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Treball Final de Grau

Optimization and validation of analytical methodologies for the determination of priority pollutants in vegetable samples.

Optimització i validació de metodologies analítiques per a la determinació de contaminants orgànics prioritaris en mostres vegetals.

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Universitat de Barcelona

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Investigar es ver lo que todo el mundo ha visto, y pensar lo que nadie más ha pensado.

Albert Szent-Györgi

Primer de tot, volia fer una menció especial al Dr. Josep Maria Bayona i a tot l'equip de Biogeoquímica Ambiental del CSIC - IDAEA, per tenir sempre la voluntat d'ajudar-me, per la paciència que han tingut i per donar-me l'oportunitat d'adquirir un infinitat de nous coneixements durant aquests mesos.

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Per altre banda, voldria donar les gràcies als meus amics i amigues que m'han estat en tots els moments importants i finalment, m'agradaria donar un molt sentit agraïment a la meva família, que gràcies al seus esforços i al seu suport constant, he aconseguit arribar al final d'aquest llarg i dur viatge de 4 anys plens d'emocions.

REPORT

CONTENTS

1. SUMMARY

Polycyclic aromatic hydrocarbons (PAHs) are a large class of organic compounds widespread in the environment and are generated mainly by incomplete combustion of fossil fuels or by pyrolysis in industrial processes. According to IARC, some of these compounds have been catalogued as carcinogenic (Group 1), probably carcinogenic (Group 2A), possibly carcinogenic (Group 2B) and not classifiable as to carcinogenicity (Group 3) to humans, who are exposed to these compounds by inhalation of contaminated air or via dietary intake. Nowadays, there is no analytical methodology that guarantees the extraction and the detection of PAHs at a trace level in horticultural crops. For this reason, this project attempts the optimization of an analytical procedure in different vegetable matrices (lettuce, tomatoes, cauliflowers) using the ultrasound assisted extraction technique, followed by a clean-up step employing adsorption chromatography with alumina as a stationary phase and its detection with gas chromatography coupled to a tandem mass spectrometry (GC – MS/MS). With this analytical procedure developed, the limits of detection (LODs) were calculated for each vegetable as 432 ng/g - 16000 ng/g (lettuce in fresh weight), 439 ng/g - 16257 ng/g (tomato in fresh weight) and 456 ng/g - 16257 ng/g (cauliflower in fresh weight) but the recoveries and precision were poor for all the matrices evaluated (0.8% - 37.3% and 83.6%, respectively) stressing the complexity.

Keywords: PAHs, vegetables, sonication, periurban agriculture, GC – EI – MS/MS.

2. RESUM

Els hidrocarburs policíclics aromàtics (PAHs) constitueixen una amplia classe de compostos orgànics que es troben en el medi ambient i que es generen principalment per combustions inacabades de combustibles fòssils o per piròlisis en processos industrials. Segons l'IARC, alguns d'aquests compostos han estat catalogats com cancerígens (Grup 1), probablement cancerígens (Grup 2A), possiblement cancerígens (Grup 2B) i no classificats com cancerígens (Grup 3) pels éssers humans que estan exposats a aquests compostos ja sigui per la inhalació d'aire contaminat o per al seva ingesta via dieta. Actualment, no és disposa d'una metodologia analítica que permeti duu a terme l'extracció i la detecció de PAHs a nivell de traça en cultius hortícoles. Per aquest motiu, aquest treball es basa en la posada a punt d'un procediment analític en diferents matrius vegetals (enciam, tomàquet i coliflor) utilitzant la tècnica d'extracció assistida per ultrasons, seguida d'una etapa de purificació emprant la cromatografia d'adsorció en columna amb alúmina com a fase estacionaria i realitzant la seva determinació amb cromatografia de gasos acoblada a l'espectrometria de masses en tàndem (GC – MS/MS). Mitjançant el procediment analític desenvolupat, es van obtenir els següents límits de detecció (LODs) per cada vegetal: 432 ng/g - 16000 ng/g (enciam en pes humit), 439 ng/g - 16257 ng/g (tomàquet en pes humit) i 456 ng/g - 16257 ng/g (coliflor en pes humit) però, la recuperació i la precisió foren baixes per totes les matrius avaluades (0.8% - 37.3 i 86.3%, respectivament) posant de manifest la complexitat d'aquestes matrius.

Paraules clau: PAHs, vegetals, sonicació, agricultura periurbana, GC – EI – MS/MS.

3. INTRODUCTION

Pollution levels especially in developing countries and emerging economies, generated by industrial processes, inappropriate waste management, intensive agriculture and farming, rise worldwide. Among the numerous classes of chemical pollutants, polycyclic aromatic hydrocarbons (PAHs) constitute a big group of carcinogenic, teratogenic and mutagenic compounds [1]. Moreover, multiple human exposures to these contaminants occur through contaminated food, ambient air, tobacco smoke among others [2].

During the last decades, due to the growing food demand, the so-called periurban agriculture, where crop production is located nearby cities, has grown substantially. This activity has brought about many economical and social benefits besides of providing fresh food supply to urban centres. However, due to the proximity to point and nonpoint pollution sources (industrial, transportation infrastructures), food products can harbour high levels of PAHs. For this reason, foodstuff from these periurban areas require a pollution assessment to be accepted as healthy products.

Consequently, the development of an analytical methodology that allows the extraction and detection of PAHs in different food matrices is sought. Currently there are many different proposed methods for the extraction of PAHs in food matrices but, for the specific detection processes, the range of options is more limited as it is necessary to use techniques that could be developed at trace levels. Among them, the detection techniques more commonly used are High Performance Liquid Chromatography (HPLC) coupled to fluorescence (FLD) or coupled to a mass spectrometry (MS) and gas chromatography (GC) coupled to a mass spectrometry (MS) or to tandem mass spectrometry (MS/MS) [3].

3.1. POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

PAHs constitute a wide class of organic contaminants which structure contains at least two fused aromatic rings together without the presence of heteroatoms. These compounds could present different origins, among them, the incomplete combustion of fossil fuels, pyrolysis

processes, industrial emissions like thermo-electrical power plants or aluminium smelting processes, urban waste incineration and emissions of vehicles [3,4].

On the other hand, large molecular weight (MW) PAHs are poorly soluble in water but they have a great adsorption and absorption affinity for the organic matter and/or soot particles. As a consequence, they behave as persistent, and consequently, they can be transported by air or water reaching soil, sediments, irrigation waters and crops areas far from the different sources of origin [5]. Thus, the main routes of exposure of human beings to these compounds are the inhalation of contaminated air, and food (fish, cereals, fruits and vegetables) [3,6]. However, in 1998 WHO/IPCS has reported that up 4 aromatic ring PAHs can be easily biodegraded under aerobic conditions by volatilization or photolysis in water and by sunlight or by oxidant agents air [7].

Countless researches have been carried out in order to determine how PAHs enter into vegetables. At first, absorption processes in roots and leaves were considered to be the main exposure routes, but recent studies have shown that there is no direct correlation between the concentration of PAHs in soil and the concentrations of PAHs found in the roots [8,9]. Nevertheless, there is a strong linear relationship between the foliar uptake through the cuticles, which are the lipophilic membranes that cover the leaf surface, and the concentration of PAHs in the ambient air [8,10].

Therefore, from these data it can be stated the PAHs can be easily sorbed in the vegetables most commonly consumed, such as tomatoes, lettuce, etc. In order to control the presence of PAHs in vegetables, different analytical methodologies have been developed [Table 1].

Different extractions, clean-up and detection PAHs in foodstuff				
Sample	**Extraction**	**Clean-up**	**Detection**	**Reference**
Apples	HS-SPME[1]	SPE[6]	GC – MS	Poinot et al., 2014 [11]
White clover	LLE[2]	Silica	GC – FID[7]	Yanzheng et al., 2009 [12]
Food and oils	ASE[3]	SPE	GC – MS	Veynard et al., 2007 [13]
Maize plants	Soxhlet	Silica	GC – MS	Lin et al., 2006 [8]
Lettuce	UAE[4]	SPE	GC – MS	Dugay et al., 2002 [5]
Rice plants	LLE	Silica	GC – MS	Tao et al., 2005 [14]
Olive oil	HS[5]	-	GC – MS	Arrebola et al., 2006 [6]

[1]Headspace-Solid Phase Microextraction,[2]Liquid-Liquid Extraction,[3]Accelerated Solvent Extraction,[4]Ultrasound Assisted Extraction, [5]Headspace,[6]Solid-phase Extraction,[7]Gas Chromatography coupled to Flame Ionization Detector.

Table 1. Different extractions, clean-up and detection PAHs in foodstuff.

3.2. Determination of Polycyclic Aromatic Hydrocarbons in Vegetables

Due to the high matrix complexity, the determination of PAHs in vegetables makes necessary to use a high resolution chromatography technique (GC or LC) coupled to a high sensitivity detection system such as MS. These analytes can be determined by HPLC – FLD or HPLC – MS, although the detection of PAHs by GC – MS and more recently with GC – MS/MS is the most widely documented [4].

HPLC – FLD has a high specificity and sensitivity when compared to other detection techniques like HPLC coupled to an ultraviolet-visible (UV-vis) detector. Nevertheless, FLD has some limitations, such as not being able to use deuterated internal standard (surrogates) [15,16]. Furthermore, the FLD detector generates no signal for analytes that are not fluorescent.

3.2.1. Mass Spectrometry (MS)

The first part is the input system of the sample; usually this input system refers to the sample that comes from the GC, HPLC or the Capillary Electrophoresis (CE) that can be coupled to the MS. Then, the transfer line and ion source where Electronic Impact (EI) is the most popular ionization technique in GC and electrospray in HPLC. The following part is the analyser and the most commonly used are the Single Quadrupole (SQ), Time of Flight (TOF), Ion Trap (IT) and Orbitrap (OT). The election of the mass spectrometry technique will depend on

the analytical objectives (target or non-target analysis) and analyte concentration (trace or major component).

HPLC – MS is an option with clearly higher sensibility and specificity when photoionization ion source is used but it is not commonly available. The GC – EI – MS option is one the most commonly available to detect PAHs [4]. This technique has significant improvements over the FLD detector by offering a greater selectivity, sensibility and providing structural information.

3.2.2. Tandem Mass Spectrometry (MS/MS)

A wide variety of analysers have been used in MS/MS, for example, triple quadrupole (QqQ), quadrupole coupled of time of flight (qTOF) and quadrupole-Orbitrap but due to the lack of fragmentation of PAHs their applicability is limited [17,18].

MS/MS or tandem mass spectrometry presents, a high analytical flexibility (product, precursor or neutral loss scan, and Multiple Reaction Monitoring (MRM)) and selectivity. However, between all these options, the highest sensitivity is obtained in the MRM mode selected in this work.

3.2.3. Sample preparation for food analysis

Different extraction methods (ASE, MAE, UAE), clean-up (C18 cartridges, silica, alumina, Florisil) procedures and integrated methods (Quick, Easy, Cheap, Effective, Rugged and Safe (QuECheRS)) have been developed for PAH determination in foodstuff [Table 1]. In this work, Ultrasound Assisted Extraction, alumina for clean-up and GC – MS/MS for determination was selected due to the robustness and simplicity.

3.3. HUMAN HEALTH RISK AND PAH REGULATION IN FOODSTUFF

A large number of studies have been performed in order to identify which are the toxicological effects of PAHs and which is the maximum acceptable daily intake of these compounds that food can contain against the possible harmful effects they can cause to human health [2]. In this regard, the European Union (EU) established in 2011 the maximum concentrations of a set of PAHs present in certain foodstuffs by using Benzo[a]pyrene, Benz[a]anthracene, Benzo[b]fluoranthene and Chrysene as markers [19]. This list is composed with PAHs that the European Union considered as the most dangerous ones based on a study carried out by the Joint Committee of the Food and Agriculture Organization of the United

Nations (FAO) and the World Health Organization (WHO): Expert Committee on Food Additives (JECFA).

Some examples of maximum levels for these PAHs in food are: 2.0 µL/Kg (Benzo[a]pyrene) and 10.0 µL/Kg (sum of Benzo[a]pyrene, Benz[a]anthracene, benzo[b]fluoranthene and chrysene) in oils and fats, 6.0 µL/Kg (Benzo[a]pyrene) and 35.0 µL/Kg (sum of Benzo[a]pyrene, Benz[a]anthracene, Benzo[b]fluoranthene and Chrysene) for processed cereal-based foods and baby foods for infants and young children [2]. However, no guidelines for vegetables have been established yet.

On the other hand, the Environmental Protection Agency of the United States (US-EPA) [20] formulated a list of PAHs slightly different from the one designed by the EU, as the US-EPA only shows the most common PAHs in the environment but not the most harmful ones [Appendix 1].

4. OBJECTIVES

As was commented in the previous section, this work has 1 global objective and 2 specific ones.

The global is as following:

- To develop an analytical procedure for the detection of PAHs in vegetable samples.

The specific ones are as following:

- To optimize the sample preparation mainly extraction and clean-up steps.
- To optimize the detection of PAHs in GC-MS/MS.

The PAHs determination in vegetables involves three different steps. The first one is the extraction of PAHs, the second is the extract clean-up and the last one is the detection.

5. EXPERIMENTAL SECTION

5.1. MATERIALS, REAGENTS, SOLUTIONS AND INSTRUMENTATION

- PAHs – US-EPA:

A PAH mixture containing (Acenaphthene (ACP), Acenaphthylene (ACY), Anthracene (ANT), Fluoranthene (FA), Fluorene (FLR), Naphthalene (NPH), Phenanthrene (PHE), Pyrene (PYR), Benz[a]anthracene (BAA), Benzo[b]fluoranthene (BBF), Benzo[k]fluoranthene (BKFc), Benzo[ghi]perylene (B(ghi)P), Benzo[a]pyrene (BAP), Chrysene (CHR), Dibenzo[a,h]anthracene (DB(ah)A), Indeno[1,2,3-cd]pyrene (IP)) (US-EPA list) [Appendix 1] at 10 ng/µL each in cyclohexane was supplied by Dr. Ehrenstorfer (Augsburg, Germany).

- Glassware:

20 mL scintillation vials, 12x32 mm amber vials, centrifuge tubes, glass wool, glass column chromatography (30 cm x 0.8 cm), Hamilton syringes (100, 50, 25 µL) and Pasteur pipettes.

- Reagents:

Neutral aluminium oxide (activity 90), 0.063-0.200 mm Merck KGaA (Darmstadt, Germany), potassium hydroxide 85% Panreac Química S.A (Castellar del Vallès, Spain), sodium chloride 99.5% SIGMA-ALDRICH, CO. (St Louis, USA) and anhydrous sodium sulfate for analysis 99% EMSURE® Merck KGaA (Darmstadt, Germany).

- Vegetables:

Samples of lettuce, tomato and cauliflower were collected in 2016 by the CSIC – IDAEA, in the Parc Agrari (Baix Llobregat, NE, Spain) [21]. Samples were collected in different plots irrigated with waters of different quality, zone 1 is the least polluted and zone 3 is the most polluted. The samples were introduced in scintillation vials with their pertinent reference and stored at -20ºC without pre-treatment.

- Solvents:

Suprasolv grade acetone, n-hexane and methanol were obtained from Merck (Darmstadt, Germany). High purity water produced with a Milli-Q Arium®pro UF/VF/DI/UV System (Sartorius, Barcelona, Spain) was used.

❖ Solutions:

The working PAH standard was obtained from the stock solution by appropriate dilution in acetone to reach a concentration of 4 µg/mL and it was stored at -20ºC until its use.

Solution for saponification was prepared with KOH 1 M and methanol 80:20 [v/v].

❖ Calibration mixture and quantification:

Calibration Standard solutions in n-hexane at different concentration levels (n=8) from 700 ng/mL to 0.5 ng/mL, were prepared from the standard stock PAHs solution indicated in previous sections on this work.

Quantification of analytes by GC–MS/MS was performed using the internal calibration method, using Triphenylamine (TPhA) as an Internal Standard (IS).

❖ Instrumentation:

Centrifuge 3-16 (Sigma Laborzentrifugen, Osterode am Harz, Germany). Ultrasound DL 156BH (Bandelin Electronic, Berlin, Germany). Vortex mixer REAX top (Heidolph Instruments, Schwabach, Germany). Bruker 450-GC gas chromatograph coupled to a Bruker 320-MS triple-stage quadrupole mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA).

5.2. OPTIMIZATION OF SAMPLE PREPARATION

5.2.1. Spiking samples

To validate a method, the best option is to use a certified reference material of the same matrix that the samples. If not available, spiked samples at two different concentrations provided that an equilibration with the matrix is often used. For this reason, in order to validate the method, spiked samples and un-spiked samples have been analysed [22]. Also, to simplify the study, spiking was performed at a single level. With the vegetables analysed in this project, 3 samples were spiked with 100 ng/g, 3 samples un-spiked and 2 blanks.

5.2.2. Extraction

Vegetable extraction was adapted from a previously method [3,5]. Briefly, 1 g of vegetable matter was placed in a glass tube. Subsequently, 3 samples were spiked with 100 ng of mixture of 16 PAHs. Then, vortexed to homogenize the spikes and then, the samples were left for equilibration over approximately 1 hour.

In the next step, the saponification was performed with 10 mL of the saponification solution. Once this is done, the samples were sonicated for 30 min at 40ºC. In the next step, 1 g of NaCl for salting out and 5 mL of hexane were added to begin the extraction of PAHs. In order to facilitate the phase separation, the samples were centrifuged at 3000 rpm for 15 min. Finally, the recovered organic phase in the centrifugation tubes was removed, transferring them to scintillation vials.

To achieve the largest PAH recovery, 5 mL of hexane was added in the aqueous phase present in the centrifugation tubes, performing the same procedure as described above. Once the second extraction is done, the two organic phases were combined in the pertinent scintillation vial.

Before performing the clean-up step, the organic phase was concentrated with a gentle stream of N_2 which allows reducing the initial volume present in the vial to about 200 µL by evaporation. On the other hand, if the next step was not immediately performed, the samples were stored in the freezer [Appendix 2].

5.2.3. Clean-up

Clean-up was made by adsorption chromatography in an open glass column (30 x 0.8 cm) packed with 1 g of anhydrous sodium sulfate in the top and 5 g of neutral alumina (activated at 400ºC deactivated with 5% Milli-Q water).

The eluted extract was evaporated under a gentle nitrogen stream until ca. 200 µL, and then 122.5 ng of TPhA was added as an IS. Finally, the amber vials were stored in the freezer until their analysis [Appendix 2].

5.3. GC – MS/MS ANALYSIS

Analytical GC – MS/MS condition used for the detection of PAHs, are indicated below [Table 2]:

Conditions of GC – MS/MS	
Column	SAPIENS.5MS (20 m × 0.18 mm × 0.36 µm) (5% diphenyl 95% dimethyl polysiloxane) Teknokroma (Sant Cugat del Vallès, Spain).
Injector Temperature	290ºC
Injection Mode	Splitless
Purge activation time	1.0 min
Pressure pulse / Pulse duration	35 psi / 1.0 min
Injection Volume	1 µL
Carrier Gas / Flow Rate	Helium / 0.6 mL/min
Temperature Ramps	60ºC isotherm 1 min 60ºC to 190ºC at 15ºC/min 190ºC to 265ºC a 7.5ºC/min 265ºC to 310ºC 3ºC/min 310ºC isotherm 2 min
Ionization Mode	EI – 70 eV
Acquisition Mode	MRM

Table 2. Conditions used for GC – MS/MS.

The MRM mode was selected to obtain the calibration curve and the subsequent detection of PAHs in the vegetable samples. This acquisition mode allows working with different transitions from the father ion (precursor) to the corresponding daughter ion.

For each PAH determination, it is necessary to select two transitions, one for quantification and the other for the unequivocal identification of the ion to study [22]. The following table shows the different transitions for each analyte and the energies that are used for fragmentation and retention time for each one [Table 3] [23,24]. As illustrated, many of the transitions involve a

loss of molecular mass H_2^+, $[C_2H_4]^+ + [H_2]^+$. Because, the largest PAHs are more difficult to perform their fragmentation due to their molecular stability, the ionized parent molecules were used instead of the daughter ion [4,17].

Parameters for the GC – MS/MS determination of PAHs					
PAH	**Retention time** [min]	**Transition of quantification**	**Collision energy** [eV]	**Transition of identification**	**Collision energy** [eV]
Naphthalene	6.37	128>128	8	128>102	21
Acenaphthylene	8.11	152>151	19	152>150	29
Acenaphthene	8.37	154>153	19	154>152	32
Fluorene	9.21	166>165	22	166>164	27
Phenanthrene	11.11	178>152	19	178>151	32
Anthracene	11.21	178>152	19	178>151	32
Fluoranthene	11.70	202>202	12	202>200	34
Pyrene	11.75	202>202	12	202>200	34
Benz[a]anthracene	17.32	228>226	32	228>228	10
Chrysene	17.48	228>226	32	228>228	10
Benzo[b]fluoranthene	20.77	252>250	33	252>252	9
Benzo[k]fluoranthene	20.82	252>252	9	252>250	33
Benzo[a]pyrene	21.70	252>252	9	252>250	33
Indeno[1,2,3-cd]pyrene	24.32	276>274	34	276>276	8
Dibenzo[a,h]anthracene	24.41	278>278	9	278>276	35
Benzo[ghi]perylene	24.81	276>276	8	276>274	34

Table 3. Parameters for the GC – MS/MS determination of PAHs.

All the acquired data were processed by using MS Workstation 8MS (Bruker).

With the samples analysed, the corresponding limits of detection (LODs) and limits of quantification (LOQs) of the method and the instrument were calculated for each PAHs.

These LODs and LOQs were obtained from the equations shown below, in which the following parameters were implemented: parameters of the calibration curve (y-interception (A) and slope (B)), blank signals (Y_{BL}), standard deviation of blanks in the range of signals for each analyte (s_{BL}) and standand deviation of y-inteception (s_A).

The LODs of the method were calculated from the following equations [Equation 1, 2 and 3]:

$$Y = A + Bx$$

Equation 1. Calibration curve.

$$Y_{LOD} = Y_{BL} + 3s_{BL}$$

Equation 2. Calculation of Y_{LOD} using Y_{BL} and S_{BL}.

$$LOD = \frac{(Y_{LOD} - A)}{B}$$

Equation 3. Calculation of LOD using Y_{LOD} and parameters of calibration curve.

The LOQs of the method were obtained with these equations [Equation 1, 4 and 5]:

$$Y_{LOQ} = Y_{BL} + 10s_{BL}$$

Equation 4. Calculation of LOQ using Y_{LOD} and S_{BL}.

$$LOQ = \frac{(Y_{LOQ} - A)}{B}$$

Equation 5. Calculation of LOQ using Y_{LOQ} and parameters of calibration curve.

For calculations of LOD and LOQ of the instrument, the following equations were employed [Equation 1, 6 and 7]:

$$LOD = \frac{(3 \times S_A)}{B}$$

Equation 6. Calculation of LOD using S_A and parameters of calibration curve.

$$LOQ = \frac{(10 \times S_A)}{B}$$

Equation 7. Calculation of LOQ using S_A and parameters of calibration curve.

6. RESULTS

6.1. OPTIMIZATION OF SAMPLE PREPARATION

The volume of the sample was selected from previous works where the main object to analyse similar matrices to the ones studied in this research [5,6,15].

Vegetable samples usually contain lipids, carbohydrates and pigments (chlorophylls, carotenoids) that can compromise the analytical instrument performance, obviously it must be avoided or minimized. Consequently, a **saponification** process is carried out to eliminate the fatty acids present in the samples [4,10]. Usually, this reaction must be performed for a long period of time to ensure a complete elimination of these compounds. However, in order to accelerate and homogenize this process throughout the whole sample, the saponification reaction was carried out in an ultrasound bath for 30 min at 40ºC [15].

Before establishing the saponification reaction, a preliminary assay was performed with all the vegetal matrices to evaluate the saponfication performance. In this assay, a variety of vegetal matrices with different lipidic content and pigments were selected namely, broad beans, lettuce, tomato and cauliflower. Once the saponification was performed, 5 mL of hexane was introduced to each reaction vessel in order to separate the organic phase. At this point, a large emulsion occurs in all the samples [Figure 1].

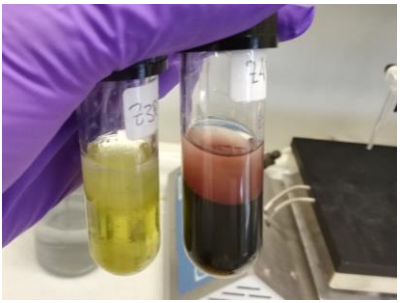

Figure 1. Sample of lettuce (left) and sample of broad bean (right). Both with emulsion but more in bean sample.

In order to minimize the emulsion formation, 1 g of NaCl as solid, was introduced in the centrifuge vials. In fact, NaCl is a strong electrolyte that gets completely dissociated in the aqueous phase increasing the ionic strength of the aqueous solution breaking the emulsions.

Accordingly, it improves the interface present between aqueous phase and organic phase when the hexane is introduced, known as salting out effect [Figure 2].

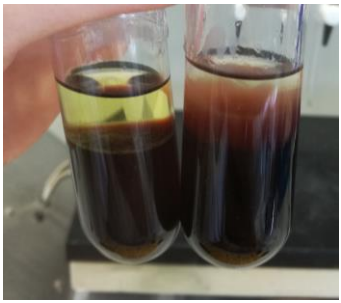

Figure 2. Beans extract with NaCl (left) and without NaCl (right). Both with 1 g of broad beans, 10 mL saponification solution and 5 mL of hexane.

Based on this trial, 1 g of NaCl, before the introduction of hexane liquid-liquid extraction was added.

Regarding to the **clean-up**, column chromatography of the samples was performed with 5 g of silica gel (activated at 400ºC, deactivated with 3% water) one of the most frequently adsorbent used to carry out the clean-up of the samples [13,14]. The analytes were eluted with 9:1 hexane/ethyl acetate (v/v).

One of the biggest inconvenient about silica gel is the column packaging. This fact caused that some of the sample extracts never got proper, elution or, if they managed to get eluted, the samples obtained had an intense coloration suggesting that they are not useful for GC-MS. Due to this disadvantage, some parameters were adjusted to improve the clean-up process.

Three tests were simultaneously performed with different columns, where some conditions were modified (silica gel deactivation and eluent composition). One of them was a decrease in the deactivation from 3 to 1%, which was expected to allow silica to have higher activity that could retain better all the co-extracted interferences. In another column, the only parameter modified was the of the eluent proportions at 9.5/0.5 hexane/ethyl acetate (v/v), decreasing the polarity of the eluent which facilitated the elution of PAHs. Finally, in the third column both modifications previously mentioned were combined. Nevertheless, none of these adjustments improved the initial results.

Accordingly, neutral alumina (5 g, activated at 400ºC, deactivated with 5% water) was evaluated as adsorbent by using hexane as eluent.

By comparing the two adsorbents media indicated above (silica and alumina), alumina exhibited better performance than silica to pack columns. Moreover, it minimized the intensity of the extract colour of the lettuce, tomato and cauliflower (pigments remained retained in the upper part of the alumina phase) [Figure 3].

Figure 3. Column chromatography clean-up showing how red pigments of tomato sample are retained on the top of the alumina phase.

6.2. OPTIMIZATION OF GC – MS/MS MRM METHOD

Before performing the detection of PAHs in the samples, it is necessary to carry out the calibration for each of the 16 targeted PAHs. The calibration curves were evaluated at 8 concentration levels from 10000 ng to 100 ng.

Once the calibration curve was obtained for every analyte, certain compounds with a higher MW show poor response at lower concentrations [Figures 4], differing from the lower MW counterparts.

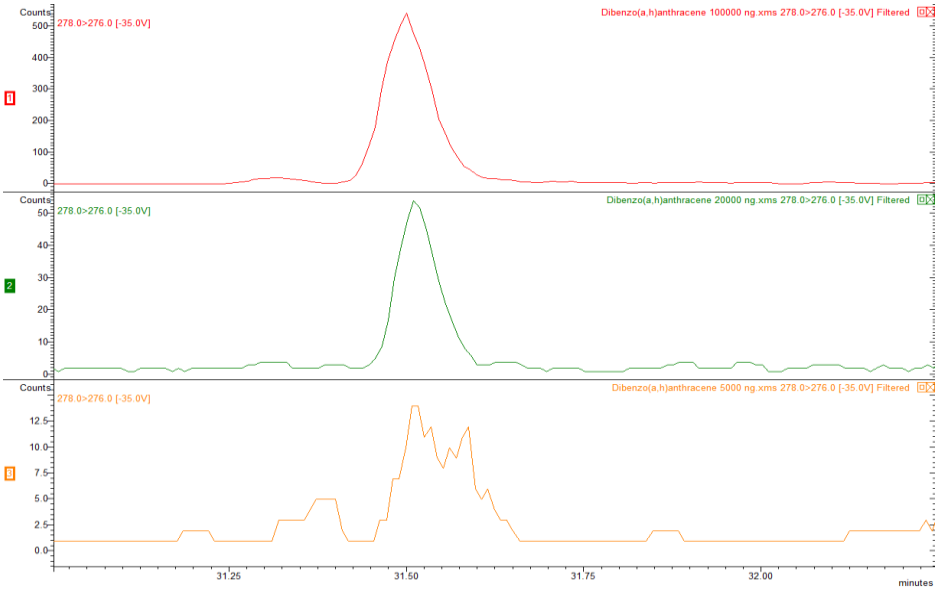

Figure 4. Dibenzo[a,h]anthracene from 1000 ng at 500 ng in 278>274.

Due to these instrumental results, several acquisition conditions were modified in order to improve the detection of higher MW PAHs. To evaluate if these adjustments were successful, the detection of the PAH solution with 1 ng/µL under the initial conditions was previously performed [Table 2]. This will allow obtaining clear peaks that will facilitate the comparison with values obtained in other tests.

The first modification was the **transfer line temperature**, which is a section that allows that the separated analytes by GC reach the MS/MS ions source. This transfer line is usually at a high temperature to avoid a possible condensation of the analytes.

During the first assay, transfer line temperature was increased from 290ºC to 300ºC to ensure that the PAHs with higher MW remain in gaseous state in the transfer line, which might have caused the low sensitivity in the previous detection. Notwithstanding, the results showed a decrease in response even for the analytes with lower MW [Figure 5].

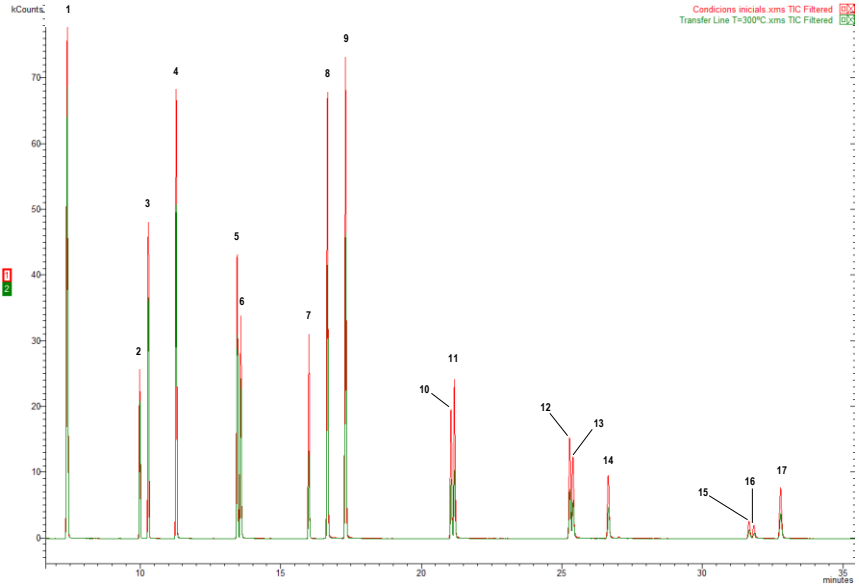

Figure 5. Total Ion Current (TIC) to initials conditions (red) and with transfer line at 300ºC (green). 1. Naphthalene, 2. Acenaphthylene, 3. Acenaphthene, 4. Fluorene, 5. Phenanthrene, 6. Anthracene, 7. Triphenylamine (IS), 8. Fluoranthene, 9. Pyrene, 10. Benz[a]anthracene, 11. Chrysene, 12. Benzo[b]fluoranthene, 13. Benzo[k]fluoranthene, 14. Benzo[a]pyrene, 15. Indeno[1,2,3-cd]pyrene, 16.Dibenzo[a,h]anthracene, 17. Benzo[ghi]perylene.

The next test was performed increasing the transfer line temperature up to 310ºC, to ensure that the former results are confirmed. However, the response of the higher MW analytes did not increase [Figure 6].

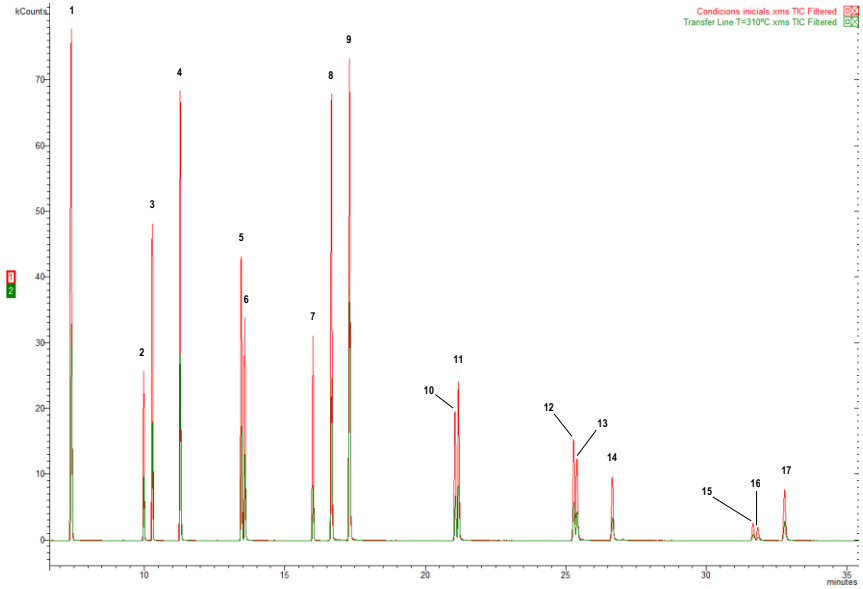

Figure 6. Total Ion Current (TIC) to initials conditions (red) and with transfer line at 310ºC (green). 1. Naphthalene, 2. Acenaphthylene, 3. Acenaphthene, 4. Fluorene, 5. Phenanthrene, 6. Anthracene, 7. Triphenylamine (IS), 8. Fluoranthene, 9. Pyrene, 10. Benz[a]anthracene, 11. Chrysene, 12. Benzo[b]fluoranthene, 13. Benzo[k]fluoranthene, 14. Benzo[a]pyrene, 15. Indeno[1,2,3-cd]pyrene, 16.Dibenzo[a,h]anthracene, 17. Benzo[ghi]perylene.

After these tests, it was concluded that the transfer line temperature was not a parameter that could improve the results. Then, the **temperature of the injector** was increased up to 300ºC and the temperature of the transfer line was hold to its initial value (290ºC). Nevertheless, with this new test there was no improvement with the final results if compared with the initial conditions of the method [Figure 7].

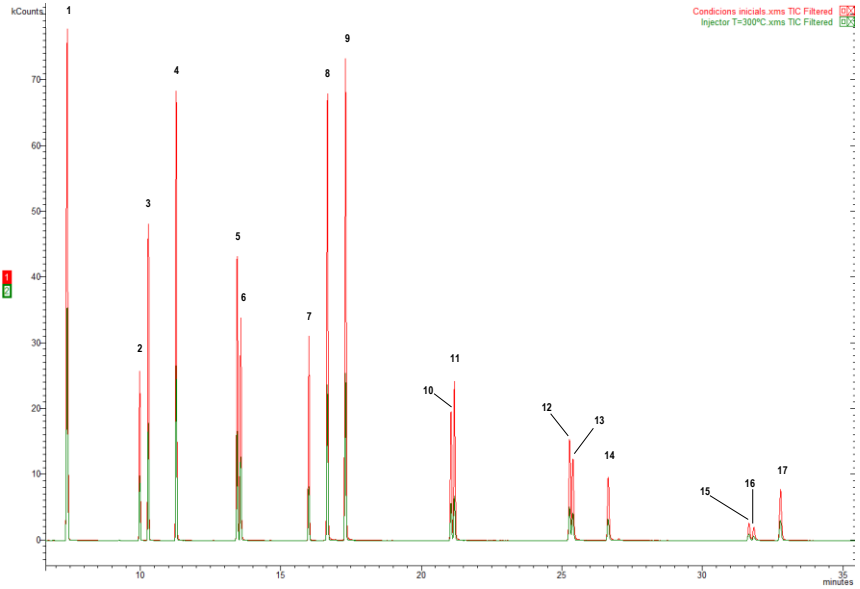

Figure 7. Total Ion Current (TIC) to 290ºC initial conditions (red) vs injector at 300ºC (green). 1. Naphthalene, 2. Acenaphthylene, 3. Acenaphthene, 4. Fluorene, 5. Phenanthrene, 6. Anthracene, 7. Triphenylamine (IS), 8. Fluoranthene, 9. Pyrene, 10. Benz[a]anthracene, 11. Chrysene, 12. Benzo[b]fluoranthene, 13. Benzo[k]fluoranthene, 14. Benzo[a]pyrene, 15. Indeno[1,2,3-cd]pyrene, 16.Dibenzo[a,h]anthracene, 17. Benzo[ghi]perylene.

Due to the results obtained from the three different tests performed, the following research was finally carried out following the initial conditions, and the linearity, limit of detection and quantitation (method and instrumental) and recovery were determined for every matrix [Tables 4-6]:

LODs and LOQs, linearity and recoveries for lettuce						
PAH	**Method**		**Instrument**		**R^2**	**% Recovery**
	LOD [ng/g]*	**LOQ** [ng/g]*	**LOD** [ng]	**LOQ** [ng]		
Naphthalene	1490	2949	227	757	0.9999	0.8
Acenaphthylene	557	1030	773	2577	0.9998	Not detected
Acenaphthene	4253	13491	572	1906	0.9998	Not detected
Fluorene	432	929	315	1051	0.9999	Not detected
Phenanthrene	1798	4512	623	2077	0.9999	Not detected
Anthracene	2149	5185	643	2143	0.9993	Not detected
Fluoranthene	472	504	910	3033	0.9996	Not detected
Pyrene	493	566	1049	3498	0.9994	Not detected
Benz[a]anthracene	721	766	1698	5659	0.9993	Not detected
Chrysene	878	898	2002	6674	0.9990	Not detected
Benzo[b]fluoranthene	2478	2558	6955	29216	0.9959	Not detected

Benzo[k]fluoranthene	3674	4792	8236	27454	0.9943	Not detected
Benzo[a]pyrene	3099	3403	6571	21903	0.9963	Not detected
Indeno[1,2,3-cd]pyrene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Dibenzo[a,h]anthracene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Benzo[ghi]perylene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

*LOD and LOQ with lettuce in fresh weight.

Table 4. Limit of detection (LOD), limit of quantification (LOQ), linearity and recovery of lettuce. Recoveries were calculated with the ng obtained from spiked samples, to which the ng present in un-spiked samples was deducted to determine the real ng obtained from spike.

LODs and LOQs, linearity and recoveries for tomato						
PAH	**Method**		**Instrument**		**R^2**	**% Recovery**
	LOD [ng/g]*	**LOQ** [ng/g]*	**LOD** ng]	**LOQ** [ng]		
Naphthalene	1513	2997	227	757	0.9999	9.4
Acenaphthylene	556	1046	773	2577	0.9998	16.4
Acenaphthene	4321	13708	572	1906	0.9998	10.3
Fluorene	439	944	315	1051	0.9999	29.9

Phenanthrene	1827	4584	623	2077	0.9999	33.8
Anthracene	2183	5269	643	2143	0.9993	2.9
Fluoranthene	480	512	910	3033	0.9996	37.3
Pyrene	501	575	1049	3498	0.9994	Not detected
Benz[a]anthracene	733	778	1698	5659	0.9993	Not detected
Chrysene	892	912	2002	6674	0.9990	Not detected
Benzo[b]fluoranthene	2518	2599	6955	29216	0.9959	Not detected
Benzo[k]fluoranthene	3733	4869	8236	27454	0.9943	Not detected
Benzo[a]pyrene	3148	3457	6571	21903	0.9963	Not detected
Indeno[1,2,3-cd]pyrene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Dibenzo[a,h]anthracene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Benzo[ghi]perylene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

*LOD and LOQ with tomato in fresh weight.

Table 5. Limit of detection (LOD), limit of quantification (LOQ), linearity and recovery of tomato. Recoveries were calculated with the ng obtained from spiked samples, to which the ng present in un-spiked samples was deducted to determine the real ng obtained from spike.

LODs and LOQs, linearity and recoveries for cauliflower						
PAH	**Method**		**Instrument**		**R^2**	**% Recovery**
	LOD [ng/g]*	**LOQ** [ng/g]*	**LOD** [ng]	**LOQ** [ng]		
Naphthalene	1573	3115	227	757	0.9999	17.9
Acenaphthylene	1045	1088	773	2577	0.9998	13.7
Acenaphthene	4435	14251	572	1906	0.9998	11.5
Fluorene	870	981	315	1051	0.9999	9.0
Phenanthrene	2410	4766	623	2077	0.9999	23.9
Anthracene	784	5477	643	2143	0.9993	1.2
Fluoranthene	1281	533	910	3033	0.9996	1.8
Pyrene	1390	598	1049	3498	0.9994	4.8
Benz[a]anthracene	1673	809	1698	5659	0.9993	Not detected
Chrysene	1722	948	2002	6674	0.9990	Not detected
Benzo[b]fluoranthene	3513	2702	6955	29216	0.9959	Not detected

Benzo[k]fluoranthene	4314	5062	8236	27454	0.9943	Not detected
Benzo[a]pyrene	5166	3594	6571	21903	0.9963	Not detected
Indeno[1,2,3-cd]pyrene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Dibenzo[a,h]anthracene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Benzo[ghi]perylene	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected

*LOD and LOQ with cauliflower in fresh weight.

Table 6. Limit of detection (LOD), limit of quantification (LOQ), linearity and recovery of cauliflower. Recoveries were calculated with the ng obtained from spiked samples, to which the ng present in un-spiked samples was deducted to determine the real ng obtained from spike.

As expected, these results show that the PAHs with the highest MW (Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene and Benzo[ghi]perylene) can not be detected at lower concentrations. Therefore, neither their corresponding calibration curves nor neither their detection and quantification limits can be determined.

Moreover, cauliflower samples show slightly higher recovery values compared to samples of other vegetables [Figure 8]. However, low values are obtained in any of the matrices, even by PAHs with lower MW, which presence was expected to be bigger. In view of this fact, it can be affirmed that the experimental method developed is not successful for PAH determination in all the matrices evaluated.

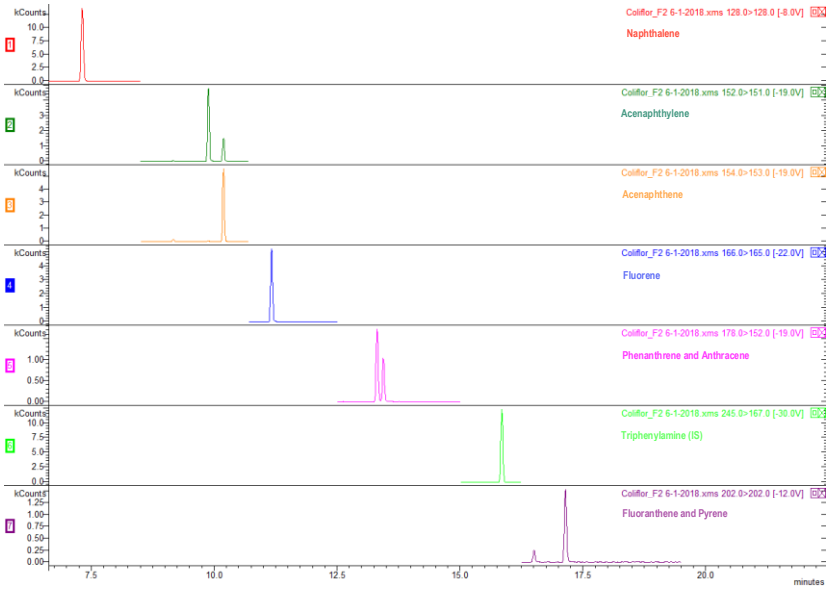

Figure 8. Cauliflower spiked sample. Naphthalene (128>128), Acenaphthylene (152>151), Acenaphthene (154>153), Fluorene (166>165), Phenanthrene (178>152), Anthracene (178>152), Triphenylamine (IS, 245>167), Fluoranthene (202>202), Pyrene (202>202).

7. DISCUSSION

The poor PAH recoveries obtained in this work could be attributed to different factors. One possibility could be the emulsion formation since the salting out effect of NaCl leads to an insufficient ionic strength in the aqueous phase to eliminate completely the emulsions generated during the saponification reaction. This fact could cause the ineffective extraction of PAHs from the aqueous phase with n-hexane. In order to eliminate emulsions, a stronger electrolyte, such as $MgSO_4$, could be used which increases the ionic strength of the aqueous phase and, as a consequence, it could decrease the chances of emulsion formation.

Nevertheless, the clean-up step is considered one of the most critical of the analytical procedure. In fact, since n-hexane was used as a mobile phase to elute the analytes, which is

probably non polar enough to elute quantitatively the targeted PAHs. Consequently, in the future, the clean-up process should be revaluated by increasing the polarity of the elution solvent used to elute the analytes in the chromatography column. Moreover, Florisil® could be evaluated instead of alumina as an alternative to the alumina as stationary phase as provides strong retention of plant pigments as chlorophylls. [6,15].

Regarding to the detection step, with the results indicated previously, it is concluded that the initial conditions are the most appropriate ones to conduct the quantification and the identification of PAHs, as there is no improvement on the results obtained when several adjustments either in the GC or MS were performed.

In this regard, the response reduction obtained by increasing the transfer line temperature can be related to the thermal degradation when operated at higher temperatures, resulting in a decrease in the abundance of low MW PAHs; while analytes with higher MW are less affected as their boiling points are higher than the temperature of the transfer line.

On the other hand, when increasing the injector temperature, as with the transfer line, there is no improvement in the results, but in this case the explanation may be due to that solvent undergoes an explosive volatilization in the splitless injection affecting to PAH losses by condensation in the cold spots of the injector (gas tubing and valves).

In order to increase the response of high MW PAHs, samples could be analysed mainly with a GC-MS/MS device but using a Programmed Temperature Vaporization (PTV) injector instead of splitless that allows the injection of higher volumes and is less prone to the mass discrimination of the high boiling compounds, which would enhance the sensitivity of all PAHs studied, particularly the high boiling compounds [4,25].

Nevertheless, it is important to mention that each vegetable sample has matrices with different properties, which difficult the obtention of an universal analytical procedure for any vegetable sample suggesting the need to adjust the extraction and clean up steps to the matrix specificity.

10. CONCLUSIONS

- The analytical method developed was not successful for the determination of PAHs in any of the vegetable matrices studied in this project.
- The saponification reaction generates the presence of emulsions that can not be completely removed, despite the introduction of a strong electrolyte in the samples. This fact affects the extraction recovery of PAHs.
- The low polarity of the eluent used during the clean-up step could generate an uncomplete elution of the analytes of interest.
- From the parameters evaluated, the acquisition method used in the GC-MS/MS is the most optimal for the detection of PAHs, since after several modifications of the initial parameters, the detection of analytes was not improved.

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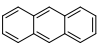
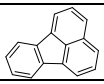
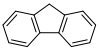
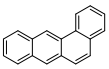
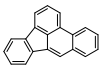
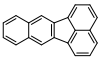
12. ACRONYMS

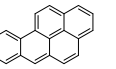
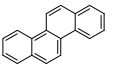
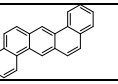
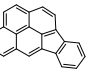
ASE	Accelerated Solvent Extraction
CE	Capillary Electrophoresis
EI	Electronic Impact
EPA	Environmental Protection Agency
EU	European Union
FAO	Food and Agriculture Organization
FID	Flame Ionization Detector
FLD	Fluorescence Detector
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HS	Headspace
HS-SPME	Headspace-Solid Phase Microextraction
IPCS	International Programme on Chemical Safety
IS	Internal Standard
IT	Ion Trap
LLE	Liquid-Liquid Extraction
LOD	Limit of detection
LOQ	Limit if quantification
MAE	Microwave Assisted Extraction
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MW	Molecular Weight

OT	Orbitrap
PAHs	Polycyclic Aromatic Hydrocarbons
PTV	Programmed Temperature Vaporization
QqQ	Triple Quadrupole
qTOF	Quadrupole coupled of Time of Flight
QuECheRS	Quick, Easy, Cheap, Effective, Rugged and Safe
SPE	Solid-phase Extraction
SQ	Single Quadrupole
TIC	Total Ion Current
TOF	Time of Flight
UAE	Ultrasound Assisted Extraction
UV-Vis	Ultraviolet-Visible
WHO	World Health Organization

APPENDICES

APPENDIX 1: LIST OF PAHS – US-EPA

List of PAHs – US-EPA				
PAH	**Abbreviation**	**CAS number**	**Molecular mass**	**Structure**
Acenaphthene	ACP	83-32-9	154.1	
Acenaphthylene	ACY	208-96-8	152.2	
Anthracene	ANT	120-17-7	178.23	
Fluoranthene	FA	206-44-0	202.3	
Fluorene	FLR	86-73-7	166.2	
Naphthalene	NPH	91-20-3	128.2	
Phenanthrene	PHE	85-01-8	178.2	
Pyrene	PYR	129-00-0	202.3	
Benz[a]anthracene	BAA	56-55-3	228.3	
Benzo[b]fluoranthene	BFF	205-99-2	252.3	
Benzo[k]fluoranthene	BKFc	207-99-2	252.3	
Benzo[ghi]perylene	B(ghi)P	191-24-2	276.3	

Benzo[a]pyrene	BAP	50-32-8	252.3	
Chrysene	CHR	218-01-9	228.3	
Dibenzo[a,h]anthracene	DB(ah)A	53-70-3	278.3	
Indeno[1,2,3-c,d]pyrene	IP	193-39-5	276.3	

APPENDIX 2: SAMPLE PREPARATION