves reported in **Figure 6**, the matrix effect was found to be strongly dependent on acrylamide-d_3 concentration compared to the analyses carried out on standard water solutions. Higher signal suppression effect was found for "coffee solutions" with higher analyte amounts in the range -30.65 ±3 to -13.54 ±2 %, while at 0.05, 0.1 and 0.3 μg/mL concentrations the matrix effect resulted almost inaudible. Reported data are in agreement with what reported by several literature studies whose indicated a signal suppression between 30 and 40% for coffee extracts in LC-MS/MS analysis, thus confirming that it is an unavoidable issue, regardless of the sample treatment used, and the necessity of internal standards labelled with stable isotopes employment to allow quantitation accuracy. (Pugajeva *et al.*, 2015; Mastovska and Lehotay, 2006).

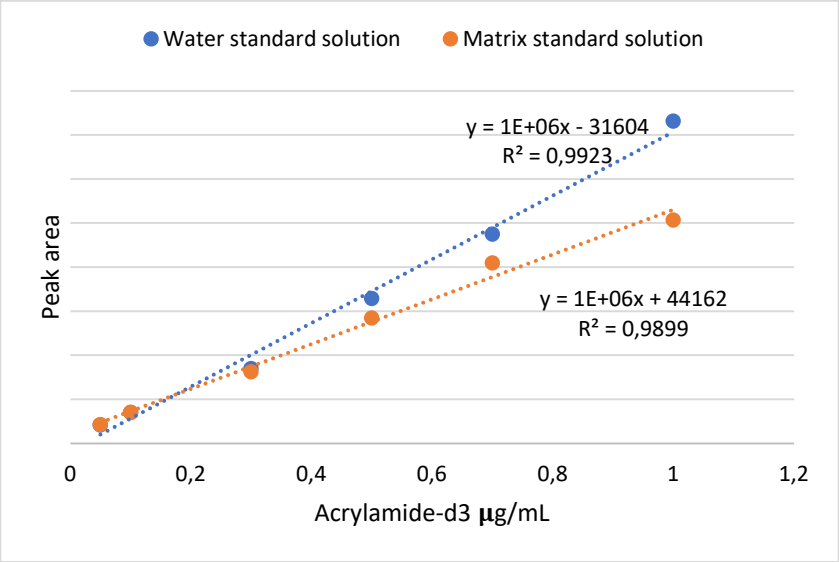

Figure 6. Calibration curves, obtained from acrylamide-d_3 water and water coffee extracts solutions, evidencing the matrix effect on the analyte detection in MS/MS spectrometry.

The described analytical method was then employed to test as both different percentage of unripe beans and post-harvest treatments can affect acrylamide values in roasted samples of Arabica and Robusta coffees deriving from different geographical origins. Since the acrylamide amount in coffee is substantially influenced by roasting procedure, the same roasting level (medium) was applied for all the samples.

Table 5 shows results obtained from Arabica (Brazil) and Robusta (Vietnam) samples both obtained by dry method and containing different percentages of unripe beans (0, 10, 50, 100%). No significant differences were revealed between the two cultivars containing 0% unripe beans, with slightly higher levels in the Arabica one (358.01 *vs* 304.32 μg/kg). On the other hand, the amount of acrylamide detected rises with the increase of the unripe beans amount supporting in this way the idea that a careful selection of green beans could be an effective method for reducing the development of acrylamide during roasting. Specifically, an amount of 10% unripe beans in coffee samples imply an average increase of 11% for Arabica and 19% for Robusta while, when the content rise to 50% a major acrylamide amount of 60 and 80% was respectively found. Finally, in samples consisting only of immature green beans contaminant levels reached higher values, with a more significant increase for Robusta (+160%) compared to Arabica (+99%).

Table 5. Acrylamide quantification expressed as μg/kg in coffee samples containing different percentages of unripe beans.

	Arabica (Brazil) Acrylamide μg/kg ±SD [a]	**Robusta (Vietnam)** Acrylamide μg/kg ±SD [a]
0% unripe beans	358.01 ±7	304.32 ±10
10% unripe beans	397.53 ±8	363.3 ±7
50% unripe beans	573.25 ±10	547.11 ±13
100% unripe beans	712.9 ±9	792.46 ±11

[a]SD calculated over 6 replicates of each sample.

Arabica samples from Brazil subjected to dry and semi-dry processing, and Robusta samples from India, obtained by dry and wet methods, were then analysed to investigate if they can be considered as potential tool to increase final product safety. Since it is not common for all green beans to undergo all kinds of post-harvest treatments, but this is a function of the different cultivars and geographical origins, it was not possible to proceed with an evaluation of the totality of treatments for each origin selected in this trial.

Results reported in **Table 6** show as both Arabica and Robusta natural samples contain higher acrylamide amounts compared to beans subjected to semi-dry and wet methods, specifically in Arabica coffee acrylamide level is reduced of about 20% in semi-dry process, while in Robusta sample treated with wet method its concentration is reduced of about 50%. These values can be related to the higher amount of glucose and fructose, the main reducing sugars involved in acrylamide formation, in natural coffees with respect to the washed ones Knopp et al. (2006). Anyhow, it is known that, as a consequence of the anaerobic fermentation step occurring during wet method, content of this type of sugars is drastically reduced of about 80%.

Finally, when considering samples obtained by dry method, no significant variability of acrylamide levels between Arabica (Brazil) and Robusta (India) species has been found, with slightly higher levels (~8%) detected in the second variety.

Table 6. Acrylamide quantification expressed as μg/kg in coffee samples obtained through different post-harvest methods.

	Arabica (Brazil) Acrylamide μg/kg ±SD	**Robusta (India)** Acrylamide μg/kg ±SD
Dry method	364.2 ±10	396.7 ±9
Semi-dry method	293.4 ±9	-
Wet method	-	204.6 ±8

SD calculated over six UPLC-MS/MS analyses of each sample.

This correlation between post-harvest treatments and acrylamide levels in roasted coffee reflects the reported chemical changes on precursors occurring in green beans, even if literature currently available on this topic is few and some authors did not find significant differences on acrylamide levels between dry and wet processed coffee bean samples.

IV. Decaffeination of green coffee beans

Growing concerns as to the use of organic solvents has stimulated the development of greener techniques for more sustainable and efficient caffeine extraction. Of the alternative green processes available, supercritical CO_2 extraction (SFE–CO_2) can be considered the technique of choice for decaffeination and the recovery of high-value lipophilic compounds from natural matrices.

However, being the SFE-CO_2 extraction characterized by a very slow kinetic (97% decaffeination achieved between 11.5 -22 hours) we hypothesized that the intensification of the mass transfer, enhanced by acoustic cavitation could have significantly improved the diffusion obtaining higher extraction yields and shorter extraction times. We therefore undertook a preliminary study aimed at evaluating the applicability of the ultrasound (US)-SFE-CO_2 hybrid technology for decaffeination. In this work, an SFE unit equipped with a tightly fixed sonotrode with an immersion horn was employed under conditions of sonication and silence. The analysis of the extract was performed using ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS-MS). Caffeine extractions from Arabica green coffee beans were performed with SFE-CO_2 and US-SFE-CO_2 under different experimental conditions (reported in **Table 7**). Comparison of the results obtained shows that, in 1 hour of extraction time, the effect of acoustic waves can lead to a doubling of the caffeine extraction yield and an approximately 10% higher extract purity. The best experimental parameters (US-SFE-CO_2, 75°C, 250 bar, 185 g of sample) were also tested with longer processing times, obtaining a 63.1% decaffeination in 4 hours. These experimental data lay the foundation for further studies on US-SFE-CO_2 caffeine from coffee beans, as an improvement of the decaffeination process in the coffee industry, and as an effective technique to produce purer extracts in shorter time for industrial use.

Table 7. Experimental conditions for caffeine extraction from Arabica green coffee beans and caffeine determination using UPLC-MS/MS analyses. Reported data are the mean of two experiments.

Entry	Sample (g)	Temperature (°C)	Pressure (bar)	Extraction method[1]	Total caffeine (mg)	Extract caffeine %	Caffeine mg /g coffee beans	Decaffein. %	Time (h)
1	185	75	250	a	155.1 ± 11	83.2 ± 6	0.83 ± 0.06	8.86 ±0.6	1
2	185	40	250	a	59.2 ± 4	76.2 ± 6	0.32 ± 0.02	3.38 ± 0.3	1
3	185	20	70	b	26.3 ± 4	80.2 ± 12	0.14 ± 0.02	1.50 ± 0.2	1
4	185	75	250	c	318.3 ± 20	90.1 ± 6	1.72 ± 0.1	18.2 ± 1	1
5	185	40	250	c	126.5 ± 10	83.9 ± 7	0.68 ± 0.06	7.2 ± 0.6	1
6	185	75	125	c	279.8 ± 16	74.1 ± 4	1.51 ± 0.08	16.0 ± 0.9	1
7	50	75	250	c	148.5 ± 9	69.0 ± 4	2.97 ± 0.2	31.4 ± 2	1
8	185	75	250	c	673.8 ± 26	87.3 ± 3	3.64 ± 0.1	38.5 ± 1	2
9	185	75	250	c	906.5 ±31	91.6 ± 3	6.04 ± 0.2	51.8 ± 2	3
10	185	75	250	c	1104.3 ± 40	93.4 ± 3	5.97 ± 0.2	63.1 ± 2	4

[a]Supercritical CO_2 extraction (SFE–CO_2); [b]extraction with liquid CO_2 (LE–CO_2); [c]ultrasound supercritical CO_2 extraction (US–SFE–CO_2).

V. Polycyclic aromatic hydrocarbons in coffee

Polycyclic aromatic hydrocarbons (PAHs) make up a group of more than 200 hydrophobic compounds that are formed of two or more fused aromatic rings. Deriving from the incomplete combustion and pyrolysis of organic matter, they are well known to be ubiquitous environmental pollutants that can be introduced into the food chain and can be formed during food processing. As a result, environmental sources (from air, soil or water), industrial food processing, domestic cooking procedures and, less often, contaminated packaging materials can promote PAHs contamination in food.

The European Union (EU) and the Environmental Protection Agency (EPA) have included 16 PAHs in the list of priority pollutants due to their toxicity, frequency of occurrence and human exposure, as these compounds are potentially genotoxic and carcinogenic to humans. Their presence in various environmental samples, including soil, water and air has been extensively evaluated and also their content in various food samples and related processing.
4PAH (Benzo[a]pyrene, BaP; Chrysene, CHR; Benzo[a]anthracene, BaA; Benzo[b]fluoranthene, BbF) have been indicated by the European Commission as markers suitable for PAHs in food indicating BaP as the most relevant (EFSA, 2008; Commission Regulation (EU) No. 835/2011). The sum of PAH markers in foods should not exceed 10 μg/kg for oils and fats, 35 μg/kg for cocoa beans and derived products, 30 μg/kg for meat and fishery products, and 1.0 μg/kg for baby foods and dietary foods for special medical protocols.
However, as far as we know, there are no regulations indicating the maximum limits of PAH recommended in any drink, including coffee for which the assessment must take into account the preparation technique and consumption habits of the population. Therefore, the aim of the published review article was to describe the source, chemistry and biological activities of PAHs, discuss the generation of PAHs during coffee roasting focusing on different process variables (e.g., temperature, time, cultivar and origin of the coffee), provide a synopsis of the extraction and analytical procedures for the determination of PAHs on coffee beans and on the infusion, with particular attention to the complexity of the matrix.

Conclusions

In conclusion, this thesis mainly concerned the study of some coffee contaminants by means of research focused on the determination of pollutants and on the optimization of analytical methods suitable for their detection.
Our experimental results demonstrated that 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. As far as the determination of OPP is concerned, it was demonstrated that it can potentially form lipophilic conjugates that can mask its presence. We have highlighted how essential is the extraction of the coffee matrix with an apolar solvent followed by basic hydrolysis of the extract before the application of QuEChERS method to allow exaustive OPP detection in coffee samples avoiding the false negatives. As concerns acrylamide quantification, the preliminary outcomes obtained on increasing amounts of unripe beans and different post-harvest treatments need further investigation to better provide a clear correlation between these parameters and acrylamide generation during roasting. The experimental data obtained from the decaffeination tests of coffee beans with US-SFE-CO_2 lay the foundations for further studies both as an improvement of the decaffeination process in the coffee industry, and as an effective technique to produce purer extracts in shorter times for industrial use.

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Abstract

In recent years, public health issues affecting the food chain have triggered health authorities to increase their concern on contaminants and the industry to guarantee the highest quality. Coffee is a popular highly consumed beverage; along its production chain, with roasting being the crucial step, can be subjected to different types of contamination or alteration that may compromise its safety.

Hence, this project was mainly focused on the study of contaminants in green and roasted coffee both to evaluate environmental contamination (e.g., pesticides) and the study of process contaminants generation occurring during thermal treatment. At the same time, analytical and sample preparation methods were developed for the determination of compounds of interest.

The presence of phthalimide residues (ppb levels) in roasted coffee samples was studied since this compound is considered the degradation product of the pesticide Folpet which is not applicable to coffee cultivation. The goal was to prove that the detection of phthalimide in roasted coffee is not necessarily diagnostic for Folpet as it can also originate during roasting from the reaction between phthalic anhydride, derived from phthalates, and amino acids, as a NH_3 source.

Another point concerning coffee safety was considered studying the determination of the fungicide *o*-phenylphenol (OPP), here as well, in roasted coffee while no trace of this compound has been found in green beans. As it is highly unlikely that this biocide can be produced during roasting from precursors naturally present in coffee, the aim was to demonstrate how the presence of OPP in green beans is hidden by the generation of its conjugates with natural components of coffee wax, and that they are the origin of the fungicide release after heat treatments.

Acrylamide represents a process contaminant of great concern in food due to its potential carcinogenic effect. For the coffee industry, the mitigation of this contaminant represents a major challenge due to the limited technological modifications applicable, making the selection of raw material of prime importance. Thus, it has been demonstrated the influence of green coffee parameters, such ripening and the post-harvest treatments, on acrylamide generation by comparing Arabica and Robusta samples. Furthermore, the issues in the analytical determination of this contaminant were also studied.

The work carried out on coffee contaminants also involved the drafting and publication of a review article on polycyclic aromatic hydrocarbons (PAHs), compounds deriving from the incomplete combustion and pyrolysis of organic matter and considered to be potentially genotoxic and carcinogenic in humans. Since the main cause of coffee PAHs contamination is thought to be their generation during processing stages, the focus of this work was that to summarise and discuss the knowledge and recent advances in PAHs formation during roasting considering process parameters' influence and the PAHs residue in different coffee samples. The literature on coffee powder and brew extraction and purification techniques as well as the primary analytical chromatographic techniques was also carefully reviewed.

Alongside, the application of improved green methods in decaffeination process of green beans was evaluated. This process is usually carried out through supercritical CO_2 extraction which provides several advantages (e.g., selectivity towards caffeine, lower toxicity) yet it commonly requires between 12-22 hours. In this study, acoustic cavitation was employed performing an ultrasound-assisted supercritical CO_2 extraction which allowed to considerably enhance the extraction efficiency.

1. Introduction

1.1. Coffee's Origin

The origins of coffee are uncertain, but undoubtedly very remote and distant in time.
According to an ancient legend, the discovery of coffee would be the merit of an Ethiopian shepherd named Kaldi of the 9th century, who walking his flock had noticed a certain hyperactivity in it following the ingestion of particular red berries. Kaldi tasted them and experienced the same energizing effect on him. Hence, at the beginning, coffee berries have been used more and more often as an invigorator of 100% natural origin.
Another legend attributes the discovery of coffee beverage to Sheikh Omar, exiled from the city of Mocha in Yemen to a cave in the desert near to actual Wusab. The story tells that Omar after having tasted the very bitter berries try to toast them to improve their flavour and, following the decoction, he obtained a brown and fragrant liquid with a revitalizing effect.
The first evidence of coffee consumption or knowledge of the coffee plant appears in the mid-15th century in Ahmed al-Ghaffar's accounts in Yemen. It was in Arabia that coffee seeds were initially roasted and produced in a similar way to how it is prepared now.
The cultivation of coffee was restricted to Arabia, and mainly centred in the highlands of Yemen, becoming economically important for both producers and traders. In the 16th century, coffee reached the rest of the Middle East, Persia, Turkey and North Africa. From the Middle East, coffee reached Italy, and from Venetian harbour it was spread throughout Europe where coffee houses started flourishing. At the same time, detailed information on coffee reached Europe through travel reports of scientists, books' prints with drawings of the plant and descriptions of its consumption.
Despite all of the protective restrictions adopted in the Arab world, the expanding interest in the beverage encouraged a search for a similar tropical climate for growing coffee plants, and the first expansion was to the west coast of India. Then, in 1715 coffee entered the French colonies in the Americas and in other places.
Large-scale coffee cultivation began in Central and South American countries in the second half of the nineteenth century with Brazil as the largest producer in the world as early as 1852. The rapid growth of coffee production was accompanied by the growth of consumption in developed countries (Oestreich-Janzen, 2010; Weinberg and Bealer, 2001).

1.2. Coffee plant characteristics and growth

The coffee plant belongs to the botanical family *Rubiaceae*, genus *Coffea*, which grows under tropical conditions. It is a perennial tree or treelet, which can reach heights of 10-15 m with a single main trunk and horizontal branches where dark green elliptical leaves grow in opposite pairs (**Figure ~~Figure~~ 1**). Periodic pruning is carried out to optimize the plant shape for good fruiting, easy harvesting, as well as for effective disease and pest control.

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Flowers development in plants takes several weeks and several months and it is followed by cherry-like fruits growth called drupes (Figure ~~Figure~~ **1**).

Figure 1. Coffee plant on the left and its fruits on the right. https://sca.coffee/research/coffee-plants-of-the-world

Inside the soft pulp, each drupe contains two seeds known as green coffee beans covered by a pellicle named silverskin and by a membrane called parchment (**endocarp**) (Figure ~~Figure~~ **2**). In detail, the **exocarp** is commonly called the skin or peel; it is the outermost tissue of the coffee fruit characterized by a green colour at the beginning, and by a red colour, or also yellow according to the variety, toward the end of maturation. The **mesocarp** is the also called mucilage or 'pulp' rich in sugars and pectins, then the endocarp constitutes the seed envelope.

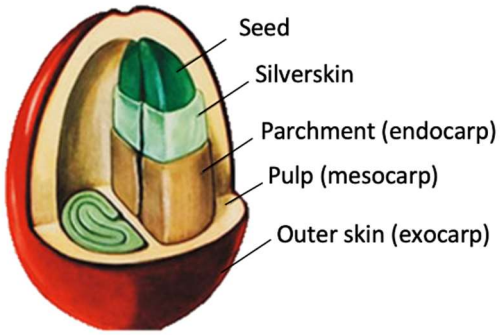


Figure 2. Section of a coffee fruit consisting of the outer skin (exocarp), the pulp (mesocarp), the parchment (endocarp), and the silverskin wrapping the coffee seed.

Coffee seeds consist in the **endosperm** and **embryo** and are generally distinguished by an elliptical and plane-convex shape, with a longitudinal groove on the plane surface. The silverskin, also called the **perisperm**, is the outermost layer of the seed and its colour is useful to determine the presence of immature coffee beans (**Figure 3**) (Farah, 2019).

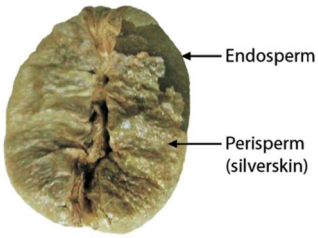


Figure 3 *Coffea arabica* seed (endosperm) and the perisperm called silverskin. From the book: Coffee: Production, Quality and Chemistry (pp.1-25). Farah A., Publisher: Royal Society of Chemistry.

The first attempts at coffee species classification were as early as 1623, when Bauhin mentioned *Coffea* in his illustrated exposition of plants *Pinax theatri botanici* (Bauhin, 1623), while a comprehensive descriptive systematization was proposed by Linne´ a century later in which the overall genus for the coffee species was index as *Coffea* L. (Linnaeus, 1735).
Since their discovery, coffee plants were spread to countries throughout the world, as a result of which several new cultivars have been created reaching more than 100 catalogued species (Davis, Govaerts, Bridson and Stoffelen, 2006).
The tropical areas of Africa host different coffee cultivars, and the first to gain economic importance was the Ethiopian *C. Arabica*. Nowadays, the principal commercial species used for beverage preparation are C. *arabica* L. (Arabica) and C. *canephora* better known as *Coffea Robusta* (Robusta), originally from Congo, Angola, Uganda. Arabica accounts for about 64%, whereas Robusta accounts for ca. 35% of the world coffee production. Both species are chemically distinct and characterized by different sensory features influenced, within the same species, by the coffee variety.
Other species of lesser importance, cultivated and used mainly in the areas of origin, are *Coffea Liberica*, *Coffea Mauritania*, *Coffea Bengalensis* e *Coffea Fragrans*.
In general, Arabica coffee is characterised by soft flavour and notes of exotic fruit, it can be employed as 100% or in blends with Robusta which is stronger and harsh in aroma.

1.3. Green coffee

1.3.1. Chemical composition

Green coffee has a mild and bean-like aroma and is characterized by a complex chemical composition that mainly includes carbohydrates, lipids, acids, minerals, and proteins, plus other nitrogen-containing compounds like caffeine and trigonelline. The amount of these components is influenced by the coffee species and varieties, cultivation conditions, maturation degree, presence of defective beans and the post-harvesting processing method employed. As raw material, its features determine the properties and quality of the roasted coffee and brew, and in this regard extensive research has been conducted.

- **Carbohydrates**

Carbohydrates are products of photosynthesis in plants. During coffee fruit development, they are produced in both the leaves and the pericarp, transported to the perisperm and the endosperm contributing to sucrose accumulation in the coffee seeds. Robusta accumulates about 30% less sucrose than Arabica.

Green coffee beans are composed by soluble and insoluble carbohydrates, the latter are the most abundant. Green beans contain about 50% w/w insoluble polysaccharides, mainly cellulose and hemi-cellulose.

The soluble carbohydrates present in green beans are:

- Monosaccharides: fructose, glucose, galactose and arabinose,
- Oligosaccharides: sucrose (accounting for over 90% of the oligosaccharides), raffinose and stachyose,
- polymers of galactose, mannose, arabinose and glucose.

These compounds are essential substances involved in aroma development during roasting, e.g. through caramelization and in the well-studied Maillard reaction. The sucrose of the green coffee disappears totally with roasting, while small amounts of monosaccharides and other disaccharides are released by roasting and in coffee brewing. A significant portion of the total insoluble carbohydrates is transformed into soluble in roasting. In addition, soluble carbohydrates act by binding aroma, stabilizing foam, and increasing viscosity of the brew (Arya and Rao, 2007; Belitz, Grosch and Schieberle, 2009).

- **Lipids**

Lipids are mainly found in the endosperm of green coffee beans; their content in Arabica is about 15 %, while Robusta contain namely around 10 % (Speer and Kölling-Speer, 2006).

Coffee oil is composed mainly of triacylglycerols with fatty acids, phospholipids, sterols, tocopherols, diterpenes of the kaurane family, mainly cafestol and kahweol and 16-O-methylcafestol, and the respective esters with fatty acids. Kahweol esters are mainly present in Arabica beans, those of cafestol in both Arabica and Robusta, and 16-O-methylcafestol esters only in Robusta, the latter being stable during roasting (**Figure 4**).

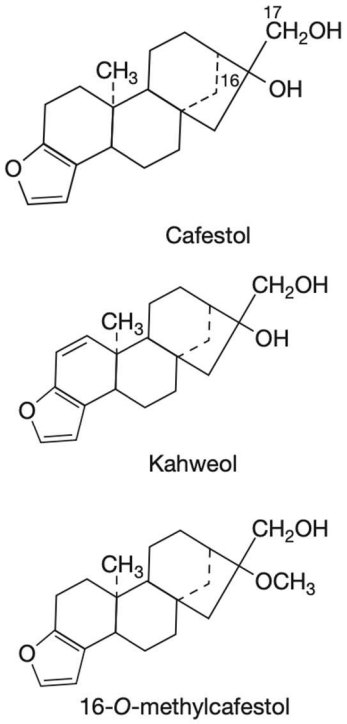


Figure 4. Main diterpenes in green coffee.

In both coffee species the main free fatty acids are $C_{18:2}$ and C_{16}. Linoleic acid ($C_{18:2}$) is the predominant fatty acid, followed by palmitic acid (C_{16}). The content stearic acid (C_{18}) and oleic acid ($C_{18:1}$) are different between Arabica and Robusta, and it is a valuable parameter, together with 16-O-methylcafestol, in differentiating the two species or blending (Belitz, Grosch and Schieberle, 2009).

The so-called coffee wax is located on the outer layer of the beans and corresponds to a small amount (0,2-0,3% w/w) of the total lipid fraction. It is mainly composed by fatty acids with chain length up to C-22, linked as amide to the amino-group of 5-hydroxytryptamine (serotonin). It is noteworthy that the extraction of such compounds, that can exert an irritating effect on the gastric mucosa, can be practiced on green beans in order to obtain a more digestible coffee brew (named dewaxed coffee) (Oestreich-Janzen, 2010).

- **Amino acids and proteins**

Proteins, peptides and amino acids are precursors of roasted coffee aromas. Peptides and proteins'

amount is about 8.7-12.2% of the coffee bean dry weight (Montavon, Mauron and Duruz, 2003), while free amino acids collectively constitute less than its 0.5%, with a content that vary greatly between Arabica and Robusta, being therefore considered a discriminating parameter (**Table 1**).

Amino acids are largely transformed upon roasting, e.g., they take part in the Maillard reaction, resulting in components that contribute to flavour and colour of the coffee brew. Levels of asparagine, aspartic acid, cysteine, histidine, lysine, serine, threonine, and

methionine are largely decreased after roasting, thus implying a clear different composition between green and roasted coffee.

Table 1. Free amino acids content in green Arabica and Robusta coffee.

Amino acid	Robusta (mg/kg)	Arabica (mg/kg)
Alanine	363,5	343,9
Glycine	69,5	53,7
Valine	120,6	100,1
Proline	255,5	271,2
Isoleucine	71,7	65,6
Leucine	539	80
Aspartic acid	539	515,3
Glutamic acid	924,2	1314,7
Phenylalanine	260,5	165,4
Lysine	100,6	76,3
Tyrosine	124,4	69,2
Tryptophan	337,7	178,8

The peptide and protein profile also changes during roasting, they are both fragmented and polymerized, and integrated into melanoidins, which contribute to coffee browning and flavour (Casal, Alves, Mendes, Oliveira and Ferreira, 2003; Preedy, 2014; Belitz, Grosch and Schieberle, 2009).

- **Principal alkaloids: caffeine and trigonelline**

Among the alkaloids present in coffee the main are caffeine (1,3,7-trimethyl-xanthine) and trigonelline, each account for about 1-2.6 % of the dry weight of green coffee and are responsible for much of the bitter and stimulating properties (Belitz, Grosch and Schieberle, 2009).
Caffeine (**Figure 5**) is a methylxanthine with purine-based structure and is biosynthesised as secondary metabolite by the coffee plant for intrinsic chemical defence against herbivory, insects, or microorganism.

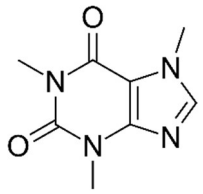


Figure 5. Chemical structure of caffeine.

Its content in beans depends on species and variety, and is typically around 0.9-1.4% in Arabica and 1.5-2.6% in Robusta coffees. Moreover, caffeine levels are not affected by postharvest processing, neither by the roasting. Readily and completely absorbed from the gastrointestinal tract towards its consumption, it is a physiologically active compound with several effects such as the well-known stimulation of the central nervous system and enhancement of blood circulation. However, ascertained negative effects as stimulation of the gastric and urinary systems, increased heart rate and blood pressure, led to an increasing consumption of decaffeinated coffee, generating the development of various processes to remove this alkaloid from coffee beans (Higdon and Frei, 2006; Nawrot, Jordan, Eastwood, Rotstein, Hugenholtz and Feeley, 2003).
Trigonelline (**Figure 6**) is a pyridine derivative known to contribute indirectly to the formation of desirable flavour products, including furans, pyrazine, alkyl-pyridines, and pyrroles, during coffee roasting.

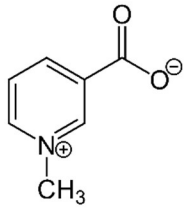


Figure 6. Chemical structure of trigonelline.

The importance of trigonelline, not only as a precursor of flavour and aroma compounds but also as a beneficial nutritional factor, has been well documented. Reports on the thermal degradation of trigonelline have revealed nicotinic acid and nicotinamide, as well as their *O*- and N-methylderivatives; N-methylpyridinium and nicotinic acid increased continuously during the roasting, with the N-methylpyridinium as the major thermal product. In particular, nicotinic acid, which is an important vitamin (Niacin, Vitamin B3), was positively correlated with the roasting degree (Ashihara, 2006; Wei and Tanokura, 2015).

- **Organic acids and chlorogenic acids**

Organic acids present in green coffee contribute to acidity as well as to the flavour and aroma following roasting. They comprise a large fraction of the total mass, about 11% of dry matter in green beans, and their composition is affected for instance by climate, maturation, post-harvest processing and of course species, with notably higher acidity in Arabica than in Robusta coffee. Acids in coffee are generally divided into two categories: organic acids and chlorogenic acids (Yeager, Batali, Guinard and Ristenpart, 2021).
Organic acids comprise less than 3% of dry weight both in green and roasted coffee. The most prominent and important for flavour development in green coffee are citric, malic, ascorbic, and acetic acids, which serve as precursors to other acidic compounds in roasting. Although phosphoric acid is an inorganic structure, it is noteworthy as it also affects the acidity of the beverage. In roasted coffee, further acids are formed by carbohydrate degradation with a rise in overall acidity attributed to an increase of lactic, glycolic, formic and acetic acids that are formed, though the latter are partly lost at higher roasting temperatures due to high volatility (Farah, 2019; Ginz, Balzer, Bradbury and Maier, 2000).
Acids are undoubtedly one of the most significant elements in coffee from a sensory perspective, and constitute one of the primary criteria used by professionals in the coffee

industry to rate the quality. According to estimations, the contribution to the beverage's total acidity can be so ascribed: 25% to acetic acid, 20% to citric acid, 8% to chlorogenic acids, 6% to formic acid, and 6% to malic acid (Brollo, Cappuccio and Navarini, 2008).
Chlorogenic acids are bioactive compounds, and the most abundant secondary metabolites in coffee accounting up to about 10-11 % of dry matter, they accumulate in the bean as the fruit matures.
Despite the "chloro" of the name, chlorogenic acids contain no chlorine. Instead, the name comes from the Greek χλωρός (khloros, light green) and -γένος (ghenos, a suffix meaning "giving rise to"), pertaining to the green color produced when chlorogenic acids are oxidized.
They are polyphenolic compounds, extensively studied due to a wide range of biological functions and excellent antioxidant potential, with coffee representing one of the richest dietary sources. Literature describes the growing epidemiological evidence and support for the protective effects of coffee on health against degenerative diseases like cancer, cardiovascular disease, and type 2 diabetes (Preedy, 2014).
There is an entire family of chlorogenic acids, which primarily consists of hydroxycinnamic acids (caffeic acid, ferulic acid and p-coumaric acid) esters with quinic acid (**Figure 7**). The most common chlorogenic acid in coffee is caffeic acid (5-*O*-caffeoylquinic acid) and its structural isomers (3- and 4- *O*-caffeoylquinic acid), followed by feruloylquinic acid and and *p*-coumaroylquinic acid including their isomers present in lower concentrations. Furthermore, several isomeric di-esters of quinic acid are present in coffee beans, e.g., di-caffeoylquinic acid, and mixed esters like caffeoyl-feruloylquinic acid. Di-esters compounds have negative effects on coffee's sensory quality thus represent crucial harvesting management indicators, as they are converted into monoesters as the beans mature (De Menezes, 1994; Oestreich-Janzen, 2010)

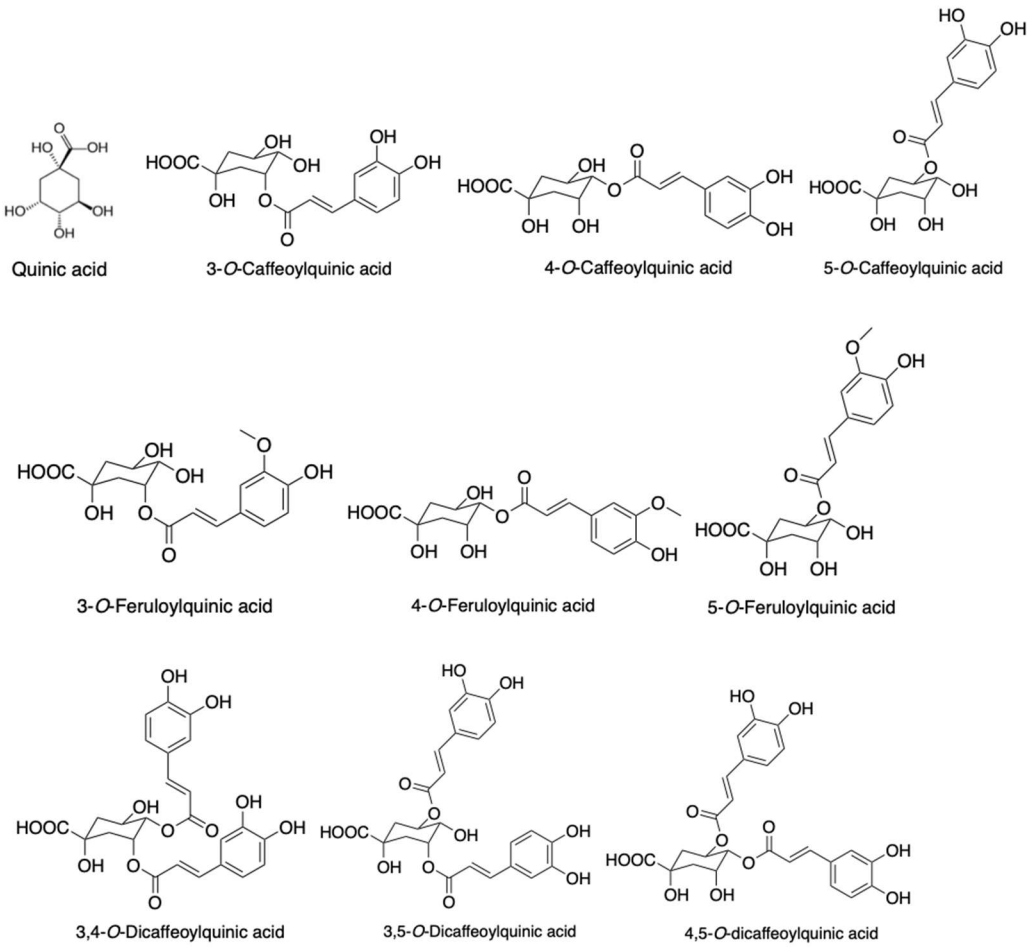


Figure 7. Quinic acid and most common chlorogenic acids in coffee.

A key factor in the flavour differences between coffees is the difference in chlorogenic acids concentrations between Arabica and Robusta (4.1% dry matter and 11.3%, respectively), as they contribute to the acidity, sourness, and bitterness of the beverage. Coffee's level of free chlorogenic acids gradually decreases during roasting, being largely converted into more complex macromolecules, such as melanoidins, or undergoing several transformation products that are peculiar to coffee (Leloup, Louvrier and Liardon, 1995).

- **Volatiles in green coffee**

The aroma of coffee is provided by volatile aromatic chemicals. Despite green coffee has more than 200 volatile compounds, the typical coffee flavour is produced during the beans roasting and will be discussed in the relative section.
However, some of volatile compounds present in green beans like 2-methylisoborneol, 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine enable Robusta and Arabica coffees to be distinguished from one another (Czerny and Grosch, 2000; Blank and Grosch, 2002)

- **Green coffee bean moisture**

Ideally, water should represent 10-12.5% of green coffee weight and changes to humidity or exposure to sunlight, during either transport or storage, could affect its content. Determining the moisture level of coffee beans is crucial for safe transport and storage as well as to reduce the danger of fungal growth affecting the quality.

1.3.2. Coffee harvesting and processing

Coffee plant (or tree) prefers a warm and temperate climate on mountainous soils, preferably of a volcanic nature and rich in nitrogen. It thrives well in countries with a hot and humid climate, with temperatures between 18 and 22 degrees. The ideal would be not to exceed 20 degrees. It is grown in about 90 countries and the main areas are located between the Tropic of Cancer and that of Capricorn (**Figure 8**). In the subtropical areas, the plants have been cultivated in the open field, while in the tropical ones, as they need shade and have to be protected from currents, trees were planted next to taller and larger plants, such as banana and castor trees, to ensure them plenty of shade (Oestreich-Janzen, 2010; Davis, Govaerts, Bridson and Stoffelen, 2006).

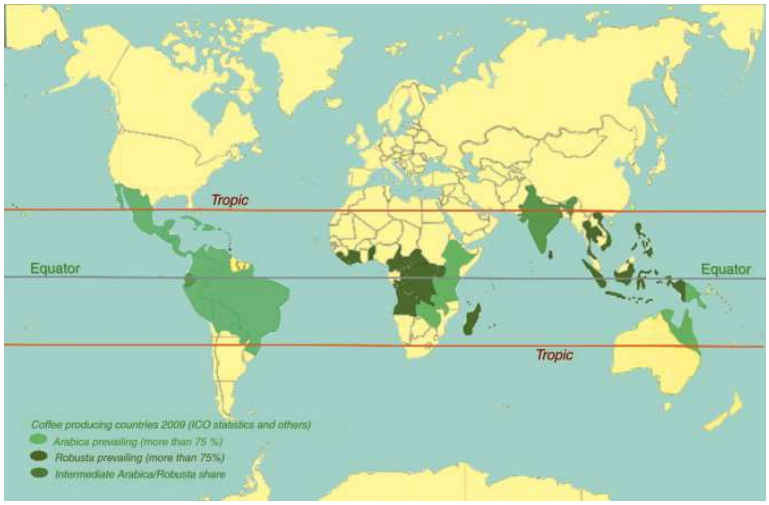


Figure 8. Distribution of Arabica and Robusta coffee cultivation in the world. From: Oestreich-Janzen, S. (2010) Chemistry of Coffee. CAFEA GmbH, Elsevier Ltd., Hamburg, Germany, Vol. 3, 1085-1096.

The coffee supply chain is quite complex and is divided into numerous phases that initially concern the processing of the fruits and subsequently that of the green beans.
The chemical composition of the product is impacted by each process, including seed maturation, postharvest processing, storage and shipping on land and sea, roasting, home brewing or commercial extraction, and any subsequent intermediate treatments.

- **Cherries harvesting**

The coffee plant propagates by sowing or by cuttings, and starts to produce fruit (called drupes or cherries) in clusters along its branches after 4-7 years when it reaches maturity.
The coffee flowering and fruit cycle may occur at various times throughout the year in equatorial regions, while in non-equatorial regions, which represent most of the worldwide

coffee production, they follow a single annual cycle. The coffee plant does not bear fruit uniformly, but rather over a period of 3 to 6 weeks which varies by region and altitude.
Since the fruits do not develop at the same time, on the same plant the berries can be ripe (red), unripe (green) or sometimes even dry or overripe (brown) as shown in **Figure 9.**

Figure 9. Coffee cherries at different ripening degree.

It is consequently necessary a manually harvest to have a better fruit selection, essentially every day of the harvest time, choosing just the mature drupes in order to create a coffee that is as homogeneous as possible; this collecting technique is known as *picking*. In terms of both time and effort, it is unquestionably the most expensive procedure, and is typically applied to all Arabica coffees cultivated in mountains (e.g., Colombia, Mexico, Ethiopia).
The second most popular technique is called "stripping," which consists in removing all of the drupes from a branch either manually or mechanically using machinery as occurs for example in the Brazilian highlands (**Figure 10**). Undoubtedly, it is a quicker and more affordable method, but because there is no chance for a proper sorting during the harvest, ripe fruits are harvested alongside dry and immature cherries.

Figure 10. Coffee harvesting technique through machinery called "stripping".

- **Post-harvest processing**

In order to prevent an uncontrolled fermentation in the drupe's moist mucilage, which would result in undesirable "off-flavours" in the cup, the fruits need to go through primary processing, which involves separating the seeds from the rest of the fruit and sorting them based on the quantity and types of defects, size, and other factors to help classify the coffee lots.
To prevent microbial spoilage and chemical degradation during storage prior to roasting and distribution, green coffee beans must be dried to a final water content of <12.5% weight. Conventionally, there are two types of drying techniques used in the coffee processing that is sun drying and mechanical drying. This step is one of the most important factors influencing

coffee quality; in fact, studies show how different drying techniques alter antioxidant activities, bioactive components, fatty acid composition, and volatile profiles in green coffee. For this purpose, freshly harvested coffee drupes can be processed through two main traditional procedures: the dry method, named "natural", or wet method, also known as “washed”. The dry processing is predominantly employed for Arabica beans in Brazil, Ethiopia and Yemen, as well as for most of Robusta coffee, while the wet process is predominant for Arabica.

There is also a third technique, called semi-wet or semi-dry method, more widespread in the processing of the finest Arabica coffee (commercially called specialty coffee). The choice between these three techniques (summarized in **Figure 11**) depends on different factors such as regional climatic conditions, consumer demand for specific characteristics, water and equipment availability.

Moreover, recently, digestive bioprocessing is practised on a small scale to produce the world’s most expensive coffee (kopi luwak and black ivory coffee) (Ghosh and Venkatachalapath, 2014.; DAS, 2022).

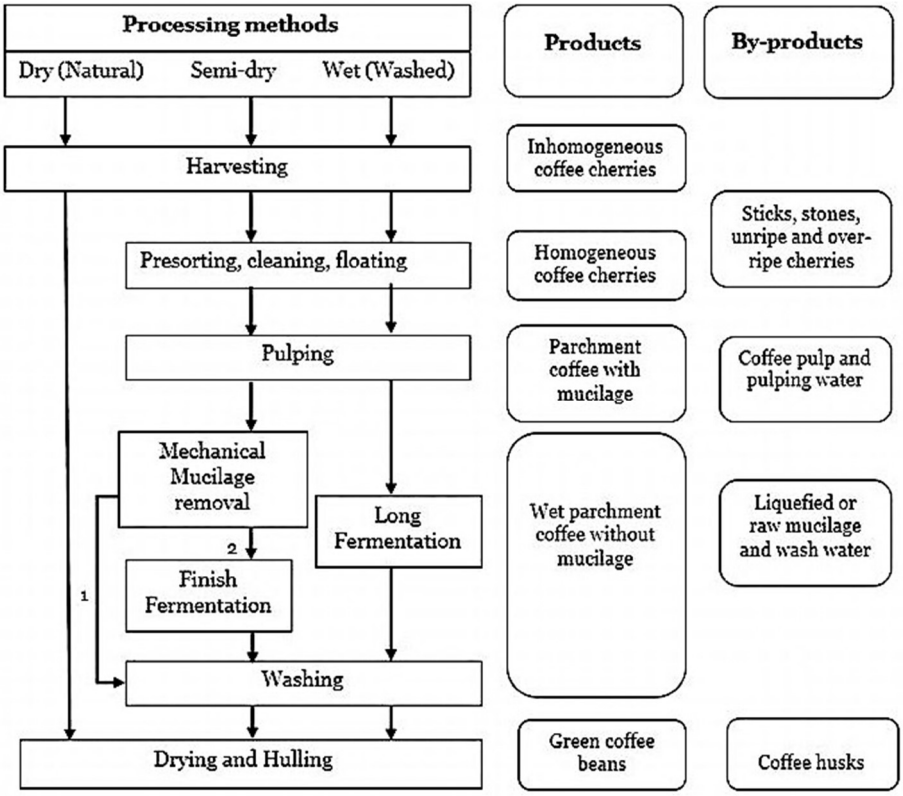


Figure 11. Scheme of coffee post-harvest processing through dry, semi-wet/dry and wet methods.

Dry/natural processing

Simpler from the other methods, the dry processing could take 3-6 weeks. In the traditional natural process collected drupes are dried, by exposing them to the sun in concrete *patios* or lifted beds, until their moisture content drops to between 10 and 12%. To prevent the cherries from spoiling, during drying they are raked and turned throughout the day (**Figure 12**).

Figure 12. Coffee drupes drying through natural method in lifted beds (on the left) and in patios (on the right). https://badgeranddodo.ie/natural-processing/

The pericarp is then removed from the dried cherry by hulling it, either by hand using a pestle and mortar or using a mechanical huller. Finally, the obtained beans are sieved and selected according to their size and defective beans are removed.
Until a few years ago, this process was mainly used for Robusta coffees, while today more and more farmers are using the dry method with Arabica coffee, including those belonging to the specialty category: the seed in fact absorbs sugars and other aromatic substances from the peel and from the pulp, resulting in less acid, more full-bodied and sweeter.

Wet processing

The wet processing is more sophisticated and used to produce a beverage of higher quality. This technique, which exclusively use ripe cherries, has numerous variations. Generally, the first step is the drupes selection mechanically or in floating tanks. After sorting, the material is mechanically depulped to remove the peel, then followed by removal of the sticky mucilage layer by fermentation or mechanical means.
Fermentation is employed to get rid of the residual pulp and silver skin. It can happen naturally or with the assistance of microorganisms or enzymes. Acidity rises during fermentation, and pH can fall as low as 4.5. Beans so obtained are next washed in tanks of clean water or with washing machines to remove residues (**Figure 13**), and then submitted to drying in yards under the sun (3-16 days) or suspended in ventilated tables with hot air (1-2 days).
This process is typically applied in regions, including Colombia, Asia, and Central America, where coffee is manually picked and in which there is currently an abundance of water. Numerous farms in nations with higher volume production, like Brazil, have also adopted it as a result of the increased market value.

Figure 13. Coffee beans washing in wet processing technique. https://crema.co/guides/coffee-processing-methods

The main distinction between wet and dry processing is that most of the outer sections of the seeds are removed in wet processing before drying, including the silver skin which is removed both during and after the fermentation process.
It is widely recognised that the so-called washed coffees, which are green coffees produced by the wet process, produce roasted beans that are characteristically different from those produced with the dry method. These flavour variations can be partially attributable to variations in the level of complexity involved during either technique of post-harvest treatment as well as the fact that only completely ripe coffee cherries are used for wet processing, while fruits at various stages of ripeness are used for dry processing.

Semi-washed/dry processing

The technique, known as the semi-washed or semi-dry method, has been developed in Brazil and combines both the dry and wet processes and, in recent years, it has been renamed to honey process. In this procedure, drupes are first depulped as in a washed coffee, removing the outer skin of the fruit and exposing the sticky mucilage. The process differs from the washed one as the drying process begins directly after depulping rather than undergoing fermentation in water. As the pulp and mucilage dry in contact with the coffee beans, their colour changes from clear to amber, giving the coffee beans a honey-like appearance.
While the initial pulping gives more speed and control to the drying process than leaving all the skin and pulp intact as in the natural process, in this case special care must be taken to avoid unwanted mold and fermentation as the coffee dries.
Once the peel has been removed, the coffee is hung out to dry in the sun with different levels of pulp and mucilage, thus obtaining different types of honey coffee: yellow and white coffee beans have less mucilage after being mechanically washed and are more similar to washed coffees. On the other hand, golden, red, and black honey coffees gradually have more mucilage and consequently this leads to a sweeter and more full-bodied coffee.
Because they tend to offer more body and sweetness to the beverage than wet processed seeds, coffee treated using the dry and semi-dry methods, which preserve the polysaccharide-rich silver skin, are frequently used in espresso coffee blends.
After processing green coffee is sorted to remove defective beans (i.e., black, immature, broken), and then stored in jute, sisal bags, or big-bags with different capacity, under controlled temperature and relative humidity.

Following the method of treatment, the beans (**Figure 14**) are stored after mechanically, manually, or electronically selection to separate and identify defective beans. This step could be also followed by additional sorting, by the use of UV light to identify defects generated during dry, wet or semi-dry processing.

Figure 14. How the beans appear after the different post-harvest treatments. https://www.caffeernani.com/conoscere-il-caffe/come-viene-lavorato-il-caffe-dopo-il-raccolto/

1.3.3. Green coffee storage and shipping

Coffee beans need to be stored, and certain measures must be taken to keep them from deteriorating or losing quality before they can be shipped. Paying special attention to humidity, storage facility location, and storage time are just a few examples.

Until recent years, all the coffee was packaged in jute bags and shipped in containers by ship, arriving at the roasters months after the coffee was processed. Roasters and importers often had the experience of tasting a coffee at the origin, and perhaps tasting and approving a sample before shipping, then receiving a coffee spoiled by exposure to bad weather during storage or transport.

Over the past decade, numerous quality roasters have led a revolution in the packaging and transport of green coffee. Many roasters, even some of the smaller ones, now buy coffee directly from farmers, share information about cupping (internationally standardized tasting method, also called "Brazilian tasting") and the classification of green coffee with farmers, asking for a fast delivery of coffee in packaging designed to preserve its freshness and quality. Jute bags are the most common and economical option for packaging and transporting green coffee, however, do not protect coffee from moisture or odours, so coffee is vulnerable to damage during transport and storage, as well as potentially attackable by mould and pests (**Figure 15**).

Figure 15. Jute bags employed in coffee storage. https://www.rawmaterial.coffee/colombian-defect-quality-classification-article

Vacuum bags are the best packaging available for green coffee as they protect the beans from humidity, odours, and oxygen, significantly slowing down the aging of green coffee. Before vacuum sealing, care must be taken to measure the moisture of the beans to prevent the development of mold during storage. Vacuum packing is more expensive than jute, requires special equipment and skills to implement, and often delays the shipment of green coffee, so it is not without cost and risk.
Hermetically sealed bags protect the coffee from moisture and odours and are cheaper and easier to use than vacuum packaging. GrainPro bags are commonly employed to store coffee, they are hermetic bags made of polyethylene with excellent water and air barrier being significantly more efficient than jute bags. GrainPro bags are often the best and most practical option for quality-conscious roasters. As in the case of vacuum sealing, it is important to measure the moisture of the beans before packing them to prevent the development of mold and other microorganisms during storage (**Figure 16**).

Figure 16. GrainPro bags for green coffee storage and shipping. https://www.grainpro.com/fr/grainpro-twist-tie

Sacks are stored for up to several months before being loaded on the major cargo ships, inside of standard shipping containers at temperatures between 18 and 25 °C. After two to three weeks, the coffee will arrive in ports all over the world and arrive at the factory for further processing. All deliveries should go through a preliminary inspection to look for damage, evidence of rotting beans, bag integrity, and symptoms of pests before releasing the material for production. Coffee consignments are first sampled following a predefined sampling plan and then subject to physical testing and sensorial analysis. These tests enable a grading of the coffee (confirmation that delivery complies with a specification) and include quality characteristics such as defects, moisture (according to the International Organization for Standardization's standard (ISO 6673).

1.3.4. Decaffeination process

The properties of caffeine are numerous, and coffee is its major source in the daily modern human diet. In addition to its most popular role as a stimulant to the central nervous system, Parkinson and Alzheimer's disease risk has been found to be primarily lowered by caffeine consumption.
The average amount of caffeine in green coffee depends on species and variety, and it is typically around 0.9-1.4% in Arabica and 1.5-2.6% in Robusta coffees. Moreover, its levels are not affected by postharvest processing, neither by the roasting. In general, Robusta coffee

beans contain 40-50% more caffeine than Arabica. Caffeine content may also vary considerably in a cup, depending on cup size and method of preparation (Burdan, 2015).
Despite many positive effects, this xanthine may be undesired by certain consumers (e.g., affected by cardiovascular diseases) and, to satisfy this need, different decaffeination techniques have been developed and applied to green coffee beans able to substantially reduce caffeine content.
According to legislation within the EC, caffeine content is reduced to 0.1% or less in decaffeinated roasted coffee beans and to 0.3% or less in decaffeinated soluble/instant coffee (DIRECTIVE 1999/4/EC).
Currently, the coffee decaffeination process can be divided into two main groups: the natural one, where water or carbon dioxide are employed, and the organic solvent decaffeination process where methylene chloride or ethyl acetate are the extraction solvents. In general, the process should avoid the loss of aroma.

- **Decaffeination Process Using Organic Solvents**

To this purpose, the green coffee beans are placed in a battery of extractors and are steamed for between 15 and 60 minutes at a temperature of 105-110 °C to provide a bean moisture content of at least 16%. The steamed green coffee beans are then soaked in water to increase their moisture content up to a final value of 50% and subjected to extraction under direct contact with methylene chloride for 8 to 12 hours at temperatures between 60 and 100 °C and pressures of 0.35-0.55 MPa.
At the end of the extraction, the coffee beans are removed, and steam treated from 1 to 4 hours to strip the solvent residues. On average, the amount of solvent required to decaffeinate 1 kg of green coffee beans is less than 8 kg (Farah, 2019).
The main problem with the use of dichlorine methane is linked to the fact that it is a solvent considered harmful and potentially carcinogenic. Another choice of solvent in extracting caffeine is represented by ethyl acetate that acts in direct contact with the coffee beans. With the advantage of not being regarded as dangerous to human health, the ethyl acetate decaffeination operation follows to the same rules as those of the methylene chloride direct approach (Berry and Walters, 1941).
When organic solvents are employed, residues may be present in the coffee beans. The European Directive 2009/32/EC fixes a maximum limit of 2 ppm of residual methylene chloride content in roasted coffee.

- **Water or Swiss Water Decaffeination**

The rationale behind using only water in the decaffeination process is based on the need of a chemical-free, natural procedure that calls for the use of an odourless and tasteless solvent. When compared to the aforementioned organic solvents, water has a far lower selectivity towards caffeine, making it a poor solvent for decaffeination. In fact, proteins, amino acids, chlorogenic acids, carbohydrates and alkaloids are water soluble and the loss of these compounds extremely affect the organoleptic properties in roasted beans.
Water was first tested as a decaffeination solvent in 1933, but at the beginning of the 1980s, this process emerged.
In this application the green coffee beans were soaked in an extraction vessel in hot water, 70-90°C to extract caffeine. The green coffee extract is then forced to pass over a pre-loaded activated carbon bed, where ideally caffeine is adsorbed. After 6 to 12 h extraction, the green coffee beans are dried by hot air to 30% of the moisture content. The green coffee extract is

then concentrated under vacuum distillation and mixed with dried beans for 3 h at 70 °C to absorb completely all the solution. Finally, the green coffee beans are dried to 10% of the moisture content.
This process has seen numerous advancements over the years, including new caffeine adsorption filters, enhanced hydrodynamics for less water use, and improved drying control of the green coffee beans.

- **Supercritical CO2 caffeine extraction**

The process conditions used in caffeine extraction using supercritical carbon dioxide are different from those used in other procedures, particularly for the high pressures required.
This technique has been ongoing since the 1960s when discovered that carbon dioxide at supercritical conditions could dissolve caffeine. Later resulting in the first patent in which carbon dioxide saturated with water above its critical point was used to remove caffeine from moist green
coffee beans (Zosel, 1964).
Coffee beans are first moistened to a water content of between 40 and 50%. Then, the expanded beans are put into a battery of high-pressure vessels, where fresh carbon dioxide saturated with water is passed through the fixed bed as long as necessary to eliminate the caffeine contained. To decaffeinate 1 kg of green coffee beans, it takes between 200 and 270 kg of carbon dioxide, depending on the type of beans.
In this process, water acts as a polarity co-solvent modifier, while carbon dioxide acts as a transport vehicle to enhance the mass transfer. Moreover, it should be emphasized that important compounds in coffee, for example chlorogenic acids and trigonelline, show a much lower affinity for CO_2 than caffein (Azevedo, Mazzafera, Mohamed, Melo and Kieckbusch, 2008; Mills, Oruna-Concha, Mottram, Gibson and Spencer, 2013).
In the supercritical CO_2 decaffeination process the caffeine solubility and extraction rates are dependent on temperature and pressure. **Figure 17** shows a simplified process flow sheet for coffee decaffeination using carbon dioxide in which the pre-wet coffee beans are loaded to the high-pressure extractor, and carbon dioxide is continuously being fed to the extractor by a circulation pump. The aqueous droplets remove the caffeine from the CO_2 stream. The recycled CO_2 stream passes through activated carbon filters before being fed again to the coffee extractors, while the caffeine can be concentrated by vacuum distillation or by reverse osmosis from the aqueous solution.
Thanks to a very high selectivity for caffeine, this technique allows different types of green coffee beans to maintain their identity. Product quality is claimed to be comparable to caffeinated coffee due to the avoidance of any aroma/flavour precursor loss during the decaffeination process. Moreover, carbon dioxide is a gas at ambient pressure and temperature, so no chemical residue is left in the beans after the decaffeination process.

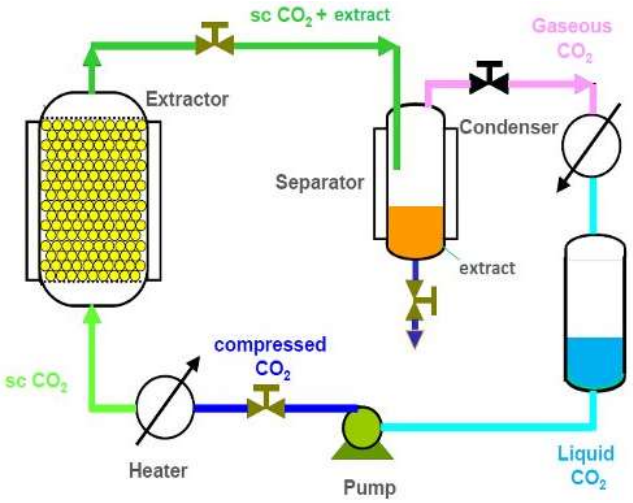


Figure 17. Simplified scheme of a supercritical CO_2 device for coffee decaffeination.

1.4. Roasting process

Roasting is an intense thermal treatment during which coffee beans are heated at temperatures higher than 200 °C for different times, depending on the desired colour, aroma and taste of the final product which is very complex and variable (**Figure 18**).

Figure 18. Spectrum of the different tastes and aromas of roasted coffee.

The roasting process can be divided into three main phases:

- A preliminary **endothermic drying step** during which most moisture is eliminated before coffee reaches 150°C losing between 8 and 10 % weight. In this stage, a few steps of Maillard reactions take place between amino acid groups and carbonyl groups of reducing sugars (see **Appendix 1**). A distinct colour and aroma begin to develop at this stage. In fact, due to the breakdown of chlorophyll, the beans turn from green to yellow within the first few minutes of roasting. Moreover, the Maillard reactions play a major role in the beans' gradual transition from yellow to light brown as the roasting process continues. The aroma shifts from herbaceous to buttery and bread-like. Still, the temperatures are too low to initiate pyrolysis.
- The actual **roasting process (Figure 19)** starts with an *initial stage* at temperatures between 150 and 180°C in which the Maillard reactions are still occurring, and the coffee beans are beginning to darken. Sugars caramelization starts with sucrose being the most abundant. The beans' acidity increases during this phase because carbohydrates degrade into carboxylic acids. Large volumes of carbon dioxide are released from the beans, significantly changing their chemical composition, and hundreds of compounds related to coffee's aroma and flavour are developed as the beans turn dark brown colour. The brown colour deepens owing to caramelization in the latter stages of roasting, when the beans get close to the first crack as described below.

  In the *second stage* of the roasting process (180-230°C), colour continues getting darker as temperature increases. The process is initially exothermic as pyrolysis takes place at this stage leading to organic substances degradation, producing heat as result of chemical transformations. When volatile chemicals are completely released, the process becomes endothermic again and can reach a maximum temperature of 230°C in case of dark roasting.

  There are two moments during roasting when coffee beans emit “cracks”. *First crack* occurs at an internal bean’s temperature of approximately 200 °C and coincides with the release of steam and gases. Each bean experiences a first crack. After about two minutes, first crack ceases and there is an acoustically inactive time. *Second crack* occurs when the temperature reaches about 230 °C and additional gas is emitted along with increased fracturing of bean material. Again, a few beans begin second crack and over about two minutes second crack stops. The beans can ignite and burn beyond this phase and hence roasting rarely continues over the end of second crack.

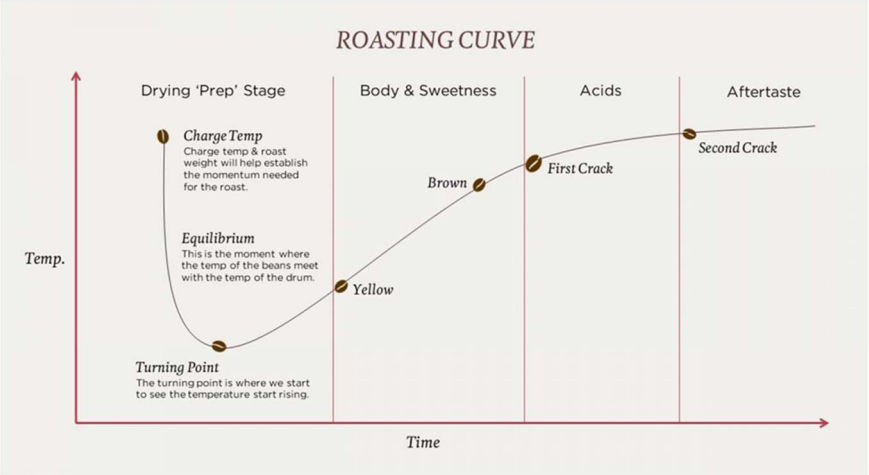


Figure 19. Roasting curve plotting time and temperature applied describing the three main phases of the process (drying, roasting, cooling).

While in the first stages acidity increases, in the final step of roasting it decreases with a pH increase at around 7.0 as roasting gets darker. Shortly before, or immediately after the start of the second crack, the oils come out on the surface of the bean (Wilson, 2014). These changes make beans expand and became more permeable facilitating the extraction of the soluble components during coffee brewing (**Figure 20**). In a dark roast, charring can make the beans black.

Figure 20. Section of coffee beans: a) green bean, b) roasted at 70°C, c) roasted coffee bean. The picture shows the physical changes in the beans structure evidencing the incresed porosity in case of roasted bean. From: Illy, A.; Viani, R. (2005) Espresso coffee: the science of quality, 2nd ed.; Elsevier Academic Press: San Diego.

- A final rapid **cooling** phase is necessary to stop roasting reactions, using air or water as the cooling agent. Companies tend solely to use cool air as water can increase beans moisture and promote undesirable effects.

Roasting is carried out using roasters (**Figure 21** and **22**), machines consisting of a pre-loading chamber where green coffee is put on hold, and the roasting chamber where the coffee beans are roasted at a temperature around 200 °C for 15/20 min. The treatment varies according to the humidity of the product (higher the humidity, the longer the roasting time), and finally a cooling tank where the product is mechanically stirred with a flow of forced air at room temperature.

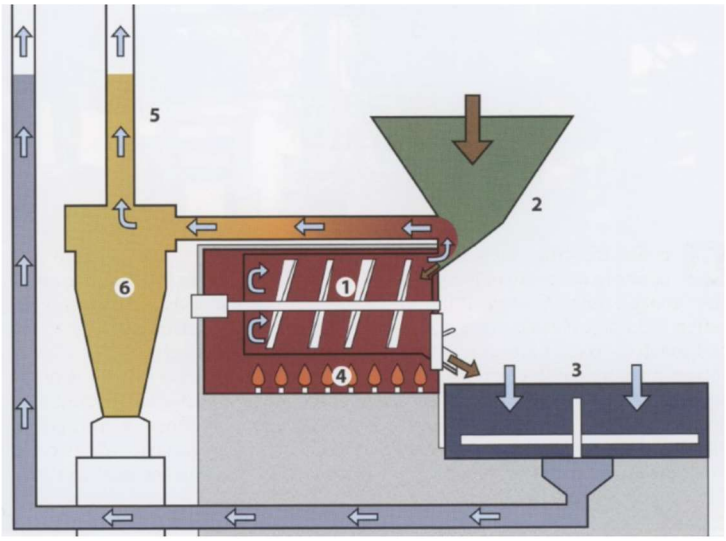


Figure 21. Schematic representation of a rotary drum roaster. 1) Cooking chamber, 2) loading hopper, 3) cooling and mixing tank, 4) gas burner, 5) exhaust pipe for the fumes and gases produced, 6) silverskin collector.

Figure 22. Example of industrial roaster machine.

The roasting level is likely the single most crucial element in producing high-quality coffee beans. The degree of roasting is usually described as being 'light', 'medium' or 'dark'. The final roasting level in industrial applications is determined by colour, which is checked in the laboratory. Colorimeters are commonly used to measure the coffee's colour after it has been ground in order to ensure product quality.

Nowadays, there is no standardization for the colour scale and industries work with diverse types of colour gradations. However, in 1995 the Specialty Coffee Association of America created a new colour classification method, the Agtron system, which is becoming more and more popular as a universal procedure for colour definition. The Agtron number (from 90 for light to 45 for dark roasting degrees) is a narrowband infrared reflectance measurement that is used to assess the degree of caramelization of sucrose. On the other hand, many roasters classify the roasting degree according to the different intensity and shade of brown that the

coffee bean assumes during the process. In this classification the lighter roast is called Cinnamon, followed by darker ones we find New England, American City, Full City, Vienna, French, Italian and Spanish (**Figure 23**).

Figure 23. Different coffee roasting degrees classification.

1.4.1. Chemical changes of coffee beans during roasting

Coffee beans are subjected to an overall change in their chemical composition after roasting as shown in **Figure 24**. Although coffee beans of various species, origins, or roasting degrees have different compositions, the degradation of polysaccharides, oligosaccharides (especially sucrose), chlorogenic acids, and trigonelline is effectively observed. The breakdown of polysaccharides and proteins, as well as the alteration of substances like sucrose, free amino acids, chlorogenic acids, and trigonelline, are what provide roasted coffee beans their distinctive aroma, flavour, taste, and colour.

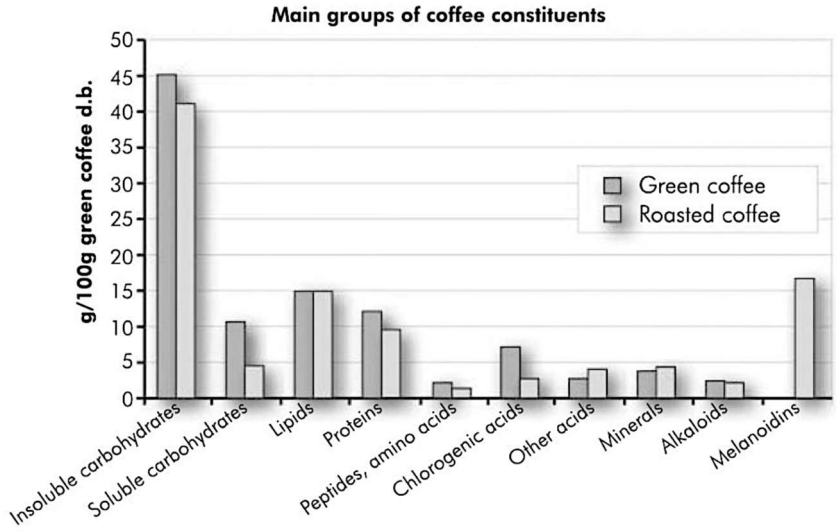


Figure 24. Chemical composition of green and roasted coffee beans comparing the amount of different compounds expressed as g/100g of dry matter. From: Illy, A.; Viani, R. (2005) Espresso coffee: the science of quality, 2nd ed.; Elsevier Academic Press: San Diego.

- **Changes of carbohydrates**

Only a very small amount of free sugars are left after roasting of the mono- and disaccharides present in green coffee. Sucrose, the most prevalent simple carbohydrate in green coffee beans, is supposed to undergo partial hydrolysis and complete pyrolysis (caramelization). It works as an aroma precursor during roasting and produces a variety of classes of compounds, furans and aldehydes, which have an impact on coffee flavour (Sanz, Maeztu, Zapelena, Bello and Cid, 2002). Furthermore, it has been shown that the aliphatic acids (formic, acetic, glycolic, and lactic), which strongly influence the product acidity, originate mainly from the degradation of sucrose.
5-Hydroxymethyl-2-furfural (HMF, **Figure 25**) is one of the major degradation products of carbohydrates and it has been studied extensively as an indicator of heat damage. Formation of HMF from carbohydrate has been found to depend on many factors, such as time, water activity and temperature, and it is decomposed rather quickly upon further roasting (Murkovic and Bornik, 2007).

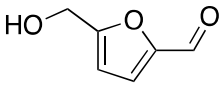


Figure 25. Chemical structure of 5-hydroxymethyl-2-furfural (HMF).

On a dry-weight basis, almost half of green coffee beans are reported to be made of polysaccharides, which include cellulose, mannan, and arabinogalactan. As the bean swells, the cell wall structure becomes more malleable, and the polysaccharides mannan and arabinogalactan depolymerize; as the bean roasts the solubility of both arabinogalactans and mannans is increased. The mouth-coating, creamy sensation known as "body" is a result of the water-soluble polysaccharides, which increase the viscosity of the coffee brew after roasting and are essential for the retention of volatile compounds (Illy and Viani, 2005)

The non-enzymatic browning that occurs through the Maillard reaction that, as described in Appendix 1, comprises a sequence of various reactions between reducing sugars and compounds with a free amino group, forms a variety of products, which can be classified as early stage, intermediate stage and final stage products; the latter are referred as melanoidins, generally defined as high molecular weight brown nitrogen compounds. Little information is available regarding their chemical structures, although they form during the heat treatment of a wide range of food products other than coffee.
Melanoidins are typically assessed by difference, which involves subtracting the total fraction of known compounds from 100% in order to account for the fact that they cannot be directly evaluated due to the uncertainty of their chemical composition. Using this criterion, they were estimated to account for up to around 25% w/w of the dry weight of roasted coffee beans (Bekedam, Schols, Van Boekel and Smit, 2006; Moreira, Nunes, Domingues and Coimbra, 2012). The effects of melanoidins on human health are very interesting since coffee brew is one of their major sources in the human diet. In fact, several biological activities, such as antioxidant, antimicrobial, anticariogenic, anti-inflammatory, antihypertensive, and antiglycative activities, have been attributed to coffee melanoidins.

- **Changes of chlorogenic acids**

At least 30 chlorogenic acids, including caffeoylquinic acids, dicaffeoylquinic acids, feruloylquinic acids, and *p*-coumaroylquinic acids, have been found to be present in coffee beans. Their levels significantly drop during roasting, to the point in which the composition of chlorogenic acids can be used as a measure of roasting degree.
The most of chlorogenic acid is assumed to be hydrolysed, producing astringent phenolic compounds that contribute to the "coffee body". In addition, isomerization and, to a smaller extent, lactonization takes place. Lactones of chlorogenic acids are formed during roasting as demonstrated for caffeoylquinic acids, feruloylquinic acids, and *p*-coumaroylquinic acids. Their formation was demonstrated to be highly dependent on the roasting degree. The optimal level of roasting to produce the most lactones in coffee, which are what give the beverage its distinct bitterness, is a light to medium roast (Farah, de Paulis, Trugo and Martin, 2005)
Quinic acid is the main degradation product of chlorogenic acids, it can undergo isomerization providing the *syllo*-quinic acid or forming the lactone derivative γ-quinide, as reported in **Figure 26** in case of 5-caffeoylquinic acid. Moreover, the cinnamic acids released from degradation of chlorogenic acids may participate in the subsequent chemical reactions to form other flavour components (Wei, Furihata, Koda, Hu, Miyakawa and Tanokura, 2012).
In addition, quinic and caffeic acids are involved in the melanoidins formation (Bekedam, Schols, Van Boekel and Smit, 2008).

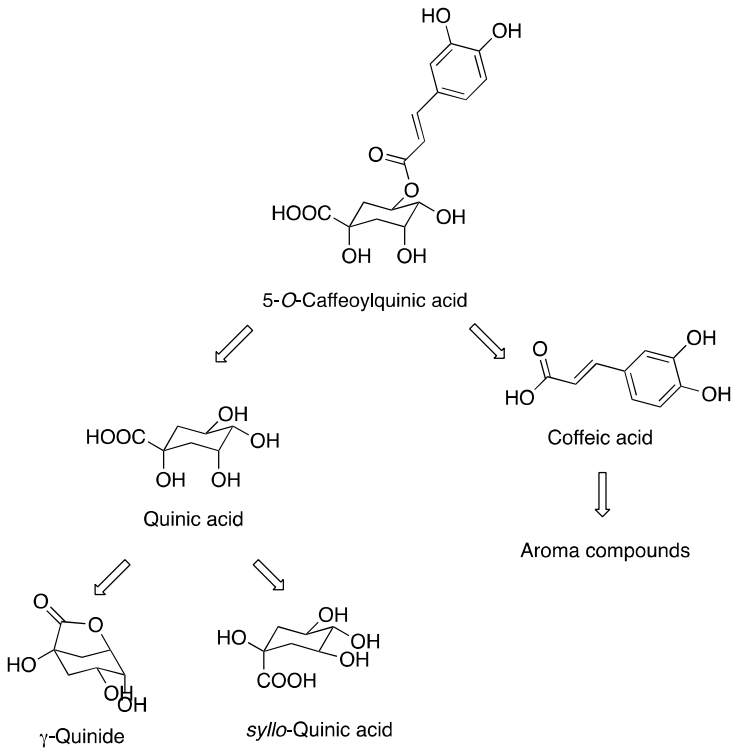


Figure 26. 5-caffeoylquinic acid degradation pathway in roasting.

- **Changes of proteins and free amino acids**

During roasting, coffee's protein composition changes; the proteins are both fragmented and polymerized and are incorporated into melanoidins.
Roasting significantly alters the green coffee's free amino acids profile and only negligible amounts are left in roasted beans. The four main free amino acids present in green beans, L-glutamic acid, L-alanine, L-asparagine, and γ-aminobutyrate, disappear after roasting (Wei , Furinata, Koda, Hu, Mivakawa and Tanokura, 2012).
Except for methionine, sulphur amino acids cystine and cysteine are largely transformed during roasting by the Maillard reaction to produce intensely aromatic volatiles, such as furfurylthiol, with a very low aroma threshold value, thiophenes, and thiazoles. Serine and threonine, two hydroxyl-amino acids, combine with sucrose to form volatile heterocyclic compounds including alkylpyrazines. Proline and hydroxyproline react with Maillard intermediates to give pyrroles, availablepyrrolizines, pyridines and also alkyl-, acyl-, and furfurylpyrroles (Oestreich-Janzen, 2010).

- **Changes of lipids**

Surprisingly, there are not too many studies examining how roasting affects the coffee's lipid composition. Overall, the available literature is conflicting regarding the compositional changes in coffee lipids during roasting. While it is well known that roasting has a substantial impact on the polar coffee components, other studies suggest that high roasting temperatures have little to no impact on coffee lipids. (Anesei, De Pillp, Massinf and Lericp, 2000; Rebeiro, Lidon, Cmpos, Pais, Leitao and Ramalho, 2016). However, even if the overall lipid profile was not studied, some research have shown considerable variations in several lipid components during roasting. (Martin, Pablos, González, Valdenebro and León-Camacho, 2001). These

compounds and their primary and secondary oxidation products greatly affect the quality and taste/aroma of coffee (Budryn, Nebesny, Żyżelewicz, Oracz, Miśkiewicz and Rosicka-Kaczmarek, 2012; Toci, Neto, Torres and Farah, 2013).
Triacylglyceroles remain unaltered throughout roasting and are hence excellent targets to transport flavor volatiles as they emerge. In fact, during extremely intense roasting, they gather at the outer bean surface and begin to "sweat," which causes the so-called "oil" to dissolve most of the volatile aromatic compounds and release them gradually. Furthermore, it is essential to consider how factors like roasting affect the quality and composition of coffee oil considering its enormous potential as a value-added component for a variety of industrial and pharmaceutical applications (Raba, Poiana, Borozan, Stef, Radu and Popa, 2015).
Because they adhere to the spent grounds and are filtered off during the preparation of the beverage, the lipids often do not get into the brew.
Strongly depending on process conditions, cafestol, kahweol, and their respective esters undergo decomposition and isomerisation in roasting, e.g. to form dehydrocafestol/-kahweol by water elimination (Oestreich-Janzen, 2010).
Lipids are a quality challenge, as they are vulnerable to oxidation and rancidity during storage of roasted beans.

- **Changes of trigonelline and caffeine**

An extremely dark roast will only include a small fraction of the trigonelline originally present in green beans because it largely degrades during roasting to form pyridines compounds and nicotinic acid.
At temperatures above 60- 230°C, where about 85% of the trigonelline is destroyed, nicotinic acid (niacin) is produced by the demethylation. According to literature studies, the process temperature more than effective roasting time influences nicotinic acid generation. Reactive intermediates and other recombination products, such as pyrroles and pyridine derivatives are also produced. These compounds have an impact on the overall aromatic perception of roast coffee and beverage.
Caffeine levels are virtually unchanged after roasting, as it is stable at typical roasting temperatures, still studies carried out after roasting showed that the highest content of caffeine was found in the medium roasted coffee (203,63 mg/L), thus evidencing a positive correlation between the caffeine levels with the degree of roasting till a certain point where the levels dropped in the dark roasted coffee (Awwad, Issa, Alnsour, Albals and Al-Momani, 2021). Additionally, even though caffeine is a molecule with a distinct taste, it only contributes minimally to the bitterness of coffee beverages.

- **Volatile compounds**

Roasted coffee aroma is a complex mixture of more than 1000 volatile compounds. There are undoubtedly many interactions between each of the pathways involved in the complex mechanisms behind the development of coffee aroma (Buffo and Cardelli-Freire, 2004). The volatile molecules within coffee are produced from various compounds present in green coffee beans (sugar, amino acids, lipid, trigonelline, chlorogenic acid and carotenoids), which, are involved in many reactions such as caramelization, Maillard reactions and thermal degradations during the roasting process.
It has also been demonstrated that modifying the roasting-grinding sequence, by reversing the order of these two phases, does not lead to significant different levels of both the 75

volatile compounds used as markers (**Table 2**) and of various physicochemical properties like colour, moisture, and pH (Lee, S. J., Kim and Lee, 2017).
Between Arabica and Robusta, some of these roasted coffee volatiles are noticeably different. After the coffee brew is prepared, the aroma is not stable and varies quickly. The quality variations are linked to losses of the volatile compounds as thiols: 2-furfurylthiol, 3-methyl-2-butenthiol, 3-mercapto-3-methylbutyl formate, 2-methyl-3-furanthiol, and methanethiol (Oestreich-Janzen, 2010)

Table 2. Volatile compounds identified in roasted coffee by Lee *et al.* (2017).

Chemical group	Compounds
Acids	Acetic acid
	3-Methylbutanoic acid
	2-Hydroxymethylbenzoic acid
	1,2,3,4-Tetrahydro-2-quinolinecarboxylic acid
Alcohols	2-Furanmethanol, acetate
	2-Furanmethanol
	Pyridinol, acetate
	Benzenemethanol
	2-Methoxyphenylethanol
	Eugenol
Aldehydes	Benzaldehyde
	2-Furancarboxaldehyde
	Benzeneacetaldehyde
	2-Methylbenzaldehyde
	2-Thiophenecarboxaldehyde
	Benzenacetaldehyde, -ethylidene
Furans	2-Pentylfuran
	2-[(Methylthio)methyl]furan
	2-Acetylfuran
	2,3,4-Trimethylfuran
	2,2'-Bisfuran
	2,2'-Methylenebisfuran
	2-(2-Furanylmethyl)-5-methylfuran
	2,3-Dihydro-6-methilthieno[2,3*c*]furan
	3-Phenylfuran
	2,2'-Oxybis(methylene)furan
Furfurals	Furfural
	5-Methylfurfural
	Furfurylmethyldisulfide
Indoles	2,3-Dihydro-4-methyl-1H-indole
	2,3-Dihydro-4-methyl-1H-indole-5-caboxaldehyde
	1H-Indole
Ketones	2-Acetylcyclopentanone
	1-(2-Pyradinyl)-ethanone
	1-(2-Furanyl)-3-pentanone
	1-(3-Methylphenyl)ethanone
	Nona-e,5-dien-2-one
	Pentylthiophene
	Propanone
	4'-Hydroxy-acetophenone
	4-Hydroxy-3-methylacetophenone
	1-(1H-pyrrol-2-yl)ethanone
Phenols	3-Amino-4-methylphenol
	2-Methoxyphenol

	4-Methoxyphenol 4-Ethyl-2-methoxyphenol 2,3,6-Trimethylphenol 2,5-Dimethylphenol 3-Ethylphenol Methylpyrazine
Pyrazines	2,5-Dimethylpyrazine Ethylpyrazine 2,3-Dimethylpyrazine 2-Ethyl-6-methylpyrazine 2-Ethyl-5-methylpyrazine 2-Ethyl-3-methylpyrazine 3,5-Diethyl-2-methylpyrazine 5-Methyl-6,7-dihydropentylpyrazine
Pyridines	Pyridine 5-Methyl-2-pyridinamine 4-Methyl-6-cyanothieno[2,3-*b*]pyridine
Pyrroles	1-Methylpyrrole 1-Ethyl-1H-pyrrole-2-carbaldehyde 2-Formyl-1-methylpyrrole 3-Acetylpyrrole 1-(2-Furanylmethyl)-1H-pyrrole 1H-Pyrrole-2-cerboxaldehyde
Other	Trimethyloxazole 4,5-Dimethyl-2-isopropyloxazole

1.5. Grinding

In order to increase the specific extraction surface area and facilitate the transfer of soluble and emulsifiable components from the coffee matrix to water during the brew extraction, whole roasted beans are reduced to small fragments by the process of grinding. The first coffee grinder, dating back to the origins of coffee, was the mortar and pestle. People in Ethiopia, Middle East, and early coffee drinking countries would use the small club and bowl to grind up coffee beans for brewing.

While the mortar and pestle are still a popular device to create powders and pastes, this approach was found to be inefficient as coffee consumption increased globally. People ground coffee in the 15th century by using manual grain or spice mills. The first coffee mill, made specifically to grind coffee beans, was invented by Nicholas Book in the late 17th century. The lever device's top was filled with coffee, which was then ground and poured in the bottom drawer (**Figure 27**).

Due to its popularity, several other creators and businesses throughout the 18th century produced their own manual devices. Until the American business Hobart manufactured the first electrical coffee grinder in the late 1800s, these were common household devices. This began a change towards the grinders we are familiar with today.

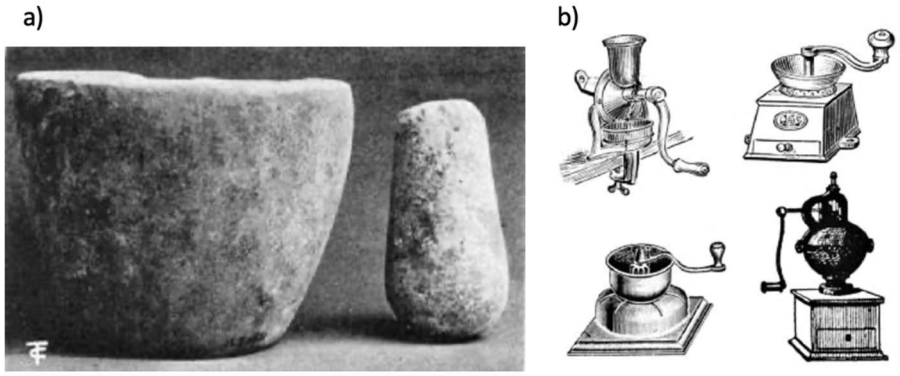


Figure 27. a) The oldest grinder, based on mortar and pestle, from ancient Egypt, probably used for grinding coffee. b) English and French coffee grinders from Nineteenth century.

Grinding process is critical as it influences the properties of the ground coffee and respective brew flavour, hence particle size must be controlled. After grinding, the coffee remains in silos for further degassing prior to packaging from 4 to 24 hours. Traditionally, the particle size of ground coffee has been measured by graded-mesh sieves evaluating size distribution, but this approach presents some limitations in controlling the quality of product. Laser diffraction (dry technique), which is faster and gives the complete particle size distribution of the samples, has been used to characterize ground coffee in order to overcome this limitation.

Particle size distribution, brewing time, and the final flavour of the coffee beverage are all related. The origin, method of processing, and roasting profile of the coffee beans have been demonstrated to have no noticeable influence on the particle size distribution of the ground coffee. Additionally, cold grinding enables a smaller particle size with a more homogeneous distribution (Uman *et al*., 2016).

Both freshly brewed coffee and the pleasant odours emitted during the grinding of roasted coffee beans are appealing. Studies into the volatile substances generated during the grinding of roasted coffee beans revealed that the nutty and smokey roast aromas predominated under all analytical conditions. (Akiyama *et al*., 2003).

The quality of the beverage depends on the extraction conditions (pressure and temperature of the water); therefore, the grinding procedure must be modified to correspond with the best extraction method for each coffee product (**Table 3**) (Farah, 2019).

Table 3. Most suitable grinding grades for the most common coffee brewing methods.

Brewing method	Grinding grade
Cafetière	Medium
Espresso	Very fine
Filter/Dip	Fine to medium
Percolator	Medium
Turkish	Pulverised

Regarding the grinding equipment, several industrial grinders offer a variety of sizes and may be employed with any sort of roasted coffee and allowing grind at capacities ranging from approximately 50 to 5000 kg per hour (**Figure 28**). These kind of equipment may use water-cooling technology to maintain a low operating temperature and preserve the sensorial properties of the coffee

Figure 28. Example of industrial coffee grinder.

1.6. Packaging and Storage

The term "packaging process" refers to the choice of packing materials and tools as well as their filling and sealing, storage, and transportation to the point of consumption. Monitoring is necessary at every stage of this productive process to ensure that the product meets both regulatory requirements and consumer expectations.

The porous structure of roasted coffee beans, which is dependent on the roasting temperature and time applied, determines the residual CO_2 content after roasting. For this reason, a degassing step is carried out on whole and ground coffee before packaging to avoid the swelling of the packages during storage.

Moreover, ground coffee is particularly sensitive to oxidation, hence the contact of oil spilled onto the surface of the beans with air dramatically increases promoting oxygen uptake, moreover oil exudation increases the stickiness of the particles which tend to aggregate making the extraction not uniform. For these reasons, storage conditions and time must be carefully considered.

Because of the interaction between the coffee matrix and the employed material, the choice of packaging methods and materials is essential for achieving the requisite shelf life. In order to maintain coffee aroma and act as a barrier against moisture and air oxygen, the packaging must also be composed of a material that is inert and suitable for food (Gloss, Schonbachler, Rast, Deuber and Yeretzian, 2014).

Tinplate, glass, aluminum, and laminated materials such flexible polymers are often used to package roasted coffee (**Figure 29**). Currently, flexible polymer-aluminum multiple laminates are the most often used materials. They are easy to handle, inexpensive, and allow for both hard and soft packs. Since they contain an aluminum foil layer in the middle, they are effective barriers.

A typical construction is poly-ethylene terephthalate (PET)/alufoil/low density polyethylene (LDPE). These containers can be equipped with a one-way valve that, while opening at a predetermined pressure to release gases, excluding ambient air from entering.

Coffee capsules for specific espresso machines commonly consist of aluminium coated with a protective film. Alternative capsule systems prevent coffee particles from migrating to the brew by using injected plastic with an aluminum layer and an inner cellulose filter. Coffee is

already available from several coffee firms in bio capsules made entirely of renewable resources.

There are different techniques used for packaging roasted coffee such as air and vacuum packaging, inert gas packaging and pressurization, which can be combined with the other methods. Although roasted coffee is a shelf-stable product, following the high temperatures employed in roasting and its low water activity (aw) no enzymatic and microbial deterioration occurs (Illy and Viani, 2005). The packaging technique must be chosen as a function of the desired shelf-life ascribed to the loss of highly volatile compounds, oil migration, chemical reactions, e.g., by oxidation with O_2, or intrinsic chemical reactions among coffee components (Cardelli and Labuza, 2001).

These unavoidable chemical and physical changes have a significant impact on the quality of the brew, while whether fast they happen depends on the amount of oxygen and moisture present, the storage temperature, the type of packaging used, and other factors. Coffee packages will likely be opened and closed a frequently when they are being used by consumers, thus that must be considered (Smrke, Adam, Mühlemann, Lantz and Yeretzian, 2022). In such situations, the rate of coffee degradation increases rapidly owing to modification of the conditions inside the package as a result of interaction with air and moisture, accelerating the loss of freshness and the development of stale smell in the coffee. Four different methods of storing whole roasted beans after opening the package were recently compared: transfer the beans to an airtight container, seal the original package with tape, close with a clip and use a package with a cap screw. The aroma was analysed by gas chromatography coupled to mass spectrometry (GC/MS), after grinding the beans, during storage. For this purpose, 2-butanone and 2-methylfuran have been used as indicators of stability and conservation. The freshness index, defined as the ratio of GC/MS signal intensities between 2-butanone/2-methylfuran, was found to be the most suitable for evaluating the "staleness" of coffee. Screw cap packaging was found to be the best performing storage method, consistent with the observed changes in oxygen and carbon dioxide content within the respective packaging (Smrke, Adam, Mühlemann, Lantz and Yeretzian, 2022).

Figure 29. Most common coffee packagings: flexible polymer-aluminum multiple laminate, tinplate packagings and pack equipped with a one-way valve.

The length of time after opening of the package during which coffee maintains acceptable quality is referred to as secondary shelf life.

1.7. Coffee brewing methods

Coffee is a beverage that can be prepared and consumed in a variety of ways. Coffee does not only mean aroma and taste, variety of notes and types, but also means history and tradition. A long history that highlights the creativity and genius of humans that can be traced through well-defined moments in time. There are many variations depending on the region, not only in terms of preparation and flavour but also in respect of how it is consumed. The following is

a list of the most popular preparation techniques that mark the birth of the main and most famous coffee extraction tools and methods.

- **Turkish coffee**

From Turkey, this method spread to Europe around 1640, thanks to the opening of a coffee shop in Venice, the first shop on the whole continent. "Turkish" coffee, also found in Greece and the Balkans, consists in boiling water inside an elongated jug (*ibrik* or *cezve*, usually covered in brass, **Figure 30**) with finely ground coffee, spices (e.g., cardamom) and, optionally, sugar. The coffee is brought to the boil again for two consecutive times, and divided into the cups redistributing foam and liquid and finally tasted letting the powder settle on the bottom.

Figure 30. Turkish coffee brewing method.

- **Arabic coffee**

To prepare Arabic coffee, the beans are roasted at temperatures ranging from 165 to 210°C and then ground, infused and flavored with cardamom. Water is boiled in a sort of tall coffee pot with a long and narrow spout named *dallah* (**Figure 31**) and a teaspoon of coffee powder is added for each guest, while stirring and put back on very low heat, repeating the operation three times. Then the coffee is served and wait for all the powder to be deposited in the cup. The resulting drink is not filtered and sweetened. To compensate for its bitter taste, coffee is usually enjoyed with sweets, dates or dried fruit. Arabic coffee is served directly in a special small cup without handles called *finjān*.

Figure 31. Coffee maker for preparation of Arabic coffee called *dallah*.

- **Syphon method**

In 1830 in Germany a new extraction method called "Syphon" was developed by Mr. Loeff of Berlin, however ten years later, James Robert Napier, Scottish engineer and scientist, reported the construction of the first example of Coffee Syphon, initially called under the name of “Vacuum Coffee Pot”. The prototype of this device, consisting of two glass ampoules, however, was never patented due to the quality of the glass used, which did not ensure heat resistance and above all its safety. Since it was not protected by patent, it was subjected to several revisions.
After the invention of borosilicate glass in 1925, "Syphon Coffee" could finally be used without risk to this day. The Siphon method is currently back in vogue, also thanks to the fact that it is very scenic. This process uses two glass vials, in the lower container the water is placed, which boils on a burner. The ground coffee is placed in the upper container. Thanks to the steam, the water is pushed into the upper bowl, mixing with the coffee (**Figure 32**). Once the lower container is removed from the burner, the pressure within the lower closed chamber will begin to decrease, then the brewed coffee in the upper part will begin to descend under the effect of gravity and the pressure difference and, passing through a filter, will collect in the container below.

Figure 32. Syphon coffee maker.

- **Espresso coffee**

In 1884, the espresso coffee was born in Turin, following the invention of the instant coffee machine patented by Angelo Moriondo, which would become the symbol of Italian coffee.
Espresso is likely one of the most appreciated coffee beverages, probably because of its very distinct characteristics when compared with coffee brews obtained by other methods. The espresso is prepared by machine according to a process of percolation under high pressure of hot water which is based on pressure induced extraction of a limited amount of hot water through a compact ground coffee cake in the brewing chamber, where the energy of water pressure is spent within the cake itself (**Figure 33**).
Extraction times are very short and should not exceed 30 seconds. Typical water temperatures applied during extraction range between 88 °C and 93 °C and optimal pressure ranges between 9 and 10 atmospheres. The combination of heat and pressure extracts soluble flavouring material, emulsifies insoluble oils and suspends both ultra-fine bean fibre particles. Altogether, these characteristics confer espresso coffee its particular sensorial properties, which include a strong body, a full fine aroma, a balanced bitter/acid taste and a pleasant lingering aftertaste.

Figure 33. Espresso coffee machine: on the left the machine patented by the Italian Angelo Moriondo in 1884, on the right a modern espresso machine.

- **French press**

In 1929 the Italian Attilio Calimani patented a more modern version of the piston coffee maker or "French press", which was in turn patented in France in 1852. It consists of a glass container with a lid equipped with a tightly meshed steel plunger. Once the ground coffee has been deposited, hot water, previously brought to the boil, is poured onto the coffee deposited on the bottom of the container and covered with the lid. After leaving it for a few minutes to infuse, slowly move the plunger down. In this way, the steel filter separates the beverage from the ground coffee (**Figure 34**).

Figure 34. French press coffee maker.

- **Moka coffee**

In 1933 the famous Moka was born, a coffee maker designed by Alfonso Bialetti, of which 105 million specimens were subsequently produced. Despite some modifications and versions made over time, its octagonal aluminum shape has remained unchanged.
The mocha consists of three parts: a base into which the water is poured, a filter into which the ground coffee is inserted, and an upper part that is used to collect the liquid coffee once it is ready.
The pot is placed on a heat source and the water is brought to its boiling point. The steam created eventually reaches a high enough pressure to gradually force the surrounding boiling water of approximately 110 °C up the funnel and through the coffee grounds. Mocha coffee is a strong brew characterized by high extraction yield (**Figure 35**).

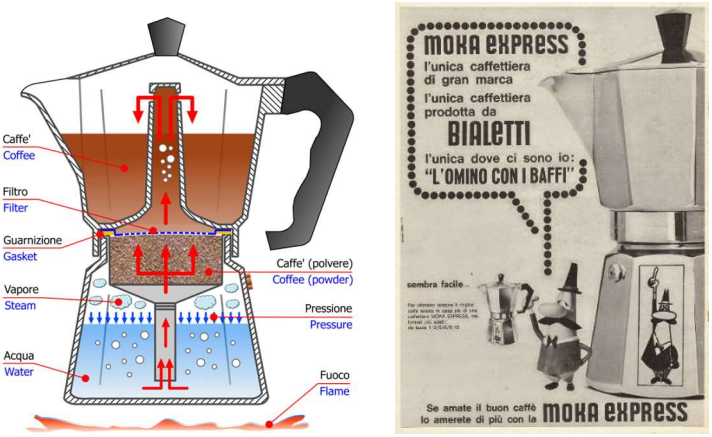


Figure 35. On the left: section of a moka coffee maker. On the right: the first Moka designed by Alfonso Bialetti.

- **Filter/Drip coffee**

Filter coffeemakers are simple devices consisting of a container that serves both as extraction chamber and as a means of separating the grounds from the resultant beverage. For this preparation, a very tightly woven paper filter is used to retain most of the solid material.
In 1908, the so-called "filtered coffee with paper filters" method was born, invented by the German Melitta Bentz. The procedure consists in slowly pouring boiling water along a layer of ground coffee, placed in a paper filter. In this way, the water dissolves the substances contained in the coffee powder and transfers them into the beverage, which is more liquid and more aromatic. This type of method is exploited by machines called “Drip coffee makers” (**Figure 36**). Among the various examples of percolation, certainly the extraction with the "Chemex" method stands out for the use of a very particular coffee maker. The Chemex invented in 1941 by Peter J. Schlumbohm, a German chemist living in New York, is one of the most popular coffee makers in the world of coffee lovers and by designers. One of his models is exhibited in the permanent collection at the MoMA in New York.

Figure 36. Drip coffee maker (left) *and "Chemex" method (right).*

- **Aeropress**

The Aeropress method was invented in 2005 in the USA and has spread very quickly. It has been defined as innovative and versatile, although it has similarities with the "French coffee" procedure; in fact, both techniques exploit an infusion chamber and a piston mechanism. The

extraction tool consists of two cylindrical containers that move inside each other and a paper filter (**Figure 37**). It acts like a syringe, the manual pressure exerted on the piston compresses the air mass making the water slide through the filter and collects the coffee in the cup below.

Figure 37. Aeropress coffee maker.

1.8. Coffee market

Coffee is the second most important commodity after petroleum, the latest report from the ICO (International Coffee Organization) notes how over the season from December 2021 to January 2022 exports of green beans reached 27.54 million 60-kg bags only in the first three months of coffee year 2021/22. The ICO Report indicates that coffee prices increased again in January 2022, reaching 204.29 US cents per libra with a constant increase in the last year (**Figure 38**).

Figure 38. ICO coffee prices report expressed as US cents per libra.

Estimates of total production for coffee year 2020/21 have been revised slightly downwards to 168.88 million bags. Robusta share of the total green beans exports increased to 37.5% in the first three months of coffee year 2021/22 as compared with 32.1% in the same period a year ago. In particular, Arabica exports amounted to 80.7 million bags compared to 81.2 million bags last year; whereas exports of Robusta were 48.31 million bags compared to 48.3 million bags the previous year.

The first area for production is South America ahead of Asia and Oceania, Africa, and Mexico and Central America. Exports in October-December 2021 saw a 29.7% increase to 11.04 million bags for Asia & Oceania, but down 19.2% for South America at 15.28 million bags in the same period (**Figure 39**).

Meanwhile, world coffee consumption has been revised upwards, now estimated to have increased to 167.68 million bags in 2020/21 as compared to 164.46 million for coffee year 2019/20, an increase of 2.0%. (ICO 2022)

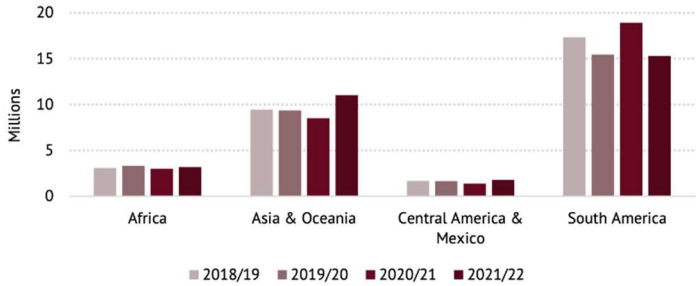


Figure 39. Comparison of the total export of coffee 60-kg bags in different regions in the world in the last four years. From: ICO 2022.

The coffee market is projected to register a compound annual growth rate CAGR of 4.28% during the forecast period (2022-2027).
The COVID-19 pandemic has increased strain on the coffee industry, which is already under stress from global price fluctuations, low productivity levels, the consequences of climate change, and harm from pests and illnesses in different parts of the world.
Retailers, roasters, and customers are currently being forced to adjust to this new reality as a result of the shift toward online purchase for consumption at home. Therefore, it is estimated that this factor will lead to an increase in coffee consumption worldwide.
The market is influenced by several variables, including rising consumer demand for certified coffee products, consumer adoption of single-serve coffee brew systems, and ongoing innovation pushed by the major coffee market players. For reasons of quality and flavour, some customers in developed economies are anticipated to convert from instant coffee to more premium options.
It has been seen over the past few years that consumers are becoming more conscious of the manufacture of the things they purchase and the origin of their purchases. This is true, particularly when it comes to the supply chain for goods like coffee that are used in food and beverages. To assure the legitimacy of their coffee purchases, buyers are therefore keenly searching for certified coffee goods.
In the food and beverage industry, there is a rising demand for certified and sustainable brands and labels due to consumer concerns about poverty, social injustice, and environmental harm. Sustainable coffee refers to diverse coffee varieties that follow a variety of social, environmental, and economic requirements and are independently verified by a reputable third party.
Several companies that offer coffee certification are involved in monitoring the methods used in coffee production and the supply chain. UTZ Certification, Fair Trade Certification, Rainforest Alliance Certification, and USDA Organic Certification are only a few of them.

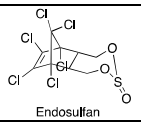
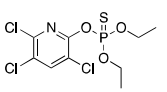
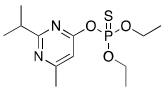
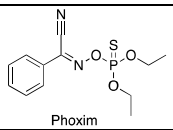
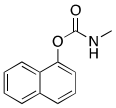
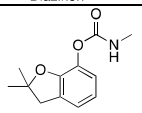
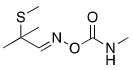
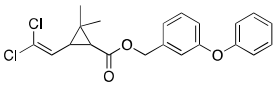
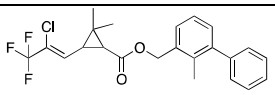
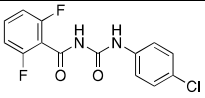
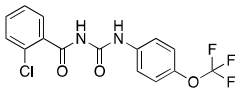
1.9. Principal contaminants in coffee

Contaminants are natural and/or chemical substances not intentionally added to food and feed but may be present in food either as a result of various stages of production, processing or transportation, or as a result of environmental contamination. Contaminants can represent a serious risk for human and animal health, whose assessment is based on the technical examination of the categories of food contaminants and specific pollutants.
The regulations on maximum residue limits (MRL, maximum residue limit) of harmful compounds, as well as the control of their presence, continue to be the main guarantees for the consumption of healthy agricultural products. However, due to the continuous evolution of environmental degradation, the monitoring and study of environmental contaminants currently represent one of the priorities for the international scientific community in order to be able to predict health emergencies, including those concerning food safety. Environmental pollution caused by human activities or unexpected natural disasters is a source of serious concern in relation to food safety, which has been highly undermined by such events. A constant study on risk assessment by all the competent international authorities is currently underway to be able to identify any new environmental pollutants as potential risk factors for food safety, which represents a priority factor for the protection of human life.
In terms of microbial risk, coffee is considered a stable food. The main contaminations are due to the presence of non-pathogenic microorganisms caused by incorrect application of hygiene standards during the harvesting and processing phase.
Like many other foods, coffee is subject to regulations that set maximum levels for substances such as pesticides, mycotoxins from fungal contamination (aflatoxins and ochratoxin) and compounds linked to processing, such as polycyclic aromatic hydrocarbons (PAHs) or acrylamide.

1.9.1. Pesticides

The World Health Organization and the Food and Agricultural Organization have defined pesticides as: any substance or a mixture of substances intended for repelling, destroying, or controlling any pest during or interfering in the production, processing, storage, or marketing of food. An ideal pesticide should be very selective, effective for pest control for a determined period of time and degrading afterwards.
The tropical climatic conditions of the areas where the coffee plants are grown, together with the time spent in the field, make this cultivation very susceptible to the attack of parasites and diseases caused by fungi, insects, nematodes and weeds which damage the development, production and the quality of the fruit (Dias, Oliveira, Madureira, Silva, Souza and Cardeal, 2013). Only 3% of the coffee available today in the world is grown using organic methods, the remaining 97% is treated with different pesticides to limit the prevalence of undesirable animal or plant species in coffee fields.
Pesticides can be applied to coffee at three different stages: during production, storage, and export involving many chemical substances such as insecticides, fungicides and herbicides.
More than 500 pesticides are admitted for the cultivation and storage of coffee beans by European legislation which sets the MRLs to coffee beans and coffee products through the Regulation EC 178/2006 along with harmonizing the pesticide registration process (Commission Regulation (EC) No 178/2006).

The main chemical classes of insecticides are organochlorine, organophosphate, carbamate compounds, pyrethroids and benzoylureas (**Figure 40**).
The most widely used synthetic fungicides belong to the chemical classes of triazoles, dithiocarbamates, anilinopyrimidines, strobilurins and benzimidazoles (**Figure 40**).
Herbicides are used to control weeds, and can be classified according to their chemical class, selectivity, nature of action and application characteristics.
Herbicides that destroy all vegetation are classified as total or non-selective, while those that control some weeds without damaging other agricultural cultures are defined as selective. Non-selective pesticides, e.g., paraquat, play a key role in the quest for sustainable coffee production by controlling weeds that would otherwise seriously reduce productivity.
Contact herbicides exert their action only on the part of the plant where they have been deposited (e.g., paraquat). On the other hand, systemic herbicides permeate the plant and can reach areas that are far from the application site.
For instance, glyphosate is a non-selective systemic herbicide that is absorbed by plant leaves and green stem tissues. In the coffee fields, it effectively eliminates a wide range of weeds, including grasses, broad-leaved plants, and woody plants (**Figure 40**).
Appendix 2 contains the table lists of the MRLs for the 32 pesticides applicable to green coffee beans and roasted coffee reported by ICO comparing data for individual exporting and importing countries, including European union, and limits of Codex Alimentarius which provides harmonized international food standards, guidelines and codes of practice, elaborating MRLs for pesticides (http://www.ico.org/documents/cy2017-18/icc-122-10-r1e-maximum-residue-limits.pdf)
Food producers and importers are responsible for food safety, this means that the pesticide use must comply with EU requirements; therefore, residue concentrations are determined by analytical methods in accredited food laboratories.
To meet regulatory requirements and protect the consumer and the environment, many analytical methods have been reported to monitor and control residue levels in coffee. With the development of new methods like SPME (solid phase microextraction) and QuEChERS (quick, easy, cheap, effective, rugged, and safe), the use of traditional extraction methods that required significant amounts of hazardous solvent has decreased. These methods are ideally suited for the powerful analytical features of GC-MS and LC-MS/MS and can provide rapid, reliable and sensitive analysis of pesticides in coffee (Pizzuttia *et al*., 2012; Reichert, de Kok, Pizzutti, Scholten, Cardoso and Spanjer, 2018; Dias, Oliveira, Madureira, Silva, Souza and Cardeal, 2013; da Silva Souza and Navickiene, 2019; Copobiango and Cardela, 2005).
Due to the high coffee consumption worldwide, it is of great importance to determine the presence of pesticide residues assessing whether it is within the maximum residue limits (MRLs) together with the evaluation of the acceptable daily intake of these substances in coffee beverage (https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/products/?event=details&p=244 24/03/2022)
In the 2015 European Union survey on pesticide residues in food, no MRL exceedance was reported for coffee beans with at least 60 samples analysed (EFSA, 2017).

Insecticides	
Organochlorine	 Endosulfan
Organophosphate	 Clhorpyrifos  Diazinon  Phoxim
Carbamates	 Carbaryl  Carbofuran  Aldicarb
Pyrethroids	 Permethrin  Bifenthrin
Benzoylureas	 Diflubenzuron  Triflumuron
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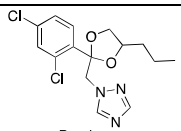
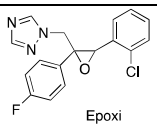
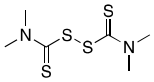
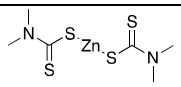
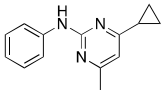
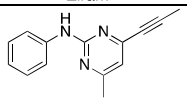
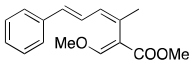
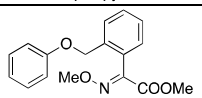
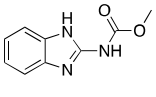
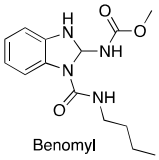
Triazoles	 Propi  Epoxi
Dithiocarbamates	 Thiram  Ziram
Anilinopyrimidines	 Cyprodinil  Mepanipyrim
Strobilurins	 Strobilurin A  Kresoxim-methyl
Benzimidazoles	 Carbendazim  Benomyl
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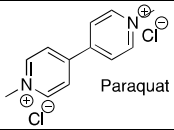
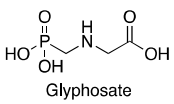
 Paraquat  Glyphosate	

Figure 40. Main classes and compounds of insecticides, fungicides and herbicides employed in agriculture.

1.9.2. Ochratoxin A

The roasting temperatures are well above those known to destroy microorganisms and even to largely decompose mycotoxins. Consequently, the presence of pathogenic bacteria is not considered a risk for roasted coffee, however Ochratoxin A (OTA, **Figure 41**) represents a major hazard for coffee.

OTA is a fungal metabolite produced by the *Aspergillus* and *Penicillum* genera, which develops in particular conditions of high humidity, and can be present in cereals, wine, coffee, beer, cocoa, dried fruit, meat, spices and fruit juices. Contamination is more widespread in some producing countries, it occurs mainly after harvesting but can form throughout the supply chain (transport, storage, production). It has marked renal toxicity and accumulates in the tissues; a possible carcinogenicity to the liver and kidneys has also been found, so much so that The International Agency for Research on Cancer has classified OTA as a possible carcinogen for humans (group 2B). Pfohl-Leszkowicz and Manderville, 2007; IARC, 1993)

It is preferable to analyse it on green coffee as, if the identification of OTA pollution occurred on roasted coffee, it would lead to the loss of the coffee lot.

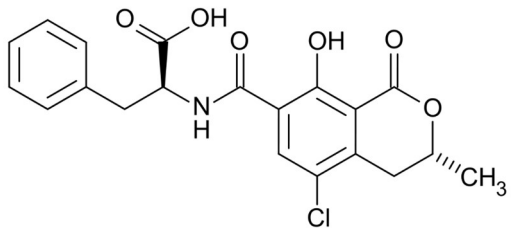


Figure 41. Chemical structure of Ochratoxin A (OTA).

Green bean moisture (regulated at a maximum level of 12.5%) has been identified as the primary risk factor for OTA generation beyond the initial processing stage. Within the European Community, maximum limits have been established for roast, ground and soluble coffees at 5 and 10 μg/kg, respectively, but there is no limit yet for green coffee (Commission Regulation (EC) No 1881/2006)

OTA presence in coffee beans can be caused by the cherry contamination at the field with toxigenic fungi and has been shown to be related to the quality of the cherries at the harvesting time. Normally, the cherry's skin protects the actual coffee bean from spore contamination, any its damage or defect could be the main sources of OTA contamination. Coffee contamination may also be caused by beans that have been damaged by insects. In these situations, sorting damaged beans enables OTA contamination to be greatly reduced (Duris, Mburu, Durand, Clarke, Frank and Guyot, 2010).

Depending on the area where the coffee is cultivated, different OTA contamination can be found in green coffee. The differences are due to the various post-harvest practices as well as the local climatic conditions. Indeed, it has been reported that green coffee bean coffee deriving from “wet” post-harvest process typically contains lower OTA concentration, especially those from Central and South America, while Robusta and Arabica “dry” green coffee beans, typically from Africa and Asia, showed higher levels (Romani, Saccheti, Lopez, Pinavia and Rosa, 2000).

OTA contamination of coffee can also occur during transport and storage when carried out under unsafe conditions and the main factors influencing the development of toxicogenic strains are temperature and activity of water (Aw) which indicates the relationship between

the vapor pressure of water in a certain material and the vapor pressure of pure water (Suarez-Quiroz, González-Rios, Barel, Guyot, Schorr-Galindoand Guiraud, 2004).
Occurrence of Ochratoxin A in coffee brews was first reported in 1988 (Tsubouchi, Terada, Yamamoto, Hisada and Sakabe, 1988) Before that time, it was generally thought that OTA decomposed during roasting. Several studies have shown that the process of roasting is effective in reducing the OTA concentration (Blanc, Pittet, MunozBox and Viani, 1998; Castellanos-Onorio et al., 2011; Heilmann, Rehfeldt and Rotzoll, 1999; Pérez de Obanos, González-Peñas and López de Cerain, 2005; Tsubouchi, Yamamoto, Hisada, Sakabe and Udagawa, 1987; Viani, 2002).
The roasting causes OTA reduction levels ranging from 25 to 90% for medium and dark roasting degrees according to process applied. However, OTA thermal degradation can result in newly generated compounds that need to be identified and evaluated (Castellanos-Onorio, Gonzalez Rios, Guyot, Guiraud, Galindo Schorr, Durand, Fontana-Tachon and Suarez-Quiroz, 2011).

1.9.3. Heat induced contaminants

The so-called thermal process pollutants, or hazardous chemicals, can arise as a result of chemical changes caused on by heat. Over the past ten years, these substances have been the subject of extensive scientific research. Understanding the presence, formation, and possible harm to public health presented by the compounds generated during heat processing of foods has improved over time, according to researchers. Regarding food safety, the presence of pollutants from thermal processing of foods continues to be a top concern for consumers, governmental organizations, and business. Consequently, there is a critical need to reduce their development in various foodstuffs (Mogol and Gökmen, 2016).
However, there is currently no regulation on law limit levels for these compounds, and only a few entities have established regulations or standards that are gradually contributing to establish reference concentrations, such as the EU regulation 2158/2017 regarding acrylamide (Commission regulation (EU) 2017/2158)

- **Acrylamide**

Since acrylamide (2-propenamide, **Figure 42**) is categorized from 1994 by the IARC as possibly carcinogenic to humans (Group 2A), a report published by the Swedish authorities in April 2002 on the presence of acrylamide in a variety of fried and oven-cooked meals garnered attention worldwide (IARC, 1994).

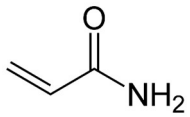


Figure 42. Chemical structure of acrylamide.

The main pathway of acrylamide formation in foods is linked to the Maillard reaction (see **Appendix 1**) and, in particular, the amino acid asparagine and reducing sugars or reactive carbonyls.
Particularly cereals, coffee and crisp bread were considered as relevant sources of human exposure to acrylamide, since they are consumed on a regular basis by a broad group of consumers with especially high exposure in certain European countries. In order to

understand the process of acrylamide formation during roasting and its fate during coffee storage and brewing, numerous food industries, regulatory authorities and institutional communities have been interested in investigating the presence of this contaminant in roasted coffee and coffee products. Considering the contribution of coffee to daily acrylamide intake, acrylamide content of coffee should be limited. Its generation has a peak in the first stages of the roasting process and its level decreases in the final step. Thus, lower levels of acrylamide are measured in dark roasted coffees compared to lighter ones. Different studies have established that the acrylamide contents vary according to the composition of the Arabica and Robusta blend, roasting degree, the storage time of the roasted coffee and the beverage preparation method (Schouten, Tappi and Romani, 2020).
A study concerning air roasting has shown how the increase in the speed of hot air circulation, regardless of the temperature, causes a massive formation of acrylamide, while the increase in the humidity of the roasting air, associated with high temperatures, reduces the amount generated (Mogol and Gökmen, 2016).
The results of another research indicate, as a promising technique, to reduce the concentration of acrylamide in soluble coffee, the incubation of the latter with sucrose and *Saccharomyces cerevisiae*; preliminary tests, in fact, have demonstrated a reduction in the concentration of acrylamide equal to 70% (Akıllıoglu and Gökmen, 2014).
Acrylamide levels in foodstuffs have been monitored in EU from 2007 to 2009 under Commission Recommendation 2007/331/EC. Results showed that the acrylamide content was less than 400 µg/kg for 82% and 92% of the roasted coffee samples in 2007 and 2008, respectively. Concerning the instant coffee samples, 82% and 87% contained less than 800 µg/kg in 2007 and 2008, respectively (EFSA, 2011).
Based on these results, the European Regulation 2017/2158 of 20 November 2017 established the application of mitigation measures and new benchmark levels for the reduction of acrylamide in foods, including coffee. Concerning coffee products, the acrylamide benchmark values are 400 µg/kg for roasted ground coffee, 850 µg/kg for soluble coffee (Commission regulation (EU) 2017/2158)
Since acrylamide is extremely soluble in water, it can be easily transferred from coffee powder to beverage. Several factors, like the coffee/water ratio used or the type of brewing method in the coffee beverage preparation have impact on its final content in the brew. In fact, the acrylamide concentration resulted lower in espresso coffee brew because of the short contact time between water and ground coffee reducing the extraction of the contaminant (Andrzejewski, Roach, Gay and Musser, 2004; Lantz, Ternite, Wilkens, Hoenicke, Guenther and van der Stegen, 2006).
Moreover, few methods are available to reduce the acrylamide content without compromising the brew's quality and sensory properties, for example “vacuum roasting” of coffee beans was proposed as alternative to conventional roasting process for the mitigation of acrylamide. It was reported that acrylamide content of medium roasted beans could be decreased approximately 50% by using vacuum roasting (Anese, Nicoli, Verardo, Munari, Mirolo and Bortolomeazzi, 2014). An option to reduce the acrylamide intake of consumers from coffee is to select commercial blends with higher Arabica contents and median or dark degrees of roasting and, simultaneously, to prefer shorter brews (i.e., espresso) instead of long ones, but this will obviously depend on the consumer preferences.

- **5-Hydroxymethylfurfural**

5-Hydroxymethylfurfural (HMF, 5-hydroxymethyl-2-furancarboxaldehyde, **Figure 43**) is a process contaminant compound which has been reported to be mutagenic, carcinogenic, and cytotoxic due to its metabolic activation to 5-sulfooxymethylfurfural (Islam, M. N., Khalil, Islam, M. A. and Gan, 2014; Choudhary, Kumar, V., Kumar, S., Majid, Aggarwal and Suri, 2020). During processing and storage of carbohydrate containing foods, important chemical modifications can take place. Under acidic conditions occurring in the foods, dehydration of carbohydrates leads to the formation of 5-hydroxymethylfurfural.
The formation of this compound with and without acid catalysis occurs during the dehydration of, for example, fructose to HMF via a series of reactions retaining the fructofuranose ring intact. The hydrolysis of sucrose, which reacts to make HMF in high yields, directly generates a required fructofuranosylation intermediate in this hypothesis (**Figure 43**) (Antal, Mok and Richards, 1990; Murkovic and Pichler, 2006).

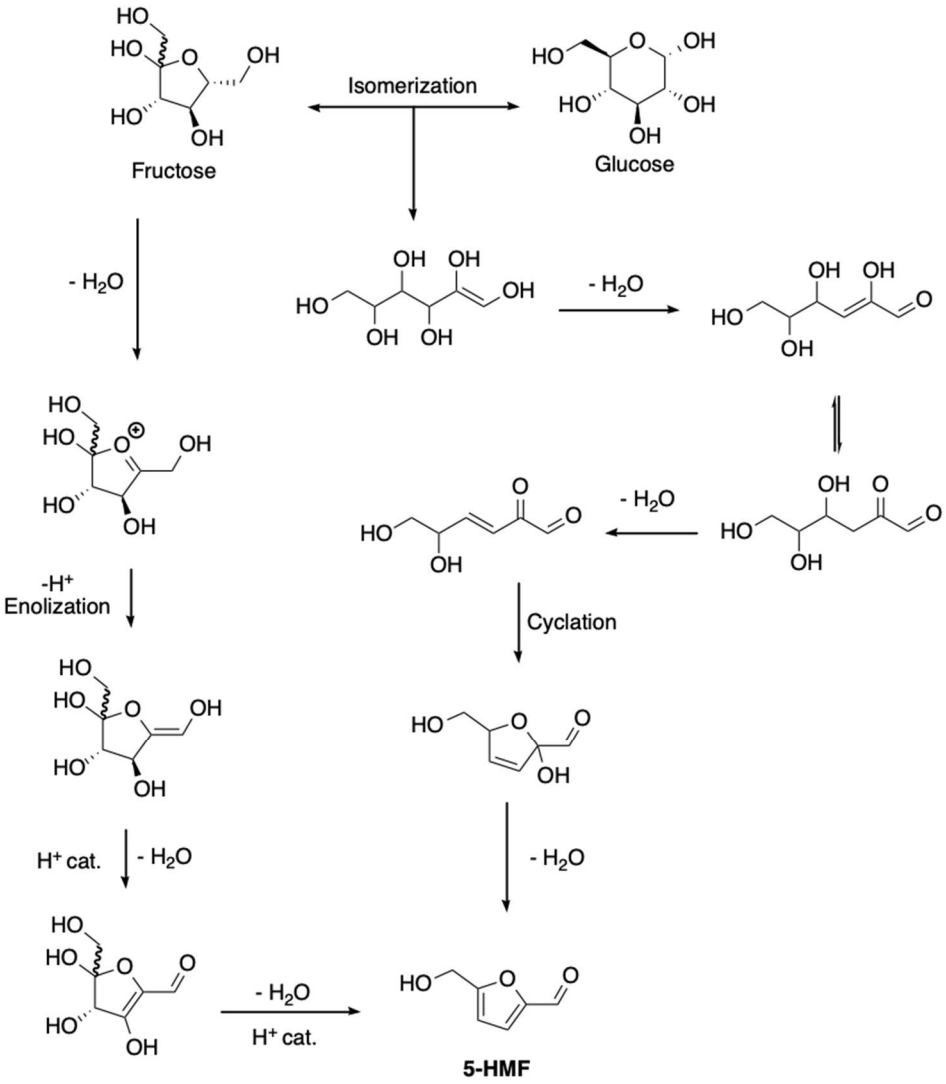


Figure 43. Proposed mechanism for catalytic dehydration of fructose and glucose to 5-HMF (Kowalski, Lukasiewicz, Duda-Chodak and Zięć, 2013).

Moreover, the Maillard reaction can also take place, giving rise to Amadori compounds during the first steps of the reaction, and HMF as a consequence of further reactions (see **Appendix 1**).

In coffee, the Amadori rearrangement products obtained from a hexose such as glucose, in a reaction at neutral pH or acidic conditions, is the Schiff base of HMF, which is formed by a 1,2 or 1,3 enolization mechanism. Subsequently an amino compound is removed, and a water molecule is added to obtain HMF (**Figure 44**) (Choudhary, Kumar, Kumar, Majid, Aggarwal and Suri, 2020).
Also caramelization of sugars is an important reaction in the generation of HMF, it is possible that the highly reactive fructofuranosyl cation is generated from fructose or sucrose, which through water loss generates HMF (Locas and Yaylayan, 2008).

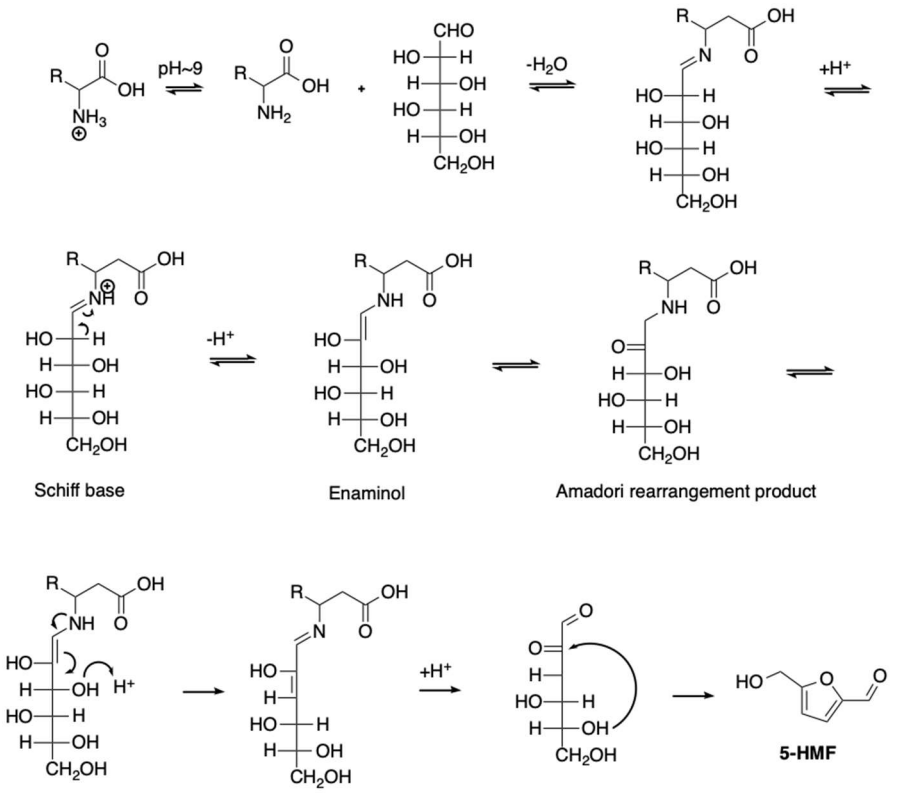


Figure 44. Maillard reaction leading to the formation of 5-HMF (De Schutter *et al*., 2008).

- **Furan**

Furan is a highly volatile compound present in many foods and drinks as a result of thermal treatment during processing. Its presence in food has been known since the 1930s (Johnston and Frey, 1938). Processing of food as cooking, roasting, frying, baking, pasteurization and sterilization are the main causes of furan formation, and it is present in coffee as part of the volatile aroma components generated during roasting of green coffee beans. From roasting to the cup, furan levels decrease because of its high volatility and low water solubility. Thus, through grinding, packaging, storage, and brewing procedures, levels of furan are lowered. Furan formation decreases at low temperatures and its evaporation loss is increased during long roasting time at 140°C. However, the best conditions to decrease the furan concentration of roasted coffee promote the formation of other contaminants as acrylamide and HMF (Lachenmeier *et al*., 2019).
It has been estimated that coffee consumption may be the highest contributor to furan exposure from dietary sources among adults. Lower amounts of furan were detected in

ground and instant coffee, with average levels of 1807 and 602 μg/kg, respectively. The highest level of furan was identified in roasted coffee beans, with mean and maximum values of 3611 and 6407 μg/kg, respectively (EFSA, 2011)
Furan is classified as possibly carcinogenic to humans (group 2B) by the IARC and considered a process contaminant in thermally treated foodstuff (IARC, 1995).
There are various pathways of furan formation, such as: (i) thermal degradation of carbohydrates alone or in presence of amino acids, (ii) thermal degradation of some amino acids, (iii) oxidation of ascorbic acid at elevated temperatures, (iv) oxidation of polyunsaturated fatty acids (PUFAs) and carotenoids (**Figure 45**) (Moro, Chipman, Wegener, Hamberger, Dekant and Mally, 2012) During the pyrolysis of amino acids such as serine, acetaldehyde and glycolaldehyde molecules are generated, which form 2-deoxyaldotetrose through aldol condensation that subsequently leads to furan.

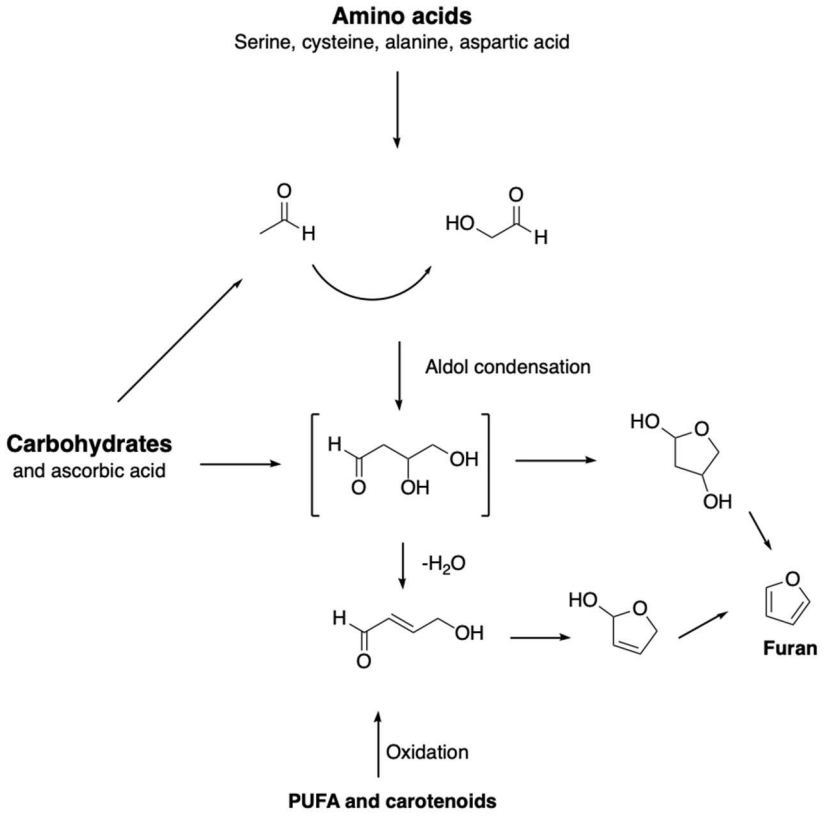


Figure 45. Reaction pathways of furan formation in thermally treated foodstuff.

In coffee beans the concentration of glucose and fructose increases in the initial phase of roasting due to the constant degradation of sucrose, these hexoses through Maillard reaction and pyrolysis generate intermediates that can trigger furan formation as result of dehydration and decarboxylation reactions (Perez Locas and Yaylayan, 2004).
Furan formation from PUFAs occurs via free radical autoxidation. Moreover, furan formation has recently been reported though 2-furoic acid decarboxylation, the latter originating from the oxidation of furfuryl alcohol which derives from the decomposition of caffeic and quinic acids (Delatour *et al*., 2020).
Furan levels increase in the darker roasting degrees, with the highest concentrations reported in the Robusta coffee beans. In fact, to reduce the development of furan in roasted coffee,

blends of Robusta with Arabica species and lighter roast levels have been suggested (Arisseto, Vicente, Ueno, Verdiani Tfouni and De Figueiredo, 2011).
In relation to coffee brew, the transfer rate of furan from ground coffee to coffee brew is mainly determined by the procedure used to prepare the beverage, the increase in the temperature when pouring hot water into coffee powder results in significant losses of furan such as in percolation or filtration methods. Lower levels of furan can be obtained during the preparation of beverages when the ground coffee is boiled with water before filtration achieving reduction of up to 98% (La Pera, Liberatore, Avellone, Fanara, Dugo and Agozzino, 2009).

- **Polycyclic aromatic hydrocarbons**

PAHs are a broad class of organic compounds which are produced by incomplete combustion or pyrolysis of organic materials at high temperatures as well as during some industrial processes.
Some of these compounds are mutagenic and carcinogenic, and it is considered that they have a role in a number of human diseases.
The PAHs endogenous contamination of coffee is primarily caused by roasting, and it has been reported to be closely related to roasting parameters, particularly the duration and temperature. The highest contamination levels have been found for roasting processes that are prolonged and conducted at high temperatures; moreover, for a correct assessment of the extent of this contamination, other aspects such as coffee cultivars or brewing procedures must be taken into consideration (Houessou, Maloug, Leveque, Delteil, Heyd and Camel, 2007; Tfouni *et al*., 2013).
PAHs in coffee can also derive from an exogenous contamination, deriving mainly from environmental pollution that affects the phases of drying and storage of green coffee beans. However, the PAHs contamination of green coffee beans is rather low or even zero (European Commission, 2002).
The PAHs pollution of roasted coffee seems to be rather restricted, and it is even lower in coffee drinks due to PAHs are poorly soluble in water, thus limiting the transfer to the coffee infusion (Duedahl-Olesen, Navaratnam, Jewula and Jensen, 2015; Houessou, Benac, Delteiland Camel, 2005).
Regulations have been set to limit PAHs levels in certain foodstuffs (European Commission Regulations (EC) No 1881/2006). Currently, the maximum levels for benzo[a]pyrene (BaP) are 2 µg/kg and 5 µg/kg in oils and fats and in cocoa bean fats, respectively. Limits in roasted coffee are not established yet but studies are underway in order to define it (Commission Regulation (EC) No 1881/2006). The European Food Safety Authority (EFSA) currently recognizes four PAHs (benz[a]anthracene, chrysene, benzo[b]fluoranthene and BaP also named PAH4) as appropriate markers for this kind of foodstuffs contamination.
The toxic potency of coffee samples is assessed in terms of BaP equivalent concentration of each individual PAH species. On roasted coffee, studies have shown that BaP contamination is often less than 5 µg/kg for the medium roasting which is by far the most common one (Jimenez, Adisa, Woodham and Saleh, 2014).

1.10. Hazards and Control Measures of green coffee

Coffee arrives at the factory or facility for further processing either in smaller units, such as jute sacks, or in bulk via containers (train and truck), or one-ton big bags. All deliveries should go through a preliminary examination to look for things like mouldy bean evidence, bag

integrity, indicators of pests, and moisture damage. Quality approval of incoming lots is a key process before releasing the material for production.
Following a predetermined sampling procedure, coffee consignments are subject to physical testing and sensorial analysis. These tests allow for the grading of the coffee (verification that the delivery conforms with the specification) and include quality parameters like defects and moisture that should be in accordance with the International Organization for Standardization's (ISO) standard (ISO 6673).
Before further processing (roasting and grinding), the green beans are passed through a magnet to not only remove metallic contaminants but also as a protection of the coffee grinder at a later stage of processing. With the use of a bean cleaner, other foreign objects including pieces of string (from bags), stones, wood, glass, plastic, etc. are eliminated before the green coffee is transported to the intermediate storage silos.
Control measures for pesticides/fumigants upstream at the supplier level are therefore mandatory and must be firmly embedded in the supplier/grower contract and identified within the Hazard Analysis and Critical Control Points (HACCP) study.
The chemical hazard includes the monitoring of OTA levels. International Coffee Organization (ICO) Resolution 420 regulates moisture and sets a maximum moisture content of 12.5% as a quality requirement for exported green coffee. Deliveries that are over 13% are refused, and depending on the supplier contract, deliveries that are between 12% and 13% may result in compensation claims being made to the supplier (https://www.ico.org/documents/iccres420e.pdf)

Appendix 1

Maillard reaction

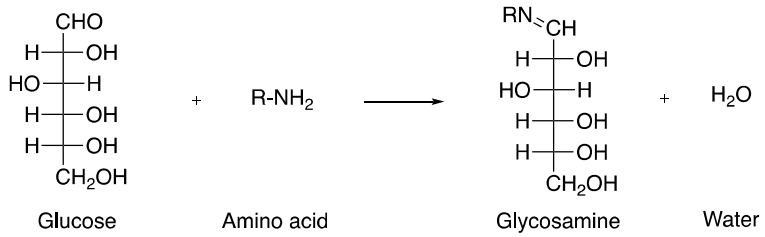
Since its discovery in 1912 by Louis Camille Maillard, the Maillard reaction (MR), also called the non-enzymatic browning reaction which typically proceeds rapidly from around 140 to 165 °C, has been widely studied because it commonly occurs during cooking, preservation and processing, leading to the formation of the so-called Maillard reaction products (Gerrard, 2006).

The MR allows to create a pleasing colour, a crusty texture, and appealing aromas to enhance food flavour through the use of various cooking techniques (baking, roasting, frying, etc.).The organoleptic properties of baked products (bread, cakes, potato-based products, meat, fish), cocoa, and coffee are all significantly enhanced by the MR.

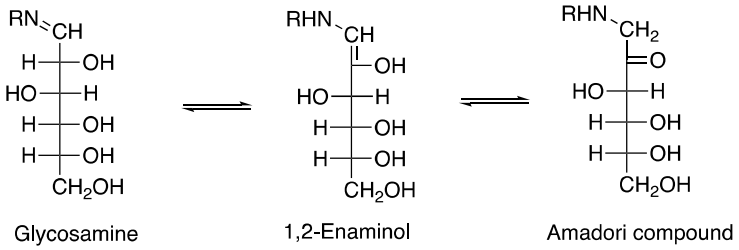
The Maillard reaction is a succession of non-enzymatic reactions whose mechanisms are still not completely elucidated but three main steps can be identified (**Figure 1**):

In the first stage, the carbonyl group of the sugar reacts with the amino group of the amino acid, producing N-substituted glycosylamine and water. In the second stage, glycosylamine compounds follow the Amadori rearrangement forming ketosamines known as Amadori compounds (Nursten, 2005).

Step 1: The carbonyl group of the sugar reacts with the amino group of the amino acid to produce N-substituted glycosylamine and water



Step 2: The glycosamine undergoes Amadori rearrangement to form ketosamines



Step 3: The ketosamines react though several pathways to form different products

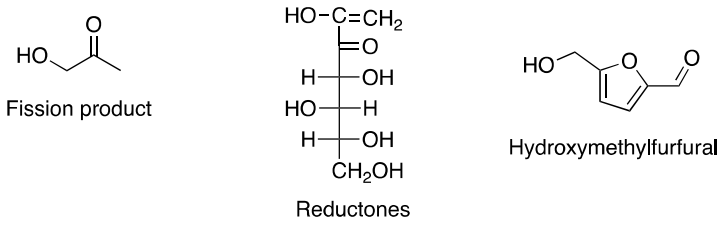


Figure 1. Main mechanisms involved in the three stages of Maillard reaction.

In the third step the Amadori products formed in this stage are then degraded into different compounds depending on the pH of the system. Low pH favors the 1,2-enolization process, which results in the formation of substances like furfural or hydroxymethylfurfural, whereas higher pH favors the 2,3-enolization pathway, which leads to reductones such as furanone (Lertittikul, Benjakul and Tanaka, 2007).

Several ways are known for the ketosamines to react further to form diacetyl, pyruvaldehyde, and other short-chain hydrolytic fission products which can be volatiles (2,3-butanedione, 2,3-pentanedione) or precursors of volatiles, which contribute to the flavour of food.

During MR together with the breakdown and fragmentation of sugars the degradation of amino acids occurs through Strecker degradation, a chemical reaction in which, in presence of a dicarbonyl compound, an α-amino acid is converted into an aldehyde, passing through the formation of an imine (**Figure 2**).

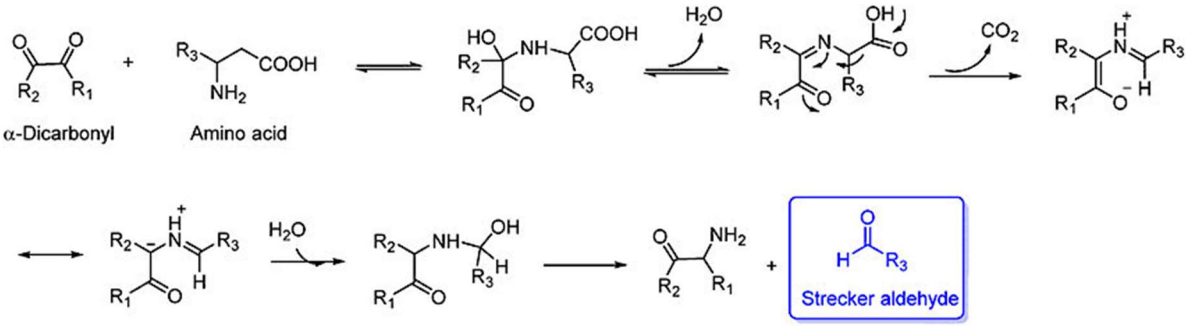


Figure 2. Mechanism of amino acids Strecker degradation.

The final stage of MR comprises aldol condensation, aldehyde-amine condensation, and the formation of heterocyclic nitrogen compounds. The highly reactive compounds created in the earlier phases are then subjected to polymerization processes to create coloured products with high molecular masses known as melanoidins. The colour development that occurs on foodstuffs during thermal processes is caused by these pigments.

Acrylamide, a possible human carcinogen, can be generated as a byproduct of Maillard reaction between reducing sugars and amino acids, especially asparagine, both of which are present in most food products. The main reaction pathways are reported in **Figure 3**.

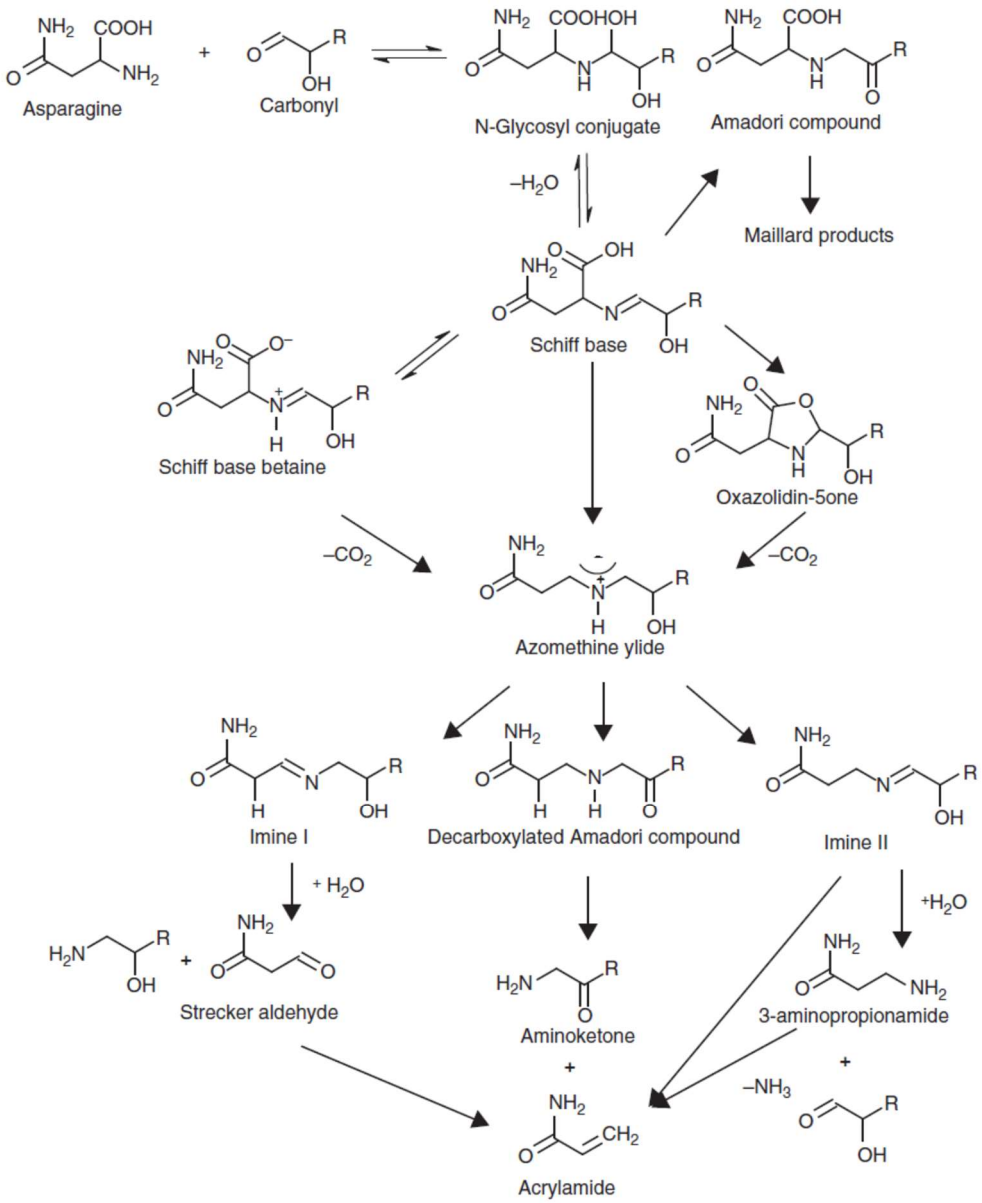


Figure 3. Main reaction pathways of acrylamide formation through Maillard reaction.

Appendix 2

Comparison between *Codex Alimentarius* and selected national standards

Pesticide	Codex Alimentarius	Year of Adoption	European Union	Japan	USA	Brazil*	DRC	Ecuador*	Ghana	Indonesia*	Kenya*	Rwanda	Uganda
Aldicarb	0.10 mg/kg		0.10	0.10	0.10	0.10		0.10		0.10	0.10	0.10	0.10
Azoxystrobin	0.03 mg/kg	2014	0.03	**0.05**									
Boscalid	0.05 mg/kg	2010	0.05	0.05		0.05							
Buprofezin	**0.40** mg/kg	2015	0.05	*0.01*									
Carbendazim	0.10 mg/kg	2001	0.10	0.10								0.10	
Carbofuran	**1.00** mg/kg	1999	*0.05*	**1.00**	0.10	0.10		0.10		0.10	0.10	**1.00**	
Chlorantraniliprole	0.05 mg/kg	2014	*0.02*	**0.40**									
Chlorpyrifos	0.05 mg/kg	2003	**0.20**	*0.05*	0.10	*0.05*		*0.05*	0.1 (EU)** / *0.05* (Japan)	*0.05*	*0.05*		
Clothianidin	**0.05** mg/kg	2011	**0.05**	**0.05**					**0.05** (EU) / *0.02* (Japan)**				
Cyantraniliprole	0.05 mg/kg	2016	0.05	0.05									
Cyhalothrin (includes lambda-cyhalothrin)	0.01 mg/kg	2016	**0.05**	0.01									
Cypermethrins (including alpha- and zeta-cypermethrin)	0.05 mg/kg	2009	**0.10**	0.05	0.05		**0.10**	0.05	**0.10** (EU) / *0.03* (Japan)**	0.05	0.05	0.05	
Cyproconazole	*0.07* mg/kg	2014	0.10	0.10									
Cyproconazole (Coffee beans, Roasted)	0.10 mg/kg	2014	0.10	0.10									
Diquat	0.02 mg/kg	2014	0.02	**0.05**									
Disulfoton	**0.20** mg/kg	1995	*0.05*	**0.20**	**0.20**	0.10				**0.20**		**0.20**	

Pesticide	Codex Alimentarius	Year of Adoption	European Union	Japan	USA	Brazil*	DRC	Ecuador*	Ghana	Indonesia*	Kenya*	Rwanda	Uganda
Endosulfan	**0.20** mg/kg	2007	0.10	0.10		*0.05*	0.10	0.10		0.10	0.10		
Fenpropathrin	**0.03** mg/kg	2015	0.02	*0.01*									
Flutriafol	0.15 mg/kg	2012	0.15	**0.20**									
Glufosinate-Ammonium	0.10 mg/kg	2013	0.10	0.10									
Haloxyfop	0.02 mg/kg	2010	**0.05**	*0.01*									
Imidacloprid	**1.00** mg/kg	2009	**1.00**	0.70	0.80	0.07	**1.00**		*0.05*				
Permethrin	0.05 mg/kg		**0.10**	0.05		*0.01*				0.05		0.05	
Phorate	**0.05** mg/kg	2006	**0.05**	*0.02*	*0.02*	**0.05**							
Propiconazole	*0.02* mg/kg	2008	*0.02*	**0.10**		0.05				**0.10**			
Pyraclostrobin	*0.30* mg/kg	2007	*0.30*	*0.30*		**0.50**							
Saflufenacil	*0.01* mg/kg	2012	**0.03**	**0.03**									
Spirodiclofen	*0.03* mg/kg	2010	**0.05**	*0.03*		*0.03*							
Tebuconazole	*0.10* mg/kg	2012	*0.10*	0.20	**0.30**	0.20							
Terbufos	**0.05** mg/kg	2006	*0.01*	**0.05**	**0.05**	**0.05**				**0.05**		**0.05**	
Thiamethoxam	**0.20** mg/kg	2011	**0.20**	**0.20**	0.05	*0.02*			0.05 (EU) / *0.02* (Japan)**				
Triadimefon	**0.50** mg/kg	2008	*0.05*	*0.05*		0.10	**0.50**	*0.05*		*0.05*	*0.05*		
Triadimenol	**0.50** mg/kg	2008	*0.05*	0.10		**0.50**		0.10		0.10	0.10	0.10	

Notes: a blank means information was not reported or not available.

* As reported in document ICC-110-3 Rev. 2, 25 February 2013.

** As reported by Ghana

1.11. Bibliography

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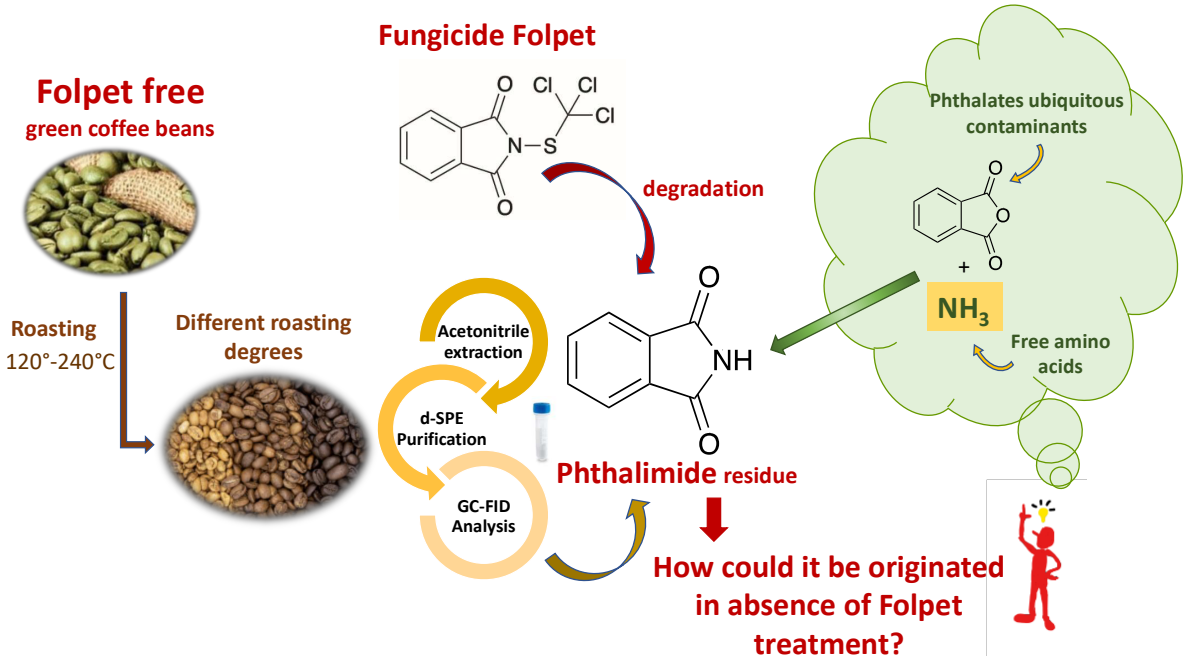
2. The aim of the project

In recent years, food-related public health problems have increased the attention of the competent authorities on the control of pollutants, adulterants, process or environmental contaminants and, at the same time, have prompted the industry to focus on the quality of products and raw materials.
The growing interest in a more protected diet, together with the developing regulatory framework, have led to the need for further analysis of high-consumption food matrices, which certainly include coffee, one of the most important products in the world and whose safety and quality are function of the entire production line.
The success of coffee brew is the result of the availability of different cultivars and combinations of flavours that can meet the different sensory expectations of consumers. Numerous scientific research works have therefore been aimed at studying the various components of this precious vegetal matrix.
From beans to the brew, the coffee production chain is a complex process that involves growing, harvesting, hulling, drying, packing, shipping, blending, roasting and grinding.
Chemical residues and pollutants can enter the food supply chain through food processing or environmental variables like air, soil, and water. The concentrations of these substances in diet can differ, and some could have potential adverse health effects if consumed at levels considered to be unsafe. Moreover, due to its high value and significant socioeconomic impact, there is a great deal of concern about coffee quality, and this requires not only safety during planting, harvesting and processing, but also frauds tracking and the control of both environmental and process pollutants.
The presence of contaminants in green and roasted coffee beans, both from environmental (Folpet and *o*-phenylphenol (OPP) and roasting (acrylamide) processes has been investigated and rationalized in collaboration with the coffee company Luigi Lavazza S.p.A. The focus of this study was about the optimization of adequate analytical and sample preparation methods for the accurate interest compounds determination in such complex matrix.
In particular, with regard to Folpet, the presence of phthalimide in samples of roasted coffee was studied as a degradation product and marker of this pesticide, which is not among those indicate by Codex Alimentarius as applicable to coffee cultivation. As concern OPP, reported as detectable only in roasted beans and not in the green ones, the objective was to investigate the possibility to have false negative results in unroasted samples by applying the common pesticide multiresidue analytical methods. In fact, given its chemical structure, OPP generation from precursors present in green beans during roasting is highly unlikely. For the coffee industry, the mitigation of acrylamide represents a major challenge due to the limited technological modifications applicable, making the selection of raw material of prime importance. Thus, the influence of green coffee parameters such ripening, and the post-harvest treatments has been studied to evaluate, on both Arabica and Robusta samples, their influence on acrylamide generation.
The Covid-19 lockdown in 2020 has been dedicated to the drafting and publication of a review article on polycyclic aromatic hydrocarbons (PAHs), compounds deriving from the incomplete combustion and pyrolysis of organic matter and considered to be potentially genotoxic and carcinogenic in humans. Since the main cause of coffee PAHs contamination is thought to be their generation during processing stages, the focus of this work was that to summarise and discuss the knowledge and recent advances in PAHs formation during roasting considering process parameters' influence and the PAHs residue in different coffee samples.

Alongside, during this project another aspect concerning the coffee production chain has been taken into consideration through the application of improved green methods in decaffeination process of green beans was evaluated. Acoustic cavitation was chosen to assist supercritical CO_2 extraction of caffeine, with concerns about time saving and extraction efficiency of the process.

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



Janet Menzio, Silvia Tagliapietra, Elena Calegari, Bianca Serito, Arianna Binello,* Giancarlo Cravotto*.
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3.1. Introduction

Folpet is a fungicide that belongs to the thiophthalimide class and is commonly used in agriculture to avoid diseases caused by fungi (Tomlin, 1997; Joint Meeting on Pesticide Residues (JMPR), 1999; http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation99/18Folpet.pdf.) 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 during both thermal processing and analytical procedures in sample preparation (EFSA, 2014; Commission regulation (EU) 2016/156; EURL- SRM, 2017).
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.

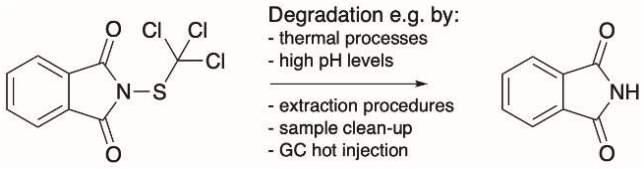

Figure 1. Degradation of Folpet into phthalimide.

Modified sample preparations and analytical methods for Folpet determination in tea have been validated in order to overcome the generation of analytical artifacts (Huertas-Perez *et al.*, 2018; Badoud *et al.*, 2018; Badoud, 2019; Gao *et al.*, 2019). 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 (Relana position paper No. 16-03), 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 posed under heating conditions such as food processing (i.e., drying), sample preparation (exothermic reactions), and analytical procedures (Relana position paper No. 17-01). Nevertheless, it should be underlined that no toxicity effects for PAI have been reported by the European Chemical Agency (ECHA) for food consumption (https://echa.europa.eu/de/registration-dossier/-/registered- dossier/13146/7/6/2.; Lorz, Towae, Enke, Jäckh, Bhargava and Hillesheim, 2012).
Phthalates (PAEs) are a group of either dialkyl or alkyl-aryl esters of 1,2-benzendicarboxylic acid (phthalic acid, PA) that are widely used as plasticizers to produce plastic- based materials. With an estimated annual global production of 11 billion pounds, these compounds are mainly employed to improve polymer characteristics such as flexibility, durability, and elasticity. For example, PVC products can contain up to 50% of PAEs by weight (Liang *et al.*, 2008).
There is great economic interest in PAEs as they also find applications in many other fields, such as cosmetics, household and building materials, toys, packaging, medical equipment,

pharmaceuticals, pesticides, lubricants, adhesives, and printing inks. In particular, some of the most commonly used PAEs are diethyl phthalate (DEP), di(2-ethyhexyl) phthalate (DEHP), di-isononyl phthalate (DINP), benzyl butyl phthalate (BBP), and di-n-butyl phthalate (DBP) (Giuliani *et al.,* 2020). As they are not covalently bound to the plastic polymers but only dispersed within them, these plasticizers can be released into the environment via leaching, for example (Heudorf, Mersch-Sundermann and Angerer, 2007).

In the case of foodstuffs, in addition to migration, another considerable source of these substances is processing, e.g., gloves used to handle food matrixes (Cao, 2010; Alp and Yerlikaya, 2020). Found in soil, air, natural water, and sediments, PAEs are potentially hazardous for both humans and the environment. Due to their environmental persistence and, therefore, bioaccumulation along the food chain, they have been defined as ubiquitous and unavoidable contaminants in food and the identification of the contamination source can be challenging (Matsumoto, Hirata-Koizumi and Ema, 2008; Dekant, 2020; Saido *et al*., 2003). It is worth noting that the thermal degradation of PAEs results in monoesters, PA and PAA, from which PAI can be obtained (Saido, Motohashi, Kuroki, Ikemura, Satomi and Kirisawa, 1979).

As the most frequently consumed beverage worldwide, coffee is a common food commodity that can be polluted by PAEs. In green coffee amino acids (AAs) are present not only in proteins but also in free form, and their content varies between different cultivars (e.g., in a range from traces to 0.13% w/w of dry green coffee beans) (Preedy, 2014). Free AAs (e.g., glycine, Gly; aspartic acid, Asp; phenylalanine, Phe) are unstable under roasting conditions, and therefore, a negligible amount of these compounds remains in roasted coffee (Illy and Viani, 2005).

Table 1 shows the main decomposition products of Gly, Asp, and Phe as described by Sato et al. (2004).The AA decomposition temperature is reported, but it has to be considered that ammonia starts to be released at the lower ones (110−180°C) (Sohn and Ho, 1995).

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 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 artifacts. High temperature coffee roasting treatments may therefore be the optimal conditions under which to evaluate the non-Folpet-related generation of PAI.

The importance of process pollutants in food products, together with the possible formation of artifacts, has focused our attention on the study of PAI generation due to the environmental PAE contamination in Folpet-free coffee samples. To the best of our knowledge, the literature presents significant omissions in regard to the mechanisms involved in the origin of PAI, making it necessary to better clarify the development of this artifact.

This work aims to demonstrate that PAI can be formed directly in coffee beans as a result of the reaction between PAA, which is generated from the thermal decomposition of PAEs, and

the NH_3 released by the degradation of AAs during the roasting process (Sato et al. ,2004; Sohn and Ho, 1995; Weiss, Muth, Drumm and Kirchner, 2018).
First, PAI generation has been monitored through tests performed on several AAs, supplemented with DEP or degradation products of PAEs (i.e., PA, PAA), posed in hermetically sealed systems and heated at different temperatures in a laboratory oven. Subsequently, Folpet-free Arabica green coffee bean samples have been subjected to the same thermal treatments in order to evaluate PAI development. The obtained results have been compared with those derived from Folpet-free DEP- or PAA-spiked samples. Quantitative determinations were performed using a GC-FID apparatus. Finally, the PAI content in Folpet-free industrially roasted coffee has been considered for discussion.

3.2. Results and discussion

3.2.1. Phthalimide Formation from amino acids and phthalic anhydride thermal treatment

As mentioned above, the presence of PAI may represent a false positive for the pesticide Folpet.
PAA is an intermediate formed during the thermal degradation of PAEs. As illustrated in **Figure 2** for DEP, in the absence or in a trace amount of water, the decomposition pathway proceeds via the formation of the monoester, an unstable compound in which neighboring group interactions can lead to the formation of the anhydride compound (Saido, Motohashi, Kuroki, Ikemura, Satomi and Kirisawa, 1979).

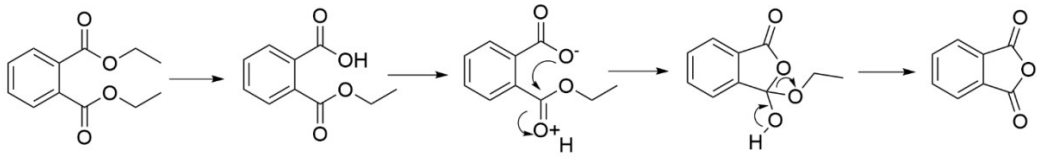

Figure 2. Thermal decomposition of diethyl phthalate to phthalic anhydride in absence or in trace amount of water.

Furthermore, PA is an indicator of the presence of monoester hydrolysis; its generation takes place in the presence of at least 10% water, while it is not formed with a lower amount of it.
Preliminary tests about PAI generation were carried out by adding PAA to Asp, Cys, Gly, Glu, Lys, and Phe (**Figure 3**).

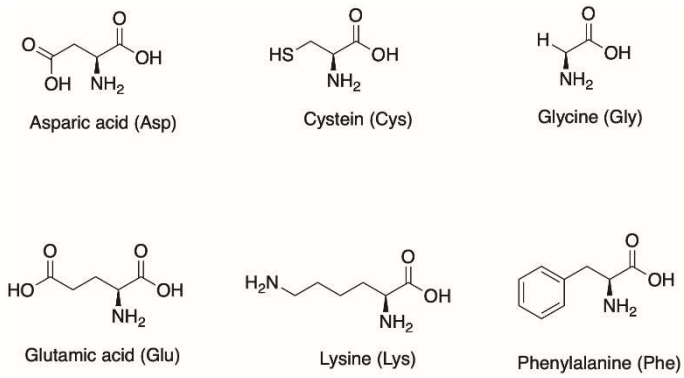

Figure 3. Amino acids chosen for trials.

These AAs (Asp, Gly, Glu, Lys, Phe, and Cys) have been selected because they have been reported to be present in free form in green coffee (Preedy, 2014); in addition, Cys and Asp were indicated by Sohn and Ho (1995) to be among the AAs with a higher NH_3 release.
The thermal treatment was performed in a laboratory oven at a range of temperatures (120, 150, 180, 200, and 220 °C) in order to emulate common coffee roasting conditions.
The quantitative data obtained from the GC-FID analyses (see **Table 5**) 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 and 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 2 h (Sohn and Ho, 1995).
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 sulfur 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 of PAI/100 mg of Cys vs 0.31 mg of PAI/100 mg of Asp).
The obtained data show that the NH_3 amounts involved are in line with those previously reported by Weiss et al. (2018). indicating that the thermal treatment of Asp, Cys, and Gly can result in at most 1/2 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

3.2.2. Phthalimide Formation from Aspartic Acid, Cysteine, and Glycine in the Presence of Phthalic Acid

As PA can dehydrate to PAA at high temperatures, PAI generation has also been evaluated in this study with PA as a reactant (**Figure 4**). In this case, Asp, Cys, and Gly were employed as they were found to be the most responsive AAs in the tests described above.
The thermal decomposition of PA (melting point 207 °C) is a complex process that heavily depends on heating. Chatterjee et al. (2002). have reported that the onset of PA melting begins at 180°C and then peaks at 197°C. This may be due to concomitant dehydration to PAA, small quantities of which depress the melting point and thus cause the physical process to begin at lower temperatures than expected. As the PAA melting point is 130 °C, its generation and evaporation occur simultaneously.

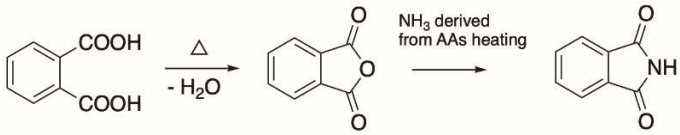

Figure 4. PAI generation from PA as a result of thermal treatment in the presence of AAs as a NH_3 source.

200°C was selected as the temperature to carry out these experiments, aiming to maximize the release of NH_3 by the AAs, guarantee the formation of PAA from PA, and fall within the temperature range used for roasting. Moreover, in the attempt to evaluate the time-of-exposure influence on this phenomenon, each reaction was performed in a sealed vial for both 30 and 60 min.

From the quantitative GC-FID analyses (see **Figure 5 showing PAI amounts generated in each experiment**), it is clear that the PAI yield is dramatically higher in the case of Asp with more than 20 mg/100 mg of AA, while Cys and Gly exhibit lower amounts of PAI. This confirms the particular behavior of Asp, which has already been evidenced in the previous trials. Furthermore, data show that an increase in residence time in the oven at 200 °C fails to lead to an improvement in PAI yield for all considered AAs.

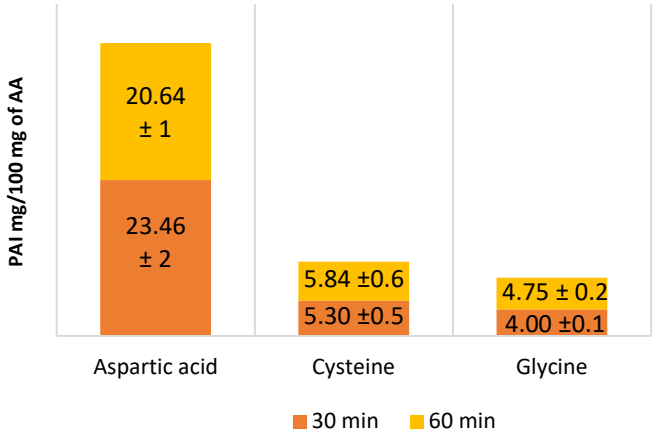

Figure 5. Phthalimide formation from AA in the presence of PA at 200°C for 30 and 60 minutes. Data indicate PAI amount expressed as mg/100mg of AA ± SD.

3.2.3. 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 (Saido *et al*., 2003). DEP was chosen as the 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 (see Phthalate Detection in Green Coffee Beans via SPME in section 3.3.2.).

Trials involving the use of DEP and Asp, Cys, and Gly were performed at three different temperatures (180, 200, and 220 °C), and 30 and 60 min of heating time were compared. The 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 rationalized if we consider the formation of PAA via the intermediate monoester (see **Figure 2**), whose decomposition, we believe, follows the trend reported by Saido et al. (1979). 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%, and therefore are able to trigger PAE degradation (http://www.ico.org/projects/good-hygiene-practices/cnt/cnt_ sp/sec_2/docs_2.1/ICO.pdf.)

Unlike the previous assays that entailed the direct addition of PAA (Section 3.2.1), a significant dependence on higher temperature occurs for DEP. In fact, only at 220 °C is it possible to observe an increase in PAI generation. In particular, the results reported in **Table 3** and **Figure 6** show that, for all temperatures, Asp gave higher PAI yields than Cys and Gly with the maximum value of 1.68 mg of PAI/100 mg of AA being observed at 220°C and 60 min 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	0.16 ±0.04	0.26 ±0.05	0.45 ±0.01	0.86 ±0.03	1.68 ±0.08
Cys	0.054 ±0.007	0.063 ±0.005	0.065 ±0.006	0.085 ±0.006	0.083 ±0.004	0.24 ±0.01
Gly	0.069 ±0.004	0.085 ±0.003	0.083 ±0.004	0.096 ±0.001	0.093 ±0.005	0.19 ±0.04

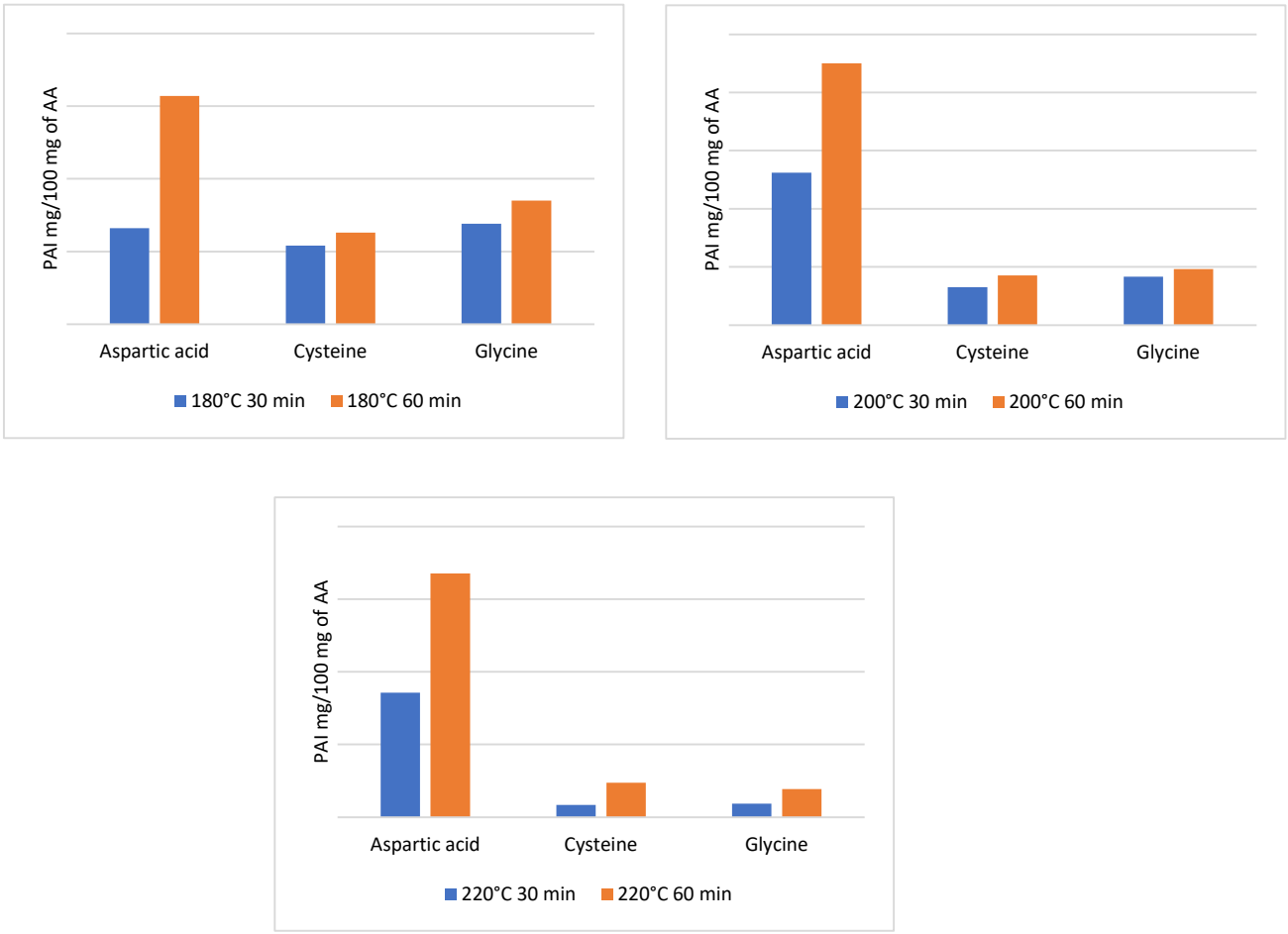

Figure 6. Phthalimide generation from aspartic acid, cysteine and glycine in presence of diethyl phthalate under heating.

3.2.4. 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 (http://www.ico.org/documents/cy2017-18/icc-122-10-r1e- maximum-residue-limits.pdf). 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 a decrease in the matrix effect on the analysis (https://www.waters.com/webassets/cms/library/docs/ 720003643en.pdf).

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 evaluated in PAI-spiked coffee samples at two different concentrations, and recovery in the range of 86− 99.9% was obtained (see Section 3.3.3). Subsequently, the green coffee samples, either used as such or spiked with PAA and DEP, were submitted to heating at different temperatures (180, 200, 220 °C) for 30 min in a laboratory oven in order to emulate a variety of roasting degrees. The results obtained from the GC-FID are reported in **Figure 7** and show that PAI was detected in all spiked samples. The formation of PAI follows a temperature-dependent trend with the maximum yield being achieved at 200 °C and remaining almost constant at 220 °C. This trend seems to reflect the results obtained in tests involving AAs in the presence of PAA. In particular, higher PAI yields (0.149 mg/g coffee) were detected in coffee samples with added PAA. For DEP enriched coffee, a maximum amount of 0.038 mg/g was observed. The trend found in tests performed on AAs heated in the presence of PAA and DEP was confirmed in this case. Surprisingly, PAI generation was also observed in non-spiked samples, which displayed a maximum PAI generation at 200 °C (0.0056 mg/g coffee, **Figure 7**). Since the samples were certified Folpet-free, it is evident that the heating process leads to PAI generation, the presence of which can only be due to environmental PAE pollution.

Of course, it cannot be assumed that this is the effective PAI concentration in commercial roasted coffee beans as the experimental conditions applied for tests are not comparable to those of a roasting process. For this reason, industrially roasted Folpet-free coffee samples (air roaster) that were obtained at four different roasting degrees (named light, medium light, medium dark, dark) were analysed. PAI was not detected in the lighter samples (light, medium light), while darker samples gave concentrations of 0.034 and 0.038 mg/kg, thus affirming that more intensive thermal conditions lead to a higher PAI residue.

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 the roasting process demonstrate a temperature dependent 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 humans and the environment, the roasting process induces their chemical modification to the nontoxic PAI. Certified Folpet-free Arabica green coffee beans, heated in laboratory oven at 180, 200, and 220 °C, showed PAI traces. Despite the experimental conditions of laboratory trials performed in hermetically sealed systems not being comparable with those of industrial roasting processes, it follows that the detection of the PAI residue 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).

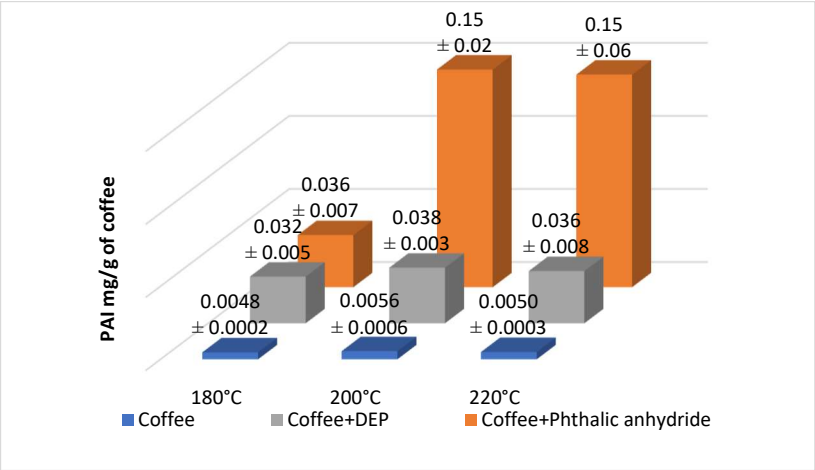

Figure 7. Phthalimide formation (expressed as mg/g of coffee) from Folpet free ground green coffee submitted to heating, either used as such or spiked with diethyl phthalate and phthalic anhydride. Data indicate PAI amount expressed as mg/100mg of AA ± SD.

3.3. Experimental section

3.3.1. Chemicals and Coffee Samples

Amino acids (aspartic acid, Asp; cysteine, Cys; glycine, Gly; glutamic acid, Glu; lysine, Lys; phenylalanine, Phe), phthalic anhydride (PAA, ≥99.9%), phthalic acid (PA, ≥99.9%), diethyl phthalate (DEP, ≥99.9%), phthalimide (PAI, Pestanal grade), and acetone (HPLC grade, lot: 61300645) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy).
Green coffee beans (Arabica, China) certified as Folpet-free (Eurofins Product Testing, Italy S.r.l., Turin, Italy) were kindly provided by LAVAZZA S.p.A (Settimo Torinese, Turin, Italy).
A DisQuE QuEChERS kit (150 mg of MgSO4, 25 mg of PSA, 25 mg of C18, and 7 mg of 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) fiber was used in green coffee SPME (solid-phase microextraction).

3.3.2. Preliminary Tests on PAI Generation

- **Thermal Treatment of AAs in the Presence of Phthalic Anhydride at Different Temperatures**

Six different AAs were chosen for the 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 min at different temperatures: 120, 150, 180, 200, 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 ionization detector (GC-FID).
Each test was repeated three times to verify method reproducibility.

- **Thermal Treatment of Amino Acids in the 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 in a vial for GC-FID analyses. Each test was repeated three times to verify the reproducibility of the method.

- **Thermal Treatment of Amino Acids in the Presence of Diethyl Phthalate at Different Temperatures**

100 mg of Asp, Cys, and Gly were, respectively, mixed with 134 µL (0.67 mmol) of DEP and 150 µL (0.0083 mmol) of H_2O, which corresponds to 1.24% mol with respect to DEP, in a 20 mL headspace vial, and the samples were shaken with a Vortex for 1 min. The hermetically sealed samples were heated in a laboratory oven at 180, 200, and 220 °C in all cases for both 30 and 60 min. After cooling to room temperature, each sample was

suspended in 1 mL of acetone, filtered through a 0.45 µm filter, and then transferred to a vial for GC-FID analyses.
Each test was repeated three times to verify the reproducibility of the method.

- **Phthalate Detection in Green Coffee Beans via SPME**

1 g of ground green coffee beans was transferred to a 20 mL headspace vial and submitted to solid-phase microextraction (SPME) employing a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/ PDMS) fiber at 80 °C for 12 h. The sample was then analyzed using gas chromatography coupled with mass spectrometry (GC-MS).
This test has been repeated three times to verify the reproducibility of the method.

3.3.3. Folpet-Free Green Coffee Beans Thermal Treatment

Tests on green beans were carried out on a 5 g quantity that was milled through a household coffee grinder. Ground coffee samples were transferred to 20 mL headspace vials and then heated in the laboratory oven for 30 min at different temperatures: 180, 200, and 220 °C. The same procedure was used for the PAA- and DEP-spiked samples: either 5 mg (0.037 mmol) of PAA or 7.5 µL (0.037 mmol) of DEP was added to 5 g of ground green coffee beans before heating.
After cooling to room temperature, 6 mL of acetonitrile was added to the samples, which were shaken with a Vortex for 1 min. In accordance with the QuEChERS method for multiresidue pesticide analysis, 1.5 mL of the obtained extract was transferred into a d- SPE tube for cleanup, and the sample was shaken for 1 min (Anastassiades, Lehotay, Stajnbaher and Schenck, 2003; https://www.waters.com/webassets/cms/library/docs/ 720003643en.pdf). The d-SPE tube was then centrifuged for 10 min at 5000 rpm (Hettich ROTOFIX 32 centrifuge), and 1 mL of the supernatant was evaporated under nitrogen flow. The residue was solubilized 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 the 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, the data on PAI content in industrially roasted Folpet- free coffee beans, which were kindly provided by LAVAZZA S.p.A. (certified and analyzed by Eurofins), were considered for discussion.

3.3.4. 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 ionization 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 0.25 µm) 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 postrun 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, 0.5, 0.35, 0.25, 0.1, and 0.05 mg mL^{-1} solutions were prepared and injected. A calibration curve was obtained with a linear correlation coefficient (R^2) of 0.9988 (LOD 0.0025 mg/mL; LOQ 0.005 mg/mL).

GC-MS analyses on ground green coffee bean samples that had undergone SPME were performed on an Agilent Technologies 6850 GC Network GC System equipped with a 7683B Automatic sampler and coupled with a 5973 Network Mass Selective detector. Separation was performed using an HP 5-MS column ((5%-phenyl)-methyl- polysiloxane, length 30 m, i.d. 0.25 mm, film thickness 0.25 µm). Helium was used as the carrier gas with a constant flow of 1.3 mL/min. The split/splitless injector was set in split mode (10:1), and its temperature was maintained at 250 °C. The oven-temperature program for the separation of the volatile compounds that were adsorbed onto the fiber was as follows: from 50°C (held 2 min) to 150 °C at 10 °C min^{-1}; then increased to 260 °C (held 15 min) at 5°C min^{-1}. The main chromatographic peaks were identified by comparing them to the mass spectra of the pure standards or using mass spectra libraries with the match quality index, as calculated by the NIST Similarity and Identity Spectrum Search algorithm (NIST 08 and Wiley MS 275).

Data analyses refer to the measurements performed in triplicate, and the results are expressed as mean data ± standard deviation (SD).

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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

Janet Menzio, Silvia Tagliapietra, Elena Calegari, Bianca Serito, Arianna Binello,* and Giancarlo Cravotto*

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ABSTRACT: Folpet, a fungicide used on 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), derived from phthalates, and amino acids (AAs), as a 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.

KEYWORDS: *phthalimide, amino acids, phthalates, coffee beans, roasting, pesticides degradation*

1. 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 byproduct of Folpet (Figure 1) can

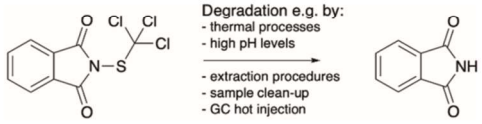

Figure 1. Degradation of Folpet into phthalimide.

occur during both thermal processing and analytical procedures in sample preparation.[3−5] 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.

Modified sample preparations and analytical methods for Folpet determination in tea have been validated in order to overcome the generation of analytical artifacts.[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 posed under heating conditions such as food processing (i.e., drying), sample preparation (exothermic reactions), and analytical procedures.[11] Nevertheless, it should be underlined that no toxicity effects for PAI have been reported by the European Chemical Agency (ECHA) for food consumption.[12,13]

Phthalates (PAEs) are a group of either dialkyl or alkyl-aryl esters of 1,2-benzendicarboxylic acid (phthalic acid, PA) that are widely used as plasticizers for the production of plastic-based materials. With an estimated annual global production of 11 billion pounds, these compounds are mainly employed to improve polymer characteristics such as flexibility, durability, and elasticity. For example, PVC products can contain up to 50% of PAEs by weight.[14] There is great economic interest in PAEs as they also find applications in many other fields, such as cosmetics, household and building materials, toys, packaging, medical equipment, pharmaceuticals, pesticides, lubricants, adhesives, and printing inks. In particular, some of the most commonly used PAEs are diethyl phthalate (DEP), di(2-ethyhexyl)phthalate (DEHP), di-isononyl phthalate (DINP), benzylbutyl phthalate (BBP), and di-*n*-butyl phthalate (DBP).[15] As they are not covalently bound to the plastic polymers but only dispersed within them, these plasticizers can be released into the environment via leaching, for example.[16]

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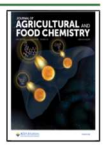

In the case of foodstuffs, in addition to migration, another considerable source of these substances is processing, e.g., gloves used to handle food matrixes.[17,18] Found in soil, air, natural water, and sediments, PAEs are potentially hazardous for both humans and the environment. Due to their environmental persistence and, therefore, bioaccumulation along the food chain, they have been defined as ubiquitous and unavoidable contaminants in food and the identification of the contamination source can be challenging.[19−21] It is worth noting that the thermal degradation of PAEs results in monoesters, PA and PAA, from which PAI can be obtained.[22]

As the most frequently consumed beverage worldwide, coffee is a common food commodity that can be polluted by PAEs. It is made from the roasted seeds of *Coffea* plants with two coffee species being of major importance for coffee production: *Coffea arabica* (known as Arabica) and *Coffea canephora* (known as Robusta).[23] In addition to caffeine, nitrogen fractions in green coffee beans include trigonelline and protein. Noteworthy, amino acids (AAs) are present not only in proteins but also in free form, and their content varies 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 and 240 °C for less than 20 min. The overall roasting treatment is characterized by a number of different steps: first, the dehydration of the product occurs; next, the main roasting process itself takes place, which produces the aroma as well as the typical color 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 protein 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; 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 AA decomposition temperature is reported, but it 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 (°C)
glycine (Gly)	ammonia, methyl amine, glycolic acid, formic acid	290
aspartic acid (Asp)	ammonia, fumaric acid, maleic acid, malic acid, succinic acid, pyruvic acid, lactic acid, acetic acid, formic acid	271
phenylalanine (Phe)	ammonia, acetic acid, formic acid	283

The aforementioned considerations provide some evidence that 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 artifacts. High temperature coffee roasting treatments may therefore be the optimal conditions under which to evaluate the non-Folpet-related generation of PAI.

The importance of process pollutants in food products, together with the possible formation of artifacts, has focused our attention on the study of PAI generation due to the environmental PAE contamination in Folpet-free coffee samples. To the best of our knowledge, the literature presents significant omissions in regard to the mechanisms involved in the origin of PAI, making it necessary to better clarify the development of this artifact.

This work aims to demonstrate that PAI can be formed directly in coffee beans as a result of the reaction between PAA, which is generated from the thermal decomposition of PAEs, and the NH_3 released by the degradation of AAs during the roasting process.[26−28]

First, PAI generation has been monitored through tests performed on several AAs, supplemented with DEP or degradation products of PAEs (i.e., PA, PAA), posed in hermetically sealed systems and heated at different temperatures in a laboratory oven. Subsequently, Folpet-free Arabica green coffee bean samples have been subjected to the same thermal treatments in order to evaluate PAI development. The obtained results have been compared with those derived from Folpet-free DEP- or PAA-spiked samples. Quantitative determinations were performed using a GC-FID apparatus. Finally, the PAI content in Folpet-free industrially roasted coffee has been considered for discussion.

2. MATERIALS AND METHODS

2.1. Chemicals and Coffee Samples. Amino acids (aspartic acid, Asp; cysteine, Cys; glycine, Gly; glutamic acid, Glu; lysine, Lys; phenylalanine, Phe), phthalic anhydride (PAA, ≥99.9%), phthalic acid (PA, ≥99.9%), diethyl phthalate (DEP, ≥99.9%), phthalimide (PAI, Pestanal grade), and acetone (HPLC grade, lot: 61300645) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy).

Green coffee beans (Arabica, China) certified as Folpet-free (Eurofins Product Testing, Italy S.r.l., Turin, Italy) were kindly provided by LAVAZZA S.p.A (Settimo Torinese, Turin, Italy).

A DisQuE QuEChERS kit (150 mg of $MgSO_4$, 25 mg of PSA, 25 mg of C18, and 7 mg of 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) fiber 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 were chosen for the 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 min at different temperatures: 120, 150, 180, 200, 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 ionization detector (GC-FID).

Each test was repeated three times to verify method reproducibility.

2.2.2. Thermal Treatment of Amino Acids in the 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 in a vial for GC-FID analyses.

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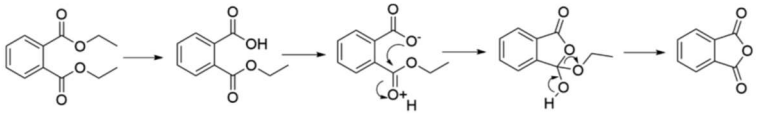
Scheme 1. Thermal Decomposition of Diethyl Phthalate to Phthalic Anhydride in the Absence or in a Trace Amount of Water



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

2.2.3. Thermal Treatment of Amino Acids in the Presence of Diethyl Phthalate at Different Temperatures. 100 mg of Asp, Cys, and Gly were, respectively, mixed with 134 μL (0.67 mmol) of DEP and 150 μL (0.0083 mmol) of H_2O, which corresponds to 1.24% mol with respect to DEP, in a 20 mL headspace vial, and the samples were shaken with a Vortex for 1 min. The hermetically sealed samples were heated in a laboratory oven at 180, 200, and 220 °C in all cases for both 30 and 60 min. After cooling to room temperature, each sample was suspended in 1 mL of acetone, filtered through a 0.45 μm filter, and then transferred to a vial for GC-FID analyses.

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

2.2.4. Phthalate Detection in Green Coffee Beans via SPME. 1 g of ground green coffee beans was transferred to a 20 mL headspace vial and submitted to solid-phase microextraction (SPME) employing a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber at 80 °C for 12 h. The sample was then analyzed using gas chromatography coupled with mass spectrometry (GC-MS).

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

2.3. Folpet-Free Green Coffee Beans Thermal Treatment. Tests on green beans were carried out on a 5 g quantity that was milled through a household coffee grinder. Ground coffee samples were transferred to 20 mL headspace vials and then heated in the laboratory oven for 30 min at different temperatures: 180, 200, and 220 °C. The same procedure was used for the PAA- and DEP-spiked samples: either 5 mg (0.037 mmol) of PAA or 7.5 μL (0.037 mmol) of DEP was added to 5 g of ground green coffee beans before heating.

After cooling to room temperature, 6 mL of acetonitrile was added to the samples, which were shaken with a Vortex for 1 min. In accordance with the QuEChERS method for multiresidue pesticide analysis,[29,30] 1.5 mL of the obtained extract was transferred into a d-SPE tube for cleanup, and the sample was shaken for 1 min. The d-SPE tube was then centrifuged for 10 min at 5000 rpm (Hettich ROTOFIX 32 centrifuge), and 1 mL of the supernatant was evaporated under nitrogen flow. The residue was solubilized 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 the 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, the data on PAI content in industrially roasted Folpet-free coffee beans, which were kindly provided by LAVAZZA S.p.A. (certified and analyzed by Eurofins), were considered for discussion.

2.4. 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 ionization 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 0.25 μm) 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 postrun 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, 0.5, 0.35, 0.25, 0.1, and 0.05 mg mL^{-1} solutions were prepared and injected. A calibration curve was obtained with a linear correlation coefficient (R^2) of 0.9988 (LOD 0.0025 mg/mL; LOQ 0.005 mg/mL).

GC-MS analyses on ground green coffee bean samples that had undergone SPME were performed on an Agilent Technologies 6850 GC Network GC System equipped with a 7683B Automatic sampler and coupled with a 5973 Network Mass Selective detector. Separation was performed using an HP 5-MS column ((5%-phenyl)-methylpolysiloxane, length 30 m, i.d. 0.25 mm, film thickness 0.25 μm). Helium was used as the carrier gas with a constant flow of 1.3 mL/min. The split/splitless injector was set in split mode (10:1), and its temperature was maintained at 250 °C. The oven-temperature program for the separation of the volatile compounds that were adsorbed onto the fiber was as follows: from 50 °C (held 2 min) to 150 °C at 10 °C min^{-1}; then increased to 260 °C (held 15 min) at 5 °C min^{-1}. The main chromatographic peaks were identified by comparing them to the mass spectra of the pure standards or using mass spectra libraries with the match quality index, as calculated by the NIST Similarity and Identity Spectrum Search algorithm (NIST 08 and Wiley MS 275).

Data analyses refer to the measurements performed in triplicate, and the results are expressed as mean data ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Phthalimide Formation from Amino Acids and Phthalic Anhydride Thermal Treatment. As mentioned above, the presence of PAI may represent a false positive for the pesticide Folpet.

PAA is an intermediate formed during the thermal degradation of PAEs. As illustrated in Scheme 1 for DEP, in the absence or in a trace amount of water, the decomposition pathway proceeds via the formation of the monoester, an unstable compound in which neighboring group interactions can lead to the formation of the anhydride compound.[22]

Furthermore, PA is an indicator of the presence of monoester hydrolysis; its generation takes place in the presence of at least 10% water, while it is not formed with a 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 (Asp, Gly, Glu, Lys, Phe, and Cys) have been selected because they have been reported to be present in free form in green coffee;[24] in addition, Cys and Asp were indicated by Sohn and Ho[27] to be among the AAs with a higher NH_3 release.

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

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

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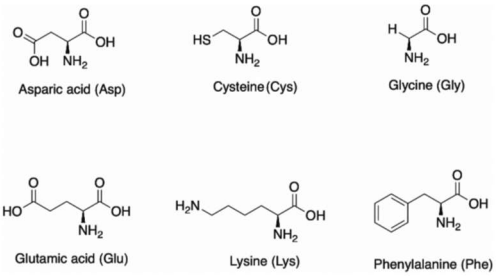

Figure 2. Amino acids chosen for the trials.

tested. Asp is stable up to 150 °C and begins to release NH_3 at higher temperatures with a significant increase at 180 °C and 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 2 h.[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 sulfur 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 of PAI/100 mg of Cys vs 0.31 mg of PAI/100 mg of 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 1/2 mol of NH_3 per mol of AA.

3.2. Phthalimide Formation from Aspartic Acid, Cysteine, and Glycine in the Presence of Phthalic Acid. As PA can dehydrate to PAA at high temperatures, PAI generation has also been evaluated in this study with PA as a reactant (Figure 3). In this case, Asp, Cys, and Gly were employed as they were found to be the most responsive AAs in the tests described above.

The thermal decomposition of PA (melting point 207 °C) is a complex process that heavily depends on heating. Chatterjee et al.[31] have reported that the onset of PA melting begins at 180 °C and then peaks at 197 °C. This may be due to concomitant dehydration to PAA, small quantities of which depress the melting point and thus cause the physical process to begin at lower temperatures than expected. As the PAA melting point is 130 °C, its generation and evaporation occur simultaneously.

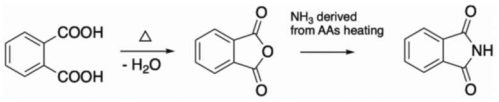

Figure 3. PAI generation from PA as a result of thermal treatment in the presence of AAs as a NH_3 source.

200 °C was selected as the temperature to carry out these experiments, aiming to maximize the release of NH_3 by the AAs, guarantee the formation of PAA from PA, and fall within the temperature range used for roasting. Moreover, in the attempt to evaluate the time-of-exposure influence on this phenomenon, each reaction was performed in a sealed vial for both 30 and 60 min.

From the quantitative GC-FID analyses (see Figure 4), it is clear that the PAI yield is dramatically higher in the case of Asp

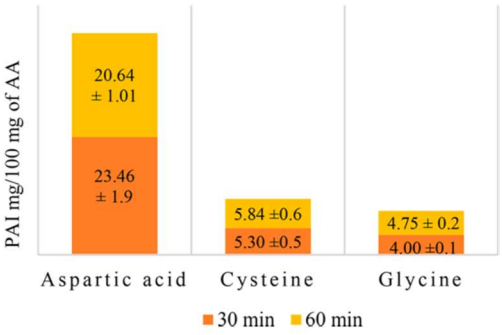

Figure 4. Phthalimide formation from AA in the presence of PA at 200 °C for 30 and 60 min. Data indicate the PAI amount expressed as mg/100 mg of AA ± SD.

with more than 20 mg/100 mg of AA, while Cys and Gly exhibit lower amounts of PAI. This confirms the particular behavior of Asp, which has already been evidenced in the previous trials. Furthermore, data show that an increase in residence time in the oven at 200 °C fails to lead to an improvement in PAI yield for all considered AAs.

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 the model compound for the tests, as it was found to be the only PAE present in detectable quantities in

Table 2. GC-FID Quantitative Results from the Thermal Treatment of Tested AAs with Phthalic Anhydride at Different Oven Temperatures[a]

	PAI mg/100 mg of 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

[a]Data indicate the phthalimide amount expressed as mg/100 mg of AA ± SD.

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Table 3. Phthalimide Generation from Aspartic Acid, Cysteine, and Glycine in the Presence of Diethyl Phthalate under Different Heating and Temperature Conditions[a]

	PAI mg/100 mg of 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

[a]Data indicate PAI amount expressed as mg/100 mg of AA ± SD.

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, 200, and 220 °C), and 30 and 60 min of heating time were compared. The 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 rationalized 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 are able to trigger PAE degradation.

Unlike the previous assays that entailed the direct addition of PAA (Section 3.1), a significant dependence on higher temperature occurs for DEP. In fact, only at 220 °C is it possible to observe an increase in PAI generation. In particular, the results reported in Table 3 and Figure 5 show that, for all temperatures, Asp gave higher PAI yields than Cys and Gly with the maximum value of 1.176 mg of PAI/100 mg of AA being observed at 220 °C and 60 min 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.

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 cleanup 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 cleanup step, which allows purer extracts with high recovery rates and a decrease in the 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 evaluated in PAI-spiked coffee samples at two different concentrations, and recovery in the range of 86–99.9% was obtained (see Section 2.3). Subsequently, the green coffee samples, either used as such or spiked with PAA and DEP, were submitted to heating at different temperatures (180, 200, 220 °C) for 30 min in a laboratory oven in order to emulate a variety of roasting degrees. The results obtained from the GC-FID are reported in Figure 6 and show that PAI was detected in all spiked samples. The formation of PAI follows a temperature-dependent trend with the maximum yield being achieved at 200 °C and remaining almost constant

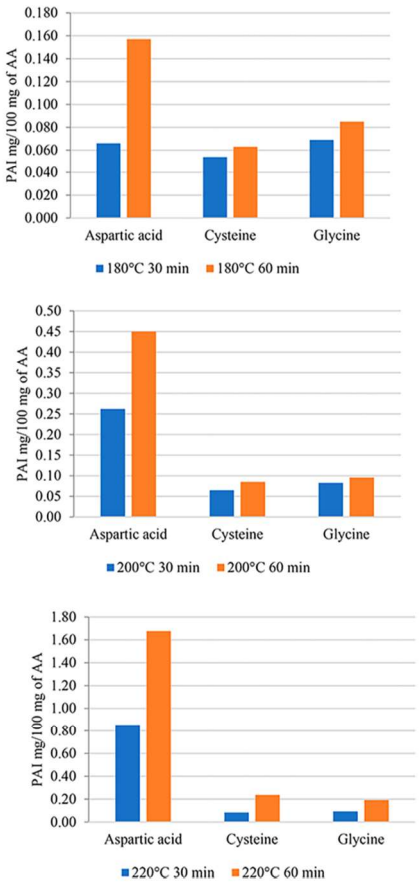

Figure 5. Phthalimide generation from aspartic acid, cysteine, and glycine in the presence of diethyl phthalate under heating.

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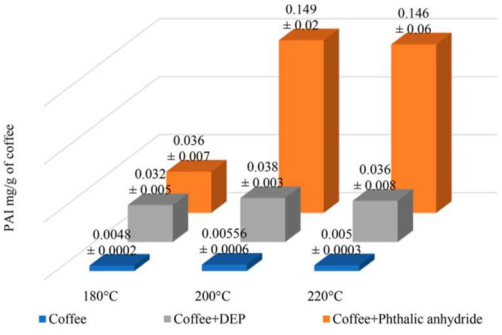

Figure 6. Phthalimide formation (expressed as mg/g coffee) from Folpet-free ground green coffee submitted to heating, either used as such or spiked with diethyl phthalate and phthalic anhydride. Data indicate the PAI amount expressed as mg/100 mg of AA ± SD.

at 220 °C. This trend seems to reflect the results obtained in tests involving AAs in the presence of PAA. In particular, higher PAI yields (0.149 mg/g coffee) were detected in coffee samples with added PAA. For DEP enriched coffee, a maximum amount of 0.038 mg/g was observed. The trend found in tests performed on AAs heated in the presence of PAA and DEP was confirmed in this case. Surprisingly, PAI generation was also observed in nonspiked samples, which displayed a maximum PAI generation at 200 °C (0.0056 mg/g coffee, Figure 6). Since the samples were certified Folpet-free, it is evident that the heating process leads to PAI generation, the presence of which can only be due to environmental PAE pollution.

Of course, it cannot be assumed that this is the effective PAI concentration in commercial roasted coffee beans as the experimental conditions applied for tests are not comparable to those of a roasting process. For this reason, industrially roasted Folpet-free coffee samples (air roaster) that were obtained at four different roasting degrees (named light, medium light, medium dark, dark) were analyzed. PAI was not detected in the lighter samples (light, medium light), while darker samples gave concentrations of 0.034 and 0.038 mg/kg, thus affirming that more intensive thermal conditions lead to a higher PAI residue.

PAI, a Folpet byproduct, 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 the roasting process demonstrate a temperature-dependent 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 humans and the environment, the roasting process induces their chemical modification to the nontoxic PAI.

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

Despite the experimental conditions of laboratory trials performed in hermetically sealed systems not being comparable with those of industrial roasting processes, it follows that the detection of the PAI residue 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).

AUTHOR INFORMATION

Corresponding Authors

Giancarlo Cravotto − *Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, 10125 Turin, Italy*; orcid.org/0000-0001-7574-7350; Phone: +39 0116707183; Email: giancarlo.cravotto@unito.it

Arianna Binello − *Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, 10125 Turin, Italy*; Phone: +39 0116707170; Email: arianna.binello@unito.it

Authors

Janet Menzio − *Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, 10125 Turin, Italy*

Silvia Tagliapietra − *Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, 10125 Turin, Italy*

Elena Calegari − *Luigi Lavazza SpA R&D, 10156 Turin, Italy*

Bianca Serito − *Luigi Lavazza SpA R&D, 10156 Turin, Italy*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.1c00462

Notes

The authors declare no competing financial interest.

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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 a flame ionization detector; GC-MS, gas chromatography coupled to a 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 fiber, divinylbenzene/carboxen/polydimethylsiloxane fiber; SPME, solid-phase microextraction

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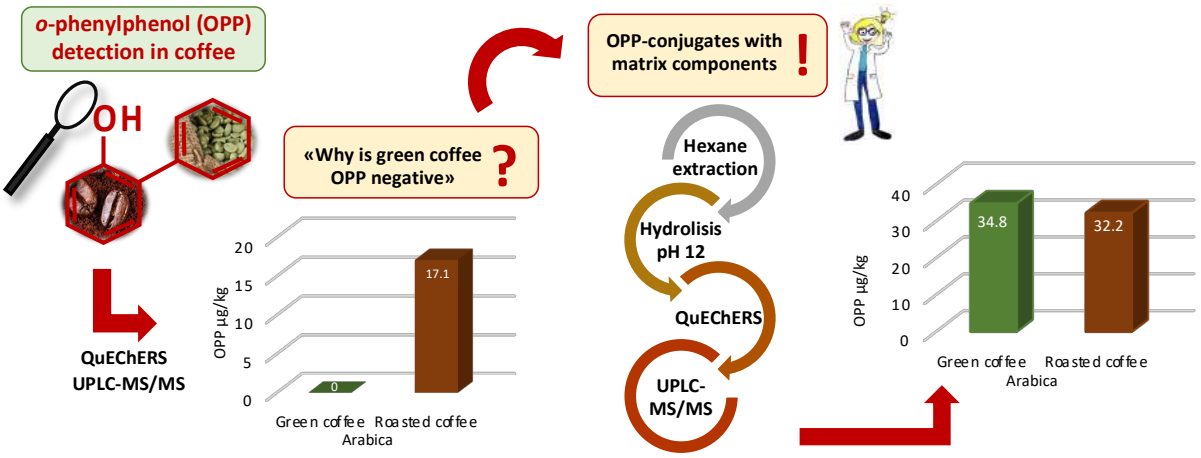
4. The challenge of *o*-phenylphenol detection in coffee: How "OPP-conjugates" hide their presence in green and roasted samples



Janet Menzio, Silvia Tagliapietra, Alessandro Barge, Bianca Serito, Elena Calegari, Arianna Binello*, Giancarlo Cravotto*.
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4.1. Introduction

There is currently a steady increase in concern about food and pollution. Coffee, as other food and beverages of vegetable origin, can suffer environmental and processing contamination affecting its quality and/or safety; it follows that control agencies, such as the European Food Safety Authority (EFSA), recommend strengthening investigations covered by specific legislation (EFSA Topics).

Biphenyl-2-ol or *o*-phenylphenol (2-phenylphenol, OPP) and its water-soluble salts (i.e., sodium o-phenylphenate, SOPP) are widely employed in the post-harvest treatment of diseases in fresh fruits and vegetables thanks to their antimicrobial activity. Initially associated with the fungistatic biphenyl used in Citrus fruits to prevent damage during storage and shipment (Gutter and Lattar, 1967), OPP acts against mold and rot as a residual contact substance that disrupts cell walls and cell membranes (EFSA Reasoned opinion, 2017). Its lipophilic derivatives (i.e., esters and amine complexes) can be incorporated into wax emulsions to reduce OPP phytotoxicity, which precludes some applications, and to provide more selective antimicrobial activity (Eckert, Esuruoso, and Carey, 1979; Gutter and Lattar, 1967). OPP and SOPP have not only been used in agriculture to control fungal and bacterial growth on stored crops but are still widely used as general surface disinfectants and in packaging materials (CARB, 1997; Patent WO 2021/ 064075A1). In general, improper use of biocides can affect human health through the skin, inhalation, and oral intake. Information on the toxicity of OPP and SOPP is limited. The European Food Safety Authority EFSA (EFSA Reasoned Opinion 2017) and the World Health Organization (WHO) (World Health Organization, 2000) have reported a consumer risk assessment, based on existing CXLs (codex maximum residue limit), with an ADI (acceptable daily intake) of 0.4 mg/kg of body weight (EFSA Scientific Report, 2008).

The lipidic profile is a key element in coffee authentication, with nine free fatty acids, evenly distributed between Robusta and Arabica species, being identified. In particular, a comparison of the contents of stearic and oleic acid allows the two species to be identified; stearic acid content is much lower than oleic in Robusta, while the percentages of these two acids in Arabica are almost equal (Farah, 2019).

Coffee is a rich source of phenolic compounds whose profile is strongly affected by roasting processes (Ky et al., 2001) which can cause substantial losses (up to 95 % of chlorogenic acids) (Farah, 2019). Despite this, OPP cannot be considered a natural phenol because it is not a component of any food.

Although the control of pests and diseases in coffee crops requires the well-regulated use of selective insecticides, fungicides and herbicides, OPP and SOPP are not listed among the pesticides for use on coffee beans, as regulated by the UE (European Commission MRLs Coffee beans,https://ec.europa.eu/food/plant/pesticides/eu-pesticides-data-base/products/?event=details&p=244; Farah, 2019). In particular, EFSA has published a reasoned opinion on the review of the existing MRLs, in accordance with Article 12 of Regulation (EC) No. 396/2005 (EFSA Reasoned Opinion 2017), recommending that the residue definition for plant products be modified and that the MRL for OPP should be 0.05 mg/kg for coffee beans.

As far as we know, OPP is detected by standard pesticide residue analysis methods only after coffee roasting and never in green beans. Theurillat et al., (Theurillat, Laborie and Schenk, 2022) have recently ruled out the possibility that the traces of OPP found in roasted coffee are caused by the possible use of the fungicide on green coffee and packaging contamination. The authors assigned the presence of OPP in coffee to its generation during roasting, with

content increasing with roasting degree. This conclusion has been supported by the facts that no OPP was detected in green coffee beans and by the differing amounts of the pesticide found in Arabica (slightly higher) and Robusta samples, indicating that production is species dependent. Nevertheless, no possible chemical pathway for OPP generation during roasting has been reported, and it is assessed that the transfer rate of the fungicide into the coffee brew is low (16 %).

The occurrence of OPP residues in green and roasted coffee beans has been investigated in this chapter due to its use as an antimicrobial agent in a variety of applications and its potential as a foodstuff contaminant (Coelhan, Bromig, Glas, and Roberts, 2006). Specifically, the aim was to demonstrate that the OPP found in roasted coffee beans can only derive from the accidental or technically unavoidable contamination of green coffee during its processing chain, and that this contamination cannot be detected in green beans using the common analytical methods for pesticide residues due to the generation of OPP conjugates. In addition to green and industrial roasted coffee samples, this study also considers green coffee beans that were heated in a laboratory oven in order to monitor the development of OPP in relation to temperature. An analytical procedure that is based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) for pesticide multiresidue determination has been optimized through dispersive solide phase extraction (d-SPE) sample clean-up. This pretreatment method has been applied to the study of several substances to isolate and clean up various analytes from complex matrices, this technique relies on the addition of a sorbent directly into the analytical solution followed by dispersion favoring the contact between the sorbent and the analytes. As a selective, reliable, and adaptable technology, d-SPE has a wide range of applications in many different fields (Lehotay, 2011). Then, OPPquantification has been performed by UPLC-MS/MS.

4.2. Results and discussion

4.2.1. OPP analysis via a QuEChERS-based method in green and industrially roasted coffee

Despite the presence of phenolic compounds in green coffee beans, the possible chemical pathway for the formation of OPP from precursors naturally present during roasting is difficult to define. This work therefore attempts to explain the presence of OPP in coffee by considering the possibility of accidental or technically unavoidable contamination during green coffee processing.

In the case of lipophilic pesticides, such as OPP, sample preparation is essential in their tendency to concentrate in the fatty fraction of the vegetal matrix, and this may have an effect on acceptable recoveries because of co-extract coffee components and adducts between the pesticide and the fatty fraction.

Sample preparation for OPP determination in both green and roasted coffee was performed using a QuEChERS-based method. This is a simple procedure for the efficient detection of multiple pesticide residues in food (Anastassiades, Lehotay, Stajnbaher and Schenck, 2003) that provides cleaner, enriched samples, which is of primary importance in complex matrixes, such as coffee, and because of the low residue levels of biocides required by authority regulations (Huertas-Perez et al., 2019). Firstly, this procedure involves matrix extraction with acetonitrile and then a clean-up step via dispersive solid-phase extraction (d- SPE) to remove interfering components. Two sorberts have been employed for this purpose: PSA and C18. Primary secondary amine (PSA) consists in a silica-based resin bearing an 2-

aminoethylethylamine and is weak anion exchanger sorbent reported as an effective sorbent for the removal of polar organic acids, fatty acids, polar pigments and some sugars as it contains secondary amino as well as primary groups. Then C18 sorbent was employed for non-polar interfering substances removal as lipids (Harmoko, Kartasasmita and Tresnawati, 2015). In recent years, QuEChERS has been evaluated on a large number of commodity/pesticide combinations, using GC, GC–MS/MS and/or LC-MS/MS analyses (European Standard Method EN 15662:2018; Theurillat, Dubois and Huertas-Perez, 2021). Because of the very low OPP concentrations, samples were prepared from 200 g of both green and roasted coffees. A Triple Quadrupole System equipped with a collision cell integrated into an UPLC-ESI-MS/MS instrument was employed for the analysis thanks to its high sensitivity and selectivity. An example of the chromatogram obtained by UPLC-MS/MS analysis of a roasted coffee sample is reported in **Figure 1**.

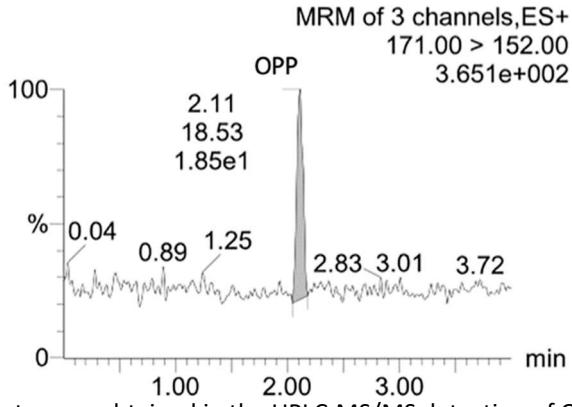

Figure 1. Example of a chromatogram obtained in the UPLC-MS/MS detection of OPP in roasted coffee samples (Arabica, Brazil).

Arabica (Brazil) and Robusta (Vietnam) green coffee beans and their related roasted samples, with different roasting degrees, were analysed; the roasting degrees are defined by ground sample colour and identified with a number on a scale between 45 (darker) to 60 (lighter). As re- ported in **Table 1**, the data show higher biocide content in samples subjected to lighter roasting, while no OPP was detected in the green samples. Moreover, a comparison of the two species showed that higher levels are found in Robusta samples than in Arabica, especially at the degrees of roasting identified, 55 and 60 (32.1 µg/kg vs 16.4 µg/kg and 34.2 µg/kg vs 17.1 µg/kg, respectively).

The results were in complete agreement with those of Theurillat et al. (2022), who found OPP concentrations ranging from < 10 µg/kg to 46 µg/kg in commercial roasted coffee. However, some differences were noted with the data shown here, as they reported an OPP increase with roasting degree and higher values for the Arabica species.

Table 1. OPP concentration in ground green and industrially roasted coffee analyzed via the QuEChERS-method and UPLC-MS/MS.

Roasting degree	Arabica µg/kg (±SD)	Robusta µg/kg (±SD)
Dark (45)	11.3 (±0.3)	traces
Medium dark (50)	11.8 (±0.2)	12.5 (±0.2)
Medium light (55)	16.4 (±0.5)	32.1 (±0.6)
Light (60)	17.1 (±0.1)	34.2 (±0.8)
Green coffee	-	-

4.2.2. Isothermal heating of Arabica and Robusta green coffee beans

To better understand the correlation between OPP detection and thermal treatment, Arabica and Robusta green coffees were subjected to isothermal heating in a laboratory oven. Using industrial roasting temperatures, tests were carried out for 1 h at: 120°C, 150°C, 180°C and 220°C. The residence time of the samples in the oven was opportunely selected, according to previous tests, to obtain a compromise between monitoring the OPP and not excessively prolonging the treatment. After heating, the beans were ground, in order to maximize the extractable surface, and were then analysed according to the procedure described above. The graph reported in **Figure 2** shows the mean values of OPP amount found at every thermal condition, with each calculated over three repetitions of the treatment.

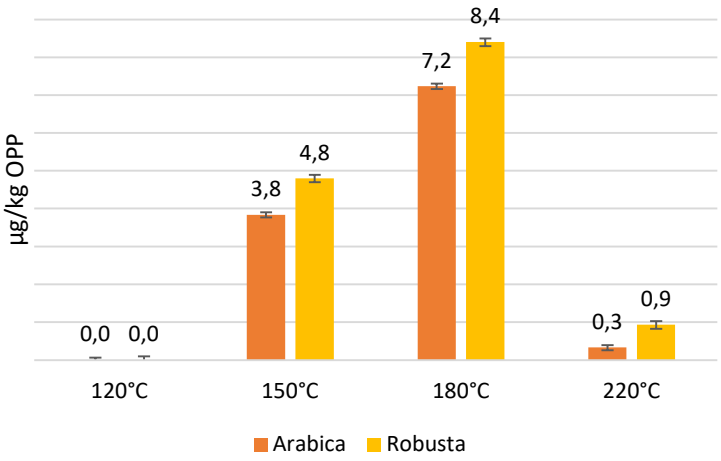

Figure 2. OPP concentration detected via the QuEChERS-method and UPLC-MS/MS in thermally treated Arabica (Brazil) and Robusta (Vietnam) green coffee samples. The treatment was carried out in a laboratory oven for 1 hour.

The obtained results show a significant increase in the OPP values in both species at 150 ◦C (Robusta 4.8 µg/kg, Arabica 3.8 µg/kg) and especially at 180 ◦C (Robusta 8.4 µg/kg, Arabica 7.2 µg/kg). Although the heating conditions were useful in making a correlation between OPP detection and temperature, the lower levels of fungicide found in these trials, compared to industrially roasted samples, can be attributed to the different levels of thermal shock suffered by the beans in the roaster (Farah, 2019). As regards the comparison between the

two species Arabica and Robusta, the trend previously established in the industrially roasted coffees is confirmed here.
A EURL-SRM (EU reference Laboratories for Residues of Pesticides) report assessed that pesticides bearing reactive chemical groups, such as carboxy-, amino- and phenol groups, tend to form covalent bonds with primary or secondary plant metabolites, generating compounds commonly defined as “conjugates” or “conjugate residues”. The plant’s amino acids, sugars, alcohols and fatty acids may be involved in this kind of conjugation and the original biocide may be released when hydrolysis processes occur. It follows that the presence of conjugates such as ester compounds must be considered when setting pesticide residue definitions, and that any analysis procedure must provide the breakup of conjugates. An alkaline hydrolysis step before the QuEChERS-based method has also been suggested for this purpose in the case of OPP. These considerations may provide a rationale for the non-detection of OPP in green coffee, despite this matrix not being specifically reported in the aforementioned EURL-SRM analytical observation. We then carried out a test by subjecting the non-roasted samples (both whole and ground beans) to basic hydrolysis under mild conditions; the addition of the 5 N NaOH solution (to reach a pH of 12/13) to the QuEChERS protocol, which involves the use of acetonitrile as extracting solvent. The hydrolysis, carried out at room temperature and at 70°C for 1 h, followed by base neutralization via the addition of the sulfuric acid 5 N solution, did not provide the desired results as the subsequent analyses were negative for the presence of OPP.

4.2.3. OPP-conjugates and their influence on OPP quantification

The coffee bean surface is covered by a thin layer made of wax that makes up 0.2-0.3 % of its total weight (Folstar, 1985; Speer and Kölling-Speer, 2006). Assuming that OPP interacts with the components of this outermost layer, we performed an extraction of green coffee beans without grinding, at room temperature for 30 min with hexane and, for the sake of comparison, with ethyl acetate, acetonitrile, methanol and water in order to obtain possible “OPP-conjugates”. To evaluate the sensitivity and efficiency of the method, tests were carried out on Arabica coffee as it showed lower OPP content in its roasted samples. All of the dry extracts were found to be negative for OPP when subjected to the QuEChERS method, both directly and after heating for one hour at 180°C, i.e., the temperature that led to the maximum detection of the fungicide under our heating conditions (**Figure 2**). However, it was only possible to detect OPP in the less polar extracts when water was added before heat treatment. The water amount was 10 % w/w (named wet heat), which is related to the natural moisture content of green coffee beans. The heating and reconstruction of the humidity of green coffee is therefore a necessary condition for detecting OPP. Moreover, under the same conditions, higher concentrations of OPP were found in hexane (5.9 µg/kg), ethyl acetate (1.8 µg/kg), and acetonitrile (traces), whereas this compound was not detected in methanol and water. This evidence confirms the hypothesis that the fungicide conjugates are localized in the lipophilic layer of coffee.
The exhaustiveness of the extraction method described above was confirmed by an analysis with a negative OPP result, which was obtained by heating (1 h, 180°C) green coffee beans that had previously been treated with hexane.
Since the extracts in methanol are rich in phenolic compounds (Oliveira, Silva, Santos and Queiroz, 2019), it is interesting to note that the presence of OPP was not observed in these trials, thus leading to the conclusion that its origin may not be linked to its generation from precursors that are naturally present in green coffee beans.

The possibility of OPP contamination being limited to the beans outermost layer was further confirmed by the repetition of the extraction in hexane, and its "wet heat" treatment, on ground green coffee samples to allow exhaustive extraction to be performed. This trial provided analytical results comparable to those obtained with whole grains (6.3 ± 0.3 μg/kg). Based on the EURL-SRM report (EU reference Laboratories for Residues of Pesticides), the preliminary hydrolytic procedure that was performed at pH 12 in acetonitrile (70°C, 1 h) on the dry hexane extract was surely found to be negative for OPP because of its insolubility in this solvent. The procedure was therefore repeated in methanol, and the analysis of the neutralized sample treated using the QuEChERS-based method made it possible to detect OPP at a concentration of 34.8 μg/kg. This result confirms that OPP is present in green coffee as lipophilic conjugates that undergo partial degradation during roasting. As the OPP levels previously detected on the corresponding industrially roasted coffee samples (17.1 μg/kg for roasting degree 60, **Table 1**) can now be considered underestimated, the hexane extraction and subsequent hydrolysis in methanol were carried out on the same sample, giving a concentration of 32.2 μg/kg. This result may demonstrate that, for full OPP quantification, it is necessary to proceed with the breakup of its "conjugates". It is worth noting that, without detracting from the efficiency of the multi-residual analytical methods, in the case of a complex matrix such as coffee, preliminary sample treatment with hexane is a key step in extracting lipophilic OPP-conjugates. In fact, only the direct hydrolysis of roasted coffee in methanol provided OPP detection of 24.8 μg/kg. Finally, the same increase in OPP concentration (64.8 ± 0.4 μg/ kg vs 34.2 ± 0.6 μg/kg) was detected when the modified sample preparation procedure was applied, for the sake of comparison between the two species, on the Robusta sample with a roasting degree 60.

In **Figure 3**, it is possible to observe the influence of sample preparation on the detected OPP levels in both green and roasted samples, and that the optimized procedure (extraction with hexane, basic hydrolysis in methanol, neutralization, QuEChERS and UPLC-MS/MS) allows comparable results to be obtained for Arabica samples both before and after the roasting process. It is also evident that roasting only causes partial hydrolysis in the OPP-conjugates, with the quantity that is detected using the simple QuEChERS method only corresponding to ~50 % of the total amount.

The possibility that OPP-conjugates may be ester compounds formed from the fungicide and free fatty acids present in green coffee beans (Speer and Kölling-Speer, 2006) was investigated by synthesizing o-phenylphenyl stearate and adding this compound to a sample of Arabica ground green coffee, at a concentration of 124 μg/kg (i.e. 48 μg/kg of OPP), before subjecting the sample to the above-described procedure. The UPLC-MS/MS analysis of the obtained sample revealed an increase in OPP amount, at 41.5 μg/kg (86% of o-phenylphenyl stearate conversion), thus confirming that the conditions for the extraction in hexane and the basic hydrolysis are sufficient to achieve a reasonable release of the fungicide from this kind of conjugate.

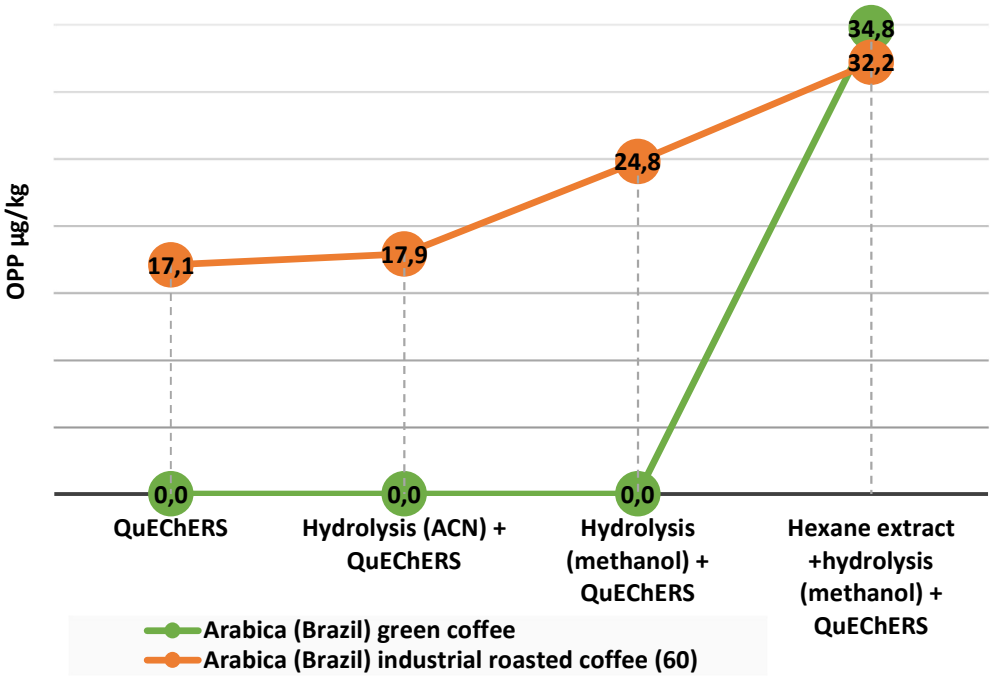

Figure 3. OPP amount (µg/kg) in green vs roasted Arabica coffee according to sample preparation method and UPLC-MS/MS analysis.

4.2.4. OPP transfer into coffee brew

In order to estimate the possible transfer of OPP into the coffee brew, the beverage was prepared by percolation using the same sample of Arabica roasted coffee (roasting degree 60) and was subjected to freeze- drying. The residue was then solubilized in methanol and hydrolyzed at pH 12 before the QuEChERS procedure, and the analysis observed no OPP, thus demonstrating that there is no risk of OPP exposure for coffee beverage consumers.
For comparison, a commercial sample of soluble coffee was also tested with the direct hydrolysis procedure, and the previous observation of an absence of OPP was therefore corroborated.

4.3. Conclusions

The low MRLs of biocides required by authority regulations have prompted the development of more reliable and sensitive analytical methods for monitoring residues in food and beverages. Coffee is one of the most widely consumed commodities in the world, and the complexity of this matrix requires specific sample enrichment methods, such as QuEChERS, for both green and roasted samples. OPP is a post-harvest fungicide that is not specifically indicated for coffee bean preservation. To the best of our knowledge, traces of OPP have been found in roasted coffee, but never in a green sample, although it is highly unlikely that this fungicide forms during roasting from precursors that are naturally present in coffee. Like other pesticides with reactive functional groups, OPP can potentially form lipophilic conjugates that can mask its presence. It follows that multiresidue analytical methods may lead to an underestimation of OPP amount if they are inefficient with respect to the conjugates. The importance of extraction with a nonpolar solvent, such as hexane, before the hydrolytic step at pH 12 and the QuEChERS method in quantitatively recovering these adducts from coffee samples has been demonstrated. Moreover, the appropriate choice of methanol for the

hydrolysis step was crucial in obtaining the correct quantification of the pesticide by UPLC-MS/MS, not only for green coffee but also for roasted samples. Indeed, the effect of the roasting process on the OPP- conjugates is not sufficient to release OPP completely, meaning that that the pesticide is underestimated by about 50 % in roasted samples. When using the optimized method on the same samples of green and industrially roasted Arabica, comparable levels of OPP were determined in both matrices, 34.8 µg/kg and 32.2 µg/kg, respectively.

In order to verify possible OPP transfer to the coffee brew, the beverage obtained by percolation and commercial soluble coffee were tested, giving negative results for OPP. The data reported in this chapter suggest that there is no significant safety risk to consumers of coffee/ coffee brew, as the detected OPP residue levels contribute minimally to the ADI of 0.4 mg/kg body weight. Furthermore, since there is no specific evidence for the use of this biocide in coffee, its detection may be due to accidental or technically unavoidable contamination during green coffee production.

Further studies on the correlation between OPP content, roasting degree and different varieties are being conducted.

4.4. Experimental section

4.4.1. Chemicals and coffee samples

o-phenylphenol (OPP, Pestanal grade) for standard solutions, formic acid (for LC-MS LiChropurTM), sulfuric acid 5 N (Titripur®), sodium hydroxide 5 N solution, hydrochloric acid 3 N solution, sodium bicarbonate (ACS reagent), anhydrous sodium sulfate (ACS reagent), 4-(dimethylamino)pyridine (DMAP, reagent plus®), N,N'-dicyclohexylcarbodiimide (DCC, ACS reagent), diethyl ether (suitable for HPLC ≥ 99.9 %), methanol (suitable for HPLC ≥ 99.9 %), hexane (puriss. p.a. ≥ 99 %), ethyl acetate (HPLC Plus ≥ 99.9 %), petroleum ether (ACS reagent), dichloromethane (ACS reagent) and acetonitrile (suitable for HPLC ≥ 99.9 %) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy).
Commercially available samples of green and roasted coffee beans belonging to the two species Arabica and Robusta with different geographical origin, named Arabica (Brazil) and Robusta (Vietnam), were investigated.
DisQuE QuEChERS cartridges (150 mg of MgSO4, 25 mg of PSA, 25 mg of C18 and 7 mg of GCB, 15 mL dispersive solid phase extraction (d- SPE) tube, 50/pk) used for coffee sample preparation were purchased from Waters SpA (Sesto San Giovanni, Milan, Italy).

4.4.2. Green and roasted-coffee sample preparation for OPP analysis

Tests on both green and roasted coffee beans of the Arabica (Brazil) and Robusta (Vietnam) species were carried out on 200 g of ground matrix. Different roasting degrees were investigated for both species. Specifically, the sample colouring grade was defined as follows: 45 (darker), 50, 55 and 60 (lighter). Each sample was transferred into a 1 L flask and 400 mL of acetonitrile was added. The mixture was submitted to mechanical stirring at room temperature for 30 min and then filtered under vacuum. The filtrate volume was reduced to 8 mL under vacuum, transferred to a d-SPE tube for clean-up and the sample was then shaken using a Vortex for 1 min. The d-SPE tube was then centrifuged for 10 min at 5000 rpm (Hettich ROTOFIX 32 centrifuge), and the supernatant was evaporated under vacuum. The residue was solubilized in 0.5 mL of eluent (acetonitrile with 0.1 % v/v formic acid), filtered through a 0.45 µm filter and transferred into a vial for UPLC-MS/MS analysis.
An OPP recovery of about 99.9 % was achieved for both the green and roasted ground coffee samples that were spiked with the OPP standard to final concentrations of 0.5 mg/kg and 0.25 mg/kg, respectively, before the extraction and clean up procedures.

4.4.3. Thermal treatment of green coffee beans

200 g of Arabica (Brazil) and Robusta (Vietnam) green coffee beans were heated in a laboratory oven (G-Therm AG-System Daily, F.lli Galli, Fizzonasco di Pieve Emanuele, Milan, Italy) for 1 h at different temperatures. Tests were performed at: 120, 150, 180 and 220 °C. Samples were then analysed according to the procedure reported in paragraph 4.4.2. The procedure was performed in triplicate for each temperature, and data are reported as mean value ± standard deviation (SD).

4.4.4. Solvent extraction of green coffee beans and thermal treatment of obtained extracts

Extractions were performed on Arabica (Brazil) green beans with solvents of different polarities (hexane, ethyl acetate, acetonitrile, methanol, water). For each test, 200 g of matrix was transferred into a 1 L flask, 400 mL of solvent was added, the solution was submitted to mechanical stirring at room temperature for 30 min and then filtered on filter paper. The filtrates were evaporated under vacuum and water extracts were lyophilized. Extraction yields: 18.92 ± 2 g/kg for hexane; 15.36 ± 2 g/kg for ethyl acetate; 12.83 ± 1 g/kg for acetonitrile; 13.61 ± 1 g/kg for methanol; 13.61 ± 2 g/kg for water.
Each obtained residue was suspended in 8 mL of acetonitrile, and the procedure described in paragraph 4.4.2. was followed before UPLC-MS/ MS analysis.
The same protocol was used to obtain extracts that were subsequently heated for 1 h in the laboratory oven prior to the sample preparation procedure (see paragraph 4.4.2.) and UPLC-MS/MS analysis. In accordance with previous trials (see paragraph 4.4.3.), thermal treatment was performed at 180°C, both with and without the addition of water (10 %w/w).
The extraction procedure with hexane, followed by extract heating at 180°C for 1 h with the 10 % w/w addition of water, was also carried out on 200 g of ground and roasted Arabica (roasting degree 60). Extraction yields: 12.87 ± 2 g/kg.
Each test was performed in triplicate and data are reported as mean value ± standard deviation (SD).

4.4.5. OPP analysis on coffee brew

Coffee brewing was achieved using the percolation method on 200 g of Arabica ground and roasted coffee (roasting degree 60) with 400 mL of boiling water, for subsequent lyophilization. The obtained residue was then submitted to sample preparation according to the procedure described in paragraph 4.4.2. and then analysed via UPLC-MS/MS. The same coffee brew preparation was carried out in order to submit the lyophilized sample to hydrolysis as described in paragraph 4.4.6.
The procedure was performed in triplicate and data are reported as mean value ± standard deviation (SD).

4.4.6. Hydrolysis of "OPP-conjugates"

- **Arabica green coffee hydrolysis**

200 g of both ground and whole Arabica green coffee beans were transferred into a 1 L flask, and 400 mL of solvent (acetonitrile or methanol) and the NaOH 5 M solution were added until pH=12 was reached. Each mixture was stirred for 1 h either at room temperature or 70 ◦C, and then the sulfuric acid 5 N solution was added to reach pH = 5 and the solution was filtered on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analysed according to procedure 4.4.2.
The samples were analysed in triplicate and data are reported as mean value ± standard deviation (SD).

- **Hydrolysis of hexane extracts**

The gummy residue obtained from Arabica green coffee extraction with hexane (as reported in paragraph 4.4.4.) was transferred into a 500 mL flask with the minimal technical addition of diethyl ether, and 200 mL of methanol and a NaOH 5 M solution were then added until pH=12 was reached. The mixture was stirred for 1 h at 70 ◦C, and then the sulfuric acid 5 N solution was added to reach pH = 5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analysed according to procedure 4.4.2.

The same treatment was used for the roasted coffee hexane extracts obtained from Arabica samples (roasting degree 60).
The samples were analysed in triplicate and data are reported as mean value ± standard deviation (SD).

- **Hydrolysis of roasted ground coffee**

200 g of Arabica ground and roasted coffee (roasting degree 60) was transferred into a 1 L flask and 400 mL of solvent (acetonitrile or methanol) and the NaOH 5 M solution were added until pH=12 was reached. The mixture was stirred for 1 h at 70°C, and a sulfuric acid 5 N solution was then added to reach pH=5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analysed according to procedure 4.4.2.
The sample was analysed in triplicate and data are reported as mean value ± standard deviation (SD).

- **Hydrolysis of coffee brew**

The coffee brew obtained by following procedure 4.4.5 was freeze-dried and the residue was transferred into a 500 mL flask, and 200 mL of methanol and the NaOH 5 M solution were added until pH=12 was reached. The mixture was stirred for 1 h at 70°C, and then a sulfuric acid 5 N solution was added to reach pH=5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analysed according to procedure 4.4.2.
The sample was analysed in triplicate and data are reported as mean value ± standard deviation (SD).

- **Hydrolysis of instant coffee**

200 g of instant coffee were transferred into a 1 L flask and 400 mL of methanol and a NaOH 5 M solution were added until pH = 12 was reached. The mixture was stirred for 1 h at 70°C, and then a sulfuric acid 5 N solution was added to reach pH=5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analysed according to procedure 4.4.2.
The sample was analysed in triplicate and data are reported as mean value ± standard deviation (SD).

4.4.7. *o*-Phenylphenyl stearate synthesis

In accordance with the procedure reported by Neises and Steglich (1978), 10 mg of DMAP (4-(dimethylamino)pyridine) and 119 mg (0.7 mmol) of *o*-phenylphenol were added to a stirred solution of stearic acid (200 mg, 0.7 mmol) in 10 mL of CH_2Cl_2. 174 mg (0.8 mmol) of DCC (N,N'-dicyclohexylcarbodiimide) were added at 0°C and the mixture was stirred for 3 hours at room temperature. The reaction was filtered and washed twice with 0.5 M HCl and saturated NaHCO3 solution, and then dried over anhydrous $NaSO_4$ and filtered. The solvent was removed by evaporation, the product was purified on a silica column (petroleum ether/ethyl acetate 9:1) and the product was characterized by 1H-NMR (Jeol ECZ-R Spectometer, 600 MHz) and FT-IR (PerkinElmer, Spectrum Two UATR). Yield: 68%.^{1}H-NMR (CDCl3): δ 7.49-7.44 (m, 4H), 7.40-7.36 (m, 1H), 7.25-7.22 (m, 2H), 6.98 (d, 2H), 2.38 (t, 1H), 2.15 (s, 8H), 1,95-1,57 (m, 6H), 1,23 (s, 18H) 0,86 (t, 2H).
FT-IR cm^{-1}: 2964 (s), 2917, 1760 (s), 1590, 1314, 1294.

4.4.8. Analysis of Green Arabica (Brazil) spiked with o-phenylphenyl stearate

200 g of Arabica ground green coffee beans was transferred into a 1 L flask and suspended in 200 mL of diethyl ether under stirring. Then, 496 µl of the 5 mg/100 mL *o*-phenylphenyl

stearate diethyl ether solution was added, corresponding to a final ester concentration of 124 µg/kg (i.e., 48 µg/kg of OPP). After solvent evaporation, the green coffee sample was submitted to hexane extraction (procedure 4.4.4.) and the residue was hydrolysed according to the procedure reported in paragraph 4.4.6. (Hydrolysis of hexane extracts).
The obtained sample was then analysed via UPLC-MS/MS (paragraph 4.4.2.).
The procedure was performed in triplicate and data is reported as mean value ± standard deviation (SD).

4.4.9. Instrumental analysis

Analyses were performed using a UPLC-MS/MS system (Acquity TQD LC-MS/MS System, Waters Corporation, Milford, MA, USA), equipped with a C8 column (BEH C8, 2.1 × 50 mm, 1.7 µm). A linear gradient elution with water (solvent A) and acetonitrile (solvent B), both acidified with 0.1 % formic acid, was carried out at 40°C.
The gradient was from70% solvent A to 50% over 1.3min, then to 100% solvent B in 0.43 min, and this was held for 2 min at a flow rate of 0.4 mL min^{-1}.
The positive electrospray ionization mode (ES+) was employed for determination, following the transitions m/z = 171 -> 152 (quantification) and m/z = 171 -> 69 (qualitative confirmation of the peak), using 23 eV as the collision energy and 30 V as the cone voltage for both ions. The two transitions were selected after a screening of all daughters detectable under different collision conditions, and the transition 171 -> 152 was the most intense signal observed. The calibration curve of the UPLC-MS/MS method was determined using OPP standard solutions in acetonitrile (from 2 to 10 µg/mL); a linear regression with R^2=0.9996 was obtained using Waters QuanLynx software (LOD 2 µg/mL, LOQ 5 µg/mL).

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The challenge of *o*-phenylphenol detection in coffee: How "OPP-conjugates" hide their presence in green and roasted samples

Janet Menzio [a], Silvia Tagliapietra [a], Alessandro Barge [a], Bianca Serito [b], Elena Calegari [b], Arianna Binello [a,*], Giancarlo Cravotto [a,*]

[a] Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, Via P. Giuria 9, 10235 Turin, Italy
[b] Lavazza SpA, Settimo Torinese, Turin, Italy

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ABSTRACT

o-Phenylphenol (OPP) is not a commonly used pesticide in the coffee production chain. Although it has only been detected in roasted coffee, it is unlikely that OPP can be formed during roasting. Its acidic nature may lead to the formation of conjugates with natural matrix components. The objective of this study is to optimize an analytical method to discover how these conjugates may mask the presence of OPP in coffee. Sample extraction with hexane followed by basic hydrolysis and then a QuEChERS method allows the presence of OPP to be quantitatively detected via UPLC-MS/MS. The optimized method was applied to the same Arabica coffee (Brazil), and the quantification of comparable amounts of OPP was observed in both green and roasted samples (34.8 *vs* 32.2 μg/kg). The optimized procedure detected twice the amount of OPP in roasted samples, compared to the QuEChERS method, suggesting that roasting causes the partial hydrolysis of OPP conjugates.

1. Introduction

There is currently a steady increase in concern about food and pollution. Coffee, as other food and beverages of vegetable origin, can suffer environmental and processing contamination affecting its quality and/or safety; it follows that control agencies, such as the European Food Safety Authority (EFSA), recommend strengthening investigations covered by specific legislation (EFSA Topics).

Biphenyl-2-ol or *o*-phenylphenol (2-phenylphenol, OPP) and its water-soluble salts (i.e., sodium *o*-phenylphenate, SOPP) are widely employed in the post-harvest treatment of diseases in fresh fruits and vegetables thanks to their antimicrobial activity. Initially associated with the fungistatic biphenyl used in *Citrus* fruits to prevent damage during storage and shipment (Gutter and Lattar, 1967), OPP acts against mold and rot as a residual contact substance that disrupts cell walls and cell membranes (EFSA Reasoned opinion, 2017). Its lipophilic derivatives (i.e., esters and amine complexes) can be incorporated into wax emulsions to reduce OPP phytotoxicity, which precludes some applications, and to provide more selective antimicrobial activity (Eckert, Esuruoso, and Carey, 1979; Gutter and Lattar, 1967). OPP and SOPP have not only been used in agriculture to control fungal and bacterial growth on stored crops, but are still widely used as general surface disinfectants and in packaging materials (CARB, 1997; Patent WO 2021/064075A1). In general, improper use of biocides can affect human health through the skin, inhalation, and oral intake. Information on the toxicity of OPP and SOPP is limited. The European Food Safety Authority EFSA (EFSA Reasoned Opinion 2017) and the World Health Organization (WHO) (World Health Organization, 2000) have reported a consumer risk assessment, based on existing CXLs (codex maximum residue limit), with an ADI (acceptable daily intake) of 0.4 mg/kg of body weight (EFSA Scientific Report, 2008).

Its incredibly widespread consumption makes coffee one of the world's most important agricultural commodities, and pre- and post-harvest management activities are closely related to quality and safety. Following harvesting, coffee cherries go through a complex series of processing steps involving de-pulping, drying, hulling, cleaning, sorting, storage, roasting, grinding and cupping, among others. In order to avoid undesired fermentation, dried green coffee beans must have a residual moisture content of 8–12 % (Haile and Kang, 2019; https://www.ico.org/projects/good-hygiene-practices/cnt/cnt_sp/sec_2/docs_2.1/ICO.pdf). The lipidic profile is a key element in coffee authentication, with nine free fatty acids, evenly distributed between Robusta and Arabica species, being identified. In particular, a comparison of the contents of stearic and oleic acid allows the two species to be identified; stearic acid content is much lower than oleic in Robusta, while the percentages of these two acids in Arabica are almost equal

* Corresponding authors.

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(Farah, 2019).

Coffee is considered to be a rich source of phenolic compounds whose profile is strongly affected by roasting processes (Ky et al., 2001) which can cause substantial losses (up to 95 % of chlorogenic acids) (Farah, 2019). Despite this, OPP cannot be considered a natural phenol because it is not a component of any food.

Although the control of pests and diseases in coffee crops requires the well-regulated use of selective insecticides, fungicides and herbicides, OPP and SOPP are not listed among the pesticides for use on coffee beans, as regulated by the UE (European Commission MRLs Coffee beans,https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/products/?event=details&p=244; Farah, 2019). In particular, EFSA has published a reasoned opinion on the review of the existing MRLs, in accordance with Article 12 of Regulation (EC) No. 396/2005 (EFSA Reasoned Opinion 2017), recommending that the residue definition for plant products be modified and that the MRL for OPP should be 0.05 mg/kg for coffee beans.

As far as we know, OPP is detected by standard pesticide residue analysis methods only after coffee roasting and never in green beans. Theurillat *et al.,* (Theurillat, Laborie and Schenk, 2022) have recently ruled out the possibility that the traces of OPP found in roasted coffee are caused by the possible use of the fungicide on green coffee and packaging contamination. The authors assigned the presence of OPP in coffee to its generation during roasting, with content increasing with roasting degree. This conclusion has been supported by that facts that no OPP was detected in green coffee beans and by the differing amounts of the pesticide found in Arabica (slightly higher) and Robusta samples, indicating that production is species dependent. Nevertheless, no possible chemical pathway for OPP generation during roasting has been reported, and it is assessed that the transfer rate of the fungicide into the coffee brew is low (16 %).

The occurrence of OPP residues in green and roasted coffee beans has been investigated in this work due to its use as an antimicrobial agent in a variety of applications and its potential as a foodstuff contaminant (Coelhan, Bromig, Glas, and Roberts, 2006). Specifically, the aim was to demonstrate that the OPP found in roasted coffee beans can only derive from the accidental or technically unavoidable contamination of green coffee during its processing chain, and that this contamination cannot be detected in green beans using the common analytical methods for pesticide residues due to the generation of OPP conjugates. In addition to green and industrial roasted coffee samples, this study also considers green coffee beans that were heated in a laboratory oven in order to monitor the development of OPP in relation to temperature. An analytical procedure that is based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) for pesticide multiresidue determination has been optimized and OPP has been quantified by UPLC-MS/MS.

2. Materials and methods

2.1. Chemicals and coffee samples

o-phenylphenol (OPP, Pestanal grade) for standard solutions, formic acid (for LC-MS LiChropur^{TM}), sulfuric acid 5 N (Titripur®), sodium hydroxide 5 N solution, hydrochloric acid 3 N solution, sodium bicarbonate (ACS reagent), anhydrous sodium sulfate (ACS reagent), 4-(dimethylamino)pyridine (DMAP, reagent plus®), *N,N′*-dicyclohexylcarbodiimide (DCC, ACS reagent), diethyl ether (suitable for HPLC ≥ 99.9 %), methanol (suitable for HPLC ≥ 99.9 %), hexane (puriss. p.a. ≥ 99 %), ethyl acetate (HPLC Plus ≥ 99.9 %), petroleum ether (ACS reagent), dichloromethane (ACS reagent) and acetonitrile (suitable for HPLC ≥ 99.9 %) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy).

Commercial available samples of green and roasted coffee beans belonging to the two species Arabica and Robusta with different geographical origin, named Arabica (Brazil) and Robusta (Vietnam), were investigated.

DisQuE QuEChERS cartridges (150 mg of MgSO4, 25 mg of PSA, 25 mg of C18 and 7 mg of GCB, 15 mL dispersive solid phase extraction (d-SPE) tube, 50/pk) used for coffee sample preparation were purchased from Waters SpA (Sesto San Giovanni, Milan, Italy).

2.2. Green and roasted-coffee sample preparation for OPP analysis

Tests on both green and roasted coffee beans of the Arabica (Brazil) and Robusta (Vietnam) species were carried out on 200 g of ground matrix. Different roasting degrees were investigated for both species. Specifically, the sample coloring grade was defined as follows: 45 (darker), 50, 55 and 60 (lighter). Each sample was transferred into a 1 L flask and 400 mL of acetonitrile was added. The mixture was submitted to mechanical stirring at room temperature for 30 min and then filtered under vacuum. The filtrate volume was reduced to 8 mL under vacuum, transferred to a d-SPE tube for clean-up and the sample was then shaken using a Vortex for 1 min. The d-SPE tube was then centrifuged for 10 min at 5000 rpm (Hettich ROTOFIX 32 centrifuge), and the supernatant was evaporated under vacuum. The residue was solubilized in 0.5 mL of eluent (acetonitrile with 0.1 % v/v formic acid), filtered through a 0.45 μm filter and transferred into a vial for UPLC-MS/MS analysis.

An OPP recovery of about 99.9 % was achieved for both the green and roasted ground coffee samples that were spiked with the OPP standard to final concentrations of 0.5 mg/kg and 0.25 mg/kg, respectively, before the extraction and clean up procedures.

2.3. Thermal treatment of green coffee beans

200 g of Arabica (Brazil) and Robusta (Vietnam) green coffee beans were heated in a laboratory oven (G-Therm AG-System Daily, F.lli Galli, Fizzonasco di Pieve Emanuele, Milan, Italy) for 1 h at different temperatures. Tests were performed at: 120, 150, 180 and 220 °C. Samples were then analyzed according to the procedure reported in paragraph 2.2.

The procedure was performed in triplicate for each temperature, and data are reported as mean value ± standard deviation (SD).

2.4. Solvent extraction of green coffee beans and thermal treatment of obtained extracts

Extractions were performed on Arabica (Brazil) green beans with solvents of different polarities (hexane, ethyl acetate, acetonitrile, methanol, water). For each test, 200 g of matrix was transferred into a 1 L flask, 400 mL of solvent was added, the solution was submitted to mechanical stirring at room temperature for 30 min and then filtered on filter paper. The filtrates were evaporated under vacuum and water extracts were lyophilized. Extraction yields: 18.92 ± 1.83 g/kg for hexane; 15.36 ± 1.5 g/kg for ethyl acetate; 12.83 ± 1.3 g/kg for acetonitrile; 13.61 ± 0.94 g/kg for methanol; 13.61 ± 1.5 g/kg for water.

Each obtained residue was suspended in 8 mL of acetonitrile, and the procedure described in paragraph 2.2. was followed before UPLC-MS/MS analysis.

The same protocol was used to obtain extracts that were subsequently heated for 1 h in the laboratory oven prior to the sample preparation procedure (see paragraph 2.2.) and UPLC-MS/MS analysis. In accordance with previous trials (see paragraph 2.3.), thermal treatment was performed at 180 °C, both with and without the addition of water (10 %w/w).

The extraction procedure with hexane, followed by extract heating at 180 °C for 1 h with the 10 % w/w addition of water, was also carried out on 200 g of ground and roasted Arabica (roasting degree 60). Extraction yields: 12.87 ± 1.94 g/kg.

Each test was performed in triplicate and data are reported as mean value ± standard deviation (SD).

2.5. OPP analysis on coffee brew

Coffee brewing was achieved using the percolation method on 200 g of Arabica ground and roasted coffee (roasting degree 60) with 400 mL of boiling water, for subsequent lyophilization. The obtained residue was then submitted to sample preparation according to the procedure described in paragraph 2.2. and then analyzed via UPLC-MS/MS. The same coffee brew preparation was carried out in order to submit the lyophilized sample to hydrolysis as described in paragraph 2.6.4.

The procedure was performed in triplicate and data are reported as mean value ± standard deviation (SD).

2.6. Hydrolysis of "OPP-conjugates"

2.6.1. Arabica green coffee hydrolysis

200 g of both ground and whole Arabica green coffee beans were transferred into a 1 L flask, and 400 mL of solvent (acetonitrile or methanol) and the NaOH 5 M solution were added until pH = 12 was reached. Each mixture was stirred for 1 h either at room temperature or 70 °C, and then the sulfuric acid 5 N solution was added to reach pH = 5 and the solution was filtered on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analyzed according to procedure 2.2.

The samples were analyzed in triplicate and data are reported as mean value ± standard deviation (SD).

2.6.2. Hydrolysis of hexane extracts

The gummy residue obtained from Arabica green coffee extraction with hexane (as reported in paragraph 2.4.) was transferred into a 500 mL flask with the minimal technical addition of diethyl ether, and 200 mL of methanol and a NaOH 5 M solution were then added until pH = 12 was reached. The mixture was stirred for 1 h at 70 °C, and then the sulfuric acid 5 N solution was added to reach pH = 5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analyzed according to procedure 2.2.

The same treatment was used for the roasted coffee hexane extracts obtained from Arabica samples (roasting degree 60).

The samples were analyzed in triplicate and data are reported as mean value ± standard deviation (SD).

2.6.3. Hydrolysis of roasted ground coffee

200 g of Arabica ground and roasted coffee (roasting degree 60) was transferred into a 1 L flask and 400 mL of solvent (acetonitrile or methanol) and the NaOH 5 M solution were added until pH = 12 was reached. The mixture was stirred for 1 h at 70 °C, and a sulfuric acid 5 N solution was then added to reach pH = 5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analyzed according to procedure 2.2.

The sample was analyzed in triplicate and data are reported as mean value ± standard deviation (SD).

2.6.4. Hydrolysis of coffee brew

The coffee brew obtained by following procedure 2.5 was freeze-dried and the residue was transferred into a 500 mL flask, and 200 mL of methanol and the NaOH 5 M solution were added until pH = 12 was reached. The mixture was stirred for 1 h at 70 °C, and then a sulfuric acid 5 N solution was added to reach pH = 5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analyzed according to procedure 2.2.

The sample was analyzed in triplicate and data are reported as mean value ± standard deviation (SD).

2.6.5. Hydrolysis of instant coffee

200 g of instant coffee were transferred into a 1 L flask and 400 mL of methanol and a NaOH 5 M solution were added until pH = 12 was reached. The mixture was stirred for 1 h at 70 °C, and then a sulfuric acid 5 N solution was added to reach pH = 5 before filtration on filter paper. The obtained filtrate was evaporated under vacuum and the residue was analyzed according to procedure 2.2.

The sample was analyzed in triplicate and data are reported as mean value ± standard deviation (SD).

2.7. *o*-Phenylphenyl stearate synthesis

In accordance with the procedure reported by Neises and Steglich (1978), 10 mg of DMAP (4-(dimethylamino)pyridine) and 119 mg (0.7 mmol) of *o*-phenylphenol were added to a stirred solution of stearic acid (200 mg, 0.7 mmol) in 10 mL of CH_2Cl_2. 174 mg (0.8 mmol) of DCC (*N, N′*-dicyclohexylcarbodiimide) were added at 0 °C and the mixture was stirred for 3 h at room temperature. The reaction was filtered and washed twice with 0.5 M HCl and saturated $NaHCO_3$ solution, and then dried over anhydrous $NaSO_4$ and filtered. The solvent was removed by evaporation, the product was purified on a silica column (petroleum ether/ethyl acetate 9:1) and the product was characterized by ^{1}H NMR (Jeol ECZ-R Spectometer, 600 MHz) and FT-IR (PerkinElmer, Spectrum Two UATR). Yield: 68 %.^{1}H NMR ($CDCl_3$): δ 7.49–7.44 (m, 4H), 7.40–7.36 (m, 1H), 7.25–7.22 (m, 2H), 6.98 (d, 2H), 2.38 (t, 1H), 2.15 (s, 8H), 1,95–1,57 (m, 6H), 1,23 (s, 18H) 0,86 (t, 2H).

FT-IR cm^{-1}: 2964 (s), 2917, 1760 (s), 1590, 1314, 1294.

2.7.1. Analysis of green Arabica (Brazil) spiked with *o*-phenylphenyl stearate

200 g of Arabica ground green coffee beans was transferred into a 1 L flask and suspended in 200 mL of diethyl ether under stirring. Then, 496 µl of the 5 mg/100 mL *o*-phenylphenyl stearate diethyl ether solution was added, corresponding to a final ester concentration of 124 µg/kg (i.e., 48 µg/kg of OPP). After solvent evaporation, the green coffee sample was submitted to hexane extraction (procedure 2.4.) and the residue was hydrolyzed according to the procedure reported in paragraph 2.6.2.

The obtained sample was then analyzed via UPLC-MS/MS (paragraph 2.2.).

The procedure was performed in triplicate and data is reported as mean value ± standard deviation (SD).

2.8. Instrumental analysis

Analyses were performed using a UPLC-MS/MS system (Acquity TQD LC-MS/MS System, Waters Corporation, Milford, MA, USA), equipped with a C8 column (BEH C8, 2.1 × 50 mm, 1.7 µm). A linear gradient elution with water (solvent A) and acetonitrile (solvent B), both acidified with 0.1 % formic acid, was carried out at 40 °C. The gradient was from 70 % solvent A to 50 % over 1.3 min, then to 100 % solvent B in 0.43 min, and this was held for 2 min at a flow rate of 0.4 mL min^{-1}.

The positive electrospray ionization mode (ES+) was employed for determination, following the transitions m/z = 171 -> 152 (quantification) and m/z = 171 -> 69 (qualitative confirmation of the peak), using 23 eV as the collision energy and 30 V as the cone voltage for both ions. The two transitions were selected after a screening of all daughters detectable under different collision conditions, and the transition 171 -> 152 was the most intense signal observed. The calibration curve of the UPLC-MS/MS method was determined using OPP standard solutions in acetonitrile (from 2 to 10 µg/mL); a linear regression with R^2 = 0.9996 was obtained using Waters QuanLynx software (LOD 2 µg/mL, LOQ 5 µg/mL).

3. Results and discussion

3.1. OPP analysis via a QuEChERS-based method in green and industrially roasted coffee

Despite the presence of phenolic compounds in green coffee beans, the possible chemical pathway for the formation of OPP from precursors

naturally present during roasting is difficult to define. This work therefore attempts to explain the presence of OPP in coffee by considering the possibility of accidental or technically unavoidable contamination during green coffee processing.

In the case of lipophilic pesticides, such as OPP, sample preparation is essential in their tendency to concentrate in the fatty fraction of the vegetal matrix, and this may have an effect on acceptable recoveries because of co-extract coffee components and adducts between the pesticide and the fatty fraction.

Sample preparation for OPP determination in both green and roasted coffee was performed using a QuEChERS-based method. This is a simple procedure for the efficient detection of multiple pesticide residues in food (Anastassiades, Lehotay, Stajnbaher and Schenck, 2003) that provides cleaner, enriched samples, which is of primary importance in complex matrixes, such as coffee, and because of the low residue levels of biocides required by authority regulations (Huertas-Perez et al., 2019). Firstly, this procedure involves matrix extraction with acetonitrile and then a clean-up step via dispersive solid-phase extraction (d-SPE) to remove interfering components. Primary secondary amine (PSA), which has been reported as an effective sorbent for the removal of polar organic acids, fatty acids, polar pigments and some sugars, and the C18 sorbent, employed for non-polar interfering substances as lipids, were used for sample purification (Harmoko, Kartasasmita and Tresnawati, 2015). In recent years, QuEChERS has been evaluated on a large number of commodity/pesticide combinations, using GC, GC–MS/MS and/or LC-MS/MS analyses (European Standard Method EN 15662:2018; Theurillat, Dubois and Huertas-Pérez, 2021). Because of the very low OPP concentrations, samples were prepared from 200 g of the both green and roasted coffees. A Triple Quadrupole System equipped with a collision cell integrated into an UPLC-ESI-MS/MS instrument was employed for the analysis thanks to its high sensitivity and selectivity. An example of the chromatogram obtained by UPLC-MS/MS analysis of a roasted coffee sample is reported in Fig. 1.

Arabica (Brazil) and Robusta (Vietnam) green coffee beans and their related roasted samples, with different roasting degrees, were analyzed; the roasting degrees are defined by ground sample color and identified with a number on a scale between 45 (darker) to 60 (lighter). As reported in Table 1, the data show higher biocide content in samples subjected to lighter roasting, while no OPP was detected in the green samples. Moreover, a comparison of the two species showed that higher levels are found in Robusta samples than in Arabica, especially at the degrees of roasting identified, 55 and 60 (32.1 μg/kg *vs* 16.4 μg/kg and 34.2 μg/kg *vs* 17.1 μg/kg, respectively).

The results were in complete agreement with those of Theurillat et al. (2022), who found OPP concentrations ranging from < 10 μg/kg to 46 μg/kg in commercial roasted coffee. However, some differences were noted with the data shown here, as they reported an OPP increase with roasting degree and higher values for the Arabica species.

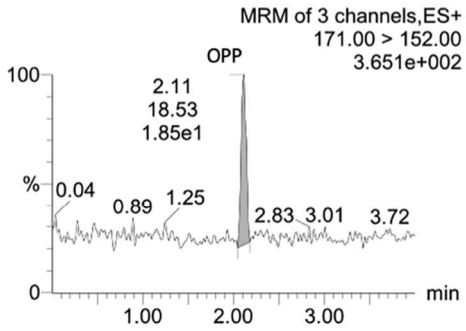

Fig. 1. Example of a chromatogram obtained in the UPLC-MS/MS detection of OPP in roasted coffee samples (Arabica, Brazil).

Table 1
OPP concentration in ground green and industrially roasted coffee analyzed via the QuEChERS-method and UPLC-MS/MS.

Roasting degree	Arabica μg/kg (±SD)	Robusta μg/kg (±SD)
Dark (45)	11.3 (±0.3)	traces
Medium dark (50)	11.8 (±0.2)	12.5 (±0.2)
Medium light (55)	16.4 (±0.5)	32.1 (±0.6)
Light (60)	17.1 (±0.1)	34.2 (±0.8)
Green coffee	–	–

3.2. Isothermal heating of Arabica and Robusta green coffee beans

To better understand the correlation between OPP detection and thermal treatment, Arabica and Robusta green coffees were subjected to isothermal heating in a laboratory oven. Using industrial roasting temperatures, tests were carried out for 1 h at: 120 °C, 150 °C, 180 °C and 220 °C. The residence time of the samples in the oven was opportunely selected, according to previous tests, to obtain a compromise between monitoring the OPP and not excessively prolonging the treatment. After heating, the beans were ground, in order to maximize the extractable surface, and were then analyzed according to the procedure described above. The graph reported in Fig. 2 shows the mean values of OPP amount found at every thermal condition, with each calculated over three repetitions of the treatment.

The obtained results show a significant increase in the OPP values in both species at 150 °C (Robusta 4.8 μg/kg, Arabica 3.8 μg/kg) and especially at 180 °C (Robusta 8.4 μg/kg, Arabica 7.2 μg/kg). Although the heating conditions were useful in making a correlation between OPP detection and temperature, the lower levels of fungicide found in these trials, compared to industrially roasted samples, can be attributed to the different levels of thermal shock suffered by the beans in the roaster (Farah, 2019). As regards the comparison between the two species Arabica and Robusta, the trend previously established in the industrially roasted coffees is confirmed here.

A EURL-SRM (EU reference Laboratories for Residues of Pesticides) report assessed that pesticides bearing reactive chemical groups, such as carboxy-, amino- and phenol groups, tend to form covalent bonds with primary or secondary plant metabolites, generating compounds commonly defined as "conjugates" or "conjugate residues". The plant's amino acids, sugars, alcohols and fatty acids may be involved in this kind of conjugation and the original biocide may be released when hydrolysis processes occur. It follows that the presence of conjugates such as ester compounds must to be considered when setting pesticide residue definitions, and that any analysis procedure must provide for the breakup of conjugates. An alkaline hydrolysis step before the QuEChERS-based method has also been suggested for this purpose in the

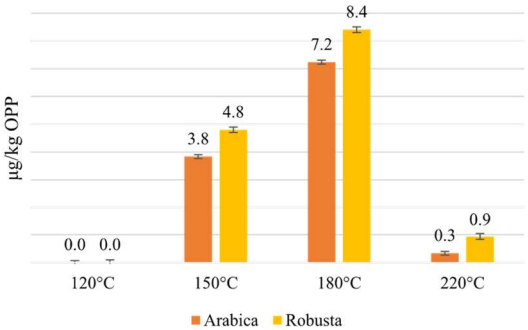

Fig. 2. OPP concentration detected via the QuEChERS-method and UPLC-MS/MS in thermally treated Arabica (Brazil) and Robusta (Vietnam) green coffee samples. The treatment was carried out in a laboratory oven for 1 h.

case of OPP. These considerations may provide a rationale for the non-detection of OPP in green coffee, despite this matrix not being specifically reported in the aforementioned EURL-SRM analytical observation. We then carried out a test by subjecting the non-roasted samples (both whole and ground beans) to basic hydrolysis under mild conditions; the addition of the 5 N NaOH solution (to reach a pH of 12/13) to the QuEChERS protocol, which involves the use of acetonitrile as extracting solvent. The hydrolysis, carried out at room temperature and at 70 °C for 1 h, followed by base neutralization via the addition of the sulfuric acid 5 N solution, did not provide the desired results as the subsequent analyses were negative for the presence of OPP.

3.3. OPP-conjugates and their influence on OPP quantification

The coffee bean surface is covered by a thin layer made of wax that makes up 0.2–0.3 % of its total weight (Folstar, 1985; Speer and Kölling-Speer, 2006). Assuming that OPP interacts with the components of this outermost layer, we performed an extraction of green coffee beans without grinding, at room temperature for 30 min with hexane and, for the sake of comparison, with ethyl acetate, acetonitrile, methanol and water in order to obtain possible "OPP-conjugates". To evaluate the sensitivity and efficiency of the method, tests were carried out on Arabica coffee as it showed lower OPP content in its roasted samples. All of the dry extracts were found to be negative for OPP when subjected to the QuEChERS method, both directly and after heating for one hour at 180 °C, i.e., the temperature that led to the maximum detection of the fungicide under our heating conditions (Fig. 2). However, it was only possible to detect OPP in the less polar extracts when water was added before heat treatment. The water amount was 10 % w/w (named wet heat), which is related to the natural moisture content of green coffee beans. The heating and reconstruction of the humidity of green coffee is therefore a necessary condition for detecting OPP. Moreover, under the same conditions, higher concentrations of OPP were found in hexane (5.9 μg/kg), ethyl acetate (1.8 μg/kg), and acetonitrile (traces), whereas this compound was not detected in methanol and water. This evidence confirms the hypothesis that the fungicide conjugates are localized in the lipophilic layer of coffee.

The exhaustiveness of the extraction method described above was confirmed by an analysis with a negative OPP result, which was obtained by heating (1 h, 180 °C) green coffee beans that had previously been treated with hexane.

Since the extracts in methanol are rich in phenolic compounds (Oliveira, Silva, Santos and Queiroz, 2019), it is interesting to note that the presence of OPP was not observed in these trials, thus leading to the conclusion that its origin may not be linked to its generation from precursors that are naturally present in green coffee beans.

The possibility of OPP contamination being limited to the beans outermost layer was further confirmed by the repetition of the extraction in hexane, and its "wet heat" treatment, on ground green coffee samples to allow exhaustive extraction to be performed. This trial provided analytical results comparable to those obtained with whole grains (6.3 ± 0.3 μg/kg).

Based on the EURL-SRM report (EU reference Laboratories for Residues of Pesticides), the preliminary hydrolytic procedure that was performed at pH 12 in acetonitrile (70 °C, 1 h) on the dry hexane extract was surely found to be negative for OPP because of its insolubility in this solvent. The procedure was therefore repeated in methanol, and the analysis of the neutralized sample treated using the QuEChERS-based method made it possible to detect OPP at a concentration of 34.8 μg/kg. This result confirms that OPP is present in green coffee as lipophilic conjugates that undergo partial degradation during roasting. As the OPP levels previously detected on the corresponding industrially roasted coffee samples (17.1 μg/kg for roasting degree 60, Table 1) can now be considered underestimated, the hexane extraction and subsequent hydrolysis in methanol were carried out on the same sample, giving a concentration of 32.2 μg/kg. This result may demonstrate that, for full OPP quantification, it is necessary to proceed with the breakup of its "conjugates". It is worth noting that, without detracting from the efficiency of the multi-residual analytical methods, in the case of a complex matrix such as coffee, preliminary sample treatment with hexane is a key step in extracting lipophilic OPP-conjugates. In fact, only the direct hydrolysis of roasted coffee in methanol provided OPP detection of 24.8 μg/kg. Finally, the same increase in OPP concentration (64.8 ± 0.4 μg/kg *vs* 34.2 ± 0.6 μg/kg) was detected when the modified sample preparation procedure was applied, for the sake of comparison between the two species, on the Robusta sample with a roasting degree 60.

In Fig. 3, it is possible to observe the influence of sample preparation on the detected OPP levels in both green and roasted samples, and that the optimized procedure (extraction with hexane, basic hydrolysis in methanol, neutralization, QuEChERS and UPLC-MS/MS) allows comparable results to be obtained for Arabica samples both before and after the roasting process. It is also evident that roasting only causes partial hydrolysis in the OPP-conjugates, with the quantity that is detected using the simple QuEChERS method only corresponding to ~50 % of the total amount.

The possibility that OPP-conjugates may be ester compounds formed from the fungicide and free fatty acids present in green coffee beans (Speer and Kölling-Speer, 2006) was investigated by synthesizing *o*-phenylphenyl stearate and adding this compound to a sample of Arabica ground green coffee, at a concentration of 124 μg/kg (i.e. 48 μg/kg of OPP), before subjecting the sample to the above-described procedure. The UPLC-MS/MS analysis of the obtained sample revealed an increase in OPP amount, at 41.5 μg/kg (86 % of *o*-phenylphenyl stearate conversion), thus confirming that the conditions for the extraction in hexane and the basic hydrolysis are sufficient to achieve a reasonable release of the fungicide from this kind of conjugate.

3.4. OPP transfer into coffee brew

In order to estimate the possible transfer of OPP into the coffee brew, the beverage was prepared by percolation using the same sample of Arabica roasted coffee (roasting degree 60), and was subjected to freeze-drying. The residue was then solubilized in methanol and hydrolyzed at pH 12 before the QuEChERS procedure, and the analysis observed no OPP, thus demonstrating that there is no risk of OPP exposure for coffee beverage consumers.

For comparison, a commercial sample of soluble coffee was also tested with the direct hydrolysis procedure, and the previous observation of an absence of OPP was therefore corroborated.

4. Conclusions

The low MRLs of biocides required by authority regulations have

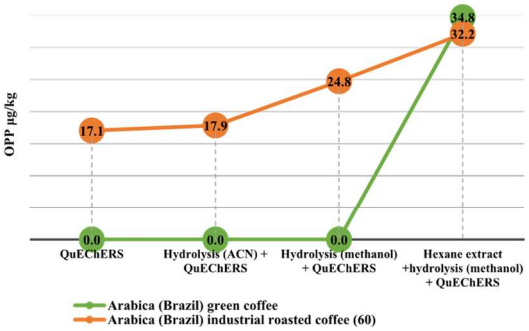

Fig. 3. OPP amount (μg/kg) in green *vs* roasted Arabica coffee according to sample preparation method and UPLC-MS/MS analysis.

prompted the development of more reliable and sensitive analytical methods for monitoring residues in food and beverages. Coffee is one of the most widely consumed commodities in the world, and the complexity of this matrix requires specific sample enrichment methods, such as QuEChERS, for both green and roasted samples. OPP is a post-harvest fungicide that is not specifically indicated for coffee bean preservation. To the best of our knowledge, traces of OPP have been found in roasted coffee, but never in a green sample, although it is highly unlikely that it this fungicide forms during roasting from precursors that are naturally present in coffee. Like other pesticides with reactive functional groups, OPP can potentially form lipophilic conjugates that can mask its presence. It follows that multiresidue analytical methods may lead to an underestimation of OPP amount if they are inefficient with respect to the conjugates. The importance of extraction with a nonpolar solvent, such as hexane, before the hydrolytic step at pH 12 and the QuEChERS method in quantitatively recovering these adducts from coffee samples has been demonstrated. Moreover, the appropriate choice of methanol for the hydrolysis step was crucial in obtaining the correct quantification of the pesticide by UPLC-MS/MS, not only for green coffee but also for roasted samples. Indeed, the effect of the roasting process on the OPP-conjugates is not sufficient to release OPP completely, meaning that that the pesticide is underestimated by about 50 % in roasted samples. When using the optimized method on the same samples of green and industrially roasted Arabica, comparable levels of OPP were determined in both matrices, 34.8 μg/kg and 32.2 μg/kg, respectively.

In order to verify possible OPP transfer to the coffee brew, the beverage obtained by percolation and commercial soluble coffee were tested, giving negative results for OPP. The data reported in this work suggest that there is no significant safety risk to consumers of coffee/coffee brew, as the detected OPP residue levels contribute minimally to the ADI of 0.4 mg/kg body weight. Furthermore, since there is no specific evidence for the use of this biocide in coffee, its detection may be due to accidental or technically unavoidable contamination during green coffee production.

Further studies on the correlation between OPP content, roasting degree and different varieties are being conducted.

CRediT authorship contribution statement

Janet Menzio: Methodology, Validation, Writing – original draft, Writing – review & editing. **Silvia Tagliapietra:** Conceptualization, Writing – original draft. **Alessandro Barge:** Methodology, Validation. **Bianca Serito:** Methodology, Data curation, Supervision. **Elena Calegari:** Data curation, Supervision. **Arianna Binello:** Conceptualization, Writing – original draft, Supervision. **Giancarlo Cravotto:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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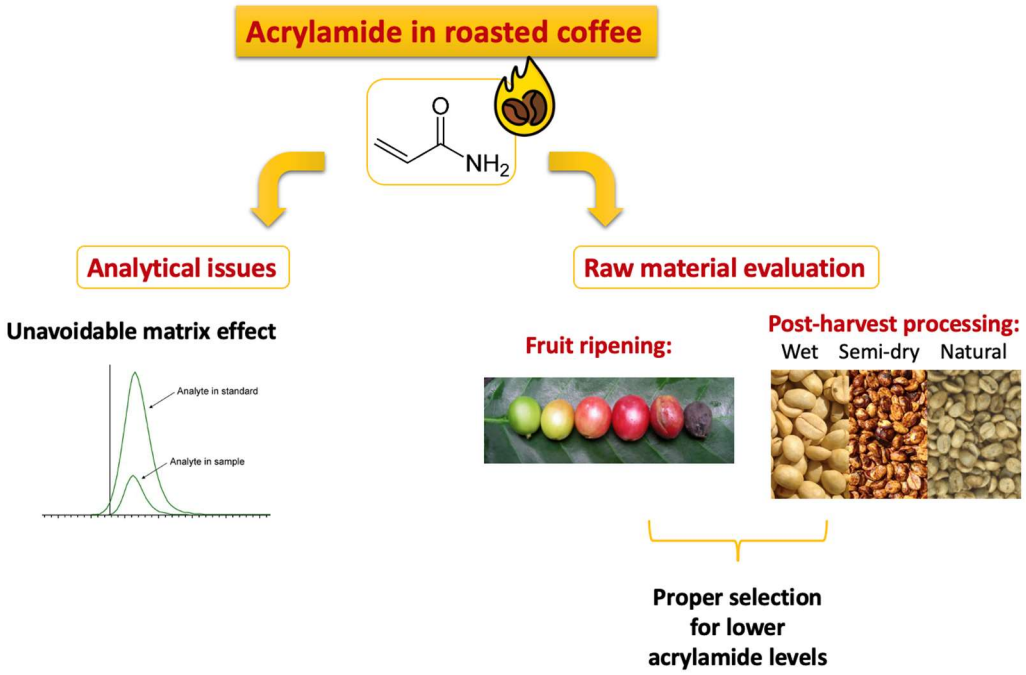
5. The big issue of acrylamide in roasted coffee: preliminary evaluation of beans ripening and different post-harvest processing methods influence



5.1. Introduction

The roasting usually performed at temperatures between 120 and 250°C involves a complex chemistry exerting processes as Maillard reaction, Strecker degradation, lipid oxidation and sugar decomposition (Farah, 2019). These chemical modifications can be responsible of undesired compounds generation, known as heat-induced contaminants, such as acrylamide, furans and hydroxymethylfurfural, which are currently mostly studied because of their high toxicological potential (Rannou, Laroque, Renault, Prost and Serot, 2016; Zhu *et al*., 2022). Classified in 1994 by the International Agency for Research on Cancer (IARC) as a compound "probably carcinogenic to human" (group 2A), the first study that demonstrates how acrylamide is generated in cooking food dates back to 2002 (Tareke, Rydberg, Karlsson, Eriksson and Törnqvist, 2002; EFSA Scientific Opinion 2015). Currently, there is no regulatory limit defined for this compound, but the European Food Safety Authority (EFSA) set recommended levels for different foodstuffs. In particular, acrylamide benchmark values in roasted coffee are 400 μg/kg, 850 μg/kg for instant coffee and 500 μg/kg for coffee substitutes (Commission Regulation (EU) 2017/2158)

Owing to the growing concern for this contaminant, and with the aim of meeting the recommended levels imposed by regulatory agencies, several studies have been reported on acrylamide generation in coffee, often pursuing the challenge of finding appropriate conditions able to mitigate its generation (Schouten, Tappi and Romani, 2020; Strocchi, Rubiolo, Cordero, Bicchi and Liberto, 2022).

Even if the Maillard reaction involving the amino acid asparagine and reducing sugars (e.g., glucose or fructose) is considered the main route of acrylamide production, other possible pathways are also contemplated including asparagine degradation, reaction between ammonia and acrylic acid deriving from aspartic acid degradation or fatty acids/glycerol oxidation, and interaction between hydroxymethylfural and asparagine (Yaylayan and Stadler, 2005; Schouten, Tappi and Romani, 2020).

As reported before, the two main coffee species, Arabica and Robusta, are characterized by different sensory properties and distinct chemical composition that can be influenced by many parameters as variety, cultivation climatic conditions and post-harvest treatments (dry and semi-dry/semi-wet and wet methods). (Joet et al., 2010; Knopp, Bytof and Selmar, 2006; Bagdonaite and Murkovic, 2004). It follows that all these variables affect the levels of acrylamide precursors in green beans, making the choice of the raw material of essential importance as reduction strategy for the contaminant. Generally, literature reports that, compared to Arabica samples, Robusta coffees show higher levels of asparagine and, despite the presence of lower sugar amounts, they give rise to a relatively higher acrylamide average level, thus evidencing as this amino acid represents the main criterion to be monitored in obtaining safer products, also when Arabica/Robusta ratio of the coffee blend is evaluated (Bagdonaite, Derler and Murkovic, 2008; Bertuzzi, Martinelli, Mulazzi and Rastelli, 2020; Esposito, Fasano, De Vivo, Velotto, Sarghini and Cirillo, 2020).

An increase in acrylamide content linked to the number of defective green beans has also been reported. In particular, it has been found that defective beans, and especially the unripe ones, contain significantly higher amounts of free asparagine and reducing sugars than ripe beans (Mazzafera, 1999). Moreover, it has also been observed that asparagine is present at more significant levels in unripe beans submitted to dry method as post-harvest treatment (Dias, Borém, Pereira and Guerreiro, 2012), and that a reduction of acrylamide levels occurs during coffee storage (Michalak, Gujska, Czarnowska, Klepacka and Nowak, 2016; Baum *et al*., 2008; Lantz, Ternite, Wilkens, Hoenicke, Guenther and Van Der Stegen, 2006).

Certainly, an important role in determining acrylamide amount is played by the roasting conditions; it has been shown that during this process its content reaches a peak early, while toward the end it drops off considerably, thus underlining as relatively lower amounts of acrylamide are present in fully roasted coffee samples (Bertuzzi *et al.,* 2020). Enzymatic treatments of raw material, vacuum or steam roasting, roasted beans supercritical fluid extraction, and final beverage treatments as yeast fermentation and amino acids/additive additions have been proposed as innovative procedures for acrylamide control in coffee samples, even if these strategies substantially affect the organoleptic properties of the brew, thus making them not applicable at industrial level (Porto, Freitas-Silva, de Souza and Gottschalk, 2019; Anese, Nicoli, Verardo, Munari, Mirolo and Bortolomeazzi, 2014; Rattanarat, Chindapan and Devahastin, 2021; Kocadağlı and Gökmen, 2022).

Due to its high polarity, low molecular weight, and high reactivity, acrylamide also raises the challenge regarding its accurate quantitative determination in food products, thus highlighting the necessity for reliable techniques to be adopted. The complexity of food matrices requires an intensive clean-up step after sample extraction to eliminate or reduce the presence of interfering compounds. The particularly difficult issue for acrylamide determination in coffee has resulted in numerous studies related to sample treatments and aimed in limiting the risk of analyte underestimation. The most used extraction solvent is water, while different purification steps are employed, including precipitation of co-extractives with organic solvents or Carrez reagent which consists in a well-established sample preparation process for food analysis through two complexation steps: Carrez reagent I (zinc acetate solution) forming complexes with high mass compounds such as proteins, and Carrez reagent II (potassium ferrocyanide solution) allowing to precipitate excess zinc. Other techniques consist in defatting with *n*-hexane, single or multiple solid phase extraction (SPE) with different sorbents. Moreover, QuEChERS methods fro acrylamide determination have been recently reported (Delatour, Perisset, Goldmann, Riediker and Stadler, 2004; Mastovska and Lehotay, 2006; Rosen, Nyman and Hellenas, 2007).

Commonly, analytical procedures involve liquid chromatography (LC) and gas chromatography (GC) combined with mass spectrometry (MS). In particular, LC with tandem mass spectrometric (MS/MS) detection methods are of election thanks to their superior performance in sensitivity, selectivity, and easier sample preparation procedures when compared to GC protocols (Pugajeva, Jaunbergs and Bartkevics, 2015; Rosen *et al*., 2007; Bertuzzi, Rastelli, Mulazzi and Pietri, 2017; Tekkeli, Onal, C. & Onal, A., 2012).

Despite the use of very sensitive analytical methods, the accuracy of acrylamide detection at low levels remains still difficult, not only as regards the sample preparation owing to the high reactivity of this molecule, but also for the so called “matrix effect”, namely the interference caused by the matrix components in the MS detector. This phenomenon, that occurs when co-eluting molecules with the compound of interest alter the ionization efficiency of the electrospray interface, is commonly observed as suppression or enhancement of analyte signal (Kebarle and Tang, 1993) and implies the need for its assessment during the development and validation of LC-MS/MS methods (Matuszewski, Constanzer and Chavez-Eng, 2003; Ciric, Prosen, Jelikic-Stankov and Durdevic, 2012), commonly through the adoption of compensation strategies as the use of internal standard isotopically labelled (Yang *et al*., 2015; Stuber and Reemtsma, 2004) that mimics perfectly the response of the corresponding unlabelled analyte, independently from the dissolving system (pure solvent or matrix) (Yarita, Aoyagi and Otake, 2015.; Cortese, Gigliobianco, Magnoni, Censi and Di Martino, 2020).

Given the need for the coffee industry to have useful tools actually applicable for acrylamide levels mitigation, a more in-depth and complete study of the raw material characteristics and

their influence is required. This chapter evaluates the correlation between acrylamide levels in Arabica and Robusta roasted coffee samples with the influence of some variables such as green beans ripening and different post-harvest treatments. A protocol has been optimized for this purpose based on a single extraction step with water, followed by a two-stage solid phase extraction clean-up and then by UPLC–MS/MS analysis. Deuterium labelled acrylamide (acrylamide-d_3) has employed to compensate, through the estimation of the recovery %, both the analyte losses and matrix effect during sample preparation and quali-/quantitative analysis and one way Analysis of Variance (ANOVA) was applied to evaluate significant differences between Arabica and Robusta coffee samples.

5.2. Results and discussion

5.2.1. Analytical Method and SPE purification trials

The complexity of the matrix, as well as the need for a sample enrichment due to the necessity to detect low levels of acrylamide, make the analysis of this process contaminant in coffee a complex challenge. For this study, UPLC-MS/MS has been chosen as quantitative analytical system, and its reliability was evaluated in terms of calibration linearity, repeatability, sensibility and matrix effect.

Following instrumental conditions reported in paragraph 5.4.10, standard solutions of acrylamide (m/z 55) and acrylamide-d_3 (m/z 58) at different concentration levels (0.05, 0.1, 0.3, 0.5, 0.7, 1 μg/mL) have been analysed three times each, and good linearity was found, with the determination coefficients being >0.99. The deviations of the obtained concentrations from the nominal values were examined for all calibration points and in all cases were <10%. The limit of detection (LOD) and the limit of quantification (LOQ) were determined in 0.02 μg/mL and 0.05 μg/mL, respectively.

Following what reported by Mastovska and Lehotay (2006), QuEChERS sample preparation procedure, consisting in the use of water and acetonitrile partitioning for extraction, and a dispersive SPE (PSA sorbent) clean-up step of the organic phase, has been initially performed (procedure 5.4.2.). Unfortunately, despite many advantages ascribed to this procedure as simplicity of execution and timesaving, it did not result a performing purification method on our samples. In fact, probably due to the greater affinity of acrylamide for water, these first trials evidenced a consistent analyte loss (~60%) during the extraction and partitioning steps, with a final recovery results that are quite in accordance with De Paola et al. (2017), who reported a decrease in acrylamide recovery of about 36% by applying QuEChERS procedure in other foodstuff as dried fruits and edible seeds (De Paola, Montevecchi, Masino, Garbini, Barbanera and Antonelli, 2017).

Other most common sample preparation methods for LC-MS/MS determination of acrylamide in coffee and coffee products, comprising also the ISO method (ISO 18862:2016), imply water extraction of ground coffee samples followed by an effective SPE clean-up step due to the presence of co-extracted components (Bortolomeazzi, Munari, Anese and Verardo, 2012). In particular, polysaccharides, proteins, acids, caffeine, trigonelline, and also colloidal particles and dark coloured negatively charged melanoidins, may affect the outcome of the quali-/quantitative analysis. Therefore, different SPE stationary phases have been employed for the retention of matrix interferences after water coffee extracts filtration on 0.45 μm syringe filters. In details, silica-based C18 bonded phase (C18), anion exchange (QMA), strong cation exchange (SCX), weak cation-exchange (CM) and amino (DSC-NH_2) sorbents were selected and singularly tested for this purpose.

To verify the percentage of analyte recovery after the elution through each SPE cartridges, all the stationary phases were firstly tested on aqueous standard solution of both acrylamide and acrylamide-d_3 (0.5 and 1 μg/mL), and obtained results showed the efficacy of all the trials with an average recovery greater than 99%. The same procedure was then applied to evaluate how coffee matrix could affect acrylamide detection. Given the impossibility of having acrylamide-free roasted coffee samples, acrylamide-d_3 aqueous standard solution was employed to spike two samples of water coffee extract (1 ml each), previously obtained from grinded roasted coffee (procedure 5.4.3.), in order to reach final concentrations equal to 0.5 and 1 μg/mL. Following elution through the selected cartridges, the average analyte recovery was evaluated after UPLC-MS/MS analysis. Results shown in **Figure 1** indicated that acrylamide-d_3 amount in coffee solutions significantly decreased after SPE, with best recovery data for C18 (65.2 ±1) and QMA (63.8 ±2) sorbents.

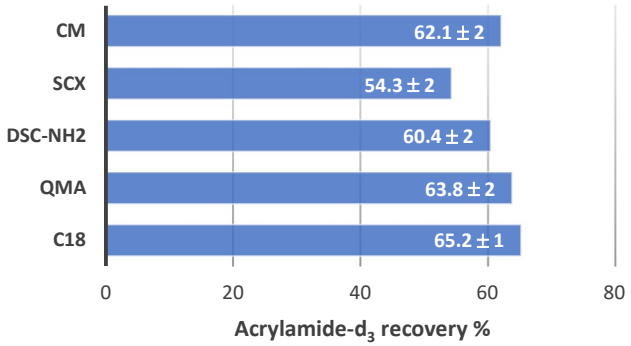


Figure 1. Average acrylamide-d_3 recovery % from elution through different SPE cartridges (silica-based C18 bonded phase, anion exchange QMA, strong cation exchange SCX, weak cation-exchange CM and amino DSC-NH_2) of water coffee extract spiked at 0.5 and 1 μg/mL. SD were obtained from 6 replicates of each sample.

However, all the obtained samples still resulted with a brown color very similar to the initial extract. Therefore, to obtain a cleaner sample and limit the matrix effect, both in analyte recovery during sample preparation and in its quantification via UPLC-MS/MS. The two more performing stationary phases (C18 and QMA) were employed in a two-step SPE clean-up procedure (see paragraph 5.4.6.) with improved results in terms of extract clearing and analyte recovery (77.3 ±1%).

The effectiveness of the two-step clean-up method was then evaluated by directly spiking the ground-roasted coffee with acrylamide-d_3 before water extraction in order to obtain two different concentration levels (250 and 500 μg/kg).

This clean-up strategy did not allow to sample concentration, on the contrary, a dilution occurs after elution through the cartridges. Therefore, to obtain LOQ as sensitive as possible, each sample has been firstly submitted to freeze-drying and then solubilized in 500 μL of water before UPLC-MS/MS analysis (see paragraph 5.4.7.).

Both Arabica and Robusta coffee samples were analysed six times for method performance evaluation at the two different spiking levels and the results indicated an internal standard average recovery of 69.3 ±4 %, a value which, as discussed below, highlights how the coffee matrix determines an underestimation of acrylamide during the MS/MS analysis.

The stability of the analyte in the final extract provides information about the storage requirements for the samples until further analysis. This is crucial for acrylamide since pH conditions, light, microbes, and co-extractives can negatively act on its quantification (Roach, Andrzejewski, Gay, Nortrup and Musser, 2003). The need to perform the quantitative analysis

immediately after sample preparation was demonstrated by a set of experiments carried out within the sample preparation day, after 24h and after 48h, in six replicates for each acrylamide-d_3 spiking level (250 and 500 μg/kg) and the recovery % was evaluated (**Table 1**). The extracts were stored at 4°C and the mean percentage reduction in acrylamide-d_3 recovery was found always less than 5% within the first day, about 17% on average after 24 hours and about 50% on average after 48 hours for both spiking levels.

Table 1. Acrylamide-d3 recovery % in coffee samples at two spiking levels after different time from sample preparation.

AC-d_3 recovery % at two spiking levels after sample preparation*		
	250 μg/kg	**500 μg/kg**
Within sample preparation day	67.5 ±2%	69.7 ±2%
24h after sample preparation	57.8 ±2%	56.8 ±1%
48h after sample preparation	35.8 ±1%	34.7 ±2%

Data obtained from 6 replicates of each sample.

5.2.2. Matrix effect in MS/MS detection of acrylamide in coffee samples

As above mentioned, acrylamide detection on food samples via MS/MS can suffer of matrix effect affecting analytical precision and other parameters such as accuracy, linearity, LOD and LOQ (Kebarle and Tang, 1993; Cortese *et al*., 2020). Roasted coffee is not exempt from this problem and the presence of interfering co-eluents as N-Acetyl-ß-alanine and valine strongly depends on the applied sample preparation method and analytical conditions (Desmarchelier, Hamel and Delatour, 2020; Mastovska and Lehotay, 2006).
Although the optimization of the sample cleaning conditions applied to spiked coffee extracts allowed to achieve discrete percentage of acrylamide-d_3 recovery, the previously reported results evidenced as it was not possible to go beyond 69.3 ±4%. Therefore, the entity of signal suppression during the quantification via UPLC-MS/MS was investigated.
By comparing the MS/MS response of an analyte in the spiked sample extract with the MS/MS response of the same analyte present in the standard solution, at various concentration levels, it is possible to examine the matrix effect during the development of the analytical method (Ciric *et al*., 2012). This is expressed as matrix effect % (ME%) and calculated with the following equation:

$$ME\% = \frac{peak\ area\ of\ post\ extraction\ standard\ addition - peak\ area\ from\ calibration\ curve}{peak\ area\ from\ calibration\ curve} \times 100$$

To this aim, not previously acrylamide-d_3 spiked coffee samples were extracted with water and purified following the two-phase procedure with C18 and QMA cartridges before freeze drying. Obtained powders, redissolved in 500 μL of distilled water, were then added with acrylamide-d_3 water standard solution only before the UPLC-MS/MS analysis to obtain the final concentrations of 0.05, 0.1, 0.3, 0.5, 0.7, 1 μg/mL as those employed for method calibration (see procedure 5.4.9.).

The matrix effect evaluated using this sample preparation, which meant using the aqueous coffee extract as a solvent, was found to be strongly depended on acrylamide-d_3 concentration. Compared to the analyses carried out on standard water solutions, a major signal suppressive effect in the range -30.65 ±3 to -13.54 ±2 % was found for "coffee solutions" with higher contaminant amounts (1-0.5 μg/mL), while at 0.05, 0.1 and 0.3 μg/mL of acrylamide-d_3 the matrix effect resulted almost inaudible showing a very small increase in signal enhancement (**Table 2**).

Table 2. Average values of matrix effect % ±SD on acrylamide-d_3 solutions obtained using water coffee extracts as solvent.

Acrylamide-d_3 concentration μg/mL	Coffee matrix effect % [a]
0.05	0.98 ±0.2
0.1	0.83 ±0.2
0.3	-4.36 ±2
0.5	-13.54 ±2
0.7	-13.76 ±2
1	-30.65 ±3

[a]SD calculated over six UPLC-MS/MS analyses of each sample.

Calibration curves obtained both with water acrylamide-d_3 solution and the one achieved employing coffee extracts (matrix-matched calibration), having R^2 of 0.9923 and 0.9899 respectively, are reported in **Figure 1** and show how this phenomenon affects acrylamide quantification through MS detection despite significant sample clean-up.

The matrix effect found is in agreement with several literature data reporting a signal suppression between 30 and 40% for coffee extracts in LC-MS/MS analysis, thus confirming that it is an unavoidable issue, regardless of the sample treatment used (Pugajeva *et al*., 2015; Mastovska and Lehotay, 2006.; Delatour *et al*., 2004).

In those circumstances and without the availability of blank matrix, the addition of acrylamide-d_3 before the coffee extraction step allows to correct all random errors occurring during both the preparative step and instrumental analysis (Berg and Strand, 2011; Cortese *et al*., 2020). Taking into account the recovery values of the labelled internal standard leads to improve method ruggedness, precision, and accuracy in acrylamide detection.

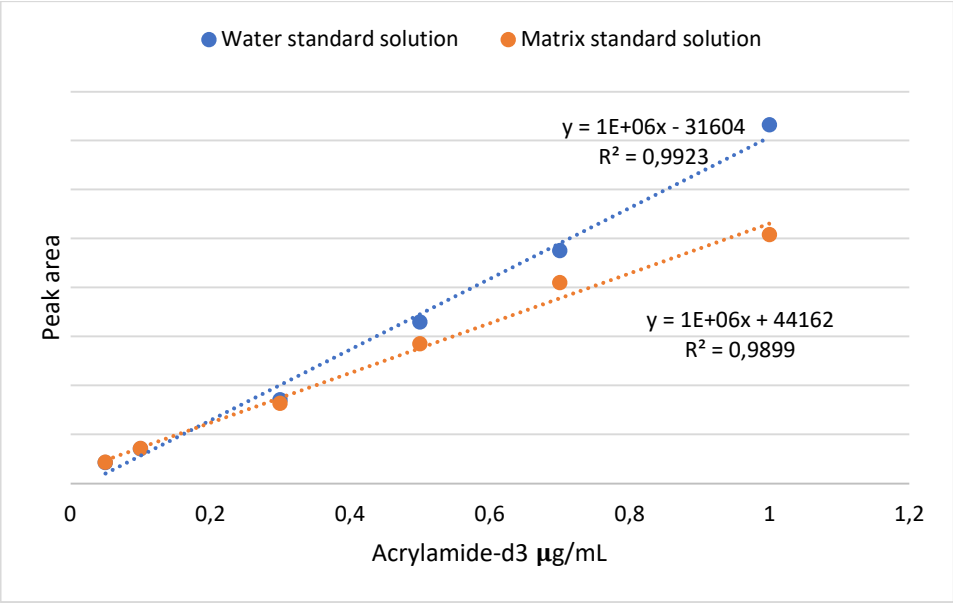


Figure 1. Calibration curves, obtained from acrylamide-d_3 water and water coffee extracts solutions, evidencing the matrix effect on the analyte detection in MS/MS spectrometry.

5.2.3. Influence of green coffee parameters on acrylamide levels in roasted coffee

The growing attention to acrylamide generation, and the limited possibilities from a technological point of view to reduce its levels whitout affecting the organoleptic properties of coffee brew, would call for the evaluation of strategies that allow to control the development of this process contaminant during roasting and, at the same time, to guarantee a product capable of satisfying consumer habits. A possible way currently viable could be to make a careful selection of green beans in function of their features that can be influenced not only by the type of cultivar, but also by climate cultivation conditions, maturation and processing even if, in this regard, data available in the scientific literature is very often discordant probably due to matrix variability requiring further evaluation (Bertuzzi *et al.*, 2020; Lantz, I.; Ternite *et al.*, 2006; Bagdonaite *et al.*, 2008; Kleinwachter and Selmar, 2010; Alves *et al.*, 2010).

Arabica and Robusta coffees normally employed alone (e.g., 100% Arabica) or in blend according to the consumers preferences, present distinct specific chemical composition (Farah, 2019) that can influence the acrylamide amounts found in the final products; in this contest, higher concentrations in Arabica roasted samples are generally reported (Esposito *et al.*, 2020).

With the aim to provide relevant tools for this contaminant mitigation, and to encourage the selection of specific green coffee features rather than technological changes in the roasting process, this study intended to compare acrylamide content in Arabica and Robusta samples based on different geographical origins, different percentages of unripe beans, post-harvest processing method used to obtain the green beans to be roasted.

Moreover, since the acrylamide amount in coffee is substantially influenced by roasting procedure, the same roasting level (medium) was applied for all the samples.

5.2.3.1. The effect of increasing amounts of unripe beans on acrylamide levels

Although green beans are often sorted and categorized according to their stage of maturation, it is inevitable that some unripe ones, which contain significantly higher amounts of free asparagine and reducing sugars than ripe beans (Mazzafera, 1999), may be present throughout processing.

Results obtained through acrylamide UPLC-MS/MS analysis of Arabica (Brazil) and Robusta (Vietnam) samples, both submitted to dry method and containing different percentages of unripe beans (0, 10, 50, 100%), are reported in **Table 3**. No significant difference between the two cultivars has been revealed in roasted coffee containing 0% unripe beans, with slightly higher levels in the Arabica one (358.01 *vs* 304.32 μg/kg). These outcomes are in accordance with results published by Bertuzzi et al. (2020) who reported greater formation of acrylamide in Arabica coffee despite lower levels of asparagine, thus ascribing it to the higher concentration of reducing sugars in this specie respect to Robusta coffee. On the other hand, the amount of acrylamide detected rises with the increase of the unripe beans amount in coffee samples. Results were statistically significant as found by one way ANOVA both for Arabica ($F_{3,20}$=2100,5; p=3,71x10^{-25}) and Robusta ($F_{3,20}$=2505,2; p=6,41x10^{-26}), supporting the idea that a careful selection of green coffee, with fewer immature beans, could be an effective method for reducing the development of acrylamide during roasting. Specifically, an amount of 10% unripe beans in coffee samples implies an average increase of 11% for Arabica and 19% for Robusta while, when the content rise to 50% a major acrylamide amount of 60 and 80% was respectively found. Finally, in samples consisting only of immature green beans contaminant levels reached higher values, with a more significant increase for Robusta (+160%) compared to Arabica (+99%).

Table 3. Acrylamide quantification expressed as μg/kg in coffee samples obtained by dry processing containing different percentages of unripe beans.

	Arabica (Brazil) Acrylamide μg/kg ±SD [a]	**Robusta (Vietnam)** Acrylamide μg/kg ±SD [a]
0% unripe beans	358.01 ±7	304.32 ±10
10% unripe beans	397.53 ±8	363.3 ±7
50% unripe beans	573.25 ±10	547.11 ±13
100% unripe beans	712.9 ±9	792.46 ±11

[a]SD calculated over six UPLC-MS/MS analyses of each sample.

5.2.3.2. Post-harvest treatments influence on acrylamide levels

Green beans derive from processing of coffee plant fruits, known as cherries or drupes, each one containing two seeds. Depending on the climate of the country and farm tradition, after collection, ripe coffee cherries are processed to reach a green bean moisture of around 11–12% and provide a stable product for exportation. The beans metabolism is affected by the post-harvest procedures implying variations in the green bean composition with regard to secondary metabolites, carbohydrates, proteins and amino acids (Hameed, Hussain, Ijaz, Ullah, Pasha and Suleria, 2018; Pereira *et al*., 2019; Bytof, Knopp, Schieberle, Teutsch and Selmar, 2005; Scholz, Prudencio, Kitzberger and da Silva, 2019). It has also been considered that the washed method involves a careful selection of the cherries; only the fully ripe ones

can be treated by this process, thus exerting a positive impact on the quality of the beverages. Otherwise, simple drying and hulling of the cherries employed in the natural coffee involves ripe, unripe and overripe fruits being processed together (Toledo, Pezza, L., Pezza, H. R. and Toci, 2016). Moreover, when the immature coffee beans were treated using the wet technique the asparagine levels were much lower whit respect to what exerted by the dry process, probably due to the existence of a more active metabolism in coffee beans that are treated using the washed technique. (Dias *et al.*, 2012). This trend can be confirmed by results reported in paragraph 5.2.3.1. as samples analysed were obtained by dry processing showing significant higher acrylamide levels when increasing unripe beans amount.

Since it is not common for all green beans to undergo all kinds of post-harvest treatments, but this is a function of the different cultivars and geographical origins, it was not possible to proceed with an evaluation of the totality of treatments for each origin selected in this trial. Therefore, two Arabica samples from Brazil subjected to dry and semi-dry processing, and Robusta samples from India obtained by dry and wet methods were employed to investigate this topic.

Results reported in **Table 4** show as both Arabica and Robusta natural samples contain higher acrylamide amounts compared to beans subjected to semi-dry and wet methods, specifically in Arabica coffee acrylamide level is reduced of about 20% in semi-dry process, while in Robusta sample treated with wet method the resulting acrylamide is reduced of about 50%. These values Results were statistically significant as found by one way ANOVA both for Arabica ($F_{1,10}$=183,75; p=9,2x10^{-8}) and Robusta ($F_{1,10}$=1314,2; p=6,06x10^{-12}) and can be correlated to the higher amount of glucose and fructose, the main reducing sugars involved in acrylamide formation, in natural coffees with respect to the washed ones as it is reported that the content of this type of sugars is drastically reduced in wet method of about 80% due to the anaerobic fermentation step occurring in this process (Knopp et al., 2006).

Table 4. Acrylamide quantification expressed as μg/kg in coffee samples obtained through different post-harvest methods.

	Arabica (Brazil) Acrylamide μg/kg ±SD	**Robusta (India)** Acrylamide μg/kg ±SD
Dry method	364.2 ±10	396.7 ±9
Semi-dry method	293.4 ±9	-
Wet method	-	204.6 ±8

Data obtained from 6 replicates of each sample.

Finally, when considering samples obtained by dry method, no significant variability of acrylamide levels between Arabica (Brazil) and Robusta (India) species has been found, with slightly higher levels (~8%) detected in the second variety.

Based on this preliminary outcome, results affirm a correlation between post-harvest treatments and acrylamide levels in roasted coffee, reflecting the reported chemical changes on precursors occurring in green beans. On the other hand, literature currently available on this topic is few and some authors did not find significant differences on acrylamide levels between dry and wet processed coffee bean samples. In fact, the work published by Lantz *et al.* (2006) reports a comparison of acrylamide levels after roasting between different Arabica and Robusta coffees: Arabica from Brazil (dry-processed) and Colombia (wet-processed), and Robusta from Vietnam (dry-processed). On average, a higher acrylamide level was found in Robusta coffee than in Arabica, while no difference appeared for the two different green

processing methods (dry or wet). Also Alves *et al.* (2010) reported that there were no appreciable variations between Brazilian dry-processed samples and other wet-processed Arabica samples (Alves *et al*., 2010).
Based on these considerations and given the high variability of the matrix, a further evaluation will be performed taken into account a greater number of samples belonging to different varieties within the same species for a broader evaluation of the correlation with acrylamide, and together with the results obtained they will be the subject of a future publication.

5.3. Conclusions

Albeit numerous studies on the topic, the analytical determination of acrylamide levels in roasted coffee is still a challenge. Taking into account the need for sample enrichment and the matrix effect that affects the quantitative analysis via MS/MS detection, a procedure was optimized consisting of a water extraction, a two-step SPE clean-up procedure and UPLC-MS/MS analysis. As signal suppression of acrylamide in LC-MS/MS still remained unavoidable, it was anyway necessary the use of the labelled internal standard.
The growing attention to this process contaminant, and the limited possibilities from a technological point of view to reduce its levels, call for an in-depth study on the influence of raw material features may have on its generation during roasting. For this purpose, both Arabica (Brazil) and Robusta (Vietnam and India) roasted coffee samples were analysed to evaluate the effect of increasing amounts of unripe beans and, on the other hand, to assess how post-harvest (dry, semi-dry and wet) treatments affect acrylamide values.
Results showed a strong correlation between the percentage of unripe beans and the contaminant level, with 60 and 80% increase in case of coffee samples consisting in 50% defective beans for Arabica (Brazil) and Robusta (Vietnam) respectively.
As concern post-harvest tretments, a consistent decrease in acrylamide levels in case of semi-dry and wet processed samples was demonstrated by preliminary results, thus evidencing a chemical modification in the contaminant precursors in both Arabica (Brazil) and Robusta (India) species. In particular, when compared to the respective dry samples, the wet approach produces around a 50% reduction in acrylamide in the second cultivar, while the semi-dry process reduces it by roughly 20% in Arabica one.
Owing to the significant variability of the matrix, and considering the limited and conflicting information currently avalilable in the literature, these preliminary outcomes need further investigation to create a suitable strategy useful to the industry in order to properly select raw material.

5.4. Experimental section

5.4.1. Chemicals and Coffee Samples

Acrylamide (analytical standard), acrylamide-d_3 standard solution (500 mg/L in acetonitrile), formic acid (for LC-MS LiChropurTM), acetonitrile (suitable for HPLC ≥99.9%) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy).
SPE cartdriges: Silica-based C18 bonded phase (C18; Sep-Pak C18 3 cc Vac Cartridge, 200 mg Sorbent per Cartridge, 55 - 105 µm, 50/pk), anion exchange (QMA; Sep-Pak Accell Plus QMA 3 cc Vac Cartridge, 500 mg Sorbent per Cartridge, 37-55 µm, 50/pk), weak cation-exchange (CM; Sep-Pak Accell Plus CM 3 cc Vac Cartridge, 500 mg Sorbent per Cartridge, 37-55 µm, 50/pk) cartridges were purchased from Waters SpA (Sesto San Giovanni, Milan, Italy). Amino cartridges (DSC-NH2; Supelco DSC-NH2 SPE Tube bed wt. 500 mg, volume 3 mL, pk of 54) were purchased from Sigma-Aldrich (Sigma-Aldrich, Milan, Italy). Strong cation exchange cartridges (SCX; SEClute P-SCX SPE Cartridge, 100mg, 3 ml, pk.50) were purchased from Sepachrom (Rho, Milan, Italy).
DisQuE QuEChERS cartridges (150 mg $MgSO_4$, 50 mg PSA, and 50 mg C18, 2 mL dispersive solid phase extraction (d-SPE) tube, 100/pk) were purchased from Waters SpA (Sesto San Giovanni, Milan, Italy).
Both Arabica (Brazil) and Robusta (Vietnam and India) roasted coffee samples were kindly provided by LAVAZZA S.p.A (Settimo Torinese, Turin, Italy). Samples containing different amount of unripe coffee beans and deriving from dry, semi-dry and wet processing were employed.

5.4.2. QuEChERS sample preparation procedure

1 g of ground roasted coffee was weighed into 50-mL polypropylene tubes and spiked with acrylamide-d_3 water standard solution (10 µg/mL) at 500 µg/kg. 10 mL of deionized water and 10 mL of acetonitrile were added for the sample extraction, then 4 g of $MgSO_4$ and 0.5 g of NaCl were added to the mixture and the sealed tube was shaken vigorously for 1 min. The sample was centrifuged at 5000 rpm for 10 min (Hettich ROTOFIX 32 centrifuge) obtaining solvent partitioning. 1 mL of the acetonitrile phase was transferred into a 2 mL d-SPE tube (150 mg $MgSO_4$, 50 mg PSA, and 50 mg C18) and submitted to vortex for 30 s, then centrifuged at 5000 rpm for 10 min. The obtained supernatant was transferred into an vial for LC-MS/MS analysis.

5.4.3. Coffee extraction procedure

For trials performed on coffee extracts, 2 g of ground roasted coffee were weighed into 50-mL polypropylene tubes and 10 mL of deionized water were successively added at room temperature, then the whole mixture was shaken at vortex for 3 min and sonicated for 15 minutes at temperature $< 40°C$ in an ultrasonic bath (Sonica 1200 S3, Soltec, ultrasound power 160 W). After centrifugation at 5000 rpm for 10 min, a 2 mL aliquot of the aqueous phase were filtered through 0.45 µm syringe filters in order to obtain 1 ml samples to be submitted to SPE procedures.

5.4.4. SPE recovery test on standard acrylamide solution

For this recovery tests, different SPE sorbent have been employed: Silica-based C18 bonded phase (C18), anion exchange (QMA), strong cation exchange (SCX), weak cation-exchange (CM) and amino (DSC-NH2) sorbents.
For each trial, 1 mL of acrylamide standard water solution (0.5 and 1 μg/mL) was eluted with 1 mL of deionized water through each SPE cartridge previously conditioned with 2 mL of methanol and 2 mL of deionized water. The eluate obtained was then analysed through UPLC-MS/MS for acrylamide recovery assessment.
To verify the reliability of the procedure, each test was repeated 6 times and the results were reported as mean value ± SD.

5.4.5. SPE recovery test on coffee extracts spiked with acrylamide-d_3

Tests described in paragraph 5.4.4. were also performed with coffee extracts obtained through sample extraction method as above mentioned (procedure 5.4.3.). Only before SPE procedures, 1 mL of the aqueous extract was spiked with acrylamide-d_3 water standard solution (10 μg/mL) in order to reach final concentration levels of 0.5 and 1 μg/mL. The sample was loaded on each SPE cartridge previously conditioned (2 mL of methanol and 2 mL of water) and then eluted with 1 mL of deionized water. The eluate was analysed through UPLC-MS/MS for acrylamide recovery assessment.
To verify the reliability of the procedure, each test was repeated 6 times and the results were reported as mean value ± SD.

5.4.6. Two-step SPE clean-up of coffee extracts spiked with acrylamide-d_3

Based on results obtained with preliminary recovery tests (paragraphs 5.4.4. and 5.4.5.), two different SPE cartridges (C18 and QMA) have been subsequently used for this trial. Only before SPE procedure, 1 mL of the aqueous coffee extract obtained through sample extraction method reported in procedure 5.4.3. was spiked with acrylamide-d_3 water standard solution (10 μg/mL) in order to reach final concentration levels of 0.5 and 1 μg/mL. Firstly, the sample was loaded on the C18 SPE cartridge after conditioning (2 mL of methanol and 2 mL of water) and eluted with 1 mL of deionized water. The obtained eluate was then loaded on the QMA cartridge, previously conditioned with methanol (2 mL) and water (2 mL) and eluted with 1 mL of deionized water. The purified sample resulted in a clarified, slight yellow coloured solution that was analysed through UPLC-MS/MS for acrylamide recovery assessment.
To verify the reliability of the protocol, the entire procedure was repeated 6 times and the results were reported as mean value ± SD.

5.4.7. Sample preparation of ground coffee samples for acrylamide-d_3 recovery assessment

For sample analysis, 2 g of ground roasted coffees were weighed into 50-mL polypropylene tubes, spiked with a water standard solution of the internal standard acrylamide-d_3 (10 μg/mL) in order to reach final concentration levels of 250 and 500 μg/kg, and then thoroughly mixed. Water (10 mL) was successively added, and the whole mixture was shaken at vortex for 3 min and sonicated for 15 minutes at temperature <40°C in an ultrasonic bath. After centrifugation at 5000 rpm for 10 min, an aliquot of the aqueous phase was filtered through 0.45 μm syringe filter and 1 ml of the obtained sample was then submitted to two consecutive SPE clean-up steps as reported in procedure 5.4.6.

The purified sample resulted in a clarified, slight yellow coloured solution was freeze-dried. The solid residue obtained was solubilized in 500 µL of water, filtered through 0.45 µm syringe filters and then analysed via UPLC-MS/MS for acrylamide recovery assessment.
To verify the reliability of the protocol, the entire procedure was repeated 6 times and the results were reported as mean value ± SD.

5.4.8. Sample preparation method for acrylamide quantification in ground roasted coffee samples

For sample analysis, 2 g of ground roasted coffees were weighed into 50-mL polypropylene tubes, spiked with a water standard solution of the internal standard acrylamide-d_3 (10 µg/mL) in order to reach final concentration level of 500 µg/kg, and then thoroughly mixed. Water (10 mL) was successively added, and the whole mixture was shaken at vortex for 3 min and sonicated for 15 minutes at temperature <40°C in an ultrasonic bath. After centrifugation at 5000 rpm for 10 min, an aliquot of the aqueous phase (2 mL) was filtered through 0.45 µm syringe filters and 1 ml of the obtained sample was then submitted to two consecutive SPE clean-up steps as reported in procedure 5.4.6.
The purified sample resulted in a clarified, slight yellow coloured solution was freeze-dried. The solid residue obtained was solubilized in 500 µL of water, filtered through 0.45 µm syringe filters and then analysed via UPLC-MS/MS for acrylamide quantification.
To verify the reliability of the protocol, the entire procedure was repeated 6 times and the results were reported as mean value ± SD.

5.4.9. Sample preparation for matrix effect assessment on UPLC-MS/MS analysis

2 g of ground roasted coffees were weighed into 50-mL polypropylene tubes and 10 mL of water were successively added, then the whole mixture was shaken at vortex for 3 min and sonicated for 15 minutes at temperature <40°C in an ultrasonic bath. After centrifugation at 5000 rpm for 10 min, an aliquot of the aqueous phase was filtered through 0.45 µm syringe filters and 1 ml of the obtained sample was then submitted to two consecutive SPE clean-up steps as reported in procedure 5.4.6.
The purified sample resulted in a clarified, slight yellow coloured solution was freeze-dried. The solid residue obtained was solubilized in 500 µL of water and filtered through 0.45 µm syringe filters. The obtained sample was then added with acrylamide-d_3 water standard solution (10 µg/mL) to obtain concentrations equal to 0.05, 0.1, 0.3, 0.5, 0.7, 1 µg/mL for monitoring the entity of the signal suppression through UPLC-MS/MS analysis.
To verify the reliability of the protocol, the entire procedure was repeated 6 times and the results were reported as mean value ± SD.

5.4.10. Instrumental analysis

Analyses were performed using a UPLC-MS/MS system (Acquity TQD LC/MS/MS System, Waters Corporation, Milford, MA, USA), equipped with a Acquity UPLC HSS T3 column (1.8 µm, 2.1 mm x 100 mm). A linear gradient elution with water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid, was carried out at 30°C. The gradient was from 100% solvent A to 50% within 2.6 min, then to 100% solvent B in 0.43 min and then hold for 2 min. The flow rate was set at 0.4 ml min^{-1} and the injection volume was 5 µL.

For the quantification of acrylamide and acrylamide-d_3, the positive electrospray ionization mode (ES+) was employed, following the transitions m/z = 72 -> 54.9 and m/z = 75 -> 58 respectively, using 10 eV as the collision energy and 20 V as cone voltage in both cases. The calibration curve of the UPLC-MS/MS method was determined using water standard solutions of acrylamide and acrylamide-d_3 at 0.05, 0.1, 0.3, 0.5, 0.7, 1 μg/mL; a linear regression with R^2 = 0.9996 was obtained using Waters QuanLynx software (LOD 0.02 μg/mL, LOQ 0.05 μg/mL). To verify the quantitative data reliability, all the UPLC-MS/MS analysis have been repeated 6 times and the results were reported as mean value ± SD.

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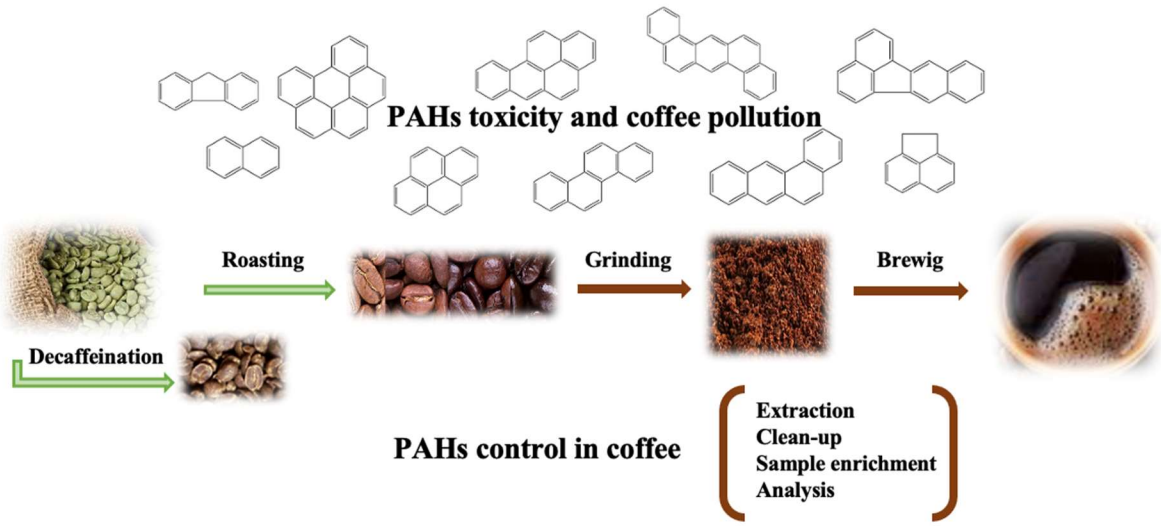
6. Review paper: "Polycyclic aromatic hydrocarbons in coffee samples: Enquiry into processes and analytical methods"



Arianna Binello, Giancarlo Cravotto*, Janet Menzio, Silvia Tagliapietra.
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6.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) make up a group of more than 200 hydrophobic compounds that are formed of two or more fused aromatic rings. Deriving from the incomplete combustion and pyrolysis of organic matter, they are well known to be ubiquitous environmental pollutants that can be introduced into the food chain, and generally occur in complex mixtures. As a result, environmental sources (from air, soil or water), industrial food processing, domestic cooking procedures and, less often, contaminated packaging materials can promote PAHs contamination in food (Harvey, 1997; Purcaro, Moret & Conte, 2016).

PAHs are constantly evaluated by numerous international organisations, the U.S. Agency for Toxic Substances and Disease Registry (ATSDR) and the United States Environmental Protection Agency (US EPA) are required to prepare, and constantly revise, a priority list of substances, and 16 PAHs (see **Table 1** for their names, abbreviations, chemical-physical properties, and IARC group) have been placed onto the list because of their toxicity, occurrence frequency and human exposure.

These compounds are potentially genotoxic and carcinogenic to humans, possibly inducing mainly breast, lung and colon cancers, meaning that the Scientific Committee on Food (SCF) consider them to be a priority group for the assessment of the risk of long-term adverse health effects from dietary intake. In particular, in 2002, this EU organisation recommended that 15 PAHs - namely BaA, BbF, BjF, BkF, BghiP, BaP, CHR, CPP, DBA, DBaeP, DBahP, DBaiP, DBalP, IND and 5-MC, 8 of which are also on the EPA list, should be monitored. Moreover, the SCF indicated, on the basis of PAHs profile in food commodities and carcinogenetic studies in mice (Culp, Gaylor, Sheldon, Goldstein, & Beland, 1998), that BaP is a food marker of PAHs impact. It is worth noting that the Joint FAO/WHO Expert Committee on Food Additives (JECFA) re-evaluated these substances in 2005, and concluded that 13 PAHs, the same as those indicated by the SFC with the exception of BghiP and CPP, are clearly genotoxic and carcinogenic. They confirmed the use of BaP as a marker of exposure to carcinogenic PAHs, and also suggested that benzo [c]fluorene be monitored, as it may contribute to the formation of lung tumours (Stadler & Lineback, 2009).

Results from the detection of PAHs level, as performed by the EFSA (EFSA, 2008) on several food commodities (almost 10,000), found that BaP was detected in about 50% of the analysed samples, and that other carcinogenic and genotoxic PAHs (mainly CHR) were observed in about 30% of the remaining samples, despite the negative test for BaP. Moreover, the EFSA has also grouped these compounds as follows: PAH2 (BaP and CHR), PAH4 (PAH2 plus BaA and BbF), and PAH8 (PAH4 plus BkF, IND, DBA and BghiP).

The European Union (EU) and the EPA have included PAHs in the list of priority pollutants, and their presence in several environmental samples, including soil, water and air has been extensively evaluated (Alegbeleye, Opeolu & Jackson, 2017) as well as their content in different food samples (Kayali-Sayadi, Rubio-Barroso, Beceiro-Roldan and Polo-Dıez, 1999.; Ishizaki, Saito, Hanioka, Narimatsu & Kataoka, 2010; Bansal & Kim, 2015;. Zelinkova & Wenzl, 2015; Adeyeye, 2020) and relative processing (Singh, Varshney and Agarwal, 2016; Gonzalez, Marques, Nadal & Domingo, 2019; Li et al., 2020).

As mentioned above, BaP is the principal marker used to evaluate the occurrence of PAHs in foodstuffs. Moreover, in accordance with the EFSA's conclusions, PAH4 have been indicated as suitable markers for PAHs in food by the European Commission (EFSA, 2008; Commission Regulation (EU) No 835/ 2011). According to this regulation, the sum of PAH markers in foods should not exceed 10 μg/kg for oils and fats, 35 μg/kg for cocoa beans and derived products,

30 µg/kg for meat and fishery products, and 1.0 µg/kg for baby foods and dietary foods for special medical protocols.

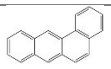
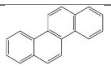
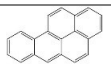
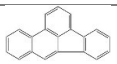
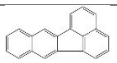
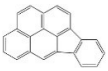
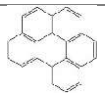
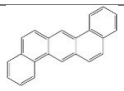
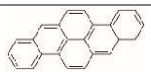
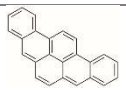
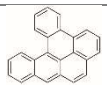
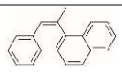
However, to the best of our knowledge, there are no regulations indicating the maximum quantity of PAHs recommended in any beverages, including coffee and coffee substitutes. It must be underlined that the presence of PAHs in food samples may be related to their preservation conditions, cooking (roasting, baking and frying) and manufacture protocols (drying and smoking) and, furthermore, can originate from direct deposition from air and water.

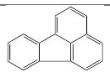
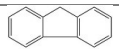
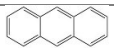
As for coffee, low PAHs level in green coffee beans, i.e., amounts that derive from environmental pollution, have been widely reported in the literature (Tfouni et al., 2013.; Houessou, Maloug, Leveque, Delteil, Heyd & Camel, 2007), which indicates that these compounds are mainly formed during roasting (Badolato et al., 2006; Stanciu, Dobrinas, Birghila & Popescu, 2008).

The pyrolysis of coffee lipids, carbohydrates and amino acids during roasting is thought to lead to PAHs formation, although the complexity of coffee matrices means that it is difficult to formulate schemes that precisely describe the chemical reactions involved (Houessou, Goujot, Heyd & Camel, 2008).

Humans are never exposed to single PAHs, as the substances are always encountered as complex mixtures, which are not always of constant composition. This fact renders health consequences hard to assess. This is also true for coffee, for which the evaluation must take into account the brewing technique and consumption habits of the population. Therefore, the aim of the published review article was that to describe source, chemistry and biological activities of PAHs, discuss PAHs generation during coffee roasting focusing on the several process variables (e.g. temperature, time, coffee cultivar and origin), provide a synopsis on extractive and analytical procedures for PAHs determination on coffee beans and brewing, with a particular attention to the complexity of matrices.

Table 1. Structures of PAHs assessed by US EPA and EU with related IARC group.

		PAH	Abbreviation and Molecular weight (g mol^{-1})	Structure	IARC group[a]	US EPA priority PAHs	EU priority PAHs	Water Solubility (mg L^{-1} at 25°C)
PAH8	PAH4	**Benzo[a]anthracene[c]**	BaA 228.3		2B	*	*	0.011
		Chrysene[c]	CHR 228.3		2B	*	*	0.0019
		Benzo[a]pyrene[c]	BaP 252.3		1	*	*	0.0015
		Benzo[b]fluoranthene[c]	BbF 252.3		2B	*	*	0.0015
		Benzo[k]fluoranthene[c]	BkF 252.3		2B	*	*	0.0008
		Indeno[1,2,3–cd]pyrene[c]	IND 276.3		2B	*	*	0.00019
		Benzo[ghi]perylene[c]	BghiP 276.3		3	*	*	0.00014
		Dibenzo[a,h]anthracene[c]	DBA 278.4		2A	*	*	0.0005
		Benzo[j]fluoranthene[c]	BjF 252.3		2B	-	*	0.0008
		Cyclopenta[cd]pyrene[c]	CPP 226.3		3	-	*	Not specified
		Dibenzo[a,e]pyrene[c]	DBaeP 302.4		3	-	*	Insoluble
		Dibenzo[a,h]pyrene[c]	DBahP 302.4		2B	-	*	Insoluble
		Dibenzo[a,i]pyrene[c]	DBaiP 302.4		2B	-	*	Insoluble
		Dibenzo[a,l]pyrene[c]	DBalP 302.4		2A	-	*	Insoluble
		5-methylchrysene[c]	5-MC 242.3		2B	-	*	Insoluble
		Benzo[c]fluorene[c]	BcF 216.3		3	-	*	Not specified

Fluoranthene[c]	FL 202.3		3	*	-	0.2
Pyrene[c]	Pyr 202.3		3	*	-	0.13
Naphthalene[b]	NAPH 128.1		2B	*	-	31
Acenaphthylene[b]	ACY 152.1		Not assessed	*	-	16
Acenaphthene[b]	ACE 154.2		3	*	-	3.8
Fluorene[b]	FLU 166.2		3	*	-	1.9
Phenanthrene[b]	PHE 178.2		3	*	-	1.1
Anthracene[b]	ANT 178.2		3	*	-	0.04

[a]International Agency for Research on Cancer classification: carcinogenic to humans, group 1; probably carcinogenic to humans, group 2A; possibly carcinogenic to humans, group 2B; not classifiable as to carcinogenicity to humans, group 3. [b]Low molecular weight (light) PAHs; [c]High molecular weight (heavy) PAHs. EFSA classification: [d]PAH4 and [e]PAH8.

6.2. PAHs chemistry and carcinogenic activity

From a toxicological point of view, experimental observations indicate, to date, that a structure in which there are at least four condensed rings is a necessary, but not exhaustive, condition for the carcinogenicity of PAHs. In fact, ring condensation decreases their aromaticity and makes metabolic epoxidation reactions easier, thus leading to the formation of compounds with higher carcinogenicity. In this regard, it should be noted that dihydrodiol epoxides, which derive from their respective PAHs, are the true carcinogens (**Figure 1**). Indeed, PAHs are generally devoid of the electrophilic features that allow covalent interactions to occur with the nucleophilic centres of DNA (nitrogen atoms, oxygen, phosphorus). The metabolic conversion of PAHs by oxidative microsomal systems, however, generates intermediates (epoxides and diol epoxides) that can form highly reactive carbocation species that are, in turn, responsible for the link with DNA.

The carcinogenic activity of BaP is attributed to metabolic products that originate in the liver; it is oxidised, forming BaP-7,8-dihydrodiol- 9,10-epoxide, which is believed to be the actual carcinogenic species that binds to DNA by interfering with its replication mechanism (Miller & Ramos, 2001; van Delft et al., 2010).

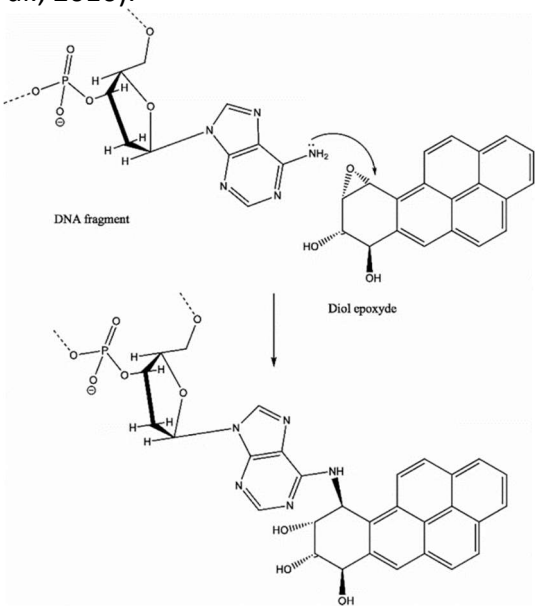


Figure 1. Reaction involving nitrogenous residues of DNA (nucleophile) and a PAH diol epoxide derivative (electrophile).

Albeit the well characterized genotoxicity of PAHs due to the generation of bulky adducts with DNA, more specific molecular features about the DNA damage in cells or tissues still remain to be elucidate (Gao, da Silva, Hou, Denslow, Xiang & Ma, 2018).

Substitutions onto the aromatic rings of PAHs can strongly condition their carcinogenic activity. Halogens decrease or suppress this effect, since dehalogenation allows hydroxy derivatives to be obtained without going through epoxide generation. Conversely, nitro-derivatives are usually stronger carcinogens, and nitration often transforms PAHs into carcinogens. Pyr, for example, is not carcinogenic, while nitropyrene acquires this feature. However, the reactive metabolites of nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are not diol epoxides, but nitro-group reduction products (N-hydroxy derivatives). Nitro-PAHs are widely distributed as environmental contaminants and can be generated both during pyrosynthesis (incomplete combustion processes) and as a consequence of reactions that take place in the atmosphere (Dusek, Hajslova` & Kocourek, 2002).

Furthermore, it has been shown that the pyrolysis of biomass and nitrogen-containing substrates, such as amino acids and proteins, causes nitrogen oxide (NOx) radical precursors to be generated (e.g. HCN and NH_3) (Hansson, Samuelsson, Tullin & Amand, 2004). As the incomplete combustion of organic products and the formation of NO_x are essential for generating nitro-PAHs in food, thermal- treatment conditions for food are crucial.
PAHs can be divided into light (low molecular weight when are formed until three aromatic rings, and heavy (high molecular weight) if their structure includes four or more of them (Vasconcelos, de Franca & Oliveira, 2011). Anyway, in some research works this kind of subdivision is carried out in function of their specific atom mass units: <202 light PAHs, and >202 heavy PAHs (Zelinkova and Wenzl, 2015).
As already specified, the contamination of food with PAHs may be a result of both general environment pollution and heat processing during food production and its preparation for consumption. Roasting, smoking, grilling and direct drying can, in particular, promote PAHs formation (Chung et al., 2011), but the exact mechanism that causes PAH generation is not yet fully understood. Nevertheless, it has been suggested that free-radical reactions, intramolecular additions and small- molecule polymerisation may be involved (Perez, Lilla & Cristalli, 1986).

6.3. Formation of PAHs and derivatives during coffee roasting

Grilled, smoked and roasted foods can all represent an elevated health risk to the consumer, especially when they possess substantial fat content, which is also due to the occurrence of higher levels of PAHs when compared to foods prepared by other cooking methods (Sundararajan, Ndife, Basel & Green, 1999). It is known that fats act as carrier compounds for hydrophobic PAHs and their content in coffee beans, that commonly ranges from 10 to 17%, make the latter subject to this kind of pollution (Ciecierska, Derewiaka, Kowalska, Majewska, Druzynska & Wołosiak, 2019).
Roasting can cause unwanted PAHs (Buffo & Cardelli-Freire, 2004), it has been reported that PHE, BaA and ANT form near 220°C, while a higher temperature (250–260°C) is necessary for the generation of Pyr and CHR (Houessou et al., 2007). Longer roasting times and higher roasting temperatures clearly result in the formation of darker beans and, when cracks appear on bean surfaces, their insides, which are rich in fat molecules, appear, promoting the production of lipophilic PAHs, especially those with higher molecular weights (Yeretzian, Jordan, Badoud & Lindinger, 2002). In particular, the partial degradation of 3-ring PAHs takes place near 260 ◦C; low molecular weight PAHs are transformed into heavy structures and BghiP is generated (Singh, Varshney & Agarwal, 2018). In general, authors reported that DBA and BaP have been found in instant coffee granules, roasted coffee beans and decaffeinated samples while this contamination was not detected when green coffees were analysed. Roasting method, degree and the desired characteristics of the final product are thus strictly related to PAHs generation in coffee.
Ciecierska et al. (2019) have recently studied how mild roasting conditions can affect PAHs contamination in their studies on green Arabica and Robusta coffee beans from different countries. Samples have been roasted with an electric heating system for 25–26 min at temperatures between 125 and 135°C and results (see **Table 2**) showed that the contamination levels of 19 PAHs (15 of which belonging to the SCF list) were mainly due to the lighter analogues (PHE, ANT, FL and Pyr), while the mutagenic and carcinogenic heavy PAHs are present in low percentages (2–24%). Nevertheless, similar qualitative profiles were

reported for both green and roasted beans, and it was calculated that BaP, BaA, BbF and CHR constitute 2–18% of all the examined PAHs in the majority of tested samples.
This study has proven that the roasting procedure can reduce PAHs contamination level. In fact, the high volatility of light PAHs means that the mild conditions tested do not lead to circumstances that are favourable for the generation of heavy PAHs. Nevertheless, the authors do not explicate the influence of their roasting procedure, at lower temperatures, on the organoleptic properties of coffee brews.

Table 2. Effect of mild roasting conditions on samples from different countries: green vs roasted coffee beans PAHs contamination level, standard deviations are not reported (Ciecierska, Derewiaka, Kowalska, Majewska, Druzynska and Wołosiak, 2019).

Arabica Coffee Sample		Sum of 4 light PAHs[a] µg kg^{-1}	Sum of 4 heavy PAHs[b] µg kg^{-1}	Sum of 19 tested PAHs[c] µg kg^{-1}	Robusta Coffee Sample		Sum of 4 light PAHs[a] µg kg^{-1}	Sum of 4 heavy PAHs[b] µg kg^{-1}	Sum of 19 tested PAHs[c] µg kg^{-1}
Brazil	Green	8.52	0.54	9.06	**Cameroon**	Green	56.19	1.42	58.36
	Roasted	4.60	0.38	4.98		Roasted	8.56	0.73	9.29
Colombia	Green	28.19	0.74	28.93	**India**	Green	24.70	0.76	25.46
	Roasted	9.40	0.52	9.92		Roasted	15.23	0.52	15.75
Cuba	Green	8.17	0.49	8.66	**Indonesia**	Green	47.71	0.58	48.52
	Roasted	3.95	0.34	4.29		Roasted	7.88	0.52	8.57
Ethiopia	Green	44.35	0.71	45.06	**Ivory Coast**	Green	75.19	1.22	76.63
	Roasted	5.09	0.32	5.41		Roasted	15.30	0.87	16.17
Indonesia	Green	45.42	1.40	47.32	**Thailand**	Green	30.16	0.68	30.84
	Roasted	4.25	1.03	5.57		Roasted	9.02	0.52	9.54
Kenya	Green	56.53	1.12	57.65	**Uganda**	Green	26.95	0.61	27.56
	Roasted	7.49	0.68	8.17		Roasted	10.55	0.48	11.03
Peru	Green	33.02	0.88	33.90	**Vietnam**	Green	9.26	0.42	9.68
	Roasted	12.56	0.64	13.20		Roasted	5.45	0.32	5.77
Tanzania	Green	57.76	1.65	59.60	**Zaire**	Green	31.68	0.69	32.37
	Roasted	7.39	0.66	8.05		Roasted	9.64	0.48	10.12

[a]PHE, ANT, FL, Pyr;
[b]BaA, CHR, BaP, BbF;
[c]CPP, BaA, CHR, 5-MC, BjF, BbF, BkF, BaP, DBA, BghiP, IND, DBalP, DBaeP, DBaiP, DBahP, PHE, Pyr, ANT, FL.

In 2018 Ko, Das, Kim and Shin (2018) attempted to elucidate the correlation between heating conditions and the presence of amino acids in nitro-PAH formation using a model coffee system. The authors studied coffee beans (sourced from Colombia, Brazil and Vietnam) that were roasted at various combinations of temperature and time: 150, 180, 210, 230 and 250°C for 5, 10 and 20 min. The results of the GC–MS analyses showed how, for all three varieties of coffee analysed, nitro-PAH concentrations gradually increased with increasing temperatures and roasting time. The reported levels of total nitro-PAHs ranged from 0.30 μg kg^{-1}, for the Vietnam variety roasted at 150° C for 5 min, to 5.74 μg kg^{-1} for the Colombia variety roasted at 250°C for 20 min. Trials carried out using model coffee systems, that were submitted to heat with the addition of several amino acids, demonstrated the correlation between the presence of these compounds and nitro-PAH generation, as higher levels of these pollutants were detected. Nevertheless, the mechanisms involved in nitro-PAH formation are still unknown.

6.4. Sample preparation and PAHs analysis

Accurate coffee-sample treatment usually involves solvent extraction followed by a suitable clean-up; accelerated solvent extraction (Rey-Salgueiro, Martinez-Carballo, Garcia-Faicon, Gonzalez-Barreiro & Simal-Gandara, 2009), liquid–liquid extraction (LLE) (Tfouni et al., 2013), Soxhlet extraction (Kukare, Bartkevics & Viksna, 2010; Grover, Sharma, Singh & Pal, 2013), and ultrasound-assisted extraction (UAE) (Orecchio, Paradiso, Ciotti & Culotta, 2009) are among the most popular methods for PAHs recovery, and can make use of solvents such as hex- ane, cyclohexane, dichloromethane, acetone, methanol, acetonitrile and ethyl acetate. Microwave assisted extraction (MAE) has been reported as a good method to extract PAHs from several foodstuffs (Moret, Conchione, Srbinovska & Lucci, 2019), nevertheless, few applications are referred to coffee matrix (Kamalabadi, Mohammadi & Alizadeh, 2018). Solid-phase extraction (SPE) with different stationary phases (e.g. C18, silica, alumina, florisil) (García- Falcón et al., 2005), and column chromatography (Bishnoi, Mehta, Sain, and Pandit, 2005), mainly with silica gel, are commonly the most chosen clean-up strategies.
Their separating capacities means that chromatographic techniques that are coupled with appropriate detection systems can allow PAHs analysis to be performed while avoiding matrix effects. Gas chromatography (GC) coupled with flame ionisation (Grover et al., 2013) or mass spectrometry (MS) (Plaza-Bolanos, Garrido Frenich & Martínez Vidal, 2010), and high performance liquid chromatography (HPLC) coupled with photometric (Bishnoi et al., 2005), fluorimetric (Lee & Shin, 2010) or MS (Rey-Salgueiro et al., 2009) detection are the most appreciated analytical techniques.

6.4.1. Solid coffee samples

In relation to the above information, García- Falcón et al. (2005) have determined seven PAHs in 12 water-soluble instant coffee samples 6 of them decaffeinated (3 of each 6 obtained by natural roasting and the others by roasting with added sugar, namely torrefy). The torrefy samples (also called “Torrefacto” coffee), refer to a particular coffee-bean roasting technique that is commonly applied in some states (e.g. Spain, France, Paraguay, Portugal, Mexico, Costa Rica, Uruguay and Argentina) and that involves the addition of a certain amount of white sugar (typically 20% of coffee weight) in the final stage of the process; thus at high temperature, the sugar burns, coating the beans in a shiny, black film, resulting in dark and bitter coffee. In this work, the authors analysed 7 PAHs in homogenised samples extracted with hexane in a shaker.

Solvent separation through nylon membranes was followed by a clean-up procedure in which the extract was eluted through a silica cartridge. After solvent removal by rotary evaporation, the obtained samples were dissolved in acetonitrile and then analysed using reversed-phase (RP) HPLC with fluorescence detection (FLD), by applying conditions selected to maximise the analyte response, while reducing possible interference. The proposed method gives recovery that is generally higher than 87%, and precision that ranges between 5% and 8%. All tested instant coffee samples showed much lower PAHs level than any potential EU legal restriction (proposal of BaP at 1 μg/kg in foods not yet accepted). In particular, these compounds were not extracted into water during the preparation of instant coffee; BbF, BkF and BaP were found in only two torrefied (high roast) decaffeinated samples at levels of 0.03–0.1 μg/kg for the first and 0.01–0.04 μg/kg for the other two.

On the basis of this previous study, a method for the simultaneous determination of the same PAHs in green coffee beans, roasted coffee beans, instant coffee granules and coffee without caffeine, all from the same producer, has been developed by Stanciu et al. (2008). Each sample was submitted to Soxhlet extraction with hexane and purified using a chromatographic column containing activated aluminium oxide and activated silica/gel (1/1). Analyses carried out by HPLC-FLD indicated a green coffee bean sample and that made up of instant coffee granules as the least and the most contaminated, respectively. When considering the sum of overall PAHs content, obtained values are as follow: 0.023 μg/kg (green coffee beans), 0.196 μg/kg (coffee without caffeine), 1.492 μg/kg (roasted coffee bean) and 2.116 μg/kg (instant coffee granules). BaP was not detected in the green coffee beans, but its presence was found in all the other samples with values in the 0.119 to 0.857 μg/kg range. These results indicate that there is a reduced possibility of contamination from environmental pollution, but confirms the influence of coffee processing, principally roasting procedures.

Different samples of light-, medium- and dark-roasted coffee, including instant and decaffeinated brands, have been evaluated for their content about 18 PAHs (Jimenez, Adisa, Woodham & Saleh, 2014). A rotor mixer and hexane were chosen for coffee extractions and, after centrifugation, supernatant clean-up was performed using SPE (silica gel). HPLC-FLD and the external standard method were used for analyses, and recoveries greater than 80% were reported for the spiked samples. The results validated how PAHs concentration is related to the roasting conditions, as values were higher in the dark-roasted samples and lower in the light- and medium-roasted ones. This study calculated that the sum of the analysed PAHs ranged from 197 μg/kg for instant coffee to 3000 μg/kg for dark-roasted coffee. BaP was observed not to be present in only one of the considered instant coffee samples, while, for the other, the values were: light blend coffees (1.4–3.4 μg/kg), medium blend coffees (2.4–4.9 μg/kg), espresso coffee (3.4 μg/kg), dark blend coffees (3.5–18.5 μg/kg), instant coffee (6 μg/kg) and decaffeinated dark blend coffee (13.7 μg/kg). PHE, FL and Pyr were identified as the major PAHs in the analysed coffees.

Liquid-liquid extraction (LLE) was used to investigate the presence of 7 PAHs in roasted coffee beans (Lee & Shin, 2010). Preliminary saponification with KOH can reduce the presence of lipids, whose amounts can reach up to 15% of the organic-extract composition of coffee. SPE (florisil cartridge) was used for sample purification, analyses were per- formed on an HPLC-FLD system, and recoveries of the spiked samples were found to be between 68 and 91%. The concentrations of the total screened PAHs in 10 commercial coffee samples were found to be from 0.62 to 53.25 μg/kg. In particular, BaA, CHR and BbF were detected in almost all samples, with respective amounts of 0.15–1.57 μg/kg, 0.14–1.90 μg/kg and 0.85–3.90 μg/kg.

Isotope dilution GC–MS has been used in an exploratory study to determine 10 of EPA priority PAHs in 24 commercial samples of roasted coffee that were extracted with simultaneous

purification involving pressurised liquid extraction, liquid–liquid partition, and clean-up by SPE (silica columns) (Pissinatti, Nunes, de Souza, Junqueira & de Souza, 2015). In the proposed method, mean PAHs recovery ranged from 87 to 111%. PAHs contamination (sum of the ten chosen compounds) was foundtobeinarangefrom1.00 μg/kg to11.29 μg/kg, with a BaP contribution in the sample with the highest level of contamination of 0.50 μg/kg.

Micro-extraction techniques are promising because of their capacity to reduce the use of chemicals; multi-walled carbon nanotube-impregnated agarose film microextraction (Loh, Sanagi, Wan Ibrahim and Hasan, 2013), headspace solid-phase microextraction (Viñas, Campillo, Aguinaga, Pérez-Cànovas & Hernàndez-Còrdoba, 2007) and stir-bar sorptive extraction (Zuin, Montero, Bauer & Popp, 2005) are some of these interesting procedures for PAHs determination in tea, but their use with coffee samples is still limited.

Dispersive SPE (e.g. QuEChERS, acronym for Quick, Easy, Cheap, Effective, Rugged and Safe) is an alternative that can be used in the clean-up operations of coffee extracts. Different bulk sorbents with diverse properties are available for this kind of procedure. A satisfactory protocol that provides a combination of different sorbent phases, followed by LLE, has been suggested by Sadowska-Rociek, Surma & Ciéslik (2015) for the determination of several PAHs in 37 commercial coffee samples including ground coffee, instant coffee and coffee substitutes. After extraction, an appropriate combination of sorbent (primary secondary amine PSA, weak anion exchanger NH2 to remove strong acids, and strong anion exchange SAX for the extraction of carboxylic acids) and $MgSO_4$ was used for sample clean-up. Analyses were carried out via GC coupled with an Ion Trap MS detector, and no significant peaks that interfered with the analytes were found in the purified extracts. Results indicated that the PSA and SAX 1/1 combination was the best variant of the clean-up system, with a mean recovery value of all tested PAHs ranging from 70 to 104%.

Magnetic solid phase extraction, which is based on magnetic nanoparticles, has recently been developed as a sample preparation technique; the target analytes can be adsorbed onto magnetic adsorbents that are directly dispersed in the sample solution (Zhao, Lu & Feng, 2013). The separation of nanoparticles is assured by a magnet that is positioned outside the solution container, and an organic solvent can be used for analyte desorption. It is worth noting that the magnetic ad- sorbents can be reused or recycled. Optimised experimental parameters for this technique were applied to rapidly (12 min) extract and enrich 7 PAHs from water solutions of coffee samples (Shi et al., 2016). Fe_3O_4@3-(Trimethoxysilyl)propyl methacrylate@ionic liquid magnetic nano- particles were synthesised and used as adsorbent materials, exploiting their strong π-π bonds and hydrophobic interactions with the organic pollutants. Analytes were desorbed from the isolated particles using small volumes of acetone under sonication. Analyses were performed using HPLC-FLD, and the recoveries of PAHs in spiked coffee samples ranged from 87.5% to 104.5%, while the real commercial Quechao, Guangdong and China coffee samples were found not to be contaminated by the selected analytes.

A fast and environmentally friendly method that is based on a pre- treatment method involving hydrophobic natural deep eutectic solvents (NADES) and ultra-small iron-oxide-based nanoferrofluids has been described for selective PAHs microextraction (Fan et al., 2019). 14 PAHs were determined in 12 coffee-bean samples roasted under different conditions (light, medium, full-city and Italian roasting). Optimal extraction conditions (using menthol, borneol and camphor as NADES, and a nanoferrofluid), which were selected thanks to a predictive model, led to improvements in selectivity and sensitivity the extraction process that only requires a few minutes (less than 3 min) to complete. The reported comparison between the time required by other pre-treatment methods, ranging from 25 to 60 min for

SPME to 15 min for liquid–liquid microextraction (LLME), is interesting. PAHs analysis was accomplished via both HPLC-FLD and GC–MS with consistent results; reported recoveries from spiked samples were between 91.3 and 121%.
MAE and dispersive liquid–liquid microextraction (DLLME) coupled with GC–MS have been applied to simultaneously determine 7 PAHs in various coffee samples, with low organic-solvent consumption (Kamalabadi, Mohammadi & Alizadeh, 2018). Spiked water samples have been microwaved at 520 W for 8 min. The protein component precipitation was then performed by adding Carrez solutions I and II and, after centrifugation, the upper aqueous phase was immediately used for DLLME. Response surface methodology, based on central composite design, was used for microextraction-parameter optimisation. The obtained relative recoveries were reported in the range between 88.1 and 101.3%. The total PAH content obtained was between 0.9 and 2.1 μg/kg.
Ultrasound-assisted extraction (UAE) in an ultrasonic bath has been used by Guatemala-Morales *et al.* (2016) to quantify the EPA's 16 priority-pollutant PAHs in roasted coffee via GC–MS. The highest extraction efficiency from ground roasted coffee was achieved using hexane/methylene chloride, followed by alkaline saponification and sample purification through a silica phase. When considering BaP, these conditions gave a recovery of 68.1% from spiked samples (100 ng/g). In the case of coffee roasted in a spouted bed reactor under different time and temperature conditions, the sum of the screened PAHs was found to be in the range between 3.5 and 16.4 μg/kg, although higher levels were found at higher roasting temperatures.

6.4.2. Coffee brew

There are only around a dozen papers in the literature, to the best of our knowledge, that focus on PAHs content in coffee brews, and the number is even lower if we only consider the last 10 years. Moreover, each work has analysed a different number of compounds, from 4 to 28 analytes, which directly reflects on the sum of PAHs concentration found. Furthermore, each paper has focused on different aspects, such as the extraction of PAHs during brewing, differences in coffee cultivar or origin, and the influence of roasting degree.
The physical and chemical properties of PAHs, e.g. molecular weight and water solubility (data reported in **Table 1**), are the main aspects that influence their transfer into the beverage. Clearly, extraction processes during brewing preparation may lead to there being different PAH patterns in coffee drinks than in the beans or ground coffee.
As BaP is considered a human carcinogen, its content in brewed coffee was studied in one of the very first works by Dekruijf, Schouten and Vanderstegen (1987). They compared two different brewing methods: filtration and traditional (infusion) methods. Due to the low levels of BaP, less than 0,5 μg/kg, found in the 55 roasted-coffee samples analysed, an over-roasted coffee sample with higher BaP content (2 μg/kg) was selected to determine the analyte-extraction yield after brewing. Showing a BaP content of approximately 1 ng/L, and an extraction yield of about 1% in both coffee beverage preparation methods, the authors stated that coffee brewing does not contribute to the daily human intake of BaP in significant way.
The influence of different coffee-manufacturing processes on PAHs brew content has been studied (Kayali-Sayadi et al., 1999) by considering torrefied (3 samples), roasted, decaffeinated and green coffees. Samples that were prepared using an electric coffee maker were analysed via RP-HPLC-FLD to determine the presence of 8 PAH (FL, Pyr, BaA, CHR, benzo[e]pyrene, BaP, DBA and BghiP). With a LOD lower than 0.4 ng/mL (LOQ not reported by authors), the means of the total PAHs concentration in the brews were reported as: 1.65

ng/L for green coffee, 1.99 ng/L for decaffeinated coffee, 2.10 ng/L for roasted and 2.87 ng/L for torrefied coffee, thus proving the influence of this particular roasting process.
More recent works that take into consideration different PAH pools have been carried out with the aim of obtaining a broader description of the pattern of compounds present in different types of samples and brewing methods.
Orecchio et al. (2009) have investigated the levels of 28 PAHs in the coffee brews from 13 coffee samples obtained by means of an aluminium Moka coffeemaker. A method that is based on saponification with a KOH–methanol solution, and LLE with small volumes of hexane was reported for PAHs determination with the exclusion of further steps of purification. Analyses were carried out by GC–MS in single ion monitoring mode, thus increasing sensitivity and selectivity. The analytical procedure indicated an average recovery of above 85%, and a precision of 4–16%. The concentration of the investigated compounds, expressed as the sum of their amounts in coffee brews, varied from 0.52 to 1.8 μg/L. The PAHs-distribution profiles were similar in most of the samples analysed in the study. PHE, ACY and FL were the three most abundant PAHs, and BaP concentration ranged from 0.001 to 0.047 μg/L in all of the brews. Generally, their higher aqueous solubility meant that the low molecular weight compounds are more common. The distribution of light and heavy PAHs was studied as a reliable tool with which to identify the different origins (petrogenic or pyrolytic) of these contaminants, and it was confirmed that those observed in most of the coffee samples originate from high temperature processes.
In a previous work (Bishnoi et al., 2005), the concentration of the EPA's 16 priority pollutant PAHs was evaluated in brews of three different coffee brands submitted to LLE with hexane and to a clean-up through C-18 cartridges; analyses were performed via RP-HPLC with a UV–VIS detector. Results varied from 16.47 to 18.24 μg/L for the total sum of the 16 PAHs. Also in this case, in agreement with Orecchio et al., lower molecular weight compounds dominated the higher molecular weight ones. In particular, the two-ring (NAPH) and three-ring (ACY, ACE, FLU, PHE) analogues were found to be the predominant PAHs. Moreover, BaP was detected in all samples, at an average amount of 0.33 μg/L.
A semi-automated, closed SPE system and GC–MS apparatus have been used to detect and quantify the same PAHs considered by Bishnoi et al. (2005) in a variety of drinks, including 4 samples of coffee (Rascón, Azzouz & Ballesteros, 2019). This method was described as accurate, precise, flexible, fast and sensitive, giving recoveries in a range between 90 and 103%. After beverage preparation in a traditional Italian coffee maker, the pH was adjusted to 4 with the aim of removing the lipid fraction (triacylglycerols, sterols, tocopherols and diterpenes of the kaurene family) and precipitate all the suspended matter. A continuous system that involves RP-C18 as the sorbent (SPE), and that consumes a low volume of organic solvents was then used to clean and enrich each sample before the GC–MS analyses. NAPH, ANT, FL, CHR and BbF were the main PAHs present in a range of concentrations from 0.55 to 4800 ng/L in almost all of the coffee samples, while BaP was only detected in one tested sample at 61 ng/L. The calculated pollutant concentrations did not vary when the PAH4 and PAH8 lists were considered, and were found to be 420–631 ng/L. This is with the sole exception of the brew containing the highest quantity of PAH4, which was found to have a PAH8 concentration of 704 ng/L.
Arabica coffee of different origins (Ethiopia, Colombia) and blended samples have been investigated to determine traces of FLU, BbF and BaP, as model compounds, with the aim of developing a reliable and fast analytical procedure to determine PAHs in coffee-brew samples (Houessou et al., 2005). All coffee brews were obtained using an electric coffee maker, and both non-spiked and spiked samples were extracted using SPE. C18-silica and a polystyrene-

divinylbenzene (PS-DVB) copolymer, were tested as hydrophobic sorbents, and the latter was found to be more suitable. Analyses were performed via RP-HPLC equipped with a FLD. BbF and BaP concentrations were in the 0–95.7 ng/L range, while FLU was not detected due to the method's lack of sensitivity. In general, the reported results showed high variability in PAHs concentration in the coffee samples. In fact, no PAHs presence was detected in the Arabica coffee samples from Colombia and Ethiopia, whereas traces were found in the blended coffee. Later, Houessou et al. (2007) studied Arabica green coffee beans from Cuba to monitor PAHs formation during roasting. Controlled processes were performed by varying the temperature from 180 to 260°C, and both dark and light roasting conditions were used. Coffee brews that were obtained with an electric coffee maker were analysed for a final estimation of PAHs-transfer coefficient to the infusion. After alkaline saponification (KOH), the beverages were submitted to LLE with cyclohexane, followed by a clean-up through SPE on disposable silica cartridges before the HPLC-FLD analyses. For the dark-roasted coffee brews, similarly to the respective ground coffee, PHE, FL and Pyr were reported as the main PAHs, with concentrations of between 0.078 and 0.79 μg/L, while only traces of BaP were detected. Transfer coefficients ranged from 0.6 to 23%, depending on the PAHs and coffee samples. In the light-roasted coffee brews, whose ground coffee presented lower PAHs level due to the shorter roasting time, results were quite similar to those observed for the dark roasted ones, meaning that transfer coefficients were noticeably higher (13.9–32.4%) in this instance.

Further considerations as to the influence of coffee cultivar on PAHs content in brew samples have been reported by Tfouni et al. (2013). This parameter, together with roasting degree and brewing procedure, was taken into account to evaluate the presence of 4 PAHs in ground roasted coffee and their transfer to the brews. Therefore, Coffea arabica and Coffea canephora beans were used to obtain samples at three different roasting degrees (light, medium, dark), and two of the main brewing methods (filtered and boiled) were applied. After clean-up analyses were performed by HPLC coupled with FLD, and results showed that at least one PAH was found in all of the tested coffee brews; detected in 94% and 83% of the samples, respectively, BbF and BaA were reported as the most representative PAHs. The roasting degree had no apparent influence in the total PAHs level in the brews. Briefly, these amounts ranged from 0.015 to 0.105 μg/L and from 0.011 to 0.111 μg/L for samples brewed using *Coffea arabica* and *Coffea canephora* beans, respectively. Moreover, mean PAHs sum values seemed to be independent of the brewing procedure used and were always at levels that would not be expected to affect human health. When considering BaP alone, its transfer seemed to be influenced by roasting degree mainly for Arabica coffee. In fact, its levels ranged from n.d. to 0.016 μg/L for light and medium roasting, whereas, in dark roasted samples, BaP was not detected with both brewing methods. With regards to the Robusta samples, this PAH was found to be n.d. for all roasting degrees and preparation methods, except for medium-roasted coffee brewed via filtration, in which it reached 0.01 μg/L. The authors concluded that caffeine amount (known to be higher in Robusta) does not seem to influence the passage of PAHs to the beverage, despite a previous study affirming that the formation of a caffeine-PAH complex could facilitate this type of transfer (Navarro, Ichikawa, Morimoto & Tatsumi, 2009). PAHs transfer in brews was later studied in coffee beans, at medium- and dark-roasting degrees and instant coffee samples that are commercially available in Denmark, by Duedahl-Olesen, Navaratnam, Jewula and Jensen (2015). The presence of 25 PAHs was investigated, with a focus on PAH4 as indicators. The extraction was performed via pressurised liquid extraction for solid samples, and with hexane under reflux for the brew. GC–MS analyses were performed after clean-up of the obtained samples by gel permeation chromatography (GPC) and SPE in ready-made silica columns.

Considering PAHs transfer to coffee brews, the average percentage of PAH4 was 5–14% of the initial concentrations in the medium- and dark-roasted ground coffees while, unexpectedly considering the data of the coinciding solid samples, 0% transfer was reported for instant coffee. The highest transfer percentages were those reported for samples submitted to lighter roasting conditions, in accordance with the findings of Houessou et al. (2007). Moreover, the margin of exposure (MOE) for coffee for Danish people was calculated using the results obtained for the transfer of PAHs to infusions. In light of the fact that the EFSA have assessed that MOE values for genotoxic and carcinogenic substances of 10,000 or more are of slight concern to public health, it can be stated that none of the MOEs obtained in the above work constitute a risk deriving from coffee brew consumption, as they are all below the critical value. Specifically, the contribution from coffee to PAH4 exposure for Danes was found to be 13% (150 ng/day).

DLLME, a process that is carried out via simple solvent partition thanks to a homogeneously distributed extraction solvent, has been described by Loh et al. (2016) as an easy, rapid, efficient and selective method for PAHs determination in several spiked beverage samples, including coffee. In their system, 1-octanol and acetonitrile were pre- mixed and injected into the coffee beverage solution. After analyte partitioning, the 1-octanol extract was recovered and then analysed via HPLC-FLD. Relative recovery provided the following values: 95.3 (BaP), 95.3 (PHE) and 99.4 (FL).

Yoshioka et al. (2018) have developed a rapid high-sensitivity analytical method that involves supercritical fluid chromatography/atmospheric pressure chemical ionisation-mass spectrometry (SFC/APCI-MS) and simple sample preparation, and analysed 16 PAHs in 11 coffee beverage samples that are commercially available in Japan. Extractions were performed using cyclohexane and a QuEChERS extraction kit. An AL-N (Normal Phase Neutral Alumina) SPE cartridge was chosen for the purification of PAHs as it has retention that is suitable for the recovery of all 16 compounds (82–115%). Interestingly, none of the 16 PAHs analysed, including BaP, were detected in the commercial products.

A study carried out by dos Santos et al. (2019) presents a new analytical method, based on cold fibre solid phase microextraction (CF-SPME) followed by GC–MS analysis, that is useful for the determination not only of PAHs, but also of their oxygenated (oxy-PAHs) and nitrated derivatives (nitro-PAHs). These are compounds that are generally neglected when considering environmental contamination, but that may have higher toxicity than PAHs. The validated CF-SPME-GC/MS method presented satisfactory linearity, as well as good precision and recovery results (82.1 to 96.3%). Brews were obtained from 27 samples of ground and roasted coffee (100% Arabica or blend) by pouring hot water over a filter containing coffee (1/10 w/v). In the obtained samples, the lowest concentration range was found for NAPH, while the highest was for BbF.

As was found in previously reported results, compounds with low water solubility, such as DBA, BghiP, IND and BaP, were not detected. The total concentration of the 16 PAHs screened was found to be between 2.169 and 1.086 µg/L, and, similarly to Bishnoi et al., BkF, BbF, Pyr, ACY and ACE were the most abundant. 5,12-naphthacenquinone and 1-nitropyrene were reported to be the most abundant oxy- and nitro-PAHs, respectively. Specifically, the presence of those derivatives was found to vary between analysed samples; in many brews, nitro-PAH content was higher than that of the oxy- species, and, in some cases, only one of them was detected. Nevertheless, PAHs amount was much higher than that of their derivatives.

6.5. Conclusions

Coffee is made up of a complex food matrix, meaning that its sample preparation, including extraction, clean-up procedures and enrichment processes, is a critical step in PAHs determination. In fact, the co- extractives obtained from coffee beans are a significant limitation in trace-level analysis because of the overlap between these analytes and PAHs peaks that can occur during chromatographic analyses.

National and international regulations all over the world require reliable and valid analytical methods that can guarantee the sensibility, reproducibility, traceability and comparability of obtained results. This is highlighted by the constant advances in both sample preparation and analysis that have been developed in recent years with aim of increasing the accuracy and efficiency of the entire process by using alternative techniques that involve automation and cause reductions in time and solvent consumption. It is important to underline that there is still a lack of regulation on PAHs in coffee, although the EFSA has indicated that this widely consumed commodity is a prominent matrix when considering food contamination by these compounds. Moreover, a realistic assessment of the significance of PAHs content in roasted coffee clearly depends on the ratio of their transfer to coffee brews, which involves the beverage preparation techniques and consumption habits.

As PAHs are ubiquitous pollutant compounds, their occurrence in green coffee beans is mainly due to environmental contamination, and the various studies that have been reviewed herein have indicated that, when present, PAHs content in such matrices is low. An overview of PAHs amount in coffee of different origins can be found in an interesting work by Ciecierska et al. (2019), which demonstrated that Kenya and Tanzania (both Eastern Africa) for Arabica green samples, and the Ivory Coast (south coast of West Africa) and Cameroon (Central Africa) for Robusta samples, were the most contaminated.

When considering roasted coffee, PAHs contamination is mainly attributed to the formation of the substances during the processing phases, including drying and roasting. It is worth noting that process parameters, such as time and temperature, have been found to strongly influence PAHs generation, making them important factors when attempting to avoid or minimise this kind of pollution. Specifically, low molecular PAHs are mainly observed under mild roasting conditions, but these light compounds lead to more toxic heavy PAHs at higher roasting temperatures. From a toxicological point of view, the decaffeination processes seem to positively influence the amounts of PAHs that develop during roasting. In fact, the data reported thus far indicate that lower PAHs content is present in decaffeinated samples, although only a few studies have covered this topic and there is a need for further evaluation. With regard to instant coffee, the reported values are variable and further studies are desirable as it has sometimes been found to be more and, other times, less contaminated than ground roasted coffee.

The PAHs content in coffee brews has generally been observed to be very low, which was expected due to the hydrophobic character of these molecules. Despite the lack of extensive data, the extraction of PAHs clear varies with the brewing methods used; the observed transfer coefficients were variable and less than 35%. Different opinions on how roasting degree influences the transfer of PAHs to the beverages can be found in the bibliography. Some of the works reported herein affirm that dark-roasted coffee demonstrates slightly lower extractability than light- and medium-roasted coffees.

The contents of the main indicators, PAH4 and, particularly, BaP, in coffee brews, as assessed in the literature to date, are very low and sometimes are even not detected, indicating that this beverage does not significantly contribute to the daily human intake of carcinogenic PAHs. However, coffee is a very popular beverage, and coffee-bean roasting and brew-preparation methods can both considerably vary, meaning that further evaluations and insights into PAHs

contamination levels in coffee are still required to assure the safety of the product. Moreover, the development of industrial processes to uniformly roast coffee beans and give minimum PAHs content is desirable, even when coffee beans are then used for other purposes, such as baking.

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Polycyclic aromatic hydrocarbons in coffee samples: Enquiry into processes and analytical methods

Arianna Binello, Giancarlo Cravotto *, Janet Menzio, Silvia Tagliapietra

Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, via P. Giuria 9, 10235 Turin, Italy

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are considered to be potentially genotoxic and carcinogenic in humans. These ubiquitous environmental pollutants may derive from the incomplete combustion and pyrolysis of organic matter. Coffee is an extensively consumed drink, and its PAHs contamination is not only ascribed to environmental pollution, but mainly to the roasting processes. Although no fixed limits have yet been set for residual PAHs in coffee, the present review intends to summarise and discuss the knowledge and recent advances in PAHs formation during roasting. Because coffee origin and brewing operations may affect PAHs content, we thoroughly analysed the literature on extraction and purification procedures, as well as the main analytical chromatographic methods for both coffee powders and brews. With regards to the safety of this appreciated commodity, the control on the entire production chain is desirable, because of coffee beverage could contribute to the daily human intake of PAHs.

1. Introduction

In the modern day, a constantly growing amount of attention is being focused on the constituents of the human diet and the analytical monitoring of pollutants in food and beverages, in order to avoid health risks.

Polycyclic aromatic hydrocarbons (PAHs) make up a group of more than 200 hydrophobic compounds that are formed of two or more fused aromatic rings. Deriving from the incomplete combustion and pyrolysis of organic matter, they are well known to be ubiquitous environmental pollutants that can be introduced into the food chain, and generally occur in complex mixtures. As a result, environmental sources (from air, soil or water), industrial food processing, domestic cooking procedures and, less often, contaminated packaging materials can promote PAHs contamination in food (Harvey, 1997; Purcaro, Moret & Conte, 2016).

PAHs are constantly evaluated by numerous organisations, such as the International Programme on Chemical Safety (IPCS), the Scientific Committee on Food (SCF), the United States Environmental Protection Agency (US EPA), the European Food Safety Authority (EFSA), the WHO International Agency for Research on Cancer (IARC), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The U.S. Agency for

Abbreviations: AL-N, Normal Phase Neutral Alumina; ATSDR, U.S. Agency for Toxic Substances and Disease Registry; CF-SPME, cold fibre solid phase microextraction; CONTAM Panel, EFSA Panel on Contaminants in the Food Chain; DLLME, dispersive liquid-liquid microextraction; DMF, N,N-dimethylformamide; EFSA, European Food Safety Authority; EU, European Union; FAO, Food and Agriculture Organisation of the United Nations; FLD, fluorescence detection; GC–MS, gas chromatography-mass spectrometry; GPC, gel permeation chromatography; HPLC-FLD/DAD, high-performance liquid chromatography with fluorescence and diode array detectors; IARC, International Agency for Research on Cancer (World Health Organisation); IPCS, International Programme on Chemical Safety; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LLE, liquid-liquid extraction; LLME, liquid-liquid microextraction; LOD, limit of detection; LOQ, limit of quantification; MAE, microwave-assisted extraction; MOE, margin of exposure; NADES, natural deep eutectic solvents; n.d., not detected; PAHs, polycyclic aromatic hydrocarbons; PAH2, BaP, and CHR; PAH4 PAH2 plus BaA, and BbF; PAH8, PAH4 plus BkF IND DBA and BghiP for the other PAHs abbreviations see Table 1; QuEChERS, Quick, Easy, Cheap, Effective, Rugged, and Safe; PSA, primary secondary amine; PS-DVB, polystyrene-divinylbenzene; RP-HPLC, reverse-phase high performance liquid chromatography; SCF, Scientific Committee on Food; *SEC*, size exclusion chromatography SFC/APCI-MS supercritical fluid chromatography/atmospheric pressure chemical ionization-mass spectrometry; SPE, solid-phase extraction; SPME, solid-phase microextraction; S-X3, neutral porous styrene divinylbenzene copolymer bead; SAX, strong anion exchange; UAE, ultrasound-assisted extraction; US EPA, United States Environmental Protection Agency; WHO, World Health Organisation.

* Corresponding author.
E-mail addresses: arianna.binello@unito.it (A. Binello), giancarlo.cravotto@unito.it (G. Cravotto), janet.menzio@unito.it (J. Menzio), silvia.tagliapietra@unito.it (S. Tagliapietra).

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Toxic Substances and Disease Registry (ATSDR) and the US EPA are required to prepare, and constantly revise, a priority list of substances, and 16 PAHs (see Table 1 for their names, abbreviations, chemical-physical properties, and IARC group) have been placed onto the list because of their toxicity, occurrence frequency and human exposure.

These compounds are potentially genotoxic and carcinogenic to humans, possibly inducing mainly breast, lung and colon cancers, meaning that the SCF consider them to be a priority group for the assessment of the risk of long-term adverse health effects from dietary intake. In particular, in 2002, this EU organisation recommended that 15 PAHs - namely BaA, BbF, BjF, BkF, BghiP, BaP, CHR, CPP, DBA, DBaeP, DBahP, DBaiP, DBalP, IND and 5-MC, 8 of which are also on the EPA list - should be monitored. Moreover, the SCF indicated, on the basis of PAHs profile in food commodities and carcinogenetic studies in mice (Culp, Gaylor, Sheldon, Goldstein, & Beland, 1998), that BaP is a food marker of PAHs impact. It is worth noting that the JECFA re-evaluated these substances in 2005, and concluded that 13 PAHs, the same as those indicated by the SFC with the exception of BghiP and CPP, are clearly genotoxic and carcinogenic. They confirmed the use of BaP as a marker of exposure to carcinogenic PAHs, and also suggested that benzo [*c*]fluorene be monitored, as it may contribute to the formation of lung tumours (Stadler & Lineback, 2009).

Results from the detection of PAHs level, as performed by the EFSA (EFSA, 2008) on several food commodities (almost 10,000), found that BaP was detected in about 50% of the analysed samples, and that other carcinogenic and genotoxic PAHs (mainly CHR) were observed in about 30% of the remaining samples, despite the negative test for BaP. Moreover, the EFSA has also grouped these compounds as follows: PAH2 (BaP and CHR), PAH4 (PAH2 plus BaA and BbF), and PAH8 (PAH4 plus BkF, IND, DBA and BghiP).

The lack of data from studies on the oral carcinogenicity of singular PAH has driven the CONTAM Panel (EFSA Panel on Contaminants in the Food Chain) to indicate PAH8 as possible indicators of the carcinogenic effects of these compounds in food, either individually or in combination. This means that DBA, BbF, IND, BghiP, CHR, BkF, BaA and obviously BaP are the main target molecules to be evaluated. In further studies, the CONTAM Panel concluded that the screening of PAH4 alone can provide exhaustive data.

The European Union (EU) and the EPA have included PAHs in the list of priority pollutants, and their presence in several environmental samples, including soil, water and air has been extensively evaluated (Alegbeleye, Opeolu & Jackson, 2017) as well as their content in different food samples (Kayali-Sayadi, Rubio-Barroso, Beceiro-Roldan and Polo-Dıez, 1999.; Ishizaki, Saito, Hanioka, Narimatsu & Kataoka, 2010; Bansal & Kim, 2015;. Zelinkova & Wenzl, 2015; Adeyeye, 2020) and relative processing (Singh, Varshney and Agarwal, 2016; Gonzalez, Marques, Nadal & Domingo, 2019; Li et al., 2020).

As mentioned above, BaP is the principal marker used to evaluate the occurrence of PAHs in foodstuffs. Moreover, in accordance with the EFSA's conclusions, PAH4 have been indicated as suitable markers for PAHs in food by the European Commission (EFSA, 2008; Commission Regulation (EU) No 835/ 2011). According to this regulation, the sum of PAH markers in foods should not exceed 10 μg/kg for oils and fats, 35 μg/kg for cocoa beans and derived products, 30 μg/kg for meat and fishery products, and 1.0 μg/kg for baby foods and dietary foods for special medical protocols. However, to the best of our knowledge, there are no regulations indicating the maximum quantity of PAHs recommended in any beverages, including coffee and coffee substitutes. It must be underlined that the presence of PAHs in food samples may be related to their preservation conditions, cooking (roasting, baking and frying) and manufacture protocols (drying and smoking) and, furthermore, can originate from direct deposition from air and water.

Coffee is a principal food commodity as it is the most frequently consumed beverage worldwide. It is made from the roasted seeds of the *Coffea* plant, and mainly two coffee species are successfully employed in commercial cultivation: *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta).

Roasting is clearly a crucial step, and leads to the development of colour, aroma and flavour, which are essential for product quality. However, we must consider that this process may also lead to the formation of undesirable compounds, including PAHs (Chu, 2012), that might cause potential health risks for consumers.

Low PAHs level in green coffee beans, i.e. amounts that derive from environmental pollution, have been widely reported in the literature (Tfouni et al., 2013.; Houessou, Maloug, Leveque, Delteil, Heyd & Camel, 2007), which indicates that these compounds are mainly formed during roasting (Badolato et al., 2006; Stanciu, Dobrinas, Birghila & Popescu, 2008), a process that is typically performed at temperatures in the range of 120–240 °C, with processing times that can vary from a few minutes to about half an hour (Farah, 2019).

The pyrolysis of coffee lipids, carbohydrates and amino acids during roasting is thought to lead to PAHs formation, although the complexity of coffee matrices means that it is difficult to formulate schemes that precisely describe the chemical reactions involved (Houessou, Goujot, Heyd & Camel, 2008).

Roasting conditions vary according to the expected characteristics of the final product and the type of roasters used. Temperature and time must therefore be optimised to achieve the desired coffee features, but also to prevent the formation of undesired compounds. Changing roasting conditions can lead to coffee having different features and PAHs content.

Humans are never exposed to single PAHs, as the substances are always encountered as complex mixtures, which are not always of constant composition. This fact renders health consequences hard to assess. This is also true for coffee, for which the evaluation must take into account the brewing technique and consumption habits of the population. Therefore, coffee is among the foods for which the presence PAHs has been checked, and that require further consideration.

To the best of our knowledge, though several information are available in the aforementioned reviews, so far an overview focused to the presence of PAHs in coffee in all its variation (green beans, roasted beans, different beverages), and dealing with the relevant impact of the roasting process on the occurrence of these contaminants, has not yet been published.

The present survey is therefore aimed to:

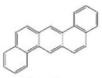
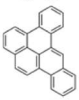
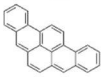
- describe source, chemistry and biological activities of PAHs;
- discuss PAHs generation during coffee roasting focusing on the several process variables (e.g. temperature, time, coffee cultivar and origin);
- provide a synopsis on extractive and analytical procedures for PAHs determination on coffee beans and brewing, with a particular attention to the complexity of matrices.

Literature concerning PAHs in food includes more than 2900 articles, between them around 480 reviews. This overview, should help to shed light on current knowledge on PAHs control in coffee on the base of the literature of the last ten years (Web of Science Core Collection (Clarivate Analytics) and SciFinder® have been chosen as scientific information platforms). In particular, PAHs occurrence in coffee roasting, in coffee brew and their analysis have been chosen as criteria for the selection of the literature to be revised using PAHs and coffee as keywords.

2. PAHs chemistry and carcinogenic activity

PAHs are naturally present in fossil carbon and oil, from which they are extracted. The anthropogenic production of these atmospheric pollutants occurs in the course of the incomplete combustion of fossil fuels, wood, fats, foliage, incense and organic compounds in general. It follows that PAHs can be found in the combustion fumes of urban waste and biomass and in tobacco smoke (Ali et al., 2020; Olsen et al., 2020; Zhang et al., 2020; Bandowe & Meusel, 2017). However, they are also present

Table 1
Structures of PAHs assessed by US EPA and EU with related IARC group.

PAH			Abbreviation and Molecular weight (g mol^{-1})		Structure	IARC group[a]	US EPA priority PAHs	EU priority PAHs	Water Solubility (mg L^{-1} at 25 °C)
PAH8	**PAH4**	**Benzo [a] anthracene**[c]	BaA	228.3		2B	*	*	0.011
		Chrysene[c]	CHR	228.3		2B	*	*	0.0019
		Benzo [a] pyrene[c]	BaP	252.3		1	*	*	0.0015
		Benzo [b] fluoranthene[c]	BbF	252.3		2B	*	*	0.0015
		Benzo [k] fluoranthene[c]	BkF	252.3		2B	*	*	0.0008
		Indeno [1,2,3–cd] pyrene[c]	IND	276.3		2B	*	*	0.00019
		Benzo [ghi] perylene[c]	BghiP	276.3		3	*	*	0.00014
		Dibenzo [a,h] anthracene[c]	DBA	278.4		2A	*	*	0.0005
		Benzo [j] fluoranthene[c]	BjF	252.3		2B	–	*	0.0008
		Cyclopenta [cd] pyrene[c]	CPP	226.3		3	–	*	Not specified
		Dibenzo [a,e] pyrene[c]	DBaeP	302.4		3	–	*	Insoluble
		Dibenzo [a,h] pyrene[c]	DBahP	302.4		2B	–	*	Insoluble
		Dibenzo [a,i] pyrene[c]	DBaiP	302.4		2B	–	*	Insoluble
		Dibenzo [a,l] pyrene[c]	DBalP	302.4		2A	–	*	Insoluble
		5-methylchrysene[c]	5-MC	242.3		2B	–	*	Insoluble
		Benzo [c] fluorene[c]	BcF	216.3		3	–	*	Not specified
		Fluoranthene[c]	FL	202.3		3	*	–	0.2
		Pyrene[c]	Pyr	202.3		3	*	–	0.13
		Naphthalene[b]	NAPH	128.1		2B	*	–	31
		Acenaphthylene[b]	ACY	152.1		Not assessed	*	–	16
		Acenaphthene[b]	ACE	154.2		3	*	–	3.8
		Fluorene[b]	FLU	166.2		3	*	–	1.9

(*continued on next page*)

Table 1 (*continued*)

PAH	Abbreviation and Molecular weight (g mol^{-1})		Structure	IARC group[a]	US EPA priority PAHs	EU priority PAHs	Water Solubility (mg L^{-1} at 25 °C)
Phenanthrene[b]	PHE	178.2		3	*	–	1.1
Anthracene[b]	ANT	178.2		3	*	–	0.04

[a] International Agency for Research on Cancer classification: carcinogenic to humans, group 1; probably carcinogenic to humans, group 2A; possibly carcinogenic to humans, group 2B; not classifiable as to carcinogenicity to humans, group 3.
[b] Low molecular weight (light) PAHs.
[c] High molecular weight (heavy) PAHs.

in food cooked at high temperatures with carbonisation processes, such as grilled meat or smoked fish (Afe et al., 2020; Singh et al., 2016).

From a toxicological point of view, experimental observations indicate, to date, that a structure in which there are at least four condensed rings is a necessary, but not exhaustive, condition for the carcinogenicity of PAHs. In fact, ring condensation decreases their aromaticity and makes metabolic epoxidation reactions easier, thus leading to the formation of compounds with higher carcinogenicity. In this regard, it should be noted that dihydrodiol epoxides, which derive from their respective PAHs, are the true carcinogens (Fig. 1). Indeed, PAHs are generally devoid of the electrophilic features that allow covalent interactions to occur with the nucleophilic centres of DNA (nitrogen atoms, oxygen, phosphorus). The metabolic conversion of PAHs by oxidative microsomal systems, however, generates intermediates (epoxides and diol epoxides) that are capable of forming highly reactive carbocation species that are, in turn, responsible for the link with DNA. The thus-formed addition products cause a distortion in the double helix structure of DNA, compromising its function and promoting the locking of the adduct in a stable genetic alteration.

The carcinogenic activity of BaP is attributed to metabolic products

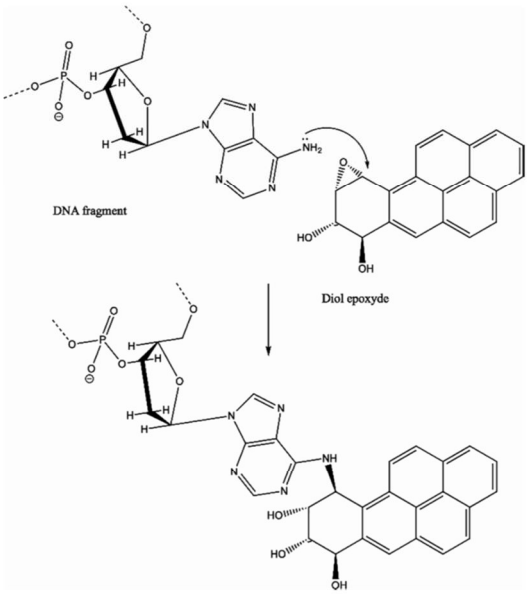


Fig. 1. Reaction involving nitrogenous residues of DNA (nucleophile) and a PAH diol epoxide derivative (electrophile).

that originate in the liver; it is oxidised, forming BaP-7,8-dihydrodiol-9,10-epoxide, which is believed to be the actual carcinogenic species that binds to DNA by interfering with its replication mechanism. (Miller & Ramos, 2001; van Delft et al., 2010)

Inhalation, ingestion and dermal contact represent the three main pathways of exposure for humans (Ma & Harrad, 2015; Ruby et al., 2016). Noteworthy, PAHs are also involved in inflammatory processes (Ferguson et al., 2017), diabetes (Yang et al., 2017), cardiovascular disease (Jomova, Baros, & Valko, 2012), poor fetal development (Sexton et al., 2011), autoimmune diseases, and in atherosclerosis (Henkler, Stolpmann, & Luch, 2012). Structural features named as "fjord" and "bay" regions are considered as related to PAHs biological activities (Fig. 2). In particular, the more studied bay region is a topological indentation in which are involved four carbons and three carbon–carbon bonds, as shown by PHE and BaP. Furthermore, the fjord region is an indentation involving five carbons and four carbon–carbon bonds present for example in DBalP, which is considered the most carcinogenic PAH occurring in the environment (International Agency for Research on Cancer IARC, 2010). PAHs with fjord regions are however rare and have not been included in the US priority pollutants EPA (Ewa & Danuta, 2017).

Albeit the well characterized genotoxicity of PAHs due to the generation of bulky adducts with DNA, more specific molecular features about the DNA damage in cells or tissues still remain to be elucidate (Gao, da Silva, Hou, Denslow, Xiang & Ma, 2018).

Substitutions onto the aromatic rings of PAHs can strongly condition their carcinogenic activity. Halogens decrease or suppress this effect, since dehalogenation allows hydroxy derivatives to be obtained without going through epoxide generation. Conversely, nitro-derivatives are usually stronger carcinogens, and nitration often transforms PAHs into carcinogens. Pyr, for example, is not carcinogenic, while nitropyrene acquires this feature. However, the reactive metabolites of nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are not diol epoxides, but nitro-group reduction products (*N*-hydroxy derivatives). Nitro-PAHs are widely distributed as environmental contaminants and can be generated both during pyrosynthesis (incomplete combustion processes)

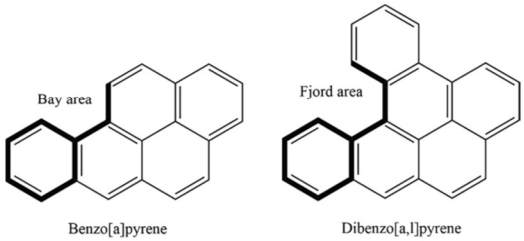


Fig. 2. Examples of "bay" and "fjord" areas in PAHs.

and as a consequence of reactions that take place in the atmosphere (Dusek, Hajslovà & Kocourek, 2002). PAHs that are adsorbed onto air particulates can react with nitrogen oxides, and thus lead to a range of diverse nitro derivatives. Furthermore, it has been shown that the pyrolysis of biomass and nitrogen-containing substrates, such as amino acids and proteins, causes nitrogen oxide (NOx) radical precursors to be generated (e.g. HCN and NH_3) (Hansson, Samuelsson, Tullin & Amand, 2004). As the incomplete combustion of organic products and the formation of NOx are essential for generating nitro-PAHs in food, thermal-treatment conditions for food are crucial.

PAHs can be divided into light (low molecular weight when are formed until three aromatic rings, and heavy (high molecular weight) if their structure includes four or more of them (Vasconcelos, de Franca & Oliveira, 2011). Anyway, in some research works this kind of subdivision is carried out in function of their specific atom mass units: <202 light PAHs, and >202 heavy PAHs (Zelinkova and Wenzl, 2015). High molecular weight PAHs, such as benzo[*e*]pyrene and BaP, are present in high quantities in tar, bitumen, pitch and in related products, such as asphalt. Moreover, they are also found in carbon black and soot. Light PAHs, such as NAPH and FLU, are ubiquitous pollutants which, due to their relative greater solubility in water, can pollute underground aquifers.

As already specified, the contamination of food with PAHs may be a result of both general environment pollution and heat processing during food production and its preparation for consumption. Roasting, smoking, grilling and direct drying can, in particular, promote PAHs formation (Chung et al., 2011), but the exact mechanism that causes PAH generation is not yet fully understood. Nevertheless, it has been suggested that free-radical reactions, intramolecular additions and small-molecule polymerisation may be involved (Perez, Lilla & Cristalli, 1986). In addition, mutagenic PAHs (e.g. PHE, FLU, methylphenanthrene and derivatives) may derive from the thermal degradation of steroids, in particular, when cholesterol, androsterone and stigmasterol (Fig. 3) are involved (Christy, Lian & Francis, 2011).

3. Formation of PAHs and derivatives during coffee roasting

Grilled, smoked and roasted foods can all represent an elevated health risk to the consumer, especially when they possess substantial fat content, which is also due to the occurrence of higher levels of PAHs when compared to foods prepared by other cooking methods (Sundararajan, Ndife, Basel & Green, 1999). It is known that fats act as carrier compounds for hydrophobic PAHs and their content in coffee beans, that commonly ranges from 10 to 17%, make the latter subject to this kind of pollution (Ciecierska, Derewiaka, Kowalska, Majewska, Druzynska & Wołosiak, 2019). Berry selection, fermentation, drying and roasting make up the entire coffee process; they cause moisture and weight loss and involve phenomena such as the Maillard reaction, caramelisation as well as fat and sugar degradation (Starowicz & Zielinski, 2019).

Many complex and still poorly elucidated reactions originate as a result of the roasting process as a whole, and lead to chemical and physical changes that are fundamental to the development of coffee's enjoyable sensorial qualities.

Roasting degree is industrially defined usually by a bean's colour, and this process can generally be divided into several steps. The first stage is the drying process, which takes place at a temperature of up to 150 °C. At this moment, most of the water contained in the beans is removed, leading to a weight loss of approximately 8–10%; the temperature is too low to cause pyrolysis but, despite this, intermediate Maillard-reaction compounds and aroma start to develop. The actual initial step of roasting ranges between 150 and 180 °C, during which Maillard products are formed, and beans become darker. At temperatures between 180 and 230 °C, coffee "cracks" happen; at this stage, pyrolysis takes place and involves exothermal reactions that degrade the substances present in coffee. More than 900 volatile compounds, together with carbohydrates, low molecular weight acids, trigonelline, lipids, caffeine and melanoidins are present in the roasted coffee beans, but unwanted PAHs also appear (Buffo & Cardelli-Freire, 2004). It has been reported that PHE, BaA and ANT form near 220 °C, while a higher temperature (250–260 °C) is necessary for the generation of Pyr and CHR (Houessou et al., 2007). Longer roasting times and higher roasting temperatures clearly result in the formation of darker beans and, when cracks appear on bean surfaces, their insides, which are rich in fat molecules, appear, promoting the production of lipophilic PAHs, especially those with higher molecular weights (Yeretzian, Jordan, Badoud & Lindinger, 2002). In particular, the partial degradation of 3-ring PAHs takes place near 260 °C; low molecular weight PAHs are transformed into heavy structures and BghiP is generated (Singh, Varshney & Agarwal, 2018). These authors have reported that low or moderate amounts of PAHs are extracted from roasted coffee during infusion. In general, DBA and BaP have been found in instant coffee granules, roasted coffee beans and decaffeinated samples while this contamination was not detected when green coffees were analysed, which demonstrates the importance of the roasting processes. Moreover, whereas dark-roasted coffee showed the highest PAHs levels, decaffeinated and often instant samples too gave lower amounts. Roasting method, degree and the desired characteristics of the final product are thus strictly related to PAHs generation in coffee.

Ciecierska et al. (2019) have recently studied how mild roasting conditions can affect PAHs contamination in their studies on green Arabica and Robusta coffee beans from different countries. Samples have been roasted with an electric heating system for 25–26 min at temperatures between 125 and 135 °C and results (see Table 3 for analytical conditions) showed that the contamination levels of 19 PAHs (15 of which belonging to the SCF list) were mainly due to the lighter analogues (PHE, ANT, FL and Pyr), while the mutagenic and

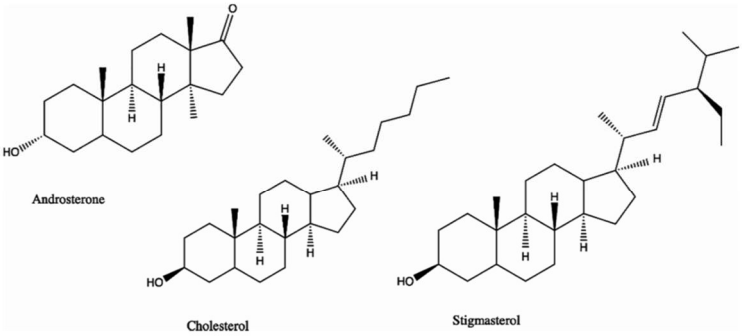


Fig. 3. Structure of androsterone, cholesterol and stigmasterol.

Table 2
Effect of mild roasting conditions on samples from different countries: green *vs* roasted coffee beans PAHs contamination level, standard deviations are not reported (Ciecierska et al., 2019).

Arabica Coffee Sample		Sum of 4 light PAHs[a] μg kg^{-1}	Sum of 4 heavy PAHs[b] μg kg^{-1}	Sum of 19 tested PAHs[c] μg kg^{-1}	Robusta Coffee Sample		Sum of 4 light PAHs[a] μg kg^{-1}	Sum of 4 heavy PAHs[b] μg kg^{-1}	Sum of 19 tested PAHs[c] μg kg^{-1}
Brazil	Green	8.52	0.54	9.06	**Cameroon**	Green	56.19	1.42	58.36
	Roasted	4.60	0.38	4.98		Roasted	8.56	0.73	9.29
Colombia	Green	28.19	0.74	28.93	**India**	Green	24.70	0.76	25.46
	Roasted	9.40	0.52	9.92		Roasted	15.23	0.52	15.75
Cuba	Green	8.17	0.49	8.66	**Indonesia**	Green	47.71	0.58	48.52
	Roasted	3.95	0.34	4.29		Roasted	7.88	0.52	8.57
Ethiopia	Green	44.35	0.71	45.06	**Ivory Coast**	Green	75.19	1.22	76.63
	Roasted	5.09	0.32	5.41		Roasted	15.30	0.87	16.17
Indonesia	Green	45.42	1.40	47.32	**Thailand**	Green	30.16	0.68	30.84
	Roasted	4.25	1.03	5.57		Roasted	9.02	0.52	9.54
Kenya	Green	56.53	1.12	57.65	**Uganda**	Green	26.95	0.61	27.56
	Roasted	7.49	0.68	8.17		Roasted	10.55	0.48	11.03
Peru	Green	33.02	0.88	33.90	**Vietnam**	Green	9.26	0.42	9.68
	Roasted	12.56	0.64	13.20		Roasted	5.45	0.32	5.77
Tanzania	Green	57.76	1.65	59.60	**Zaire**	Green	31.68	0.69	32.37
	Roasted	7.39	0.66	8.05		Roasted	9.64	0.48	10.12

[a] PHE, ANT, FL, Pyr.
[b] BaA, CIIR, BaP, BbF,
[c] CPP, BaA, CHR, 5-MC, BjF, BbF, BkF, BaP, DBA, BghiP, IND, DBalP, DBaeP, DBaiP, DBahP, PHE, Pyr, ANT,FL.

carcinogenic heavy PAHs are present in low percentages (2–24%). Nevertheless, similar qualitative profiles were reported for both green and roasted beans, and it was calculated that BaP, BaA, BbF and CHR constitute 2–18% of all the examined PAHs in the majority of tested samples. Interestingly, for green beans, the lowest content of total PAHs tested was observed for Arabica and Robusta beans from Cuba (8.66 μg kg^{-1}) and Vietnam (9.68 μg kg^{-1}) respectively, while Arabica from Tanzania (59.60 μg kg^{-1}) and Robusta from Ivory Coast (76.63 μg kg^{-1}) showed the highest amounts. As regards roasted coffee, Arabica-sample contamination was found to be in a range between 4.29 μg kg^{-1} (Cuba) and 13.20 μg kg^{-1} (Peru), whereas, in the case of Robusta, values were found to range from 5.77 μg kg^{-1} (Vietnam) to 16.17 μg kg^{-1} (Ivory coast), thus showing that levels are lower than those of their raw equivalents (Table 2). This articulated study has proven that the roasting procedure can reduce PAHs contamination level. In fact, the high volatility of light PAHs means that the mild conditions tested do not lead to circumstances that are favourable for the generation of heavy PAHs. Nevertheless, the authors do not explicate the influence of their roasting procedure, at lower temperatures, on the organoleptic properties of coffee brews. In Table 2 we reported data as indicated by Ciecierska et al. (2019), who consider Pyr and FL as light PAHs even if their atom mass units is slightly higher than 202 and they are formed by four aromatic rings.

In 2018 Ko, Das, Kim and Shin (2018) attempted to elucidate the correlation between heating conditions and the presence of amino acids in nitro-PAH formation using a model coffee system. The authors studied coffee beans (sourced from Colombia, Brazil and Vietnam) that were roasted at various combinations of temperature and time: 150, 180, 210, 230 and 250 °C for 5, 10 and 20 min . The results of the GC–MS analyses showed how, for all three varieties of coffee analysed, nitro-PAH concentrations gradually increased with increasing temperatures and roasting time. The reported levels of total nitro-PAHs ranged from 0.30 μg kg^{-1}, for the Vietnam variety roasted at 150° C for 5 min, to 5.74 μg kg^{-1} for the Colombia variety roasted at 250 °C for 20 min. Trials carried out using model coffee systems, that were submitted to heat with the addition of several amino acids, demonstrated the correlation between the presence of these compounds and nitro-PAH generation, as higher levels of these pollutants were detected (Fig. 4). Moreover, an electron

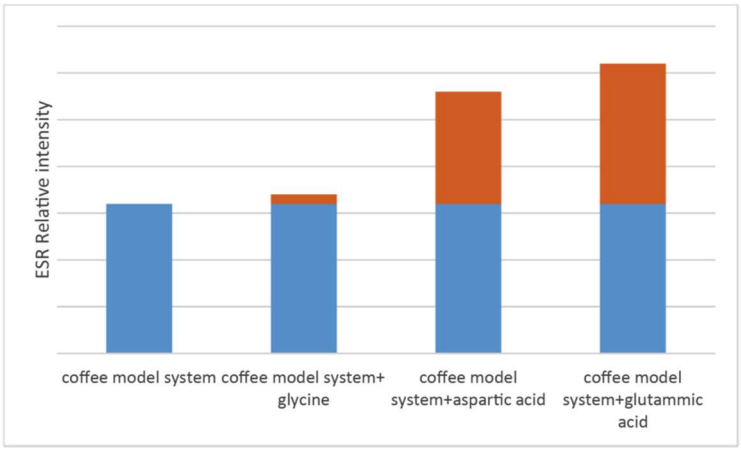


Fig. 4. ESR relative intensity in coffee model systems heated with the addition of several amino acids.

spin resonance (ESR) experiment led to the hypothesis that free radicals may be engaged in this pathway; as shown in Fig. 4, the ESR signal intensity were increased in the coffee model system when amino acids were added and results were correlated to the outcomes on the formation of nitro-PAHs. Nevertheless, the mechanisms involved in nitro-PAH formation are still unknown.

4. Sample preparation and PAHs analysis

4.1. General considerations

The presence of pollutants or contaminants in food matrices has to be monitored in compliance with permitted maximum residual limits. In order to obtain the complete release of the target molecules, often present in traces, it is commonly necessary to perform careful and laborious preparation, together with an enrichment of the sample, before setting up the appropriate analytical procedure to discern the target from interfering substances.

Ground roasted coffees are commonly produced either entirely with Arabica (*Coffea arabica)* species, or with a blend of the latter and Robusta (*Coffea canephora)*, and dark roasted coffee is the most popular and the main commercialised type. There are different procedures that are used for both coffee roasting and brewing, and they may influence the transfer of PAHs to the beverage that is directly ingested by the consumer.

An efficient chromatographic system, with low limits of detection (LOD) and quantification (LOQ), obviously requires analytical samples to be as clean as possible. Furthermore, great attention must be paid to eco-sustainable procedures that allow the use of organic solvents to be reduced and favours the choice of green replacements.

Accurate coffee-sample treatment usually involves solvent extraction followed by a suitable clean-up; accelerated solvent extraction (Rey-Salgueiro, Martinez-Carballo, Garcia-Faicon, Gonzalez-Barreiro & Simal-Gandara, 2009), liquid–liquid extraction (LLE) (Tfouni et al., 2013), Soxhlet extraction (Kukare, Bartkevics & Viksna, 2010; Grover, Sharma, Singh & Pal, 2013), and ultrasound-assisted extraction (UAE) (Orecchio, Paradiso, Ciotti & Culotta, 2009) are among the most popular methods for PAHs recovery, and can make use of solvents such as hexane, cyclohexane, dichloromethane, acetone, methanol, acetonitrile and ethyl acetate. Microwave assisted extraction (MAE) has been reported as a good method to extract PAHs from several foodstuffs (Moret, Conchione, Srbinovska & Lucci, 2019), nevertheless, few applications are referred to coffee matrix (Kamalabadi, Mohammadi & Alizadeh, 2018). Solid-phase extraction (SPE) with different stationary phases (e.g. C18, silica, alumina, florisil) (García-Falcón et al., 2005), and column chromatography (Bishnoi, Mehta, Sain, and Pandit, 2005), mainly with silica gel, are commonly the most chosen clean-up strategies.

Their separating capacities means that chromatographic techniques that are coupled with appropriate detection systems can allow PAHs analysis to be performed while avoiding matrix effects. Gas chromatography (GC) coupled with flame ionisation (Grover et al., 2013) or mass spectrometry (MS) (Plaza-Bolanos, Garrido Frenich & Martínez Vidal, 2010), and high performance liquid chromatography (HPLC) coupled with photometric (Bishnoi et al., 2005), fluorimetric (Lee & Shin, 2010) or MS (Rey-Salgueiro et al., 2009) detection are the most appreciated analytical techniques.

Table 3 summarises, in chronological order starting from 2005, the analytical procedures that will be discussed hereafter.

4.2. Solid coffee samples

In relation to the above information, García-Falcón et al. (2005) have determined seven PAHs in 12 water-soluble instant coffee samples 6 of them decaffeinated (3 of each 6 obtained by natural roasting and the others by roasting with added sugar, namely torrefy). The torrefy samples (also called "Torrefacto" coffee), refer to a particular coffee-bean roasting technique that is commonly applied in some states (e.g. Spain, France, Paraguay, Portugal, Mexico, Costa Rica, Uruguay and Argentina) and that involves the addition of a certain amount of white sugar (typically 20% of coffee weight) in the final stage of the process; thus at high temperature, the sugar burns, coating the beans in a shiny, black film, resulting in dark and bitter coffee. In this work, the authors analysed 7 PAHs in homogenised samples extracted with hexane in a shaker. Solvent separation through nylon membranes was followed by a clean-up procedure in which the extract was eluted through a silica cartridge. After solvent removal by rotary evaporation, the obtained samples were dissolved in acetonitrile and then analysed using reversed-phase (RP) HPLC with fluorescence detection (FLD), by applying conditions selected to maximise the analyte response, while reducing possible interference. The proposed method gives recovery that is generally higher than 87%, and precision that ranges between 5% and 8%. All tested instant coffee samples showed much lower PAHs level than any potential EU legal restriction (proposal of BaP at 1 μg/kg in foods not yet accepted). In particular, these compounds were not extracted into water during the preparation of instant coffee; BbF, BkF and BaP were found in only two torrefied (high roast) decaffeinated samples at levels of 0.03–0.1 μg/kg for the first and 0.01–0.04 μg kg^{-1} for the other two.

On the basis of this previous study, a method for the simultaneous determination of the same PAHs in green coffee beans, roasted coffee beans, instant coffee granules and coffee without caffeine, all from the same producer, has been developed by Stanciu et al. (2008). Each sample was submitted to Soxhlet extraction with hexane and purified using a chromatographic column containing activated aluminium oxide and activated silica/gel (1/1). Analyses carried out by HPLC-FLD indicated a green coffee bean sample and that made up of instant coffee granules as the least and the most contaminated, respectively. When considering the sum of overall PAHs content, obtained values are as follow: 0.023 μg kg^{-1} (green coffee beans), 0.196 μg kg^{-1} (coffee without caffeine), 1.492 μg kg^{-1} (roasted coffee bean) and 2.116 μg kg^{-1} (instant coffee granules). Even though the mechanisms involved have been neither explained nor explored, it appears, from these results, that the decaffeination processes may reduce PAHs formation during roasting. Of the molecules tested, those found (at the μg kg^{-1} level) in most of the samples are: BaA, BbF, BkF, BaP and IND, while BghiP was always determined to be under the quantification limit. BaP was not detected in the green coffee beans, but its presence was found in all the other samples with values in the 0.119 to 0.857 μg kg^{-1} range. These results indicate that there is a reduced possibility of contamination from environmental pollution, but confirms the influence of coffee processing, principally roasting procedures.

Different samples of light-, medium- and dark-roasted coffee, including instant and decaffeinated brands, have been evaluated for their content about 18 PAHs (Jimenez, Adisa, Woodham & Saleh, 2014). A rotor mixer and hexane were chosen for coffee extractions and, after centrifugation, supernatant clean-up was performed using SPE (silica gel). HPLC-FLD and the external standard method were used for analyses, and recoveries greater than 80% were reported for the spiked samples. The results validated how PAHs concentration is related to the roasting conditions, as values were higher in the dark-roasted samples and lower in the light- and medium-roasted ones. This study calculated that the sum of the analysed PAHs ranged from 197 μg kg^{-1} for instant coffee to 3000 μg kg^{-1} for dark-roasted coffee. Decaffeinated and instant samples showed much lower amounts of these pollutants, in accordance with a previous report by Stanciu et al. (2008), only for coffee without caffeine. BaP was observed not to be present in only one of the considered instant coffee samples, while, for the other, the values were: light blend coffees (1.4–3.4 μg kg^{-1}), medium blend coffees (2.4–4.9 μg kg^{-1}), espresso coffee (3.4 μg kg^{-1}), dark blend coffees (3.5–18.5 μg kg^{-1}), instant coffee (6 μg kg^{-1}) and decaffeinated dark blend coffee (13.7 μg kg^{-1}). Moreover, FLU, Pyr, CHR and BkF were detected in all the tested samples, while the other compounds were not always found.

Table 3
Summary of the analytical procedures in reported references for both solid coffee samples and coffee brewing.

Sample	Extraction and Clean-up	Monitored PAHs	Analysis	References
Arabica coffee brew	LLE (hexane) C18 cartridge	16 EPA's	HPLC-UV/VIS Column: RP-18 LOD: 0.0006–0.174 ng 100 μL^{-1} LOQ: 0.002–0.580 ng100 μL^{-1}	Bishnoi et al., 2005
Instant coffee (with and without caffeine)	Hexane, shaker Silica cartridge	BaA, BkF, BbF, BaP, BghiP, DBA, IND	HPLC-FLD Column: Supelcosil LC-LC-PAH LOD: 0.01–0.05 μg kg^{-1} LOQ: 0.04–0.2 μg kg^{-1}	García-Falcón et al., 2005
Coffee brew	Electric coffee maker equipped with paper filter SPE (PS-DVB cartridge)	FLU, BbF and BaP	RP-HPLC-FLD Column: LC-PAH LOD: 0.19–2.49 μg L^{-1} LOQ: 0.63–8.3 μg L^{-1}	Houessou et al., 2005
Coffee brew	Alkaline saponification (KOH), LLE with cyclohexane Silica cartridge	16 EPA's	HPLC-FLD Column: Supelcosil LC- PAH LOD and LOQ not reported	Houessou et al., 2007
Green coffee beans Roasted coffee beans Instant coffee granules Decaffeinated coffee	Soxhlet, extraction with hexane Chromatographic column (activated aluminium oxide and activated silica gel)	BaA, BkF, BbF, BaP, BghiP, DBA, IND	HPLC-FLD Column: ChromSep SS LOD: 0.02–0.04 ng kg^{-1} LOQ: 0.2 ng kg^{-1}	Stanciu et al., 2008
Coffee brew	Aluminium *Moka* coffeemaker Alkaline saponification LLE (hexane)	16 EPA's (except for NAPH), 2-methylPHE, 2-methyl ANT, 9-methylPHE, 9-methylANT, 2,4-dimethylPHE, 1,2-dimethylPHE, 1-methylPyr, Benzo[*e*]Pyr, peryleneDBalP, DBaeP, DBaiP, DBahP	GC–MS Column: Equity-5 LOD: 0.7–6.0 ng L^{-1} LOQ not reported	Orecchio et al., 2009
Roasted coffee	Saponification (1 M KOH ethanol solution), LLE (ethanol/hexane (1/1) mixture, hexane, and distilled water) SPE (florisil)	BaA, CHR, BbF, BkF, BaP, DBA, BghiP	HPLC-FLD Column: Supelcosil LC-PAH LOD: 0.016–0.497 μg kg^{-1} LOQ: 0.054–1.656 μg kg^{-1}	Lee and Shin, 2010
Coffee brews (different origins, cultivars and brewing techniques)	LLE with cyclohexane and DMF/water (9/1) mixture SPE (Silica gel)	BaA, BbF, BkF, BaP	HPLC-FLD Column: C18 LOD: 0.006–0.01 μg kg^{-1} LOQ not reported	Tfouni et al., 2013
Light, medium and dark roasted coffee Instant coffee Decaffeinated coffee	Hexane, rotor mixer SPE (Silica)	16 EPA's, 1-methylNAPH, 2-methylNAPH	HPLC-FLD Column: Pinnacle® II PAH LOD: 0.01–0.21 μg kg^{-1} LOQ: 0.03–0.71 μg kg^{-1}	Jimenez et al., 2014
Brews from roasted coffee beans and instant coffee	Hexane, reflux GPC (S-X3); SPE (Silica)	16 EPA's, BcF, CPP, 5-MC, BjF, perylene, benzo[*e*] pyrene, DBalP, DBaeP, DBaiP, and DBahP	GC–MS Column: PAH Select LOD: 0.1–0.3 μg kg^{-1} for PAH4 LOQ: 0.2–0.8 μg kg^{-1}	Duedahl-Olesen et al., 2015
Roasted coffee	Pressurized liquid extraction (hexane/dichloromethane, 85/15), liquid–liquid partition with DMF/water (9/1) SPE (Silica)	FL, Pyr, BaA, CHR, BbF, BkF, BaP, IND, DBA, BghiP	Isotope dilution GC–MS Column: DB-5MS LOD: 0.03–0.18 μg kg^{-1} LOQ: 0.11–0.59 μg kg^{-1}	Pissinatti et al., 2015
Ground coffee Instant coffee Coffee substitutes	Water, acetonitrile Dispersive SPE (PSA/SAX, 1/1) followed by solvent exchange from acetonitrile to hexane (LLE)	ACY, ANT, BaA, BbF, BkF, BaP, BghiP, CHR, DBA, FLU, IND, PHE Pyr	GC–MS Column: DB-5MS LOD: 0.18–0.33 μg kg^{-1} LOQ: 0.6–1.1 μg kg^{-1}	Sadowska-Rociek et al., 2015
Roasted coffee	UAE (hexane:methylene chloride, 9/1) Alkaline saponification, Silica phase	16 EPA's	GC–MS Column: HP-5MS LOD: 0.38–2.6 μg kg^{-1} LOQ: 1.27–7.20 μg kg^{-1}	Guatemala-Morales et al., 2016
Coffee brew	Water DLLME (1-octanol and acetonitrile)	BaP, FL, PHE	HPLC-FLD Column: C18 LOD: 0.004–0.03 μg L^{-1} LOQ not reported	Loh et al., 2016
Roasted coffee	Water Fe_3O_4@3-(Trimethoxysilyl)propyl methacrylate@ionic liquid magnetic nanoparticles	PHE, BaA, CHR, BbF, DBA, BaP, BkF	HPLC-FLD Column: Eclipse PAH LOD: 0.1–10 ng L^{-1} LOQ: 0.5–34 ng L^{-1}	Shi et al., 2016
Roasted coffee	MAE (520 W for 8 min, solvent: water) DLLME (acetone, tetrachloroethylene)	BaA, BbF, BcF, BaP, CHR, 5-MC, IND	GC–MS Column: HP-5 LOD: 0.1–0.3 ng g^{-1} LOQ: 0.3 to 0.9 ng g^{-1}	Kamalabadi et al., 2018
Coffee beverages	Cyclohexane QuEChERS, AL-N SPE cartridge	BaA, BbF, BjF, BkF, BghiP, BaP, CHR, CPP, dibenzo[*a*,*h*]ANT, DBaeP, DBahP, DBaiP, DBalP, IND, and 5-MC, BcF	SFC/APCI-MS Column: Inertsil ODS-PLOD: 0.1–0.3 μg kg^{-1} LOQ: 0.4–1 μg kg^{-1}	Yoshioka et al., 2018
Green coffee beans Roasted coffee beans	Hexane/acetone (60/40) into an US bath Chromatographic column (Silica gel) and *SEC* on TSK Gel G1000HXL column sequentially	CPP, BaA, CHR, 5-MC, BjF, BbF, BkF, BaP, DBA, BghiP, IND, DBalP, DBaeP, DBaiP, DBahP, PHE, Pyr, ANT,FL	HPLC-FLD/DAD Column: BAKERBOND PAH-16 Plus LOD: 0.06–0.47 μg kg^{-1} LOQ: 0.10–0.94 μg kg^{-1}	Ciecierska et al., 2019
Coffee brew	Filtration CF-SPME	16 EPA's, oxy-PAHs, nitro-PAHs	GC–MS Column: HP-5MS LOD: 0.008–0.068 (PAHs); 0.0031–0.048 (oxy-PAHs); 0.051–0.059 (nitro-PAHs) μg L^{-1} LOQ: 0.027–0.224 (PAHs); 0.102–0.139 (oxy-PAHs);	Dos Santos et al., 2019

(*continued on next page*)

Table 3 (*continued*)

Sample	Extraction and Clean-up	Monitored PAHs	Analysis	References
			0.170–0.198 (nitro-PAHs) μg L^{-1}	
Roasted coffee	NADES, ultra-small iron oxide based nano-ferrofluid	NAPH, ACY, ACE, FLU, PHE, ANT, FL, Pyr, CHR, BbF, BaP, DBA, BghiP, IND	HPLC-FLD Column: XB-C18 GC–MS Column: HP-5 LOD: 0.31–5.9 ng L^{-1} LOQ: 1.04–19.8 ng L^{-1}	Fan et al., 2019
Coffee brew	Italian coffee maker pH adjusted to 4, semi-automated SPE (RP C18) closed system working in continuous mode	16 EPA's	GC–MS Column: DB-5 MS LOD: 0.02–0.6 ng L^{-1} LOQ: 3.3xLOD	Rascón et al., 2019

Finally, PHE, FL and Pyr were identified as the major PAHs in the analysed coffees.

Liquid-liquid extraction (LLE) was used to investigate the presence of 7 PAHs in roasted coffee beans (Lee & Shin, 2010). Preliminary saponification with KOH can reduce the presence of lipids, whose amounts can reach up to 15% of the organic-extract composition of coffee. SPE (florisil cartridge) was used for sample purification, analyses were performed on an HPLC-FLD system, and recoveries of the spiked samples were found to be between 68 and 91%. The concentrations of the total screened PAHs in 10 commercial coffee samples was found to be from 0.62 to 53.25 μg kg^{-1}. In particular, BaA, CHR and BbF were detected in almost all samples, with respective amounts of 0.15–1.57 μg kg^{-1}, 0.14–1.90 μg kg^{-1} and 0.85–3.90 μg kg^{-1}; BaP was under the quantification limit in 4 samples and in the range of 0.09–0.36 μg kg^{-1} for the other, and neither DBA (except for 2 samples reported with trace levels) nor BghiP were detected.

Isotope dilution GC–MS has been used in an exploratory study to determine 10 of EPA priority PAHs in 24 commercial samples of roasted coffee that were extracted with simultaneous purification involving pressurised liquid extraction, liquid–liquid partition, and clean-up by SPE (silica columns) (Pissinatti, Nunes, de Souza, Junqueira & de Souza, 2015). In the proposed method, mean PAHs recovery ranged from 87 to 111%. PAHs contamination (sum of the ten chosen compounds) was found to be in a range from 1.00 μg kg^{-1} to 11.29 μg kg^{-1}, with a BaP contribution in the sample with the highest level of contamination of 0.50 μg kg^{-1}. Moreover, the most frequently detected compound was CHR, and the highest contamination was found for FL and Pyr, which reached amounts of 4.37 μg $kg^{-1}$and 3.05 μg kg^{-1}, respectively.

Despite being efficient and inexpensive, the most common sample-preparation procedures are often time-consuming and require considerable amounts of solvents; characteristics that are not well suited to the current vision of eco-sustainable processes.

Micro-extraction techniques are promising because of their capacity to reduce the use of chemicals; multi-walled carbon nanotube-impregnated agarose film microextraction (Loh, Sanagi, Wan Ibrahim and Hasan, 2013), headspace solid-phase microextraction (Viñas, Campillo, Aguinaga, Pérez-Cánovas & Hernández-Córdoba, 2007) and stir-bar sorptive extraction (Zuin, Montero, Bauer & Popp, 2005) are some of these interesting procedures for PAHs determination in tea, but their use with coffee samples is still limited.

Dispersive SPE (e.g. QuEChERS, acronym for Quick, Easy, Cheap, Effective, Rugged and Safe) is an alternative that can be used in the clean-up operations of coffee extracts. Different bulk sorbents with diverse properties are available for this kind of procedure. A satisfactory protocol that provides a combination of different sorbent phases, followed by LLE, has been suggested by Sadowska-Rociek, Surma & Cieślik (2015) for the determination of several PAHs in 37 commercial coffee samples including ground coffee, instant coffee and coffee substitutes. After extraction, an appropriate combination of sorbent (primary secondary amine PSA, weak anion exchanger NH_2 to remove strong acids, and strong anion exchange SAX for the extraction of carboxylic acids) and $MgSO_4$ was used for sample clean-up. Analyses were carried out via GC coupled with an Ion Trap MS detector, and no significant peaks that interfered with the analytes were found in the purified extracts. Results indicated that the PSA and SAX 1/1 combination was the best variant of the clean-up system, with a mean recovery value of all tested PAHs ranging from 70 to 104%. Comparing tested samples, higher BaA, CHR, BbF and BaP levels were found in natural roasted coffee (7.20–68.15 μg kg^{-1}), while instant coffee and cereal coffee showed lower amounts, in the range of 2.97–19.55 μg kg^{-1} and 8.15–15.35 μg kg^{-1}, respectively. In particular, BaP values have been reported to be as follows: not detectable (n.d.) for cereal coffee samples, 4.63–12.33 μg kg^{-1} for instant coffee samples, and 17.29–34.84 μg kg^{-1} for roasted coffee samples. Despite the higher amounts quantified in roasted coffee, it should be emphasised that only 3 out of 16 samples were found to contain BaP. Moreover, natural and instant coffees present higher levels than coffee substitutes, even when the total sum of PAHs is considered, reaching an amount of 364 and 343 μg kg^{-1}, respectively, against 265 μg kg^{-1} of roasted cereal coffee. Light PAHs, mainly PHE, were found to be the most abundant compounds, reaching a total of 209.67 μg kg^{-1}in roasted coffee.

Magnetic solid phase extraction, which is based on magnetic nanoparticles, has recently been developed as a sample preparation technique; the target analytes can be adsorbed onto magnetic adsorbents that are directly dispersed in the sample solution (Zhao, Lu & Feng, 2013). The separation of nanoparticles is assured by a magnet that is positioned outside the solution container, and an organic solvent can be used for analyte desorption. It is worth noting that the magnetic adsorbents can be reused or recycled. Optimised experimental parameters for this technique were applied to rapidly (12 min) extract and enrich 7 PAHs from water solutions of coffee samples (Shi et al., 2016). Fe_3O_4@3-(Trimethoxysilyl)propyl methacrylate@ionic liquid magnetic nanoparticles were synthesised and used as adsorbent materials, exploiting their strong π-π bonds and hydrophobic interactions with the organic pollutants. Analytes were desorbed from the isolated particles using small volumes of acetone under sonication. Analyses were performed using HPLC-FLD, and the recoveries of PAHs in spiked coffee samples ranged from 87.5% to 104.5%, while the real commercial Quechao, Guangdong and China coffee samples were found not to be contaminated by the selected analytes.

A fast and environmentally friendly method that is based on a pre-treatment method involving hydrophobic natural deep eutectic solvents (NADES) and ultra-small iron-oxide-based nanoferrofluids has been described for selective PAHs microextraction (Fan et al., 2019). 14 PAHs were determined in 12 coffee-bean samples roasted under different conditions (light, medium, full-city and Italian roasting). Optimal extraction conditions (using menthol, borneol and camphor as NADES, and a nanoferrofluid), which were selected thanks to a predictive model, led to improvements in selectivity and sensitivity the extraction process that only requires a few minutes (less than 3 min) to complete. The reported comparison between the time required by other pre-treatment methods, ranging from 25 to 60 min for SPME to 15 min

for liquid–liquid microextraction (LLME), is interesting. PAHs analysis was accomplished via both HPLC-FLD and GC–MS with consistent results; reported recoveries from spiked samples were between 91.3 and 121%. Data on real coffee samples showed a total concentration of PAHs that ranged from 0.05 to 0.58 μg kg^{-1}, and a correlation with roasting conditions was found. In particular, the partial transformation of lower molecular weight PAHs into the higher molecular analogues, as the roasting degree increased, has been observed, while BaP was only detected in medium- and Italian-roasted samples in very similar amounts (about 0.05 μg kg^{-1}).

MAE and dispersive liquid–liquid microextraction (DLLME) coupled with GC–MS have been applied to simultaneously determine 7 PAHs in various coffee samples, with low organic-solvent consumption (Kamalabadi, Mohammadi & Alizadeh, 2018). Spiked water samples have been microwaved at 520 W for 8 min. The protein component precipitation was then performed by adding Carrez solutions I and II and, after centrifugation, the upper aqueous phase was immediately used for DLLME. Response surface methodology, based on central composite design, was used for microextraction-parameter optimisation. The obtained relative recoveries were reported in the range between 88.1 and 101.3%. When real samples were analysed, BaP, BaA, BbF and CYR were found in each of them, in the ranges of 0.156–0.551, 0.082–0.180, 0.176–0.444 and 0.118–0.341 μg g^{-1}, respectively. Moreover, the total PAH content obtained was between 0.9 and 2.1 μg g^{-1}.

Ultrasound-assisted extraction (UAE) in an ultrasonic bath has been used by Guatemala-Morales, Beltrán-Medina, Murillo-Tovar, Ruiz-Palomino, Corona-González and Arriola-Guevara (2016) to quantify the EPA's 16 priority-pollutant PAHs in roasted coffee via GC–MS. The highest extraction efficiency from ground roasted coffee was achieved using hexane/methylene chloride, followed by alkaline saponification and sample purification through a silica phase. When considering BaP, these conditions gave a recovery of 68.1% from spiked samples (100 ng g^{-1}). In the case of coffee roasted in a spouted bed reactor under different time and temperature conditions, the sum of the screened PAHs was found to be in the range between 3.5 and 16.4 μg kg^{-1}, although higher levels were found at higher roasting temperatures. Of the 16 PAHs, only eight were found in detectable concentrations throughout the trials. On the basis of the reported results, the authors underlined that, except for BghiP, the most toxic compounds, such as BaP, are only found in coffee samples at trace levels, or not at all.

4.3. Coffee brew

As mentioned above, the EU has not fixed limit levels for PAHs in coffee beverages. It is therefore of prime importance that rapid and reliable analytical methods for the accurate determination of contaminants in complex matrices, such as food items, are developed in order to estimate the total amount of human exposure to PAHs. For this purpose, it is more pertinent to estimate PAHs concentration in brew samples as it is this factor that may affect the daily intake of these compounds.

There are only around a dozen papers in the literature, to the best of our knowledge, that focus on PAHs content in coffee brews, and the number is even lower if we only consider the last 10 years. Moreover, each work has analysed a different number of compounds, from 4 to 28 analytes, which directly reflects on the sum of PAHs concentration found. Furthermore, each paper has focused on different aspects, such as the extraction of PAHs during brewing, differences in coffee cultivar or origin, and the influence of roasting degree.

Brewing generally consists of the hot-water extraction of ground coffee. Therefore, the physical and chemical properties of PAHs, e.g. molecular weight and water solubility (data reported in Table 1), are the main aspects that influence their transfer into the beverage. Clearly, extraction processes during brewing preparation may lead to there being different PAH patterns in coffee drinks than in the beans or ground coffee. Moreover, different methods of preparing this beverage around the world (e.g. espresso, mocha, filtration etc.) may interfere with the transfer of these pollutants from ground coffee. However, this aspect has not yet been fully dealt with.

As BaP is considered a human carcinogen, its content in brewed coffee was studied in one of the very first works by Dekruijf, Schouten and Vanderstegen (1987). They compared two different brewing methods: filtration and traditional (infusion) methods. Due to the low levels of BaP, less than 0,5 μg kg^{-1}, found in the 55 roasted-coffee samples analysed, an over-roasted coffee sample with higher BaP content (2 μg kg^{-1}) was selected to determine the analyte-extraction yield after brewing. Showing a BaP content of approximately 1 ng L^{-1}, and an extraction yield of about 1% in both coffee beverage preparation methods, the authors stated that coffee brewing does not contribute to the daily human intake of BaP in significant way.

The influence of different coffee-manufacturing processes on PAHs brew content has been studied (Kayali-Sayadi et al., 1999) by considering torrefied (3 samples), roasted, decaffeinated and green coffees. Samples that were prepared using an electric coffee maker were analysed via RP-HPLC-FLD to determine the presence of 8 PAH (FL, Pyr, BaA, CHR, benzo[*e*]pyrene, BaP, DBA and BghiP). With a LOD lower than 0.4 ng mL^{-1} (LOQ not reported by authors), the means of the total PAHs concentration in the brews were reported as: 1.65 ng L^{-1} for green coffee, 1.99 ng L^{-1} for decaffeinated coffee, 2.10 ng L^{-1} for roasted and 2.87 ng L^{-1} for torrefied coffee, thus proving the influence of this particular roasting process. Moreover, the results showed that the most toxic BaP amounts were as follows: 2.38 ng L^{-1} in roasted coffee brew, 1.6–3.2 ng L^{-1} for torrefied samples, 3.4 ng L^{-1} in decaffeinated coffee, and it was not detected in a green coffee sample.

More recent works that take into consideration different PAH pools have been carried out with the aim of obtaining a broader description of the pattern of compounds present in different types of samples and brewing methods.

Orecchio et al. (2009) have investigated the levels of 28 PAHs in the coffee brews from 13 coffee samples obtained by means of an aluminium Moka coffeemaker. A method that is based on saponification with a KOH–methanol solution, and LLE with small volumes of hexane was reported for PAHs determination with the exclusion of further steps of purification. Analyses were carried out by GC–MS in single ion monitoring mode, thus increasing sensitivity and selectivity. The analytical procedure indicated an average recovery of above 85%, and a precision of 4–16%. The concentration of the investigated compounds, expressed as the sum of their amounts in coffee brews, varied from 0.52 to 1.8 μg L^{-1}. The PAHs-distribution profiles were similar in most of the samples analysed in the study. PHE, ACY and FL were the three most abundant PAHs, and BaP concentration ranged from 0.001 to 0.047 μg L^{-1} in all of the brews. Generally, their higher aqueous solubility meant that the low molecular weight compounds are more common. The distribution of light and heavy PAHs was studied as a reliable tool with which to identify the different origins (petrogenic or pyrolytic) of these contaminants, and it was confirmed that those observed in most of the coffee samples originate from high temperature processes.

In a previous work (Bishnoi et al., 2005), the concentration of the EPA's 16 priority pollutant PAHs was evaluated in brews of three different coffee brandssubmitted to LLE with hexane and to a clean-up through C-18 cartridges; analyses were performed via RP-HPLC with a UV–VIS detector. Results varied from 16.47 to 18.24 μg L^{-1} for the total sum of the 16 PAHs. Also in this case, in agreement with Orecchio et al., lower molecular weight compounds dominated the higher molecular weight ones. In particular, the two-ring (NAPH) and three-ring (ACY, ACE, FLU, PHE) analogues were found to be the predominant PAHs. Moreover, BaP was detected in all samples, at an average amount of 0.33 μg L^{-1}.

A semi-automated, closed SPE system and GC–MS apparatus have been used to detect and quantify the same PAHs considered by Bishnoi et al. (2005) in a variety of drinks, including 4 samples of coffee (Rascón, Azzouz & Ballesteros, 2019). This method was described as accurate, precise, flexible, fast and sensitive, giving recoveries in a range between

90 and 103%. After beverage preparation in a traditional Italian coffee maker, the pH was adjusted to 4 with the aim of removing the lipid fraction (triacylglycerols, sterols, tocopherols and diterpenes of the kaurene family) and precipitate all the suspended matter. A continuous system that involves RP-C18 as the sorbent (SPE), and that consumes a low volume of organic solvents was then used to clean and enrich each sample before the GC–MS analyses. NAPH, ANT, FL, CHR and BbF were the main PAHs present in a range of concentrations from 0.55 to 4800 ng L^{-1} in almost all of the coffee samples, while BaP was only detected in one tested sample at 61 ng L^{-1}. This study had a clear prevalence for four-ring compounds. Furthermore, the calculated pollutant concentrations did not vary when the PAH4 and PAH8 lists were considered, and were found to be 420–631 ng L^{-1}. This is with the sole exception of the brew containing the highest quantity of PAH4, which was found to have a PAH8 concentration of 704 ng L^{-1}.

Arabica coffee of different origins (Ethiopia, Colombia) and blended samples have been investigated to determine traces of FLU, BbF and BaP, as model compounds, with the aim of developing a reliable and fast analytical procedure to determine PAHs in coffee-brew samples (Houessou et al., 2005). All coffee brews were obtained using an electric coffee maker, and both non-spiked and spiked samples were extracted using SPE. C18-silica and a polystyrene-divinylbenzene (PS-DVB) copolymer, were tested as hydrophobic sorbents, and the latter was found to be more suitable. Analyses were performed via RP-HPLC equipped with a FLD. BbF and BaP concentrations were in the 0–95.7 ng L^{-1} range, while FLU was not detected due to the method's lack of sensitivity. In general, the reported results showed high variability in PAHs concentration in the coffee samples. In fact, no PAHs presence was detected in the Arabica coffee samples from Colombia and Ethiopia, whereas traces were found in the blended coffee.

Later, Houessou et al. (2007) studied Arabica green coffee beans from Cuba to monitor PAHs formation during roasting. Controlled processes were performed by varying the temperature from 180 to 260 °C, and both dark and light roasting conditions were used. Coffee brews that were obtained with an electric coffee maker were analysed for a final estimation of PAHs-transfer coefficient to the infusion. After alkaline saponification (KOH), the beverages were submitted to LLE with cyclohexane, followed by a clean-up through SPE on disposable silica cartridges before the HPLC-FLD analyses. For the dark-roasted coffee brews, similarly to the respective ground coffee, PHE, FL and Pyr were reported as the main PAHs, with concentrations of between 0.078 and 0.79 μg L^{-1}, while only traces of BaP were detected. Transfer coefficients ranged from 0.6 to 23%, depending on the PAHs and coffee samples. In the light-roasted coffee brews, whose ground coffee presented lower PAHs level due to the shorter roasting time, results were quite similar to those observed for the dark roasted ones, meaning that transfer coefficients were noticeably higher (13.9–32.4%) in this instance.

Further considerations as to the influence of coffee cultivar on PAHs content in brew samples have been reported by Tfouni et al. (2013). This parameter, together with roasting degree and brewing procedure, was taken into account to evaluate the presence of 4 PAHs in ground roasted coffee and their transfer to the brews. Therefore, *Coffea arabica* and *Coffea canephora* beans were used to obtain samples at three different roasting degrees (light, medium, dark), and two of the main brewing methods (filtered and boiled) were applied. After clean-up analyses were performed by HPLC coupled with FLD, and results showed that at least one PAH was found in all of the tested coffee brews; detected in 94% and 83% of the samples, respectively, BbF and BaA were reported as the most representative PAHs. The roasting degree had no apparent influence in the total PAHs level in the brews. Briefly, these amounts ranged from 0.015 to 0.105 μg L^{-1} and from 0.011 to 0.111 μg L^{-1} for samples brewed using *Coffea arabica* and *Coffea canephora* beans, respectively. Moreover, mean PAHs sum values seemed to be independent of the brewing procedure used and were always at levels that would not be expected to affect human health. When considering BaP alone, its transfer seemed to be influenced by roasting degree mainly for Arabica coffee. In fact, its levels ranged from n.d. to 0.016 μg L^{-1} for light and medium roasting, whereas, in dark roasted samples, BaP was n.d. with both brewing methods. With regards to the Robusta samples, this PAH was found to be n.d. for all roasting degrees and preparation methods, except for medium-roasted coffee brewed via filtration, in which it reached 0.01 μg L^{-1}. From these data, the authors concluded that caffeine amount (known to be higher in Robusta) does not seem to influence the passage of PAHs to the beverage, despite a previous study affirming that the formation of a caffeine-PAH complex could facilitate this type of transfer (Navarro, Ichikawa, Morimoto & Tatsumi, 2009).

PAHs transfer in brews was later studied in coffee beans, at medium- and dark-roasting degrees and instant coffee samples that are commercially available in Denmark, by Duedahl-Olesen, Navaratnam, Jewula and Jensen (2015). The presence of 25 PAHs was investigated, with a focus on PAH4 as indicators. The extraction was performed via pressurised liquid extraction for solid samples, and with hexane under reflux for the brew. GC–MS analyses were performed after clean-up of the obtained samples by gel permeation chromatography (GPC) and SPE in ready-made silica columns. The concentration of BaP in all the solid samples was generally found to range from 0.2 to 1.0 μg kg^{-1}, while PAH4 ranged from 0.9 to 5.1 μg kg^{-1}. Specifically, PAH4 concentrations were higher in dark-roasted and instant-coffee products than in the medium-roasted ones, and the same can be said of the concentration of BaP alone. The highest average sum of 25 PAH levels was obtained in instant coffee. When considering PAHs transfer to coffee brews, the average percentage of PAH4 was 5–14% of the initial concentrations in the medium- and dark-roasted ground coffees while, unexpectedly considering the data of the coinciding solid samples, 0% transfer was reported for instant coffee. The highest transfer percentages were those reported for samples submitted to lighter roasting conditions, in accordance with the findings of Houessou et al. (2007). Moreover, the margin of exposure (MOE) for coffee for Danish people was calculated using the results obtained for the transfer of PAHs to infusions. In light of the fact that the EFSA have assessed that MOE values for genotoxic and carcinogenic substances of 10,000 or more are of slight concern to public health, it can be stated that none of the MOEs obtained in the above work constitute a risk deriving from coffee brew consumption, as they are all below the critical value. Specifically, the contribution from coffee to PAH4 exposure for Danes was found to be 13% (150 ng day^{-1}).

DLLME, a process that is carried out via simple solvent partition thanks to a homogeneously distributed extraction solvent, has been described by Loh et al. (2016) as an easy, rapid, efficient and selective method for PAHs determination in several spiked beverage samples, including coffee. In their system, 1-octanol and acetonitrile were premixed and injected into the coffee beverage solution. After analyte partitioning, the 1-octanol extract was recovered and then analysed via HPLC-FLD. Relative recoveryprovided the following values: 95.3 (BaP), 95.3 (PHE) and 99.4 (FL).

Yoshioka et al. (2018) have developed a rapid high-sensitivity analytical method that involves supercritical fluid chromatography/atmospheric pressure chemical ionisation-mass spectrometry (SFC/APCI-MS) and simple sample preparation, and analysed 16 PAHs in 11 coffee beverage samples that are commercially available in Japan. Extractions were performed using cyclohexane and a QuEChERS extraction kit. An AL-N (Normal Phase Neutral Alumina) SPE cartridge was chosen for the purification of PAHs as it has retention that is suitable for the recovery of all 16 compounds (82–115%). In SFC/APCI-MS, an increase in the sensitivity of MS detection was achieved by varying the back-pressure in SFC, thus enhancing the amount of sample introduced to the MS. Interestingly, none of the 16 PAHs analysed, including BaP, were detected in the commercial products.

A study carried out by dos Santos et al. (2019) presents a new analytical method, based on cold fibre solid phase microextraction (CF-SPME) followed by GC–MS analysis, that is useful for the determination not only of PAHs, but also of their oxygenated (oxy-PAHs) and nitrated derivatives (nitro-PAHs). These are compounds that are generally

neglected when considering environmental contamination, but that may have higher toxicity than PAHs. In this very recent work, authors reported a significant improvement in procedure efficiency at low extraction times. The validated CF-SPME-GC/MS method presented satisfactory linearity, as well as good precision and recovery results (82.1 to 96.3%). Brews were obtained from 27 samples of ground and roasted coffee (100% Arabica or blend) by pouring hot water over a filter containing coffee (1/10 w/v). In the obtained samples, the lowest concentration range was found for NAPH, while the highest was for BbF. As was found in previously reported results, compounds with low water solubility, such as DBA, BghiP, IND and BaP, were not detected. The total concentration of the 16 PAHs screened was found to be between 2.169 and 1.086 μg L^{-1}, and, similarly to Bishnoi et al., BkF, BbF, Pyr, ACY and ACE were the most abundant. 5,12-naphthacenquinone and 1-nitropyrene were reported to be the most abundant oxy- and nitro-PAHs, respectively. Specifically, the presence of those derivatives was found to vary between analysed samples; in many brews, nitro- PAH content was higher than that of the oxy- species, and, in some cases, only one of them was detected. Nevertheless, PAHs amount was much higher than that of their derivatives.

5. Conclusions

Coffee is made up of a complex food matrix, meaning that its sample preparation, including extraction, clean-up procedures and enrichment processes, is a critical step in PAHs determination. In fact, the co-extractives obtained from coffee beans are a significant limitation in trace-level analysis because of the overlap between these analytes and PAHs peaks that can occur during chromatographic analyses.

National and international regulations all over the world require reliable and valid analytical methods that can guarantee the sensibility, reproducibility, traceability and comparability of obtained results. This is highlighted by the constant advances in both sample preparation and analysis that have been developed in recent years with aim of increasing the accuracy and efficiency of the entire process by using alternative techniques that involve automation and cause reductions in time and solvent consumption. It is important to underline that there is still a lack of regulation on PAHs in coffee, although the EFSA has indicated that this widely consumed commodity is a prominent matrix when considering food contamination by these compounds. Moreover, a realistic assessment of the significance of PAHs content in roasted coffee clearly depends on the ratio of their transfer to coffee brews, which involves the beverage preparation techniques and consumption habits.

As PAHs are ubiquitous pollutant compounds, their occurrence in green coffee beans is mainly due to environmental contamination, and the various studies that have been reviewed herein have indicated that, when present, PAHs content in such matrices is low. An overview of PAHs amount in coffee of different origins can be found in an interesting work by Ciecierska et al. (2019), which demonstrated that Kenya and Tanzania (both Eastern Africa) for Arabica green samples, and the Ivory Coast (south coast of West Africa) and Cameroon (Central Africa) for Robusta samples, were the most contaminated.

When considering roasted coffee, PAHs contamination is mainly attributed to the formation of the substances during the processing phases, including drying and roasting. It is worth noting that process parameters, such as time and temperature, have been found to strongly influence PAHs generation, making them important factors when attempting to avoid or minimise this kind of pollution. Specifically, low molecular PAHs are mainly observed under mild roasting conditions, but these light compounds lead to more toxic heavy PAHs at higher roasting temperatures. From a toxicological point of view, the decaffeination processes seem to positively influence the amounts of PAHs that develop during roasting. In fact, the data reported thus far indicate that lower PAHs content is present in decaffeinated samples, although only a few studies have covered this topic and there is a need for further evaluation.

With regard to instant coffee, the reported values are variable and further studies are desirable as it has sometimes been found to be more and, other times, less contaminated than ground roasted coffee.

The PAHs content in coffee brews has generally been observed to be very low, which was expected due to the hydrophobic character of these molecules. Despite the lack of extensive data, the extraction of PAHs clear varies with the brewing methods used; the observed transfer coefficients were variable and less than 35%. Different opinions on how roasting degree influences the transfer of PAHs to the beverages can be found in the bibliography. Some of the works reported herein affirm that dark-roasted coffee demonstrates slightly lower extractability than light- and medium-roasted coffees.

The contents of the main indicators, PAH4 and, particularly, BaP, in coffee brews, as assessed in the literature to date, are very low and sometimes are even not detected, indicating that this beverage does not significantly contribute to the daily human intake of carcinogenic PAHs. However, coffee is a very popular beverage, and coffee-bean roasting and brew-preparation methods can both considerably vary, meaning that further evaluations and insights into PAHs contamination levels in coffee are still required to assure the safety of the product. Moreover, the development of industrial processes to uniformly roast coffee beans and give minimum PAHs content is desirable, even when coffee beans are then used for other purposes, such as baking.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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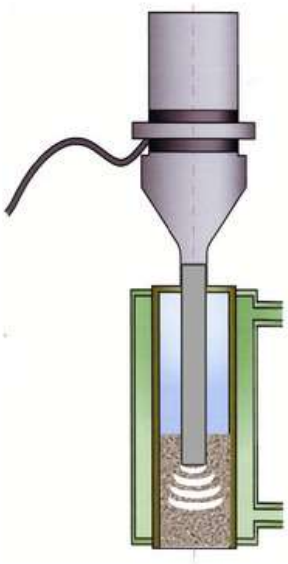
7. Highly-Efficient Caffeine Recovery from Green Coffee Beans under Ultrasound-Assisted SC–CO_2 Extraction



Janet Menzio, Arianna Binello, Alessandro Barge and Giancarlo Cravotto*
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7.1. Introduction

Caffeine is a xanthine alkaloid widely used in beverages (soft or energy drinks), as well as in pharmaceutical and cosmetic preparations (Vuong and Roach, 2014). The global caffeine market was valued at US$ 231.1 million in 2018 and is expected to reach US$ 349.2 million by 2027 expanding at a CAGR (Compound Annual Growth Rate) of 4.7% during the forecast from 2019 to 2027 (Global $350M Caffeine Market Outlook and Forecast 2020–2027). This rising demand boosts the global caffeine market. Besides production via chemical synthesis, there is an urgent need to increase caffeine production via isolation from natural sources, the principle of which is coffee beans (Shinde, R.R. and Shinde, N.H., 2017; Andreeva, Dmitrienko and Zolotov, 2012).

The decaffeination of green coffee beans commonly involves several steps, such as steaming, extraction, drying and caffeine recovery (Andreeva *et al*., 2012; Belay, Ture, Redi and Asfaw, 2008). Growing concerns as to the use of organic solvents has stimulated the development of greener techniques for more sustainable and efficient caffeine extraction. Of the alternative green processes available, supercritical CO_2 extraction (SFE–CO_2) can be considered the technique of choice for decaffeination and the recovery of high-value lipophilic compounds from natural matrices (Hasan and Farouk, 2013; Machmudah, Kitada, Sasaki, Goto, Munemasa and Yamagata, 2011). A molecular dynamics simulation study demonstrated that, unlike extraction in water, SFE–CO_2 does not favour the aggregation of the caffeine obtained, thus facilitating subsequent purification steps (Reddy and Saharay, 2019). The aim of extracting 97% caffeine from both Arabica and Robusta green coffee beans has been achieved by this technology in 11.5 h and 22 h, respectively, at 90 ◦C and 247 bar (Marco, Riemma and Iannone, 2018).

The efficiency of SFE–CO_2 can be further improved by the addition of a co-solvent, such as ethanol, in order to enhance the polarity of the system (Araújo, Paladonai, Azevedo, Hamerski, Pedersen Voll and Corazza, 2019). SFE is generally characterised by slow kinetics, meaning that mass-transfer intensification would be a welcome means to improve diffusion. Ultrasound-assisted extraction (UAE) is extremely beneficial, in this sense, thanks to the enhanced mass transfer that occurs under acoustic cavitation. Ultrasounds are mechanical waves with a frequency (>20 kHz) above the range of human audibility. (20 Hz to 20 kHz). These waves, which are made up of a series of compression and rarefaction cycles, are able to pass through solid, liquid, or gaseous media and cause the molecules to be displaced and pushed out of their initial locations. Under a strong sound wave, the molecules are pulled apart and form cavitation bubbles since the negative pressure during rarefaction is greater than the attraction force holding them together. These bubbles expand through coalescence before collapsing later during the compression phase, producing extreme local conditions such as hot spots. Up to 5000 K in temperature and 1000 atm in pressure rise are possible. The main principle employed by UAE is acoustic cavitation. The cellular matrix of the sample can undergo fragmentation, localized erosion, pore creation, shear force, enhanced absorption, and swelling as a result of collapsing cavitation bubbles and sound waves (Chemat, Rombaut, Sicaire, Meullemiestre, Fabiano-Tixier and Abert-Vian, 2017; Wen, L.; Zhang, Sun, Sivagnanam and Tiwari, 2020). In general UAE has been performed using ultrasonic bath and ultrasonic probe, which are based on piezoelectric transducer as source of ultrasound power. An ultrasonic probe is made up of a transducer and a probe or horn. The probe provides ultrasound to the media with a minimum amount of energy loss while submerged in the extraction vessel. The energy released in a probe system is concentrated in a particular sample

zone, producing a cavitation effect that is more effective and it is frequently favored over bath-based systems (Kumar, Srivastav and Sharanagat, 2021).
Farouk & Hasan (2015) have carried out a study using a high-order numerical scheme, and the data seem to confirm that the kinetics of the SFE process are significantly accelerated by cavitational effects. Higher extraction yields and shorter extraction times can therefore be achieved when SFE–CO_2 processes are assisted by ultrasound (US) (Shirsath, Sonawane and Gogate, 2012). Currently, US–SFE–CO_2 extractors are mainly available only on lab and pilot scales, and there is a distinct lack of industrial-scale data (Heilmann, 2001). Extensive analysis on the synergistic effect of US with SFE–CO_2 have been summarized by Dassoff & Li (2019), namely micro- and macro-mixing, mechanical effects on the cell wall that enhance both convective mass transfer as well as internal diffusivity. Under CO_2 pressure the oxidative degradations that may occur under sonication are negligible. A pilot-scale process using an innovative US–SFE–CO_2 system has been proposed for oil extraction from different vegetal matrices (Riera, Blanco, García, Benedito, Mulet and Gallego-Juárez, 2010).
The effectiveness of ultrasound assistance in the extraction of caffeine from green coffee beans using SFE–CO_2 was evaluated in order to improve extraction efficiency. This preliminary investigation should provide useful data to facilitate a drastic reduction in extraction time. The scalability of this hybrid technology for decaffeination will require further engineering studies. In this study, an SFE unit, equipped with a tightly fixed sonotrode with an immersion horn, has been used under sonication and silent conditions. Extract analysis was carried out using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS-MS).

7.2. Results and discussion

7.2.1. Supercritical CO_2 Extraction (SFE-CO_2), Liquid CO_2 extraction (LE–CO_2) and Ultrasound-Assisted Supercritical CO_2 Extraction (US-SFE-CO_2)

Computational simulation studies on caffeine extraction from a fixed bed of coffee beans indicate that acoustic waves could significantly accelerate the kinetics of SFE thanks to their physical/mechanical effects on the treated vegetal matrix, therefore leading to an improvement of extraction rate and yield (Reddy and Saharay, 2019). To the best of our knowledge, effective experimental works involving the hybrid technology US–SFE–CO_2 in coffee decaffeination have not been reported so far.
This study aims to evaluate the possibility of using the effect of cavitation to improve the SFE–CO_2 of caffeine from Arabica green coffee beans, in terms of reducing the process time and temperature and also enriching extracts.
Several experiments were carried out under US assistance, and silent conditions, using samples with 31% moisture. It was decided that all tests would be performed in a relatively short process time (1 h), although that does not lead to exhaustive caffeine extraction, which is an operation that usually requires several hours (De Marco, Riemma and Iannone, 2018). Nevertheless, under these conditions, a preliminary assessment of the beneficial effects of US-SFE-CO_2 was performed. The highest extraction temperature was set at 75 °C. Different conditions were applied in the first runs using SFE-CO_2 alone. The extraction vessel was filled to about 80% maximum volume with 185 g of matrix (**Table 1**, entry one and two). For the sake of comparison, LE-CO_2 (**Table 1**, entry three) was also performed. US-SFE-CO_2 was then initially carried out under the same experimental conditions as in SFE-CO_2 (**Table 1**, entries four and five). Subsequently, the influence of both lower pressure and sample amount were tested at the highest temperature (**Table 1**, entries six and seven, respectively).

Qualitative and quantitative analyses were performed using UPLC-MS/MS on the freeze-dried extracts in order to evaluate caffeine extraction yields and sample enrichment. Data (**Table 1**) were compared to the mean caffeine content in coffee (9.46 mg/g) obtained from exhaustive conventional water extraction in order to calculate the decaffeination percentage achieved.
Results clearly show that temperature plays a crucial role in extractions carried out under silent conditions (entries one, two and three). Although a poor extraction yield was observed when CO_2 was used in liquid conditions, extract purity was relatively high. Under supercritical conditions, yields were found to be directly correlated to increasing temperature and also gave better extract purity, which reached 83.2% at 75 ◦C. Despite reports by Machmudah et al. [8] on the solubility of caffeine in SC-CO_2 at pressures above 19 MPa/190 bar, caffeine solubility increases with increasing temperature, which however is in agreement with Saldana et al. (1999).

Table 1. Experimental conditions for caffeine extraction from Arabica green coffee beans and caffeine determination using UPLC-MS/MS analyses. Reported data are the mean of two experiments.

Entry	Sample (g)	Temperature (°C)	Pressure (bar)	Extraction method[1]	Total caffeine (mg)	Extract caffeine %	Caffeine mg /g coffee beans	Decaffein. %	Time (h)
1	185	75	250	a	155.1 ± 11	83.2 ± 6	0.83 ± 0.06	8.86 ±0.6	1
2	185	40	250	a	59.2 ± 4	76.2 ± 6	0.32 ± 0.02	3.38 ± 0.3	1
3	185	20	70	b	26.3 ± 4	80.2 ± 12	0.14 ± 0.02	1.50 ± 0.2	1
4	185	75	250	c	318.3 ± 20	90.1 ± 6	1.72 ± 0.1	18.2 ± 1	1
5	185	40	250	c	126.5 ± 10	83.9 ± 7	0.68 ± 0.06	7.2 ± 0.6	1
6	185	75	125	c	279.8 ± 16	74.1 ± 4	1.51 ± 0.08	16.0 ± 0.9	1
7	50	75	250	c	148.5 ± 9	69.0 ± 4	2.97 ± 0.2	31.4 ± 2	1
8	185	75	250	c	673.8 ± 26	87.3 ± 3	3.64 ± 0.1	38.5 ± 1	2
9	185	75	250	c	906.5 ±31	91.6 ± 3	6.04 ± 0.2	51.8 ± 2	3
10	185	75	250	c	1104.3 ± 40	93.4 ± 3	5.97 ± 0.2	63.1 ± 2	4

[a]Supercritical CO_2 extraction (SFE–CO_2); [b]extraction with liquid CO_2 (LE–CO_2); [c]ultrasound supercritical CO_2 extraction (US–SFE–CO_2).

7.2.2. US–SFE–CO_2 efficiency

By comparing entries one and four, both carried out at 75°C and 250 bar, it is possible to see that caffeine amount and decaffeination percentages were doubled thanks to the cavitation phenomenon. A better result if compared with data reported by Farouk & Hasan (2015), that showed an enhancement of caffeine extraction yield by around 15%–25%. Moreover, our results evidenced the effect of sonication on extract purity (90.1%). A similar trend can be observed when comparing entries two and five, which were performed at low temperature (40°C). These preliminary results demonstrate how US is a potential, efficient way of enhancing mass transfer processes in SFE. The use of US enabled higher yields in a shorter time, in agreement with Rodríguez et al. (2014) who observed 109%–150% improvement at 30 min comparing SFE–CO_2 and US–SFE–CO_2 in cocoa butter extraction.
Lower pressure (125 vs. 250 bar) and lower filling volume (50 vs. 185 g) (entry six and seven, respectively) confirmed the pivotal role of CO_2 pressure (Tang, Li, Lv and Jiang, 2010) as well as a better sound wave diffusion in the sonication cone of the horn tip. Entry four gave up to 90.1% of caffeine in the extract and 18.19% of sample decaffeination. The obtained results showed that the caffeine extraction yield was lower, by about 12%, at 125 bar (entry six). US intensity must be strong enough to induce vibrational effects (Hu, Zhao, Liang, Qiu, and Chen, 2007) when a probe system equipped with a titanium horn (40 kHz; P=90% W) was employed. In the case of a lower sample amount in the extraction vessel (entry seven) higher mass extraction was achieved; a caffeine amount of 2.97 mg per gram of coffee bean was raised, whereas 1.72 mg/g was obtained in entry four. These values reveal that there is an increase

in the sonication effect when less vessel volume was occupied by the matrix, thus allowing for better propagation of acoustic waves at lower pressure. Anyway, under these conditions, extracts showed 20% less of caffeine content. This, as observed by field emission scanning electron microscopy by Balachandran et al. (2006) that reported 200 µm surface damage in ginger particles, is more likely due to stronger cell-wall rupture and then to higher metabolite release, which results in our case in a lower selectivity for caffeine.
In order to evaluate decaffeination kinetics, the best extraction conditions (entry four) were used for longer times, 2, 3, 4 h (entries eight, nine, ten). It was found that longer extraction times gave a higher decaffeination percentage, up to 63.1% after 4 h, with no significant influence on extract purity.

7.3. Conclusions

In this preliminary study on caffeine extraction from Arabica green coffee beans, SFE-CO_2 and US-SFE-CO_2 have been compared under different experimental conditions. Results show that, in 1 h of extraction time, the effect of acoustic waves can lead to a doubling of caffeine extraction yield and around 10% higher extract purity. The best experimental parameters (US-SFE-CO_2, 75°C, 250 bar, 185 g of sample amount) were also tested at longer process times, and thus 63.1% decaffeination was achieved in 4 h. These experimental data lay the foundation for further studies into caffeine US–SFE–CO_2 from coffee beans, both as an improvement of the decaffeination process in the coffee industry, and as an effective technique to produce purer extracts in shorter times for industrial use.

7.4. Experimental section

7.4.1. Coffee Sample and Chemicals

Green coffee beans (Arabica, Brazil), that had been previously hydrated to reach 31% moisture, were kindly provided by Lavazza S.p.A (Settimo Torinese, Turin, Italy) and stored in vacuum bags. The densities of the dry coffee (710 kg/m^3) and wet coffee (550 kg/m^3) were calculated in order to select the most appropriate volume for the experiments. The more suitable amount used for the experiments was 185 g of wet coffee (water 31%) corresponding at 144.5 g of dry coffee (water 11%). A remarkable swelling of green coffee beans in water was observed during the humidification process. A caffeine standard (99.7%) was purchased from Alfa Aesar (Karlsruhe, Germany). Acetonitrile, trifluoroacetic acid (TFA) and magnesium oxide (MgO) were obtained from Merck KGaA (Darmstadt, Germany).

7.4.2. Supercritical CO_2 Extraction (SFE-CO_2) and Ultrasound-Assisted Supercritical CO_2 Extraction (US-SFE-CO_2)

The SFE–CO_2 and US–SFE–CO_2 of caffeine from green coffee beans were performed in a prototype designed and assembled by Weber Ultrasonics GA (Karlsbad, Germany) and FeyeCon B.V. (Weesp, The Netherlands). The prototype was equipped with a system that circulated fluid through the extraction chamber (11–13 kg h^{-1} of CO_2), an insulating jacket and an immersion titanium horn for US-assisted procedures.
For SFE–CO_2, green coffee beans were weighed (185 g) and placed into the 1 L-capacity extraction vessel. Different pressure and temperature conditions were tested. Moreover, SFE–CO_2 efficiency was compared to that of the extraction with liquid CO_2 (LE–CO_2), with pressure and temperature being set under the critical point. Similar conditions used in SFE–CO_2 were applied for US–SFE–CO_2. US assistance was obtained thanks to a probe system equipped with a titanium horn (40 kHz; P=90% W) that was placed in the extraction vessel. To avoid localized sample overheating, the horn was set to operate 5 min on and 2 min off for the whole extraction time (see **Table 1** for tested conditions).
Each procedure was repeated three times to verify extraction method reproducibility, and all the processes provided an aqueous extract that was subsequently freeze dried (Freeze drier LyoQuest-85, Telstar, Legnano, Italy). A 0.5 mg/mL aqueous solution of each obtained sample was prepared and transferred into a vial for UPLC-MS/MS analysis. Results are expressed as mean data ± standard deviation (SD).

7.4.3. Conventional caffeine extraction

In order to evaluate the total caffeine content in the green coffee beans used for the experiments, a conventional extraction was performed. Briefly, 100 mL of water and 4 g of MgO were added to 1 g of ground green coffee beans, and the obtained mixture was stirred for 1 hour at 90 ◦C, then filtered. The filtrate was brought to 250 mL with water and placed into a 250 mL graduated flask, and this solution was used for caffeine quantification via UPLC-MS/MS. The procedure was repeated three times to evaluate extraction-yield reproducibility. The efficiency of this conventional extraction method was confirmed by re-extracting, under the same conditions, the residual coffee sample derived from the filtration procedure.

7.4.4. Quantitative and qualitative analysis

Analyses were performed using a UPLC-MS/MS system (Acquity TQD LC/MS/MS System, Waters Corporation, Milford, MA, USA), equipped with a C-18 column (BEH C18, 2.1 × 50 mm, 1.7 µm). Isocratic elution was carried out at 50 °C with 90:10 v/v of water (0.1% TFA)/acetonitrile (0.1% TFA). The flow rate was set at 0.4 mL min^{-1}.
For determination, we operated in atmospheric-pressure chemical ionization (APCI+) mode, following the transitions m/z = 195 -> 138 (quantification) and m/z = 195 -> 110 (qualitative confirmation of the peak), using 26 eV as the collision energy. The calibration curve of the UPLC-MS/MS method was determined using aqueous caffeine standard solutions (from 0.01 to 1 mg/mL); a linear regression with R^2=0.9994 was obtained using Waters QuanLynx software (LOD 0.005 mg/mL, LOQ 0.01 mg/mL).

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Communication

Highly-Efficient Caffeine Recovery from Green Coffee Beans under Ultrasound-Assisted SC–CO_2 Extraction

Janet Menzio, Arianna Binello, Alessandro Barge and Giancarlo Cravotto *

Dipartimento di Scienza e Tecnologia del Farmaco, University of Turin, Via P. Giuria 9, 10125 Turin, Italy
janet.menzio@unito.it (J.M.); arianna.binello@unito.it (A.B.); alessandro.barge@unito.it (A.B.)
* Correspondence: giancarlo.cravotto@unito.it; Tel.: +39-011-670-7183; Fax: +39-011-670-7162

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Abstract: Natural caffeine from decaffeination processes is widely used by pharmaceutical, cosmetic and soft-drink industries. Supercritical CO_2 extraction (SFE–CO_2) is extensively exploited industrially, and one of its most representative applications is the decaffeination process, which is a greener alternative to the use of organic solvents. Despite its advantages, extraction kinetics are rather slow near the CO_2 critical point, meaning that improvements are highly sought after. The effect exerted by a combination of SFE–CO_2 and ultrasound (US–SFE–CO_2) has been investigated in this preliminary study, with the aim of improving mass transfer and selectivity in the extraction of caffeine from green coffee beans. This hybrid technology can considerably enhance the extraction efficiency and cut down process time. Further studies are in progress to demonstrate the complete decaffeination of green coffee beans of different types and origins.

Keywords: caffeine; green coffee beans; supercritical CO_2 extraction; ultrasound; hybrid technology

1. Introduction

Caffeine is a xanthine alkaloid widely used in beverages (soft or energy drinks), as well as in pharmaceutical and cosmetic preparations [1]. The global caffeine market was valued at US$ 231.1 million in 2018 and is expected to reach US$ 349.2 million by 2027 expanding at a CAGR (Compound Annual Growth Rate) of 4.7% during the forecast from 2019 to 2027 [2]. This rising demand boosts the global caffeine market. Besides production via chemical synthesis, there is an urgent need to increase caffeine production via isolation from natural sources, the principle of which is coffee beans [3,4]. The decaffeination of green coffee beans commonly involves several steps, such as steaming, extraction, drying and caffeine recovery [4–6]. Growing concerns as to the use of organic solvents has stimulated the development of greener techniques for more sustainable and efficient caffeine extraction. Of the alternative green processes available, supercritical CO_2 extraction (SFE–CO_2) can be considered the technique of choice for decaffeination and the recovery of high-value lipophilic compounds from natural matrices [7,8]. A molecular dynamics simulation study demonstrated that, unlike extraction in water, SFE–CO_2 does not favour the aggregation of the caffeine obtained, thus facilitating subsequent purification steps [9]. The aim of extracting 97% caffeine from both Arabica and Robusta green coffee beans has been achieved by this technology in 11.5 h and 22 h, respectively, at 90 °C and 247 bar [10]. The efficiency of SFE–CO_2 can be further improved by the addition of a co-solvent, such as ethanol, in order to enhance the polarity of the system [11]. SFE is generally characterised by slow kinetics, meaning that mass-transfer intensification would be a welcome means to improve diffusion. Ultrasound-assisted extraction (UAE) is extremely beneficial, in this sense, thanks to the enhanced mass transfer that occurs under acoustic cavitation [12,13]. Farouk & Hasan [14] have carried out a study using a high-order numerical scheme, and the data seem to confirm that the kinetics of the SFE process are significantly accelerated by cavitational effects. Higher extraction

yields and shorter extraction times can therefore be achieved when SFE–CO_2 processes are assisted by ultrasound (US) [15]. Currently, US–SFE–CO_2 extractors are mainly available only on lab and pilot scales, and there is a distinct lack of industrial-scale data [16]. Extensive analysis on the synergistic effect of US with SFE–CO_2 have been summarized by Dassoff & Li, [17], namely micro- and macro-mixing, mechanical effects on the cell wall that enhance both convective mass transfer as well as internal diffusivity. Under CO_2 pressure the oxidative degradations that may occur under sonication are negligible. A pilot-scale process using an innovative US–SFE–CO_2 system has been proposed for oil extraction from different vegetal matrices [18].

The aim of this work is to evaluate the effectiveness of ultrasound assistance in the extraction of caffeine from green coffee beans using SFE–CO_2. This preliminary investigation should provide useful data to facilitate a drastic reduction in extraction time. The scalability of this hybrid technology for decaffeination will require further engineering studies. In this work, an SFE unit, equipped with a tightly fixed sonotrode with an immersion horn, has been used under sonication and silent conditions. Extract analysis was carried out using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS-MS).

2. Materials and Methods

2.1. Coffee Sample and Chemicals

Green coffee beans (Arabica, Brazil), that had been previously hydrated to reach 31% moisture, were kindly provided by Lavazza S.p.A (Settimo Torinese, Turin, Italy) and stored in vacuum bags. The densities of the dry coffee (710 kg/m^3) and wet coffee (550 kg/m^3) were calculated in order to select the most appropriate volume for the experiments. The more suitable amount used for the experiments was 185 g of wet coffee (water 31%) corresponding at 144.5 g of dry coffee (water 11%). A remarkable swelling of green coffee beans in water was observed during the humidification process. A caffeine standard (99.7%) was purchased from Alfa Aesar (Karlsruhe, Germany). Acetonitrile, trifluoroacetic acid (TFA) and magnesium oxide (MgO) were obtained from Merck KGaA (Darmstadt, Germany).

2.2. Supercritical CO_2 Extraction (SFE–CO_2) and Ultrasound-Assisted Supercritical CO_2 Extraction (US–SFE–CO_2)

The SFE–CO_2 and US–SFE–CO_2 of caffeine from green coffee beans were performed in a prototype designed and assembled by Weber Ultrasonics GA (Karlsbad, Germany) and FeyeCon B.V. (Weesp, The Netherlands). The prototype was equipped with a system that circulated fluid through the extraction chamber (11–13 kg h^{-1} of CO_2), an insulating jacket and an immersion titanium horn for US-assisted procedures.

For SFE–CO_2, green coffee beans were weighed (185 g) and placed into the 1 L-capacity extraction vessel. Different pressure and temperature conditions were tested. Moreover, SFE–CO_2 efficiency was compared to that of the extraction with liquid CO_2 (LE–CO_2), with pressure and temperature being set under the critical point. Similar conditions used in SFE–CO_2 were applied for US–SFE–CO_2. US assistance was obtained thanks to a probe system equipped with a titanium horn (40 kHz; P = 90% W) that was placed in the extraction vessel. To avoid localized sample overheating, the horn was set to operate 5 min on and 2 min off for the whole extraction time (see Table 1 for tested conditions).

Each procedure was repeated three times to verify extraction method reproducibility, and all of the processes provided an aqueous extract that was subsequently freeze dried (Freeze drier LyoQuest-85, Telstar, Legnano, Italy). A 0.5 mg/mL aqueous solution of each obtained sample was prepared and transferred into a vial for UPLC-MS/MS analysis. Results are expressed as mean data ± standard deviation (SD).

Table 1. Experimental conditions for caffeine extraction from Arabica green coffee beans and caffeine determination using UPLC-MS/MS analyses. Reported data are the mean of two experiments.

Entry	Sample (g)	Temperature (°C)	Pressure (bar)	Extraction Method	Total Caffeine (mg)	Extract Caffeine %	Caffeine mg/g Coffee Beans	Decaffein. %	Time (h)
1	185	75	250	1 [a]	155.1 ± 10.6	83.2 ± 5.9	0.838 ± 0.06	8.86 ±0.61	1
2	185	40	250	1 [a]	59.2 ± 4.5	76.2 ± 5.8	0.320 ± 0.02	3.38 ± 0.26	1
3	185	20	70	2 [b]	26.3 ± 3.8	80.2 ± 11.6	0.142 ± 0.02	1.50 ± 0.21	1
4	185	75	250	3 [c]	318.3 ± 19.7	90.1 ± 5.6	1.72 ± 0.1	18.19 ± 1.1	1
5	185	40	250	3 [c]	126.5 ± 10.4	83.9 ± 6.9	0.684 ± 0.06	7.23 ± 0.6	1
6	185	75	125	3 [c]	279.8 ± 15.8	74.1 ± 4.2	1.51 ± 0.08	15.98 ± 0.9	1
7	50	75	250	3 [c]	148.5 ± 9.3	69.0 ± 4.3	2.97 ± 0.18	31.4 ± 1.9	1
8	185	75	250	3 [c]	673.8 ± 25.8	87.3 ± 3.3	3.64 ± 0.12	38.5 ± 1.4	2
9	185	75	250	3 [c]	906.5 ±30.6	91.6 ± 3.1	6.04 ± 0.2	51.8 ± 1.8	3
10	185	75	250	3 [c]	1104.3 ± 40.1	93.4 ± 3.3	5.97 ± 0.2	63.1 ± 2.3	4

[a] Supercritical CO_2 extraction (SFE–CO_2); [b] extraction with liquid CO_2 (LE–CO_2); [c] ultrasound supercritical CO_2 extraction (US–SFE–CO_2).

2.3. Conventional Caffeine Extraction

In order to evaluate the total caffeine content in the green coffee beans used for the experiments, a conventional extraction was performed. Briefly, 100 mL of water and 4 g of MgO were added to 1 g of ground green coffee beans, and the obtained mixture was stirred for 1 hour at 90 °C, then filtered. The filtrate was brought to 250 mL with water and placed into a 250 mL graduated flask, and this solution was used for caffeine quantification via UPLC-MS/MS. The procedure was repeated three times to evaluate extraction-yield reproducibility. The efficiency of this conventional extraction method was confirmed by re-extracting, under the same conditions, the residual coffee sample derived from the filtration procedure.

2.4. Qualitative and Quantitative Analyses

Analyses were performed using a UPLC-MS/MS system (Acquity TQD LC/MS/MS System, Waters Corporation, Milford, MA, USA), equipped with a C-18 column (BEH C-18, 2.1 × 50 mm, 1.7 µm). Isocratic elution was carried out at 50 °C with 90:10 *v*/*v* of water (0.1% TFA)/acetonitrile (0.1%TFA). The flow rate was set at 0.4 mL min^{-1}.

For determination, we operated in atmospheric-pressure chemical ionization (APCI+) mode, following the transitions m/z = 195 -> 138 (quantification) and m/z = 195 -> 110 (qualitative confirmation of the peak), using 26 eV as the collision energy. The calibration curve of the UPLC-MS/MS method was determined using aqueous caffeine standard solutions (from 0.01 to 1 mg/mL); a linear regression with R^2 = 0.9994 was obtained using Waters QuanLynx software (LOD 0.005 mg/mL, LOQ 0.01 mg/mL).

3. Results and Discussion

3.1. SFE–CO_2, LE–CO_2 and US–SFE–CO_2 of Green Coffee Beans

Computational simulation studies on caffeine extraction from a fixed bed of coffee beans indicate that acoustic waves could significantly accelerate the kinetics of SFE thanks to their physical/mechanical effects on the treated vegetal matrix, therefore leading to an improvement of extraction rate and yield [9]. To the best of our knowledge, effective experimental works involving the hybrid technology US–SFE–CO_2 in coffee decaffeination have not been reported so far.

This study aims to evaluate the possibility of using the effect of cavitation to improve the SFE–CO_2 of caffeine from Arabica green coffee beans, in terms of reducing the process time and temperature and also enriching extracts.

Several experiments were carried out under US assistance, and silent conditions, using samples with 31% moisture. It was decided that all tests would be performed in a relatively short process time (1 h), although that does not lead to exhaustive caffeine extraction, which is an operation that usually

requires several hours [10]. Nevertheless, under these conditions, a preliminary assessment of the beneficial effects of US–SFE–CO_2 was performed. The highest extraction temperature was set at 75 °C.

Different conditions were applied in the first runs using SFE–CO_2 alone. The extraction vessel was filled to about 80% maximum volume with 185 g of matrix (Table 1, entry one and two). For the sake of comparison, LE–CO_2 (Table 1, entry three) was also performed. US–SFE–CO_2 was then initially carried out under the same experimental conditions as in SFE–CO_2 (Table 1, entries four and five). Subsequently, the influence of both lower pressure and sample amount were tested at the highest temperature (Table 1, entries six and seven, respectively).

Qualitative and quantitative analyses were performed using UPLC-MS/MS on the freeze-dried extracts in order to evaluate caffeine extraction yields and sample enrichment. Data (Table 1) were compared to the mean caffeine content in coffee (9.46 mg/g) obtained from exhaustive conventional water extraction in order to calculate the decaffeination percentage achieved.

Results clearly show that temperature plays a crucial role in extractions carried out under silent conditions (entries one, two and three). Although a poor extraction yield was observed when CO_2 was used in liquid conditions, extract purity was relatively high. Under supercritical conditions, yields were found to be directly correlated to increasing temperature and also gave better extract purity, which reached 83.2% at 75 °C. Despite reports by Machmudah et al. [8] on the solubility of caffeine in SC–CO_2 at pressures above 19 MPa/190 bar, caffeine solubility increases with increasing temperature, which however is in agreement with Saldana et al. [19].

3.2. US–SFE–CO_2 Efficiency

By comparing entries one and four, both carried out at 75 °C and 250 bar, it is possible to see that caffeine amount and decaffeination percentages were doubled thanks to the cavitation phenomenon. A better result if compared with data reported by Farouk & Hasan [14], that showed an enhancement of caffeine extraction yield by around 15%–25%. Moreover, our results evidenced the effect of sonication on extract purity (90.1%). A similar trend can be observed when comparing entries two and five, which were performed at low temperature (40 °C). These preliminary results demonstrate how US is a potential, efficient way of enhancing mass transfer processes in SFE. The use of US enabled higher yields in a shorter time, in agreement with Rodríguez et al. [20] who observed 109%–150% improvement at 30 min comparing SFE–CO_2 and US–SFE–CO_2 in cocoa butter extraction.

Lower pressure (125 vs. 250 bar) and lower filling volume (50 g vs. 185) (entry six and seven, respectively) confirmed the pivotal role of CO_2 pressure [21] as well as a better sound wave diffusion in the sonication cone of the horn tip. Entry four gave up to 90.1% of caffeine in the extract and 18.19% of sample decaffeination. The obtained results showed that the caffeine extraction yield was lower, by about 12%, at 125 bar (entry six). US intensity must be strong enough to induce vibrational effects [22] when a probe system equipped with a titanium horn (40 kHz; P = 90% W) was employed. In the case of a lower sample amount in the extraction vessel (entry seven) higher mass extraction was achieved; a caffeine amount of 2.97 mg per gram of coffee bean was raised, whereas 1.72 mg/g was obtained in entry four. These values reveal that there is an increase in the sonication effect when less vessel volume was occupied by the matrix, thus allowing for better propagation of acoustic waves at lower pressure. Anyway, under these conditions, extracts showed 20% less of caffeine content. This, as observed by field emission scanning electron microscopy by Balachandran et al. [23] that reported 200 μm surface damage in ginger particles, is more likely due to stronger cell-wall rupture and then to higher metabolite release, which results in our case in a lower selectivity for caffeine.

In order to evaluate decaffeination kinetics, the best extraction conditions (entry four) were used for longer times, 2, 3, 4 h (entries eight, nine, ten). It was found that longer extraction times gave a higher decaffeination percentage, up to 63.1% after 4 h, with no significant influence on extract purity.

4. Conclusions

In this preliminary study on caffeine extraction from Arabica green coffee beans, SFE–CO_2 and US–SFE–CO_2 have been compared under different experimental conditions. Results show that, in 1 h of extraction time, the effect of acoustic waves can lead to a doubling of caffeine extraction yield and around 10% higher extract purity. The best experimental parameters (US–SFE–CO_2, 75 °C, 250 bar, 185 g of sample amount) were also tested at longer process times, and thus 63.1% decaffeination was achieved in 4 h. These experimental data lay the foundation for further studies into caffeine US–SFE–CO_2 from coffee beans, both as an improvement of the decaffeination process in the coffee industry, and as an effective technique to produce purer extracts in shorter times for industrial use.

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8. Final conclusions

Food-related public health concerns have shifted the emphasis of the relevant authorities in recent years to the regulation of pollutants, adulterants, process or environmental contaminants. The business has also been forced to focus on the quality of its products and raw materials.
The increasing interest in a more protected diet and the changing regulatory landscape have highlighted the need for in-depth analysis of high-consumption food matrices, such as coffee, whose safety and quality are effects of the entire production line, and the food industry has also been forced to focus on the quality of its products and raw materials.
Coffee is a widely consumed beverage that can be susceptible to contamination or alteration at any point along the production chain, with roasting being the most important. In order to assess environmental contamination (such as pesticides) and the study of process contaminants generated during thermal treatment, this research was primarily concentrated on the examination of contaminants in green and roasted coffee. Analytical and sample preparation techniques were developed concurrently for the identification of relevant substances.

In this research work it has been assessed that phthalimide may represent a false positive for the presence of the pesticide Folpet being its degradation product, especially in the case of thermally treated foods, such as roasted coffee. In fact, a possible ex novo phthalimide generation pathway in coffee during heat treatments has been suggested considering ubiquitous phthalates as precursors and some amino acids present in their free form in green coffee beans as NH_3 sources. These findings supported the hypothesis that the residues of this contaminant found in samples of roasted coffee may have originated as a process contaminant and it is not significant for the presence of folpet. However, phthalimide does not represent a health issue as it has no toxicity in humans.

In the context of coffee contaminants, *o*-phenylphenol traces amounts found in roasted coffee have been assessed as this fungicide is not specifically indicated for coffee bean preservation and it is not detected in green beans. The study led to demonstrating that OPP can form lipophilic conjugates that can mask its presence towards green coffee contamination in the production chain. It follows that common pesticide multiresidue analytical methods may lead to an underestimation of OPP amount if they are inefficient with respect to the conjugates and the method developed in this work allowed to fully determine OPP both in green and roasted coffee samples. For this reason, an adequate sample preparation procedure was developed allowing to exhaustively detect the fungicide also in green coffee samples.

Given the growing attention paid in the recent years to the process contaminant acrylamide, and the limited possibilities from a technological point of view to reduce its levels in roasted coffee, in this PhD project preliminary results have been obtained regarding the correlation between green coffee features, as beans ripening and post-harvest treatments, useful to create a suitable strategy for the industry in order to properly select raw material to reduce acrylamide levels in roasted coffee. Results obtained showed that increasing amounts of unripe beans in roasted samples imply higher acrylamide levels both in Arabica and Robusta samples, while wet and semi-wet post-harvest treatments allow a significant reduction of the contaminant.

During the project polycyclic aromatic hydrocarbons as coffee contaminants have been also evaluated through a scientific literature review comprising different aspects as environmental contamination, their formation during coffee roasting, the possible transfer in the beverage through different brewing techniques and analytical methods involved in their detection in coffee samples.
Moreover, the research on coffee also involved a preliminary study on caffeine extraction from Arabica green coffee beans employing ultrasound cavitation in supercritical CO_2 extraction process showing that, in 1 h of extraction time, the effect of acoustic waves can lead to a doubling of caffeine extraction yield and to a higher extract purity.
In general, the studies led to the understanding of some coffee contaminants and evidenced that chemistry behind coffee processing is still complex to assess and involves many mechanisms that can explain the presence of undesirable compounds compromising product's quality or safety. Moreover, the analysis of all the compounds involved in this research enhanced the complexity of both green and roasted coffee samples regarding sample preparation and analytical techniques concluding that coffee represents a challenging matrix needing proper methods development.
During this PhD project, therefore, various aspects concerning the production chain and coffee safety were dealt with which have led to the elucidation of the phenomena of formation or detection of both process and environmental contaminants, to the development of different analytical methods suitable for such a complex matrix and to the study of green techniques in the decaffeination process which have allowed us to obtain four publications on scientific journals that lay the foundations for future developments and knowledge for the management of the processes involved in obtaining roasted coffee.