Design of a method for polycyclic aromatic hydrocarbons routine determination by liquid-liquid microextraction and GC-MS/MS: Optimisation and validation.

Master in Applied Chromatographic Techniques

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Contents

Acronym list

ACN	Acetonitrile
AGC	Automatic gain control
DC	Direct current
DWTP	Drinking water treatment plant
EI	Electron ionization
EU	European union
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-MS/MS ITD	Gas chromatography coupled to tandem mass spectrometry using ion trap detector
HPLC	High performance liquid chromatography
IC	Ion chromatography
ICP	Inductively coupled plasma
ILIS	Isotope-labelled internal standard
IT	Ion trap
LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PAHs	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PF	Preconcentration factor
PSI	Pound-force per square inch
PTV	Programmable temperature vaporization
PV	Parametric value
RD	Royal decree
RF	Radiofrequency
RSD	Relative standard deviation
SPE	Solid phase extraction
SOP	Standard operating procedures
QqQ	Triple quadrupole
WPT	Water purification plants

Valero Analítica S.L.

This essay has been carried out in a company established in Zaragoza called, *Valero Analítica S.L*. (Valero Analítica, 2021). This company was founded in 2004 and consists of a physico-chemical and a microbiological laboratory. Its work focuses on health, environmental and agri-food areas; and on chemical, pharmaceutical and cosmetic industries. **Figure 1** shows the organisational chart of the company.

The laboratory has different equipments: Gas Chromatography – Tandem Mass Spectrometry (GC-MS/MS), High Performance Liquid Chromatography (HPLC), Ion Chromatography (IC), Inductively Coupled Plasma (ICP) and Fourier Transform Infrared spectroscopy (FTIR).

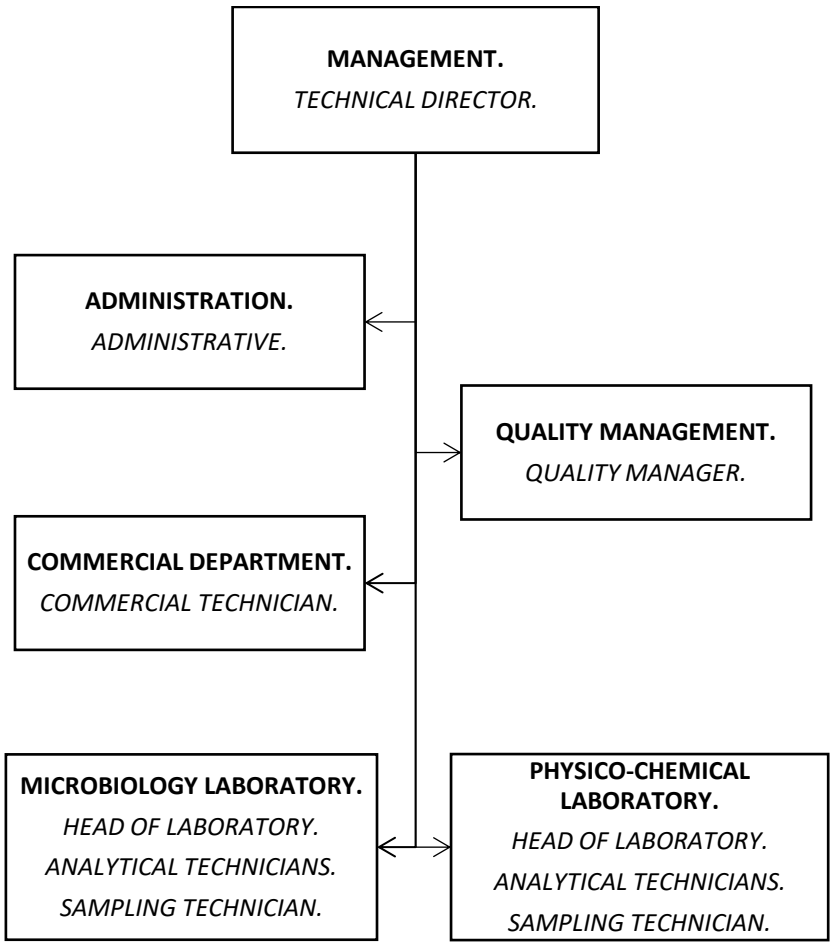


***Figure 1**. Original organisational chart of Valero Analítica S.L. (adopted from Valero Analítica S.L., 2018).*

Summary

This study focuses on the optimisation and validation of a method for the determination of Polycyclic Aromatic Hydrocarbons (PAHs) to be applied in real supply waters of the Zaragoza area. The supply water samples were analysed by gas chromatography coupled to tandem mass spectrometry using ion trap (GC-MS/MS IT) due to its great selectivity and sensitivity. Injections in GC were performed by programmable temperature vaporization (PTV) in solvent venting mode of the samples. A liquid-liquid microextraction (LLME) treatment was necessary to concentrate the sample for analysis. Moreover, in order to correct matrix effects, Isotopically-Labelled Internal Standard (ILIS) was added to the samples. Several experiments were carried out to optimise the PAHs extraction method, chromatography and MS/MS conditions. The study focused mainly on *Acenaphthylene* and *Acenaphthene* as these compounds were not included in the method in use at *Valero Analítica*. In addition, the research also focused on *Benzo(β)fluoranthene*, *Benzo(g,h,i)perylene*, *Benzo(κ)fluoranthene* and *Indeno(1,2,3-c,d)pyrene*, compounds regulated by RD 140/2003. The results of this work indicate that a comprehensive study of the PAHs of interest can be performed on the optimised method. The validation provides positive data for all compounds except for *Acenaphthylene* and *Acenaphthene*, which have an (limit of quantification) LOQ more than three times higher than the rest of PAHs. The results of this study contribute to report more complete PAHs data and therefore to understand which compounds are found in drinking water.

1. Introduction

Water is essential for life. It is a fundamental component of the nature and it is necessary for the living beings, who are composed by around 70 % of water. Furthermore, water acts as a transporter of nutrients, among other functions for the living beings. Apart from that, it is also used to cultivate lands, breeding of cattle... Therefore, for so many reasons, water should be kept free from waste and micropollutants. "According to the Health and Consumer Protection Directorate-General of the European Commission, food contaminants are substances that can be present in certain foodstuffs due to environmental contamination, cultivation practices, or production processes. If present above certain levels, these substances can pose a threat to human health" (Campo & Picó, 2015).

This work focuses on the Polycyclic Aromatic Hydrocarbons (PAHs) which are considered as contaminants and are of serious concern because of their recognised toxicity and, above all, the carcinogenic risk they present (Keyte et al., 2013), specifically *Benzo(α)pyrene* is the PAHs most studied due to its proven carcinogenic activity (Ramos-Contreras et al., 2019) and is therefore regulated in the Royal Decree 140/2003 (Ministerio de la Presidencia RD 140, 2003). PAHs enter into the environment through different routes and are usually detected in the air, soil and water (Abdel-Shafy & Mansour, 2016). **Figure 2** illustrates the dispersion of PAHs throughout air and its displacement by land and water means as a result of different processes. In particular, the present work deals with the development of a method of analysis of PAHs in drinking water and bottled water.

PAHs arrive via rainwater (Hussain et al., 2019) and groundwater to receiving water bodies, which are treated in a drinking water treatment plant (DWTP). These plants carry out different treatments and supply drinking water to villages. However, these treatments seem not to be enough to eliminate contaminants such as PAHs.

In summary, the objective of this work is to develop a method to determine the PAHs presents in drinking water samples from the analysis by gas chromatography coupled to tandem mass spectrometry using ion trap detector (GC-MS/MS ITD). Therefore, it would ensure that inhabitants of different villages in Zaragoza are receiving healthy and adequate water.

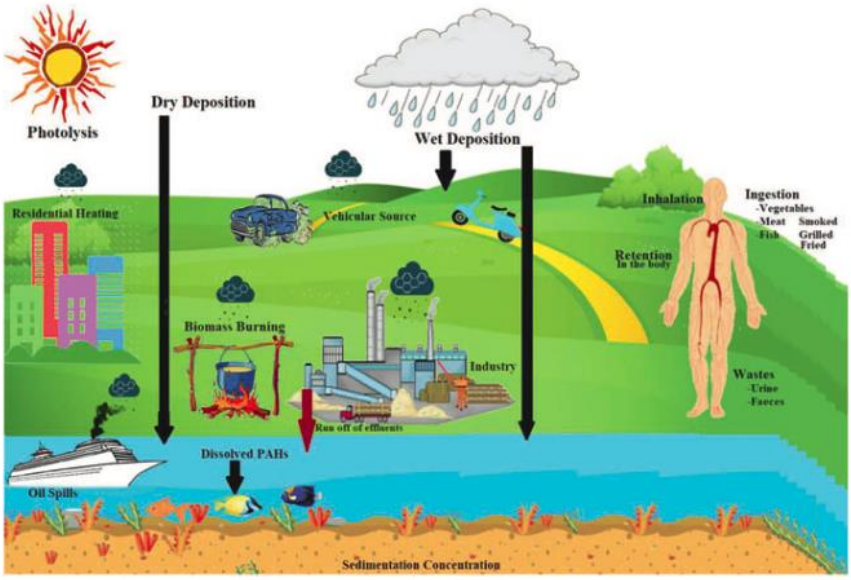


***Figure 2**. Dispersion of PAHs through the air, the terrestrial and aquatic environments. (Hussain et al., 2019)*

1.1. PAHs in water consumption

This work is devoted to the determination of PAHs in supply water. PAHs are within the group of persistent organic contaminants, which also includes pesticides, flame retardants (polychlorinated biphenyls PCB, polybrominated diphenyl ethers PBDE).

PAHs are a group of organic compounds which are characterised by containing two or more benzene rings joined together. These rings may exist in different isometric arrangements. Moreover, they are always polynuclear aromatic structures and depending on the number of rings, the boiling point varies. Therefore, higher molecular weight implies less volatility (higher boiling point) and less solubility in water.

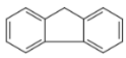
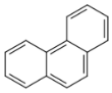
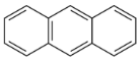
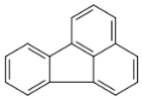
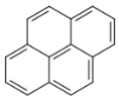
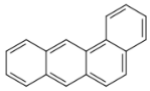
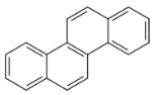
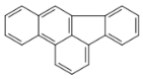
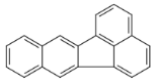
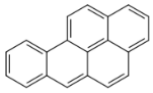
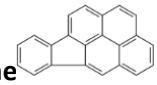
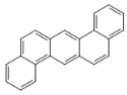
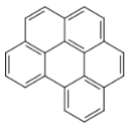
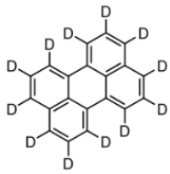
As indicated by several authors, these compounds represent an important class of hazardous organic chemicals derived from anthropogenic sources (i.e. emissions in the environment as a result of vehicle exhaust, asphalt pavements, unvented radiant and convective kerosene space heaters, heating appliances) and natural sources (i.e. all incomplete combustion at high temperature and pyrolytic processes involving fossil fuels, such as peat, coal and petroleum) (Martinez et al., 2004). Furthermore, PAHs compounds are also important pollutants due to its difficult removal in the environment and human health (Avino et al., 2017). Also, PAHs compounds stand out because they are considered as recalcitrant compounds and potentially carcinogenic with high capacity of bioaccumulation in trophic chains, in addition to being mutagenic, steroidogenic, or affecting endocrine functions of the organisms (Martinez et al., 2004).

The Royal Decree (RD) 140/2003 of 7th February (Ministerio de la Presidencia RD 140, 2003) establishes the health criteria that water intended for human consumption must fulfil in order to protect human health from adverse effects derived from any type of pollution in water. This document includes a list with different pollutants and their respective parametric values (PV) (maximum value set for each of the compounds to be controlled), from which five PAHs are studied in the present work. **Table 1** shows the complete list of studied PAHs and their parametric value.

Some of these pollutants are generally introduced into the control programmes, such as in Annex X of the Directive 2000/60/EC (Council directive 2000/60/EC, 2010), due to its wide presence and inclusion in the directives of the European Union (EU).

It is absolutely necessary to develop methods to be used in studies of PAHs presence and concentration in human consumption water with the objective of spreading the awareness of the presence of pollutants in small villages. Therefore, its healthiness, quality and cleanliness are guaranteed, as well as the protection of the health of its inhabitants from adverse effects derived from any type of water pollution (Ministerio de la Presidencia RD 140, 2003).

***Table 1**. List of the PAHs to be studied.*

Compounds	Molecular formula	Molecular mass (u)	Boiling point (°C)	CAS number (CAS, 2021)	Parametrical value (μg/L)
Acenaphthylene 	$C_{12}H_8$	152.19	265-275	208-96-8	
Acenaphthene 	$C_{12}H_{10}$	154.21	279	83-32-9	
Fluorene 	$C_{13}H_{10}$	166.22	295	86-73-7	
Phenanthrene 	$C_{14}H_{10}$	178.23	340	85-01-8	
Anthracene 	$C_{14}H_{10}$	178.23	342	120-12-7	
Fluoranthene 	$C_{16}H_{10}$	202.25	384	206-44-0	
Pyrene 	$C_{16}H_{10}$	202.25	404	129-00-0	
Benzo(α)anthracene 	$C_{18}H_{12}$	228.29	437.6	56-55-3	
Chrysene 	$C_{18}H_{12}$	228.29	448	218-01-9	
Benzo(β)fluoranthene 	$C_{20}H_{12}$	252.31	481	205-99-2	*
Benzo(κ)fluoranthene 	$C_{20}H_{12}$	252.31	480	207-08-9	*
Benzo(α)pyrene 	$C_{20}H_{12}$	252.31	495	50-32-8	0.010
Indeno(1,2,3-cd)pyrene 	$C_{22}H_{12}$	276.33	536	193-39-5	*
Dibenzo(a,h)anthracene 	$C_{22}H_{14}$	278.35	524	53-70-3	
Benzo(g,h,i)perylene 	$C_{22}H_{12}$	276.33	550	191-24-2	*
Perylene-D_{12} (ILIS) 	$C_{20}D_{12}$	264.38	467.52	1520-96-3	

*The total of the compounds must not exceed a parametric value of 0.10 μg/L, so we consider a PV 0.025 μg/L for each of the four compounds.

1.2. Analytical techniques

Considering the fact that the aim of the project has been the development of an analytical procedure for the determination of PAHs in water samples, and that the laboratory disposes gas chromatography coupled to tandem mass spectrometry (GC-MS) with ion trap (IT) analyser, this technique was selected for the method development.

Sample preparation was also studied considering that miniaturisation is a key requisite, and thus micro liquid-liquid extraction was mainly considered.

1.2.1. Liquid-liquid microextraction (LLME)

Most of the samples to be analysed are not in the adequate form in order to be injected in the chromatograph and to determine PAHs at very low concentrations. Therefore, it is necessary to carry out a treatment of the sample which needs to be developed and optimised. The sample treatment aims at extracting, preconcentrating and eliminating interferences (clean-up), so as to directly introduce the sample extract into the equipment to be analysed. This stage is very important within the analytical process because the success of the analysis depends on all its phases. In particular, the techniques of choice in most cases for the extraction of PAHs from water and wastewater are liquid-liquid extraction (LLE) and solid phase extraction (SPE) (Brum et al., 2008). In this work we will focus on LLE.

LLE is a traditional, simple, versatile and very common technique in the laboratories of routine. LLE consists of making contact (shaking) between two immiscible solvents so the analyte (typically non-polar) changes from the original phase of the sample (aqueous) into the solvent for extraction, remaining the interferences in the sample. The process requires a solvent with similar properties to those of the analyte (e.g. polarity) and also needs to be easily evaporated and immiscible with the aqueous phase. Moreover, a requisite that the laboratory indicates is that the extractant should be less dense than the water in order to facilitate the extraction.

Therefore, in LLE the analytes move from being in a larger volume and therefore less concentrated to a smaller volume and more concentrated. The preconcentration factor (PF) could be calculated according to **Equation 1**.

$$PF = \frac{Original\ Sample\ Volume\ (mL)}{Extraction\ Volume\ (mL)} \qquad \textbf{(Equation 1)}$$

Hereafter, this technique will be referred to as liquid-liquid microextraction when the volume of the extracting phase is very small in relation to the volume of the sample, and in some cases, not quantitative extraction is allowed (after internal standard correction of recovery). Therefore, it is possible to work with a small sample, gaining convenience and obtaining great sensitivity. **Figure 3** shows the liquid-liquid microextraction (LLME) carried out in the laboratory.

***Figure 3**. Liquid-liquid microextraction of PAHs.*

1.2.2. The 1079 PTV injector

The injection mechanism carried out in the development of this work is programmable temperature vaporisation (PTV), with the 1079 PTV injector. The PTV injector can be considered as the most universal injector, capable of handling a wide variety of sample types, concentrations and volumes. The sample is transferred in liquid form from the syringe to a cooled inlet (cold insert) and then a ballistic temperature gradient is applied to the sample, allowing each compound to evaporate according to its boiling point. Basically, it is designed as a Split/Splitless injector that can be heated (and cooled) rapidly. In addition, it can also operate in solvent venting mode which allows the injection of large sample volumes (up to 250 µL) removing the solvent and avoiding its entrance to the GC column, and achieve detection limits in the ppb (μg/L) range.

In solvent venting, most of the solvent is removed through the split so the compounds of interest are retained in the liner by the cold trap. The split is then closed and the insert is rapidly heated to evaporise the pre-concentrated compounds of interest and introduce them into the column head. This technique requires slow sample introduction to avoid overloading the insert. Besides, very low boiling temperature compounds will inevitably be partly removed together with the solvent, depending on insert temperatures, type of packed support and split times. In general, low-boiling solvents are preferred for best results. **Figure 4** and **Figure 5** show the 1079 PTV injector and packed liner.

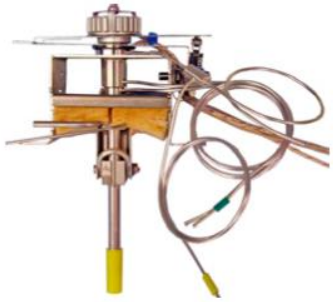


***Figure 4**. The 1079 PTV injector.*

***Figure 5**. Liner used for the PAHs study.*

1.2.3. Gas chromatography (GC)

Chromatography may be defined as a method of separation in which the mixture to resolve is introduced in a system formed by a fluid (mobile phase) that moves around in a close contact with a solid or liquid phase, which is immobile during the process (stationary phase). According to the characteristics of the mobile phase, the chromatography may be divided into three types: liquid, gaseous and supercritical fluids. This section focuses on GC, where the mobile phase (gas) carries the analytes (sample vaporised in the injector) but it does not interact with them, although the stationary phase (liquid) does. The most commonly used mobile phase is Helium, although Argon, Hydrogen and Nitrogen can also be used.

Analytes are separated according to thermodynamic parameters (they depend on the distribution, K_D) and kinetic parameters (they depend on the dispersion). According to the polarity and the boiling point of the compounds which are in the sample in the stationary phase and mobile phases, these compounds will be retained and eluted at different times. A detector is needed in order to transform these chromatographic bands into more comprehensible information. Although there are a lot of detectors, the most reliable for this type of analysis is mass spectrometry. **Figure 6** shows the equipment used for PAHs analysis.

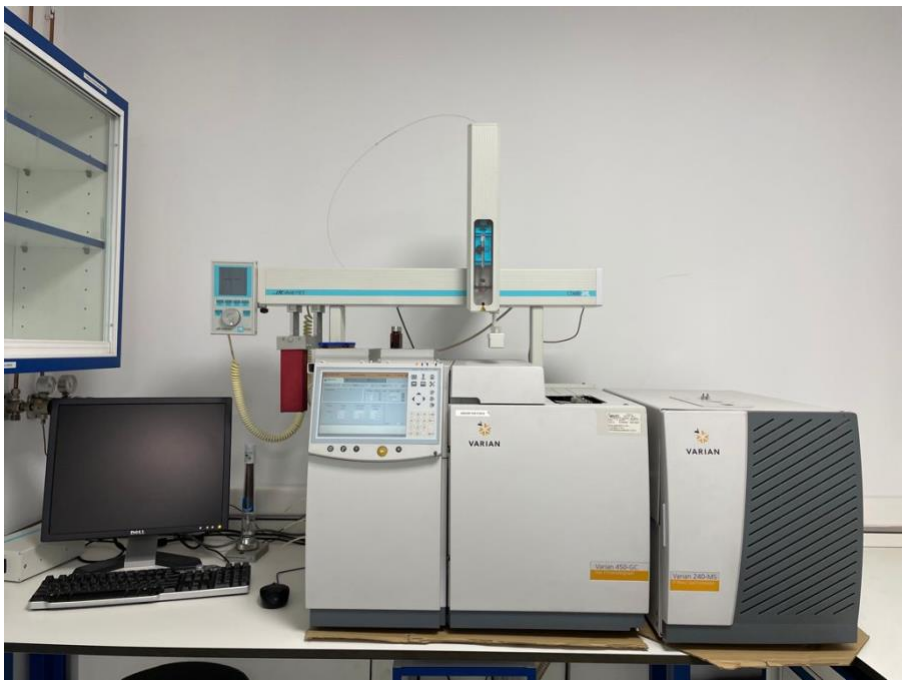


***Figure 6**. Gas chromatograph coupled to MS with autosampler used in the project.*

1.2.4. Mass spectrometry (MS). Ion trap (IT)

Mass spectrometry (MS) is an analytical technique that produces and separates ions in gas phase. MS separates the ions (in time and space) in the gaseous phase according to its m/z ratio. It is a very sensitive technique and it is commonly used in different fields, and it is especially important in environmental sciences with the purpose of studying the presence and quantification of organic pollutants (Hernández et al., 2012).

Once the sample is separated into its components, they enter the MS through a heated transfer line to prevent the sample from condensing. Then, components move to the ion source for ionisation, and then to the analyser where they are separated. In the particular case of an ion trap analyser, they are stored to be systematically ejected for analysis. Once the ions are ejected, the detector registers them.

The essential part of the MS is the analyser, which states on the resolution (i.e. capacity of differentiating between similar mass-to-charge ratios) and system's sensitivity (i.e. the ability to demonstrate that two samples have different amounts of analyte) depend. Moreover, the function of the analyser is to separate the ions based on their m/z ratio. In this study, an IT analyser has been used for the analysis (**Figure 7**). The IT consists of a 3D trap of enclosed area (with He inside, which avoids collisions) that generally contains two hyperbolic metal electrodes with confronted faces and a hyperbolic ring electrode in the middle. When assembled, they form a cavity for ionisation, fragmentation, storage, and mass analysis to occur. Firstly, potentials of direct current (DC) and radiofrequency (RF) are applied to the electrodes. With the proper RF voltage, the ion trap electrodes create a three-dimensional, hyperbolic electric field. This field traps the ions in stable orbits. In the presence of helium damping gas, the ions are cooled towards the center of the trap. As the RF voltage increases, the ion trajectories become unstable in increasing order of mass-to-charge ratio. The ion trap ejects the ions, directing them to the conversion dynode, and then to the electron multiplier, where they are finally measured. Although the expulsion is sequential, the sensitivity increases (especially when compared to quadrupole) because all the ions produced are stabilised, detected and measured. Besides, IT can be extended to tandem mass spectrometry (MS/MS) without combining with another analyser.

Furthermore, the work in MS/MS mode using IT analyser leads to the possibility of selecting an adequate precursor and product ion, and thus reducing chemical noise in the chromatograms (improving signal-to-noise ratio). Apart from that, the use of full spectra for absolute identification at trace levels in environmental samples, considering its ease of use and low cost (in comparison with a triple quadrupole (QqQ)), has made IT a widely used technique to determine organic compounds in water (Martínez Vidal et al., 2000).

The objective of connecting GC and MS techniques is to take advantage of their characteristics. More specifically, complex mixtures of pesticides can be separated through chromatography, so the corresponding compounds can be determined from their mass spectrums. This connection is easy because both techniques work in the gas phase and with an order of mass of pg – ng. However, there is also some incompatibility because the GC works at atmospheric pressure and the MS needs high vacuum. To do so, it is required an ideal interfase, which does not produce distortion in the chromatographic peaks, quantitatively transfers the analyte to the MS after being separated from the other components, and also is compatible with the exit of the column and the entrance of the MS. This is easily accomplished in GC-MS by using a transfer line, which takes the column out of the oven just before the ionisation source and consists of a resistance and insulation to avoid the condensation of the analytes.

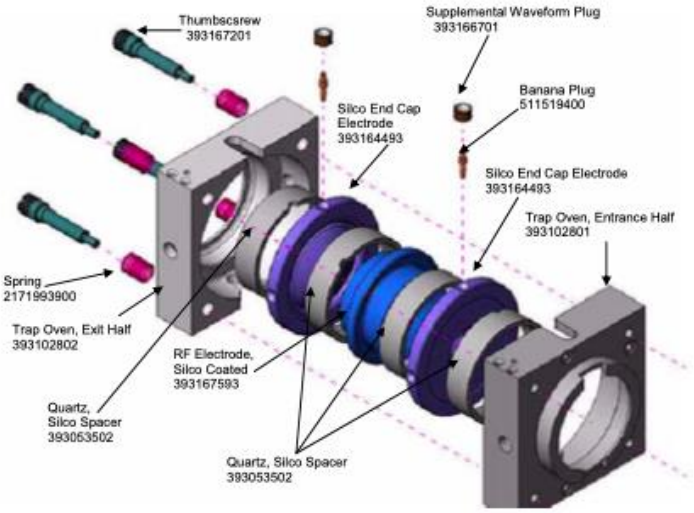


***Figure 7**. Ion trap analyzer.*

2. Objectives

The main objective of this work is the optimisation and validation of a method for the determination of PAHs by liquid-liquid microextraction and detection by GC-MS/MS. This method will be applied to real supply waters of the Zaragoza area.

2.1. Specific objectives

The main objective can be divided into following particular objectives:

- To optimise GC-MS/MS determination for Acenaphthylene and Acenaphthene PAHs and include them to the routine method.
- To increase sensitivity of the overall GC-MS/MS method.
- To optimise micro liquid – liquid extraction.
- To validate the method.
- To demonstrate my skills to plan, organise and implement the necessary analysis.
- To complement the theoretical aspects with the professional reality.
- To acquire working habits in a quality context inside the laboratory.
- To work collaboratively.

3. Materials and methods

3.1. Standards, reagents and materials

Polycyclic aromatic hydrocarbons reference standard (10 mg/L solutions) was purchased from Dr. Ehrenstorfer (Augsburg, Germany) (LGC group, 2021) as well as the solid, isotopically-labelled internal standard (ILIS) (Perylene D12). All the reference standards obtained from the aforementioned sources presented purity levels higher than 93%.

Stock standard solution of all compounds (MIX PAHs) was prepared at 1 mg/L in acetonitrile (ACN), stored at - 20 ºC and volume diluted ten times with acetone in order to prepare 100 μg/L intermediate solution. Then, the working solution was prepared by diluting intermediate solution in cyclohexane (50 μg/L), which contained all the analytes, so they were used to prepare the calibration standards. For spiking samples, another working solution was prepared from intermediate solution, with a final concentration of 5 μg/L in acetone.

A 500 mg/L solution of Perylene D12 (0.0246 g in 50 mL of chloroform) was prepared and stored at - 20 ºC, and diluted ten times with chloroform to prepare 50 mg/L stock standard solution. Furthermore, intermediate solution was prepared by diluting stock standard solution in acetone (5 mg/L) and then diluted ten times with acetone in order to prepare 500 μg/L working solution, used for calibration standards and spiking samples.

A Milli-Q water purification system (arium 611 UV, Sartorius) was used to purify water through filtration, osmosis and other processes so as to obtain LC-MS grade water. Moreover, HPLC grade methanol (MeOH), HPLC grade acetonitrile (ACN), acetone, chloroform, toluene and cyclohexane all HPLC grade were acquired from PanReac AppliChem (Castellar del Vallès, Spain).

This study has also needed: 1.5 mL/9 mm/32 x 11.6 mm and 20 mL/75.5 x 22.5 mm Ambar vials, 9 mm Ultrabond Septum Sil/PTFE caps and V0520 inserts for vials (0.1 – 0.25 mL) purchased from Análisis Vínicos (Tomelloso, Spain) (Vínicos, 2021).

3.2. Experimental

3.2.1. Sample collection

The developed method is applied to samples of consumption water, continental water and wastewater. In order to guarantee the renovation of water, the water tap is opened so as the water flows. Then, the topaz glass bottle of 250 mL, which contains 20 mg of sodium pentahydrate thiosulfate (80 mg per 1000 mL of the sample), is completely filled. All the samples were collected and stored at a cold box supplied with refrigerant blocks and transported to the laboratory once the last sample was collected. After reception in the laboratory, samples were stored at 5 $\pm$ 3 ºC in a fridge and analysed within 2 weeks.

The number of samples to be considered depends on the type of study required by the consumer. In general, the most demanded study is *Análisis Completo*, which requires a sample of the deposit, water purification plant (WPP) and of the supply network, which is commonly taken from the fountain of the village or from its bar. *Valero Analítica* has a wide customer network, but it mainly focuses on Aragón.

3.2.2. Analytical procedure

In order to prepare the samples, they are firstly tempered out of the fridge and when they are at room temperature, the extraction of specific analytes begins. To do so, 50 μL of perylene D12 (50 μg/L) are added to 250 mL of sample and then they are homogenised with a magnetic stirrer. Subsequently, 500 μL of cyclohexane, as solvent extractant, is added. Just after adding the extractant, the bottle is closed in order to avoid loss of cyclohexane, which would imply an over-concentration of analytes. With the objective of extracting the analytes, the sample is constantly stirred for 15 minutes at a speed of 20-30 (designated speed by the agitator). This speed is the maximum to avoid the sample getting in contact with the cap of the bottle. Then, it is allowed to settle during 20 minutes. Then, distilled water is added in order to increase the volume and make the cyclohexane layer, together with the extracted analytes, rise to the bottle neck. Finally, 50 μL of the upper layer (cyclohexane) were collected for the GC-MS/MS analysis. **Figure 8** shows the schematic analytical procedure carried out for the extraction of samples.

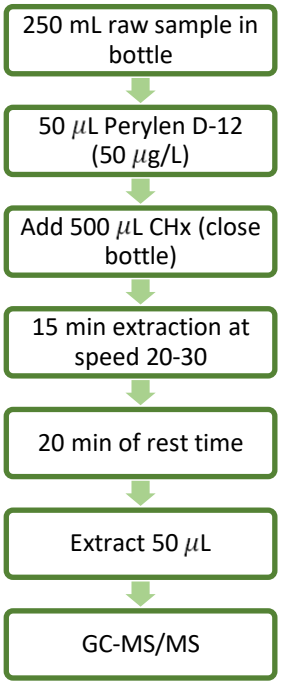


***Figure 8**. Flow chart for the analysis of the water samples.*

3.2.3. Instrumentation

GC/MS analysis was carried out with a Varian 450 GC system equipped with a 1079 injector (Varian, Walnut Creek, CA, USA), a split/splitless mode and autosampler CombiPal. A FactorFour VF-5ms column (30 m x 0.25 mm i.d., particle size 0.25 μm) (Agilent) with a constant flow rate of 1 mL/min of helium (99.99%) was used for chromatographic separation. A 6 μL extract aliquot was injected in the split mode (split ratio of 10), increased to 50 in 0.01 second to turn off at the 0.50 minutes. At 3.50 min, the split mode is activated at 100 (split ratio) and at 6.50 min, decreases to 10 and is maintained until the end of the analysis. Liner with frita (Varian) and Inlet Septa 11.5 mm are used for the 1079 injector. The injection is in sandwich mode, first 1 μL of cyclohexane is aspirated, then the 6 μL extracted and finally 1 μL of the olive oil solution (in total 8 μL are injected).

The analytes were separated using the following oven temperature programme: initial temperature 90 °C (held for 3.5 min), increased at 25 °C/min to 160 °C, increased at 3 °C/min to 290 °C and, finally, increased at 25 °C/min to 325 °C and held at this temperature for 0.97 min. Initial injector temperature 70 °C (0.50 min), increased at 200 °C/min to 300 °C (20 min) and decreased at 200 °C/min to 100 °C (held for 20 min).

The GC system was interfaced to an IT mass spectrometer IT 240 (Varian, Walnut Creek, CA, USA) and it was operated in electron ionization (EI) mode for full scan and MS/MS experiments. The manifold, trap and transfer line temperatures were set at 50, 250, 300 °C, respectively. The analysis was performed with a filament-multiplier delay of 7 min. The emission current of the ionisation filament was set at 80 μAmps generating electrons with 70 eV energy and the scan rate 0.3 s/scan. The automatic gain control (AGC) was switched on with a target fixed at 10000 counts in order to achieve the maximum sensitivity by completely filling the trap with target ions.

3.2.4. MS/MS conditions for target compounds

For GC-MS/MS, the sample extract was injected under the conditions described in the previous section. The MS/MS parameters are shown in **Table 2**.

***Table 2**. MS/MS parameters of analysis by GC-MS/MS.*

Compound	R_t (min)	Window (min)	Precursor ion (m/z)	Product ion (m/z)	Excitation amplitude (V)	Excitation storage level (m/z)
Acenaphthylene	9.4	7 - 10.20	152	150	1.80	50
Acenaphthene	9.8	7 - 10.20	153	152	2.00	50
Fluorene	11.4	10.20 - 12.50	165	163	1.80	55
Phenanthrene	15.4	12.50 - 20.50	178	152, 176	1.60	60
Anthracene	15.7	12.50 - 20.50	178	152, 176	1.60	60
Fluoranthene	22.4	20.50 - 28.00	202	198	2.50	80
Pyrene	23.8	20.50 - 28.00	202	198	2.50	80
Benzo(α)anthracene	32.6	28.00 - 37.00	228	224	3.00	90
Chrysene	32.8	28.00 - 37.00	228	224	3.00	90
Benzo(β)fluoranthene	40.2	37.00 - 46-00	252	248	4.00	110
Benzo(κ)fluoranthene	40.4	37.00 - 46-00	252	248	4.00	110
Benzo(α)pyrene	42.2	37.00 - 46-00	252	248	4.00	110
Indeno(1,2,3-c,d)pyrene	49	46.00 - 52.00	276	272	4.00	110
Dibenzo(a,h)anthracene	49.3	46.00 - 52.00	278	274	4.00	110
Benzo(g,h,i)perylene	50.2	46.00 - 52.00	276	272	4.00	110
Perylene-D_{12} (ILIS)	42.6	37.00 - 46-00	252	260	3.00	100

3.2.5. Method performance

To ensure that the new method is suitable for the analysis of PAHs in drinking water, a validation is carried out in which linearity, limits of determination and quantification, accuracy and precision are evaluated.

In order to carry out the validation, the parametric value of the compounds regulated by RD 140/2003 is taken into account. As it can be seen in **Table 1**, five of the studied compounds are regulated but only *Benzo(α)pyrene* has an individual parametric value (10 ng/L); while for *Benzo(β)fluoranthene*, *Benzo(κ)fluoranthene*, *Benzo(g,h,i)perylene* and *Indeno(1,2,3-c,d)pyrene* the parametric value can be estimated as a reference value of 25 ng/L. The Royal Decree (RD 140/2003) indicates that the used method of analysis shall be able to measure concentrations equal to the parametric value with a limit of quantification (LOQ) equal to or lower than 30% of the parametric value (LOQ).

Therefore, we planned the validation focusing on *Benzo(α)pyrene*. The parametric value of this compound is 10 ng/L and its LOQ must be equal or lower than 30% of the parametric value. Therefore, the analytical method must be able to quantify 3 ng/L in sample, which means 1.5 μg/L (1.5 ng/mL) in vial, a value that is taken into account for the calibration curve and spiked samples for validation.

The validation of the optimum method was carried out by evaluating the following parameters:

Linearity. To avoid any errors and to make it simpler to prepare the calibration, only five-point calibration curves (1.25 - 12.5 μg/L) were included and analysed in triplicate. For this reason, starting from a single stock solution, only a micropipette and a final volume of dilution will be needed. Linearity was assumed when regression coefficient (R^2) was > 0.99 with residuals lower than 30 %.

Accuracy and precision. Spiked samples consisted of real-word water, which were fortified at two concentrations levels: 3 and 10 ng/L in triplicate to estimate the accuracy. Moreover, spiked samples recoveries were considered as satisfactory when they were between 75 % and 125 % (*Valero Analítica).* For both fortified samples levels, the precision, expressed as repeatability of the method, is determined in terms of relative standard deviation (RSD).

Limits of detection and quantification. Regarding the limit of quantification (LOQ), it was considered as the lowest spiking level that was fully validated and obtained with satisfactory recovery (75 – 125 %) and precision (RSD < 25 %). The limit of detection (LOD) was calculated according to the **Equation 2** from the chromatogram at the lowest fortification level for each analyte.

In all cases (validation and sample analysis) the signal used corresponded to the relative areas of analytes to the ILIS (Perylene D-12) obtained from the extracted ion chromatogram for the product ion measured (**Table 2**).

$$LOD = 3 \cdot \frac{lower\ fortified\ conc.}{\left(\frac{peak\ h}{noise}\right)} \qquad \textbf{(Equation 2)}$$

4. Results and discussion

4.1. Optimisation

4.1.1. Optimisation of the extraction solvent

The current method used in the laboratory consists of LLME using toluene as extractant, which presents a boiling point of 110 ºC. It is possible that the use of this solvent causes a defocalisation of the most volatile PAHs, losing the compounds *Acenaphthylene* and *Acenaphthene*. For this reason, in order to provide data of *Acenaphthylene* and *Acenaphthene*, the change of the extraction solvent is necessary.

The requirements that the new extraction solvent must present are:

- A boiling point lower than the toluene.
- Miscible with ACN (solvent of reference standard). If it is not possible, an intermediate solvent miscible with ACN and extraction solvent is required.
- Immiscible with water.
- Of lower density than water in order to carry out the extraction.

Taking into account the previous requirements, cyclohexane was chosen to be used as replacement extraction solvent. This solvent is not miscible with ACN, so dichloromethane is used as an intermediate solvent. Although working with dichloromethane is difficult due its physical characteristics, it presents a low boiling point (40 ºC), so it is used instead of acetone.

Due to the change of the extraction solvent, it is needed to decrease the temperature of the injector from 100 ºC to 60 ºC. Finally, an injection temperature of 70 ºC was selected, as at 60 ºC it was observed that the time from the rest conditions to the injection conditions as well as the time among injections were very high for a routine analysis. However, an increase of 15 minutes (when using 70 ºC) was accepted.

In order to check the advantages that the cyclohexane provides, two vials were analysed in full scan mode, one of 0.1 mg/L in toluene and the other in cyclohexane (CH_2Cl_2 as intermediate solvent). As it can be seen in **Figure 9**, the signal (peak shapes) improves in all the peaks, and especially for the most volatile ones, so it means that the method is more sensitive with cyclohexane. Moreover, data of *Acenaphthylene* and *Acenaphthene* can be obtained at lowest levels.

When it was confirmed that the use of cyclohexane as solvent improves the results and moreover our first objective (i.e. provide data of *Acenaphthylene* and *Acenaphthene*) was achieved, cyclohexane as extraction solvent should be evaluated. To do so, three samples of spring water are fortified to 25 μg/L (50 ng/L) and analytes are extracted following the scheme in **Figure 8**. Moreover, a standard of the corresponding concentration (25 μg/L, which is the same as 25 ng/mL) is analysed in order to calculate the recoveries. This study is carried out in MS/MS according to the conditions set by Varian, that will be later optimised.

The first extractions show high recoveries (higher than 120 %) which may be a consequence of possible differences when preparing the fortified, so an outline is established in order to carry out the fortified and proceed with the extraction (**Figure 8**). First of all, the MIX of PAHs is added.

After, the ILIS is added and finally the cyclohexane. Then, in each addition, the sample is homogenised. Furthermore, in order to avoid the evaporation of cyclohexane during the extraction and consequently an over-concentration of the analytes, it is necessary to close the bottle immediately after adding the extraction solvent.

Figure 10 shows the *Fluoranthene* and *Pyrene* peaks in different injections (3 extractions and one standard) corresponding to 25 μg/L. It may be appreciated that the signal for the standard is slightly higher than the three extractions but the appropriate recovery between 75 % and 125 % is kept and furthermore, the good reproducibility of the extraction method is observed. Finally, due to the reported data it is considered that cyclohexane is the best extractor to carry out PAHs analysis.

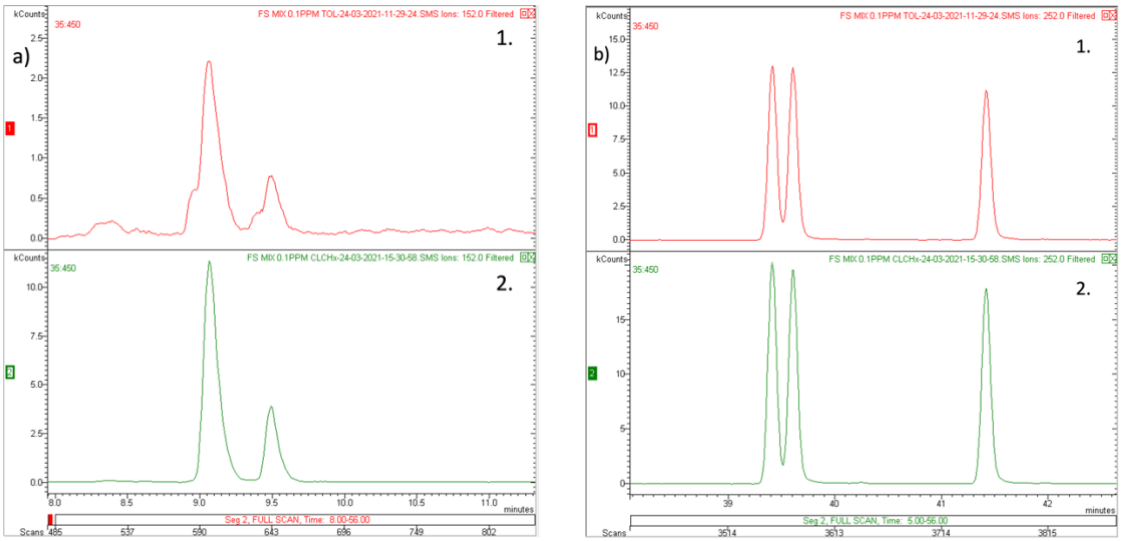


***Figure 9**. a) Acenaphthylene and Acenaphthene peaks and b) Benzo(β)fluoranthene, Benzo(κ)fluoranthene and Benzo(α)pyrene peaks in toluene and cyclohexane method. (1. FS MIX 0.1 ppm in toluene and 2. FS MIX 0.1 ppm in cyclohexane).*

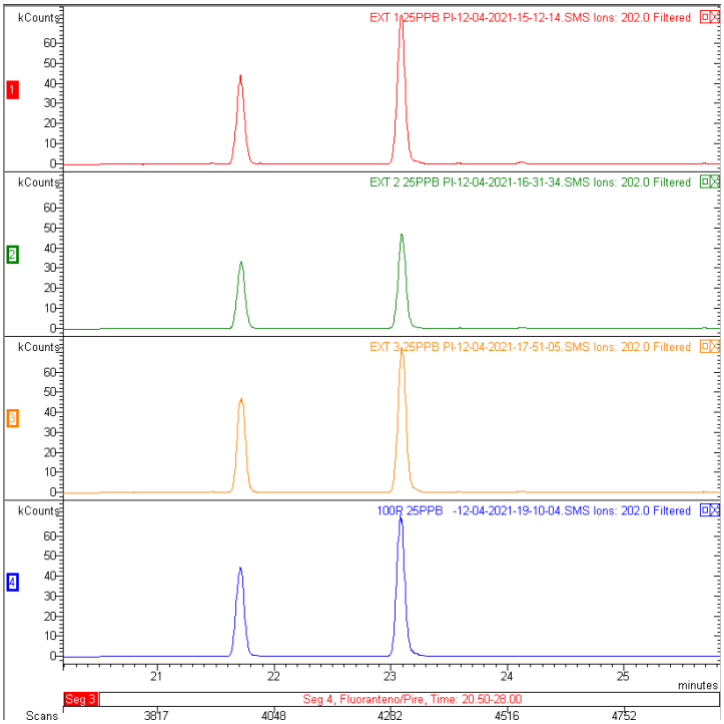


***Figure 10**. Fluoranthene and Pyrene peaks of three extractions and a standard at 25 ng/mL.*

4.1.2. Optimisation of the conditions MS/MS

Previously to the optimisation of the conditions of MS/MS, conditions used to work were those provided by Varian (**Table 4** in the Annex). The injection for the MIX PAHs to 2.5 μg/L was used to study the obtained product ion spectrums of each compound and therefore select the ion of quantification more specific for each compound and its corresponding excitation amplitude (V).

The laboratory rules considered appropriate a voltage that got an quantification ion signal of high intensity but at the same time that kept a minimum signal of precursor ion so the compound could be confirmed.

In order to obtain more sensitivity and therefore higher signal for the compounds *Acenaphthylene* and *Acenaphthene*, new quantification product ions (in relation to those used previously in the laboratory, which were 152 and 153) were assigned, 150 and 152 respectively. For the other compounds, it was confirmed that the election of ions of quantification was the appropriate one. **Figure 11** shows the spectrum of *Acenaphthylene* with the old Laboratory conditions, where it can be seen how the signal corresponding to 150 is clearly higher for this compound instead of 152 (**Table 4** in the Annex). With the changed quantification ion we gain sensitivity.

Regarding the voltage corresponding to the excitation amplitude, different voltages (different methods) were assigned for each compound depending on the necessity of obtaining a higher signal of quantification ion (i.e. increase of voltage) or observing although it was small, a peak corresponding to the precursor ion to check the compound.

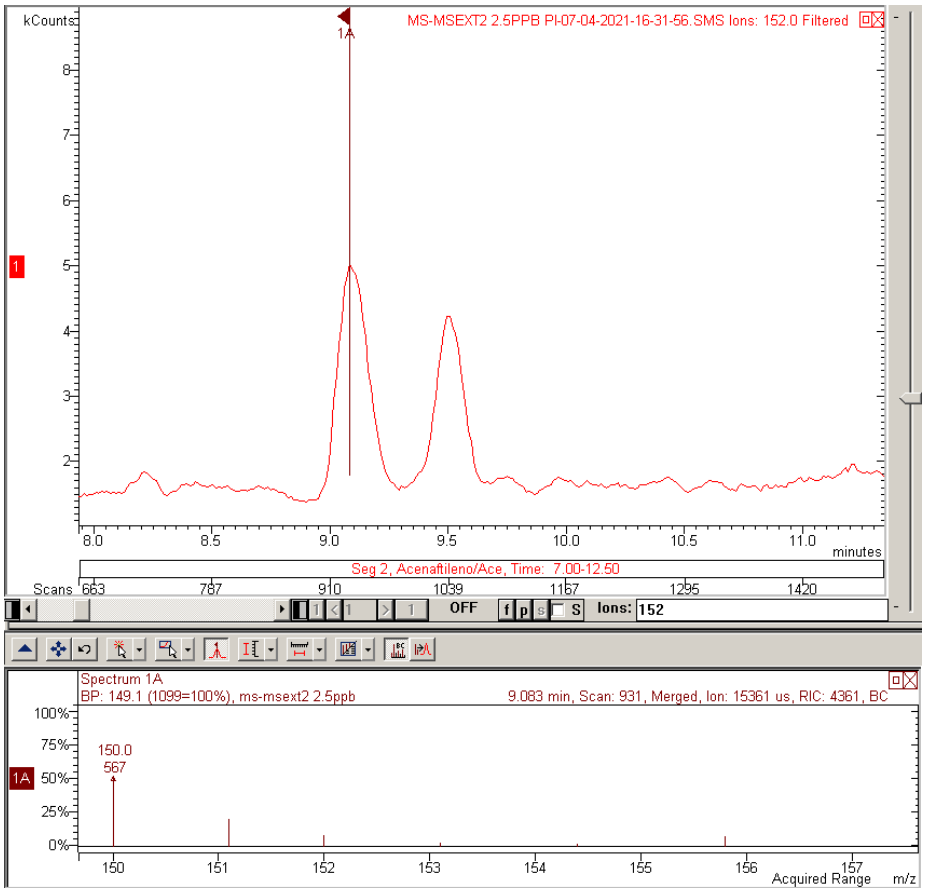


***Figure 11**. Optimisation of the quantification ion of Acenaphthylene.*

4.1.3. Chromatography optimisation

As the development of the method progresses, different problems arose which were later reflected in the chromatography, but we did not understand the origin of these problems. For example, the peaks of *Benzo(β)*fluoranthene and *Benzo(κ)fluoranthene* that were found at close retention times were not well resolved and, in addition, peaks with tails were observed in the chromatogram, especially at the end of the chromatographic analysis.

Due to those problems, we made sure that the column was correctly positioned (it should have been inserted 7.5 cm into the injector) and well cut at the ends (**Figure 12**). In addition, we changed the liner, septum and needle. However, the poor chromatographic resolution occurred and made us think that there may have be an active point in the injector that is retaining the PAHs, in particular the less volatile ones.

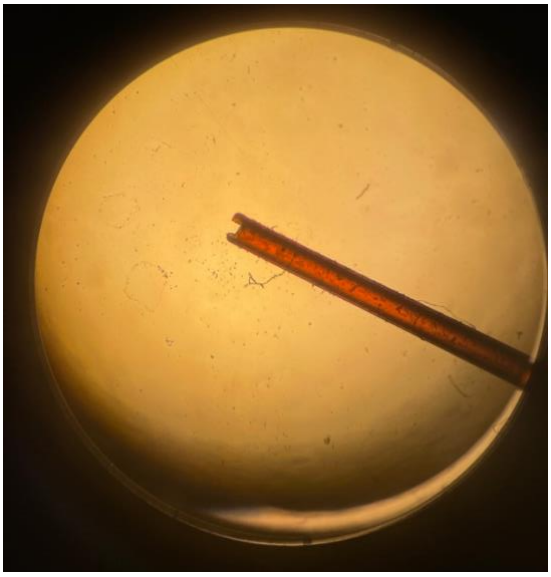


***Figure 12**. Poorly cut chromatographic column found when removing column from equipment.*

The Agilent technician told us that we could increase the PSI (pound-force per square inch) to force the release (de-retention) of the compounds retained in the possible active point. But the increase in pressure did not make any change, so no chromatographic difference was visible. Therefore, we decided to inject the matrix with olive oil. The olive oil covers the active points on the liner, which means that the compounds are not retained and enter to the column. In addition, the olive oil does not interfere with the data as it stays on the liner.

Prior to my presence in the laboratory, a method in which 0.1% of the injection extract was olive oil was developed for pesticides. Therefore, we followed the same procedure for the analysis of PAHs in order to increase the resolution and reduce the tails of the peaks.

First of all, a series of 10 standard replicates containing 12.5 ppb MIX PAHs, the corresponding ILIS and 0.1% olive oil were injected. A modest but not appreciable improvement was observed, so we decided to go up to 0.25% olive oil. In this way, higher signal peaks were observed, obtaining a higher sensitivity for the heavier (less volatile) compounds. **Figure 13** compares the first and last spike with 0.1% olive oil and 0.25% olive oil.

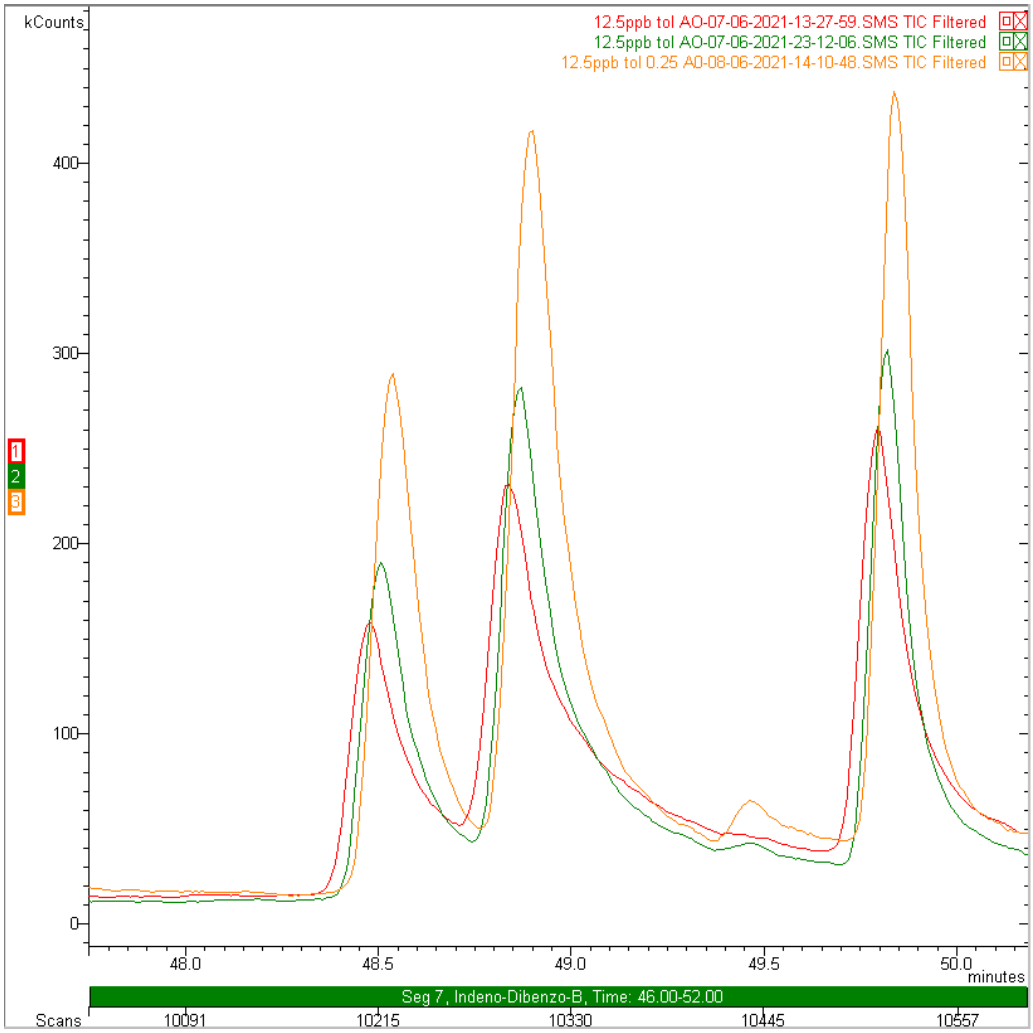


***Figure 13**. Enhancement of Indeno(1,2,3-c,d)pyrene, Dibenzo(a,h)anthracene and Benzo(g,h,i)perylene peaks by adding olive oil.*

4.2. Validation

The criteria followed for the validation of the method can be found in RD 140/2003 and in the *Guía para el funcionamiento de laboratorios de ensayo de aguas (Parte II)* (AEAS, 2016). The method validation of the determination of PAHs by GC-MS/MS was carried out through the analysis of spiked samples (n=3) in order to support the robustness, effectiveness and reliability of the method applied and also support the quantitative data reported. In addition, linearity was studied taking into account the calibration curves analysed in triplicate. The average recoveries (%) for the spiked samples prepared and LOD and LOQ are shown in **Table 3**.

As it can be seen in **Table 3**, in general, the recoveries were satisfactory with values between 75 and 125 %, acceptable range in the field of water testing (Ministerio de la Presidencia RD 140, 2003), which gives reliability to the results obtained. Recovery values were no satisfactory for *Acenaphthylene* and *Acenaphthene* at the low level, with recoveries of less than 60 %. The achievement of satisfactory results for most spiked samples was undoubtedly facilitated by the absence of complex sample treatment in the analytical process. However, the low concentrations of analytes in drinking water make this type of analysis problematic. Therefore, it was necessary to find right compromise for the application of the analytical method to a high number of compounds.

Therefore, the validation indicates that the method performs adequately in all usual concentration ranges and in the matrices to be analysed (drink water), except for the more volatile compounds (*Acenaphthylene* and *Acenaphthene*), which is in compliance with the literature (Pitarch et al., 2007). For these compounds, the LOQ objective could not be reached due to lower sensitivity. Precision was also satisfactory with values better than 25% for the majority of compounds studied, except for the compounds *Acenaphthylene* and *Acenaphthene* in fortified spiked with 3 ng/L.

***Table 3**. Average recoveries (%) and RSD (in parenthesis) of the spiked samples for validation analysis, at two levels of fortification.*

Compounds	Fortification levels (ng/L)		LOD (ng/L)	LOQ (ng/L)
	3	10		
Acenaphthylene	55 (27)	85 (17)	3.6	10
Acenaphthene	43 (32)	97 (18)	4	10
Fluorene	74 (18)	86 (18)	1.4	3
Phenanthrene	87 (14)	77 (20)	0.3	3
Anthracene	82 (12)	79 (19)	0.4	3
Fluoranthene	97 (12)	88 (16)	0.3	3
Pyrene	97 (11)	86 (17)	0.3	3
Benzo(α)anthracene	106 (8)	99 (14)	0.4	3
Chrysene	105 (10)	97 (13)	0.3	3
Benzo(β)fluoranthene	111 (10)	88 (14)	0.9	3
Benzo(κ)fluoranthene	90 (11)	99 (12)	0.9	3
Benzo(α)pyrene	112 (11)	103 (12)	0.4	3
Indeno(1,2,3-c,d)pyrene	95 (13)	84 (10)	0.3	3
Dibenzo(a,h)anthracene	99 (13)	81 (14)	0.5	3
Benzo(g,h,i)perylene	95 (10)	85 (9)	0.3	3

Once the method was validated, the standard operating procedures (SOP) called it *Determinación HAPs por microextracción con ciclohexano* was carried out.

5. Conclusions

A total of 15 PAHs compounds have been studied and therefore determined (identification and quantification) by an optimised method based on LLME-GC-MS/MS with ITD in drinking water at the Valero Analítica laboratory.

Development of a MLLE has allowed a simple extraction of samples with a very high preconcentration factor, that is related with the high sensitivity of the overall method, allowing determination of PAHs at concentration levels of ng/L.

The use of PTV-GC-MS/MS has showed as a powerful technique for the determination of PAH in water samples, with good sensitivity and accuracy. The existent method in the laboratory has been improved by introducing a matrix contamination in the extracts in order to avoid irreversible retention in the injection port. The use of olive oil as matrix has been studied and an addition of 0.25% led to satisfactory results.

Validation of the method showed good results at the two studied levels 3 and 10 ng/L for both linearity, accuracy and precision. All studied compounds were satisfactory validated at the 25 % of the PV (25 ng/L except for *Benzo(α)pyrene* which is 10 ng/L).

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Annex

***Table 4**. MS/MS conditions of PAHs analysed by SPME GC-MS/MS (Varian).*

Compounds	Precursor ion (m/z)	Excitation storage level (m/z)	Excitation amplitude (V)	Product ion (m/z)
Acenaphthylene	152	60	1.60	150
Acenaphthene	153	60	1.60	152
Fluorene	165	70	1.80	163
Phenanthrene	178	70	1.60	152 + 176
Anthracene	178	70	1.60	152 + 176
Fluoranthene	202	80	2.40	200
Pyrene	202	80	2.40	200
Benzo(α)anthracene	228	80	3.00	226
Chrysene	228	80	3.00	226
Benzo(β)fluoranthene	252	90	3.40	250
Benzo(κ)fluoranthene	252	90	3.40	250
Benzo(α)pyrene	252	90	3.40	250
Indeno(1,2,3-c,d)pyrene	276	100	4.00	274
Dibenzo(a,h)anthracene	278	100	4.00	276
Benzo(g,h,i)perylene	276	100	4.00	274