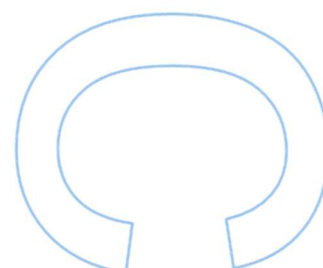
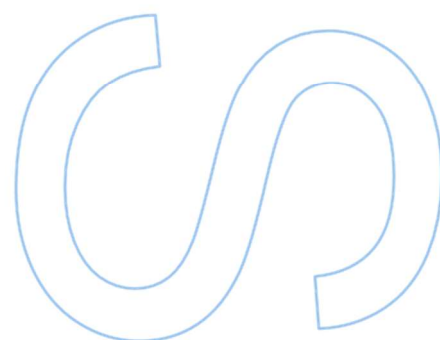
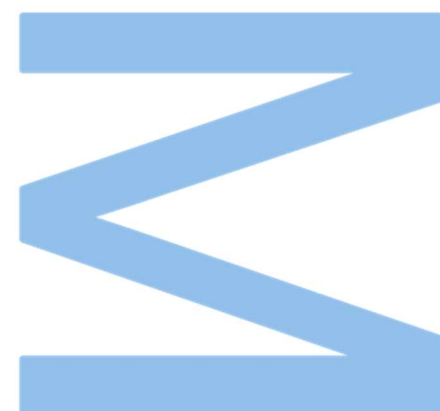




Optimization and validation of SBSE-HPLC-FLD method for determination of priority pollutants PAHs in several water matrices



Eduardo Filipe Fernandes Lage

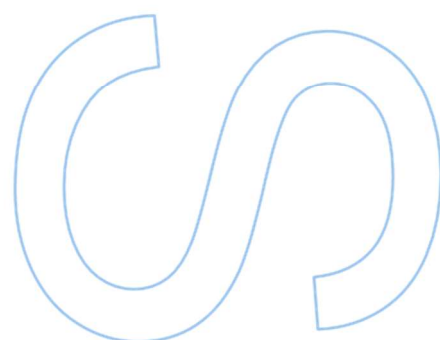
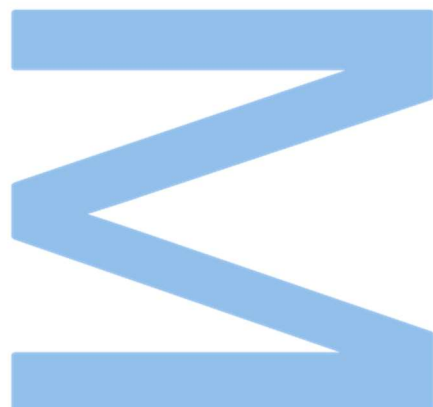
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Para a minha mãe, para a minha mulher, para o meu filho e em memória do meu pai.

“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.”

Thomas Edison

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Resumo

Os hidrocarbonetos aromáticos policíclicos (HAP) são contaminantes ambientais resultantes de emissões de uma variedade de fontes, incluindo a combustão industrial, a descarga de combustíveis fósseis e o aquecimento residencial. Devido às suas propriedades mutagénicas e carcinogénicas, o estudo dos HAP em matrizes ambientais é de grande importância. A determinação destes compostos na água é um desafio, tornando necessário o desenvolvimento de métodos específicos com limites de deteção muito baixos.

Foi otimizado e validado um método de extração rápido, preciso e económico para a determinação simultânea de hidrocarbonetos aromáticos policíclicos em diferentes matrizes aquosas por cromatografia líquida de alta eficiência (HPLC). Um passo inicial de extração foi realizado utilizando a extração sortiva em barra de agitação (SBSE) para obter um elevado enriquecimento dos analitos a partir de amostras aquosas. Esta condição foi otimizada testando a temperatura da amostra, o tempo de extração SBSE, a taxa de agitação, as matrizes de água e o tempo de dessorção.

A separação por HPLC de seis HAP (fluoranteno, benzo[b]fluoranteno, benzo[k]fluoranteno, benzo[a]pireno, benzo[ghi]perileno e indeno[1,2,3-cd]pireno) foi efetuada numa coluna C-18 de fase reversa, com partículas de 5 µm de diâmetro (tamanho 25cm x 4,6mm), com uma fase móvel composta por acetonitrilo e água (com 0,3% de acetonitrilo), em modo isocrático com um tempo de corrida de 24 minutos mais 2 minutos de condicionamento e utilizando um detetor por fluorescência (FLD). Na etapa seguinte, o método SBSE-HPLC-FLD recentemente otimizado foi validado para a análise de HAP nas matrizes água de consumo humano, água natural doce subterrânea e água natural doce superficial.

O desempenho da metodologia foi validado em termos de linearidade, limites de deteção e quantificação, exatidão, precisão (repetibilidade e precisão intermédia) e incerteza, seguindo as recomendações da norma internacional ISO/IEC 17025 e a adequação face ao fim a que se destina, de acordo com a legislação nacional portuguesa aplicável.

Palavras-chave: hidrocarbonetos aromáticos policíclicos, água de consumo humano, água natural doce subterrânea, água natural doce superficial, SBSE-HPLC-FLD, ISO/IEC 17025.

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants resulting from emissions of a variety of sources including industrial combustion, discharge of fossil fuels, and residential heating. Because of their mutagenic and carcinogenic properties, the study of PAHs in environmental matrices is of great importance. The determination of these compounds in water is challenging, making it necessary to develop specific methods with very low detection limits.

A rapid, precise, and economic extraction method was optimized and validated to simultaneously determine polycyclic aromatic hydrocarbons in different water matrices by high-performance liquid chromatography (HPLC). An initial extraction step was performed using stir bar sorptive extraction (SBSE) for achieving high enrichment of the analytes from aqueous samples. This condition was optimized by consecutively testing the sample temperature, extraction SBSE time, stirring rate, water matrices, and desorption time.

The HPLC separation of six PAHs (fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene, and indeno[1,2,3-cd]pyrene) was carried out by reverse phase C-18 column, 5 µm diameter particles (size 25cm x 4.6mm), with a mobile phase composed of acetonitrile and water (with 0.3% of acetonitrile), in isocratic mode with a runtime of 24 minutes and 2 minutes post-time, and using a fluorescence detector (FLD). In the next step, the newly developed SBSE–HPLC–FLD method was validated for the analysis of PAHs in drinking water, groundwater, and surface water.

The performance of the methodology was confirmed in terms of linearity, limits of detection and quantification, trueness, precision (repeatability and intermediate precision), and uncertainty, following the recommendations of the international standard ISO/IEC 17025 and the fitness for purpose, according to the applicable Portuguese national legislation.

Keywords: polycyclic aromatic hydrocarbons, drinking water, groundwater, surface water, SBSE-HPLC-FLD, ISO/IEC 17025.

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List of Abbreviations

BAP – benzo[a]pyrene
BBF – benzo[b]fluoranthene
BKF – benzo[k]fluoranthene
BPE – benzo[ghi]perylene
CAS – Confirmation of Acceptance for Studies
CE – capillary electrophoresis
CITAC – Co-operation on International Traceability in Analytical Chemistry
CL – central line
CLLE – continuous liquid-liquid extraction
CPE – cloud point extraction
CV – coefficient of variation
DLLME – dispersive liquid-liquid microextraction
DNA – deoxyribonucleic acid
EN – European Standard
EOCs – emerging organic contaminants
FID – flame ionisation detector
FLD – fluorescence detector
FLU – fluoranthene
GC – gas chromatography
HF-LPME – hollow fiber liquid-phase microextraction
HPLC – high-performance liquid chromatography
I.D. – internal diameter
IARC – International Agency for Research on Cancer
IEC – International Electrotechnical Commission
IND – indeno[1,2,3-cd]pyrene
IQL – intermediate quantification level
ISO – International Organization for Standardization
IUPAC – International Union of Pure and Applied Chemistry
LGC – Laboratory of the Government Chemist
LLE – liquid-liquid extraction
LOD – limit of detection
LOQ – limit of quantification
LPME – liquid-phase microextraction
MHLLE – miniaturized homogeneous liquid-liquid extraction

MS – mass spectrometer

NP – Portuguese Standard

ODS-P – octadecyl-silica phase

PA – pro analysis

PAH – polycyclic aromatic hydrocarbon

PAHs – polycyclic aromatic hydrocarbons

PDMS – polydimethylsiloxane

PMT – photomultiplier tube

POP – persistent organic pollutants

PT – proficiency tests

PTFE – polytetrafluoroethylene

QC – quality control

QUAM – Quantifying Uncertainty in Analytical Measurement

RE – residuals values

RPM – rotations per minute

RSD – relative standard deviation

RT – retention time

SBME – solvent bar microextraction

SBSE – stir bar sorptive extraction

SDME – single-drop microextraction

SDPA – standard deviation for proficiency assessment

SERS – surface enhance plasmon resonance

SFC – supercritical fluid chromatography

SPE – solid-phase extraction

SPME – solid-phase microextraction

UPW – ultrapure water

USEPA – United States Environmental Protection Agency

UV – ultraviolet

WHO – World Health Organization

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large class of pollutants with both natural and anthropogenic origins^[1] compromising a group of more than 650 compounds^[2] with two or more benzene rings bonded in linear, cluster, or angular arrangements^[3]. All the carbon and hydrogen atoms are arranged in a single plane, resulting in structures that vary from naphthalene ($C_{10}H_8$) to coronene ($C_{24}H_{12}$)^[4]. One of the main natural sources of PAHs are volcanic eruptions^[4], along with forest fires, prairie fires^[5], natural petroleum seeps^[6], biogenic formations^[7], as well as bacterial and algal synthesis^[8]. The domestic heating, power plants, waste incineration^[9], exhaust emissions from motor vehicles^[10], burning of fossil fuels, the disposal of trash, the manufacturing of coke and asphalt, the refining of oil, the creation of aluminium, as well as numerous other industrial processes, are all significant anthropogenic sources^[4].

Since their structure is thermally stable, PAHs often have high melting points, high boiling points, and low vapor pressures^[11]. In the atmosphere, PAHs are split between the gas and particle phases^[12]. Changes in air circumstances (such as temperature and relative humidity) and the physical characteristics of the PAHs themselves have a key role in controlling this phase partitioning^[13]. As a result, lighter PAHs (2-3 rings) tend to be preferentially enriched in the gas phase, whereas the heavier ones (4-6 rings) exhibit practically full interaction with particles^[14]. They can be found in wet and dry deposits and transported across great distances. The degree of soil contamination might vary greatly depending on regional emissions^[12]. The distribution of PAHs in aquatic habitats, including coastal sediments, lake and river surface water, seawater, and biota, has been extensively documented^[15]. Most PAHs have a lipophilic and hydrophobic nature, making them particularly difficult to breakdown^[16] and causing a relatively long environmental half-life^[17]. Therefore, when released into water bodies or soils, they preferentially attach to organic particles which reduces their availability for the biological breakdown in aqueous phases, and as a result, they tend to accumulate in sediments, soil^[16], and in fatty tissue of living organisms^[18]. However, most PAHs have the ability to absorb solar radiation at the surface, therefore there is a chance that they could undergo direct or sensitized photochemical processes that lead to photodegradation^[19]. As they are unstable in the environment, PAHs can photodegrade in the presence of sunlight and combine with other pollutants like ozone and nitrogen oxides to generate oxygenated and nitro/dinitro-PAHs. Since many nitro- and oxygenated PAHs frequently have more toxicity than their parent chemicals, these processes result in more complicated and dangerous combinations^[9]. PAHs are not chemically produced for industrial use.

However, several PAHs have a few commercial applications. They are mostly employed as intermediaries in the chemical, pharmaceutical, agricultural, photographic, thermosetting, and lubricant sectors^[8].

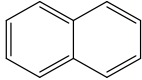
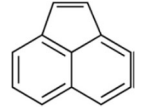
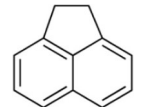
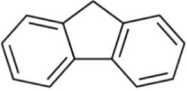
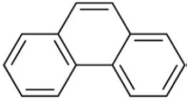
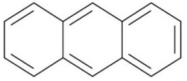
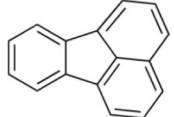
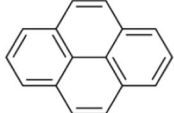
Although each PAH has a different health impact on people, some PAHs have been recognized as being of the highest concern^[20], as some of them are considered carcinogenic to humans and harmful to fish and other aquatic life^{[21] [22]}. Moreover, certain PAHs can produce many other harmful health effects, including reproductive, developmental, immunotoxic, and neurologic effects^[23]. In this sense, the United States Environmental Protection Agency (USEPA) has designated 16 unsubstituted PAHs as priority pollutants^[24], which are regularly monitored for regulatory purposes [naphthalene, acenaphthene, acenaphthylene, phenanthrene, anthracene, fluorene, benzo[a]anthracene, chrysene, fluoranthene (FLU), pyrene, benzo[b]fluoranthene (BBF), benzo[k]fluoranthene (BKF), benzo[a]pyrene (BAP), dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene (IND), and benzo[ghi]perylene (BPE)]^[12]. In the European Directive (EU) 2020/2184 of the European Parliament and of the Council of 16 December 2020, special attention is given to benzo[a]pyrene by setting a threshold limit of 10 ng/L for this single compound^[25], the most toxic and carcinogenic in the PAH class^[2], and the combined concentration of benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, and indeno[1,2,3-cd]pyrene cannot be more than 100 ng/L^[26]. The PAHs with the greatest levels found in drinking water include fluoranthene, phenanthrene, pyrene, and anthracene, although fluoranthene is the only PAH that is significantly found among the six PAHs (fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene and indeno[1,2,3-cd]pyrene) that are typically evaluated in water for regulatory purposes^[27]. According to the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC), these substances are classified as persistent organic pollutants (POP)^[28]. Due to their mutagenic and carcinogenic characteristics, it is essential to develop sensitive and selective methods for identifying them^[1].

2. Physicochemical properties of PAHs

At ambient temperature, PAHs are colourless to yellow solids^[27]. Their conjugated π -electron systems, which depend on the number of aromatic rings and their molecular weight, define their physical and chemical characteristics^[18]. Chrysene and benzo[a]pyrene are examples of four-ring and five-ring aromatic hydrocarbons that are

practically insoluble in water^[29]. With increasing molecular weight, the solubility of PAHs in water declines^[30], vapor pressure decreases^[20], their melting and boiling points rise in accordance^[30], and increases the resistance to oxidation and reduction^[8]. Chemically, the PAHs are made up of two or more benzene rings bound together in linear, cluster, or angular configurations^[8]. Molecules with a linear arrangement tend to be less soluble than angular or perifused molecules. The solubility of PAHs in water increases by a factor of three to four when the temperature rises from 5 to 30 °C^[6], and the dissolved colloidal organic substances, make PAHs that are incorporated in micelles more soluble^[6]. Therefore, the high lipophilicity of PAHs makes them miscible in organic solvents^[8], and each extra ring that is added to the PAH causes a decrease in water solubility^[20]. Due to their low volatility, most PAHs are categorised as semi-volatile organic compounds^[31]. The persistence of PAHs in the aquatic environment is determined by vapour pressure properties. Under all environmental conditions, PAHs with two to three rings are very volatile, whereas PAHs with four or more rings show insignificant volatilization loss^[32]. As the number of aromatic rings rises, biodegradation rates decrease substantially^[27], due to their stable molecular structures. Indirect photolysis caused by hydroxyl radicals is the most significant pathway for the breakdown of PAHs in air and water^[27]. Because PAHs formed at higher temperatures tend to have fewer alkylated chains than PAHs formed at lower temperatures, the temperature of formation can be a useful indicator of the location of PAH sources. For instance, the distribution of PAHs in the effluent from a power plant's stack, which is rapidly generated at extremely high temperatures, will differ from that at the site of an oil spill. Additionally, PAHs exhibit several characteristics, including light sensitivity, heat resistance, conductivity, the ability to emit, and corrosion resistance^[8]. The physicochemical properties of some priority PAHs are shown below in **Table 1**.

Table 1 - Physicochemical properties of the 16 priority PAHs according to the USEPA.

PAH	CAS number ^[33]	Nº of rings ^[11]	Chemical formula ^[33]	Molecular weight (g/mol) ^[32]	Melting point (°C) ^[6]	Boiling point (°C) ^[6]	Vapor pressure (Pa, 25°C) ^[34]	Solubility in water at 25°C (µg/L) ^[32]	IARC# group ^[6]	Structure ^[20]
Naphthalene	91-20-3	2	C ₁₀ H ₈	128.1	80.2	218	11.14	3.17x10 ⁺⁴	2B	
Acenaphthylene	208-96-8	3	C ₁₂ H ₈	152.1	92.5	280	3.87	3.40x10 ⁺³	NA	
Acenaphthene	83-32-9	3	C ₁₂ H ₁₀	154.2	93.4	279	3.07	3.93x10 ⁺³	3	
Fluorene	86-73-7	3	C ₁₃ H ₁₀	166.2	115	295	1.66	1.98x10 ⁺³	3	
Phenanthrene	85-01-8	3	C ₁₄ H ₁₀	178.2	99.2	340	1.06x10 ⁻¹	1.29x10 ⁺³	3	
Anthracene	120-12-7	3	C ₁₄ H ₁₀	178.2	215	340	8.60x10 ⁻⁴	73	3	
Fluoranthene	206-44-0	4	C ₁₆ H ₁₀	202.3	108	384	8.61x10 ⁻⁴	260	3	
Pyrene	129-00-0	4	C ₁₆ H ₁₀	202.3	151	404	5.00x10 ⁻⁵	135	3	

PAH	CAS number ^[33]	N° of rings ^[11]	Chemical formula ^[33]	Molecular weight (g/mol) ^[32]	Melting point (°C) ^[6]	Boiling point (°C) ^[6]	Vapor pressure (Pa, 25°C) ^[34]	Solubility in water at 25°C (µg/L) ^[32]	IARC# group ^[6]	Structure ^[20]
Benzo[a]anthracene	56-55-3	4	C ₁₈ H ₁₂	228.3	167	435	5.43x10 ⁻⁴	14	2B	
Chrysene	218-01-9	4	C ₁₈ H ₁₂	228.3	258	448	4.00x10 ⁻⁶	2.0	2B	
Benzo[b]fluoranthene	205-99-2	5	C ₂₀ H ₁₂	252.3	168	481	5.00x10 ⁻⁷	1.2	2B	
Benzo[k]fluoranthene	207-08-9	5	C ₂₀ H ₁₂	252.3	217	480	5.20x10 ⁻⁸	0.76	2B	
Benzo[a]pyrene	50-32-8	5	C ₂₀ H ₁₂	252.3	177	495	6.00x10 ⁻⁸	3.8	1	
Dibenz[a,h]anthracene	53-70-3	5	C ₂₂ H ₁₄	278.4	270	524	1.33x10 ⁻⁸	0.5 (27°C)	2A	
Indeno[1,2,3-cd]pyrene	193-39-5	6	C ₂₂ H ₁₂	276.3	164	536	1.27x10 ⁻⁷	62	2B	
Benzo[ghi]perylene	191-24-2	6	C ₂₂ H ₁₂	276.3	278	550	1.38x10 ⁻⁸	0.26	3	

International Agency for Research on Cancer: Group 1 consists of substances with carcinogenic potential for humans; Group 2A includes those agents that are probably carcinogenic to humans; Group 2B includes those agents that are possibly carcinogenic to humans; Group 3 contains compounds that are those not classifiable as being carcinogenic to humans

3. PAHs sources

According to their sources, PAHs are divided into two groups: pyrogenic PAHs, which are produced when fossil fuels are burned, and petrogenic PAHs, which are only found in crude oil and contaminate water following an oil spill.

Pyrolysis is the irreversible change of chemical composition and physical phase that occurs when organic material is decomposed at high temperatures in an inert atmosphere. The pyrolysis will take place at temperatures above 200 °C, fragmenting organic molecules and releasing free radicals that combine to produce stable polycyclic aromatic compounds (pyro synthesis). Pyrolysis, or the incomplete combustion of organic matter such as fat, protein, and carbohydrates at temperatures above 200 °C, is the primary cause of the production of PAHs in food products during processing^[35]. As a result, they are present in a variety of samples^[1], including grilled and edible oils, coffee, tea, and roasted meat^[36], and have frequently been found in aquatic habitats in recent years^[33].

Petrogenic PAHs are structurally distinct from pyrogenic PAHs since they are heavily alkylated or oxygenated to produce PAH quinones^[37].

4. Effects of PAHs: Environment and Human Health

4.1. Effects of PAHs on the environment

Aquatic and terrestrial species are contaminated because of PAHs' ability to spread after discharge into all environmental compartments^[38], including air, water, sediments, soil, and plants^[2], and they generally co-occur with other pollutants^[33]. Volatilization, leaching, degradation, bioaccumulation, and sequestration are the five environmental fates of PAHs^[39]. Marine plants, fish, and sedentary creatures are all exposed to PAHs in the aquatic environment. Urban runoff, oil spills, offshore drilling activities, atmospheric fallout, and industrial effluent discharges are only a few of the possible entry points for PAHs into aquatic habitats^[15]. Metabolism and photo-oxidation both have an impact on the toxicity of PAHs to aquatic organisms, as the presence of ultraviolet (UV) light usually increases toxicity^[39]. In the presence of UV light, they are typically more hazardous. For birds and aquatic life, PAHs exhibit moderate to severe acute toxicity. Except for severely contaminated soil, PAHs in soil are unlikely to have a harmful effect on terrestrial

invertebrates. Some of the negative impacts include tumours, reproduction, development, and immunity problems^[8].

In comparison to concentrations observed at remote or rural sites, atmospheric PAH concentrations are often higher in industrial, urban, or residential areas^[34]. In this context, scientific communities are currently quite concerned about the vast distribution of PAHs in the atmosphere, which has prompted their critical investigation for appropriate monitoring. Animals consume this polluted flora after plants absorb PAHs, which causes bioaccumulation and the formation of PAH derivatives^[40]. These persistent organic pollutants are frequently detected in the environment because organic matter is incompletely burned during the pyrolytic process^[41], which includes high temperatures and low oxygen levels^[42]. Temperature increases cause a directly proportional relationship between the amount of PAHs generated and the structure and variety of PAH production^[23]. The surface coating of water has the highest PAH contents. The PAH will be primarily adsorbed on the suspended particulate matter in waters that exhibit turbidity, such as rivers and lakes. Lower PAH levels are revealed by the analysis of filtered water samples^[38].

As the most pervasive and prevalent pyrogenic PAH^[43], fluoranthene has a low water solubility (260 µg/L, at 25°C^[32]), which significantly reduces its bioavailability^[44]. An activity like burning wood or gasoline can result in the production of fluoranthene. Therefore, small airborne particles with fluoranthene can be inhaled by animals and eventually settles back onto the ground, into ponds, rivers, and lakes^[33].

A prevalent component of PAH complexes produced by tobacco and fossil fuels^[33], the potential carcinogenic benzo[b]fluoranthene^[20], has been identified in rainwater, snow, and fog^[27].

The harmful carcinogenic contaminant benzo[k]fluoranthene^[20], which is present in cigarette smoke and contaminated air, seems to be accumulating in aquatic systems. This PAH has also been found as a significant toxicant affecting aquatic creatures^[33].

Incomplete combustion of organic substrates at temperatures between 300 °C and 600 °C results in the production of the high molecular weight PAH benzo[a]pyrene^[45], commonly used as an indicator for PAH exposure^[37], which is present in a variety of products, including coal tar, numerous foods, particularly smoked and grilled meats, and tobacco smoke^[45].

The class of highly conjugated polyaromatic compounds exemplified by benzo[ghi]perylene is created by combining benzenoid units with a crucial subset of

fluorescent perylene dyes. This produces a PAH molecule with a high molecular weight and six benzene rings that are extremely resistant to degradation^[33].

The harmful Indeno[1,2,3- cd]pyrene is created naturally when coal, oil, gas, wood, domestic garbage, and tobacco are burned^[46]. This PAH is the combination of an indeno and a pyrene molecule with a fluoranthene network^[20].

Therefore, it is crucial to monitor these chemicals and remove them from the environment due to their widespread distribution and hazardous potential^[18].

4.2. Human toxicity effects of PAHs

Due to the widespread distribution of these substances and their relevance in terms of toxicology^[47], biological monitoring of PAHs exposure is of utmost importance^[8]. PAHs are the greatest class of chemical substances with a history of causing cancer. Because PAHs are always found in complex combinations, there is no possibility for people to be exposed to a single PAH, and for that reason, the assessment of health effects is challenging since exposure to PAHs is always caused by a mixture^[48]. The most carcinogenic PAHs are composed of five to six fused rings, whereas smaller or bigger PAHs typically do not have a strong carcinogenic activity^[46]. Humans are exposed to PAHs through three main pathways: (a) ingestion through the skin, gastrointestinal tract, and inhalation, with the highest exposure coming from contaminated food through environmental adhesion; (b) formation in food products during cooking and processing conditions; and (c) traditional drying and curing techniques for food preservation^[35]. Therefore, PAHs can enter the human body through both dietary and non-dietary sources (e.g., inhalation and skin contact)^[16]. Dietary sources stand out as the main exposure pathway among these. Food consumption is linked to over 70% of non-smokers' exposure to PAHs^[42]. Importantly, PAHs can negatively impact human health in a variety of ways, most notably through mutagenesis and carcinogenesis^[39], as well as immunosuppressive effects. Even though not all PAHs are considered carcinogenic, they can nonetheless harm cells and have a cumulative effect on human health due to their activity as free radicals. Recent epidemiological research in humans and animals suggests that exposure to PAHs may contribute to the rising cancer incidence^[42]. Long-term exposure to combustion-related smoke, aerosols, coal dust from mines, or used motor oil in humans led to much higher rates of various cancers. Numerous studies have demonstrated that coal tars, coal tar pitches, iron and steel production, the production of coke and aluminium, coal gasification, and soot all contribute to the development of lung

cancer in people. Coal tar, coal tar pitches, non-refined mineral oils, shale oils, and soot exposure have all been linked to skin and scrotal tumours^[46]. In the human digestive system, polycyclic aromatic hydrocarbons are readily absorbed, particularly when a high-fat diet is consumed. These substances don't accumulate in the human body, and they exhibit moderate acute toxicity but extremely high chronic toxicity^[49]. Since PAHs have been discovered to be extremely dangerous even at low concentrations, it is particularly useful to understand the gas-particle distribution of these compounds when evaluating the consequences on respiratory health. The risk of inhaling carcinogenic PAHs is partially dependent on whether such PAHs reach the lungs in gas form or whether they are breathed on particles that have a higher propensity to permeate the lungs and cause cancer over an extended period of time^[13]. In addition, since PAHs could disrupt hormone systems, they may negatively impact immunological and reproductive processes. As a result, several authors have documented that exposure to PAHs causes DNA damage, as long-term exposure to PAHs may increase the risk of cardiopulmonary mortality and cellular damage caused by a gene mutation^[20]. The most well-investigated PAH is benzo[a]pyrene, a carcinogen for which toxicological data serve as quantitative benchmarks for the entire carcinogenic subgroup^[50].

5. Legislation Concerning PAHs in water

To safeguard the environment and public health, PAHs are governed by several laws, rules, and agreements. Reducing exposure is difficult because of the pervasiveness of PAHs and the sources of their emission^[21]. **Table 1** shows the 16 unsubstituted PAHs designated as priority pollutants by USEPA. However, the present work will focus on the six PAHs (fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene, and indeno[1,2,3-cd]pyrene) belonging to the Portuguese national water legislation as described in **Table 2** (the fluoranthene is not regulated in drinking water matrix).

Table 2 - Description of different water matrices and the respective criteria of the applicable Portuguese legislation.

Water Matrices	National Legislation	Description	Parameter	Unit	Parametric value	Quantification Limit	Detection Limit	Uncertainty (%)	Precision (%)	Trueness (%)
Drinking water	Decree-law nº 152/2017	Water for drinking only	benzo[a]pyrene	µg/L	1.0×10^{-2}	n/a	2.5×10^{-3}	50	25	25
			benzo[b]fluoranthene	µg/L	1.0×10^{-1}	n/a	2.5×10^{-2}			
			benzo[k]fluoranthene	µg/L	1.0×10^{-1}	n/a	2.5×10^{-2}			
			benzo[ghi]perylene	µg/L	1.0×10^{-1}	n/a	2.5×10^{-2}			
			indeno[1,2,3-cd]pyrene	µg/L	1.0×10^{-1}	n/a	2.5×10^{-2}			
			Sum of PAHs#	µg/L	1.0×10^{-1}	n/a	n/a			
Surface water*	Decree-law nº 236/98	Water that runs over soils or rocks, intended to produce water for human consumption	fluoranthene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}	n/a	50	50
			benzo[b]fluoranthene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			benzo[k]fluoranthene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			benzo[a]pyrene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			benzo[ghi]perylene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			indeno[1,2,3-cd]pyrene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
	Decree-law nº 218/2015	Water that runs over soils or rocks, and according to environmental quality standards for the water biota	fluoranthene	µg/L	1.2×10^{-1}	n/a	n/a	n/a	n/a	n/a
			benzo[b]fluoranthene	µg/L	1.7×10^{-2}	n/a	n/a			
			benzo[k]fluoranthene	µg/L	1.7×10^{-2}	n/a	n/a			
			benzo[a]pyrene	µg/L	2.7×10^{-2}	n/a	n/a			
			benzo[ghi]perylene	µg/L	8.2×10^{-4}	n/a	n/a			
Groundwater*	Decree-law nº 236/98	Water from an aquifer	indeno[1,2,3-cd]pyrene	µg/L	n/a	n/a	n/a	n/a	50	50
			fluoranthene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			benzo[b]fluoranthene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			benzo[k]fluoranthene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			benzo[a]pyrene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			
			benzo[ghi]perylene	µg/L	2.0×10^{-1}	n/a	4.0×10^{-2}			

Sum of PAHs: the parametric value corresponds to the sum of the concentrations of benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene and indeno[1,2,3-cd]pyrene; * Considering the lowest parametric value.

6. Removal of PAHs from the environment

To prevent the negative impacts of PAHs on the environment and human health, it is imperative to develop efficient removal techniques^[44]. Several processes, including as biodegradation, photochemical degradation^[8], and bioremediation, are used to remove PAHs from the environment^[51]. Physical processes, like volatilization and adsorption, are crucial in the removal of PAHs from aqueous systems because they significantly reduce the concentration of PAHs in the water. However, due to their inability to decompose these contaminants, physical processes cannot solve the problem of PAH pollution, and for this reason, the processes of biological and chemical degradation are normally selected^[18]. Due to their affordability and environmental friendliness, microorganism-based bioremediation is a practical method for the degradation of PAHs compared to traditional physicochemical methods. Since bacteria are common and simple to handle, degradation through mixed bacterial cultures is one of the best remediation options, besides they may undergo chemical degradation, adsorption, volatilization, and photolysis^[8]. Most polluted sediments and soils are oxygen-depleted, and *in situ* bioremediation of PAHs is frequently linked to anoxic or anaerobic conditions^[16]. Higher molecular weight PAHs (four or more rings) sorb strongly to soils and sediments and are more resistant to microbial degradation because of their high molecular weight and hydrophobicity, besides these PAHs are also toxic to bacteria cells. Low molecular weight PAHs (two or three rings) are relatively volatile, soluble, and more degradable than higher molecular weight PAHs^[51]. Due to the availability of potentially varied degradation pathways, effective synergism, and coordinated metabolic activities, mixed bacterial cultures are always preferable to pure cultures for attaining maximum degradation and complete mineralization of xenobiotic substances^[44]. Because PAHs are poorly soluble in water, the effectiveness of phytoremediation techniques to remove them is constrained. Thus, combining bioremediation and phytoremediation would increase the removal effectiveness, as the positive interaction between the plant and the rhizobacteria is provided by rhizoremediation, which comprises both phytostimulation and rhizodegradation, for the removal of these organic pollutants^[51]. A pollutant of concern may be partially or completely converted to its elemental components through bioremediation by microorganisms such as bacteria or fungi. The toxicity of these molecules to cells is one of the issues with PAH bioremediation since these lipophilic substances immediately affect cellular membranes^[33]. Water PAHs can be removed using conventional chemical oxidation techniques^[18], and membrane processes^[52]. The usage of common oxidants including ozone, chlorine, and potassium permanganate have been the subject of numerous investigations. However, issues could arise during

the decontamination process itself, because chlorine has been shown to react with naturally occurring organic matter in water, to produce halogenated hydrocarbons (e.g. trihalomethanes and haloacetic acids), as disinfection by-products, that are both carcinogenic and mutagenic^[18]. The removal of PAHs from potable water by membrane processes is strongly related to the type of membrane selected, but they have the disadvantage of requiring pre-treatment and energy consumption. A membrane is a material that makes a thin barrier capable of selectively resisting the movement of diverse constituents of a fluid, thereby enabling the separation of the constituents. Different membrane filtration systems such as microfiltration, ultrafiltration, nanofiltration, and reverse osmosis are employed in water and wastewater treatment^[52]. Anodic oxidation is the most widely used electrochemical technology for wastewater remediation because it has proven to be a reliable and flexible mechanism for the abatement of a variety of organic compounds^[18]. Therefore, these organic pollutants in water and wastewater can be removed using biological, physical, and chemical methods. Activated sludge procedures, anaerobic and aerobic processes, as well as physical/chemical approaches like adsorption (e.g. the use of activated carbon), advanced oxidation processes (e.g. the use of ozone), and coagulation have all been described for the removal of various organic contaminants, including PAHs^[33, 45].

7. Methods of measuring PAHs in water

7.1. Sampling

Water matrices, particularly drinking water, surface water, and groundwater are subject to changes because of physical, chemical, or biological reactions that may occur between the time of sampling and the beginning of analysis. The nature rate of these reactions is frequent, and if measures are not followed during sampling, transport, and storage (for specific analytes), the concentration obtained may be different from those existing at the time of sampling^[53]. PAHs may only represent as little as 0.01% of the total organic fraction present in the water sample, and because of that, serious errors may occur from losses or contamination during sampling, as any surface that the sample encounters have the potential to either contaminate the sample or adsorb trace pollutants^[38].

The sampling procedure is significantly influenced by the type of water matrix used. Because they typically contain few suspended particles, drinking waters are comparatively simple to sample. River, lake, and coastal waters, in comparison, show

significantly more temporal and spatial heterogeneity^[4]. Hence, the sampling strategy should reflect the system's known or predicted variability^[54].

The sampling should, whenever possible, be performed directly into the extraction vessel and extracted as soon as possible^[38]. For sampling and handling organic compounds in general, amber borosilicate glass containers with PTFE-lined tops are normally recommended. The reason is that polymers such as polyethylene and polypropylene, may leach plasticizers into the sample and produce interference issues in the chromatographic analysis that follows. Additionally, many plastics are porous to volatile substances, which could result in losses during storage and transportation. Finally, the surface of plastics usually facilitates microbial colonisation, enhancing biodegradation of some compounds^[4].

Sources of contamination such as dust, motor vehicle exhaust, or cigarette smoke, must be avoided^[38], and if samples are chlorinated, the residual chlorine must be removed using sodium thiosulfate^[38], to neutralize this residual disinfectant in the water sample^[55].

7.2. Extraction techniques

Modern analytical chemistry is progressing toward the detection of chemicals at increasingly low concentrations in progressively smaller sample sizes^[56]. Proper sample preparation for analysis is a crucial step in the analytical process. To increase the sensitivity, specificity, and selectivity of the analytical methods, sample preparation processes include clean-up and pre-concentration steps^[57]. In environmental water analysis, sample pretreatment (which must include a precise technique for analyte extraction, isolation, and concentration) is one of the most important steps involved in determining of these contaminants^[28]. The primary component influencing the extraction process in an experimental setup is the selection of the right solvent, which is dependent on the solubility of the target PAHs with respect to solvent polarity and solvent-matrix interactions. To prevent reactivity with the analytes, the chosen solvent should be non-toxic, non-flammable, chemically stable, and inert. For easy handling and storage, the solvent's viscosity, vapour pressure, and freezing point should be low, and the solvent should be suitable with chromatographic analysis and extremely selective toward PAHs in comparison to other matrix components. The extraction process is significantly impacted by the matrix and the solvent ratio^[40].

For the process to be economically viable and to reduce energy usage, the extraction time must be carefully chosen. The "green methods" would be those that use less

solvent, a shorter extraction time, and a higher yield for extraction because the amount of extracted analyte is typically directly related to the extraction time^[40].

Analyte-matrix interactions are highly influenced by the features of the matrix, such as the particle size, chemical composition, surface area, pH, water content, total organic matter content, pore size, and polarizability of the analytes^[40]. At low pH (≤ 2.5), the polycyclic aromatic hydrocarbons do not ionise and are present in neutral form through this pH range^[56]. To improve the solubility of PAHs in water, methanol, a matrix modifier, is frequently added to water samples. Additionally, the use of methanol prevents these substances from quickly adhering to the glass flask walls^[57].

As a result of their low solubility, PAHs are usually present in trace amounts in water samples^[6]. Given the low concentration of PAHs in the environment, the target analytes must first undergo pre-treatment to be isolated and enhanced before being subjected to instrumental determination^[36, 38], and concentrated to a point where the compounds can be recognised^[56]. A stir bar coated with the sorbent, commonly polydimethylsiloxane (PDMS), is used in the preconcentration and extraction process known as stir bar sorptive extraction (SBSE)^[58], allowing the preconcentration of emerging organic contaminants (EOCs), such as PAHs, from complex matrices using sorptive extraction^[59]. This method has been effectively applied to a wide range of matrices, including soils, environmental water and wastewater, solid and liquid foods, gaseous samples, and biological fluids, in the analytical domains of environmental, clinical, and food analysis^[57]. The SBSE process is substantially influenced by the extraction temperature^[56]. High temperature causes a reduction in fluid density and viscosity along with a reduction in surface tension, which increases contact between the solvent and the targeted analytes and improves extraction efficiency. The solute-matrix interactions become more unstable as the temperature rises, increasing the solubility of solutes in the organic solvent more effectively with improved mass transfer, quick diffusion, and faster recoveries^[40]. For PAHs, the extraction efficiency increases above the temperature of 40°C^[56].

The extraction by movable sorption element, SBSE, which is increasingly employed in daily practice due to its speed, accuracy, reproducibility, and selectivity, has received particular attention among the sample preparation techniques for chromatographic analysis^[56], at trace levels^[60], of environmental, food, and biomedical samples^[61]. Similar to solid phase microextraction (SPME), it is based on the same principles but improves the performance of PDMS as a sorbent material^[62]. Direct immersion and spinning of the stir bar in the liquid sample are used for extraction^[58]. Following extraction, it is possible

to desorb the trapped analytes on the bar, either thermally for gas chromatography or into a solvent for liquid chromatography^[60]. Compared to other microextraction techniques like SPME, this method employs a larger amount of sorbent phase^[58], which directly increases the sensitivity of this technique^[63]. As a result, SBSE enables low detection limits and good recovery rates due to the sorbent phase's improved sensitivity and sample capacity^[58]. Other benefit is the minimal chemical consumption, as the sample volume required for liquid-liquid extraction (LLE) to isolate PAHs is ten times greater than that required for SBSE to achieve equivalent detection limits^[56], and LLE is more time-consuming and hazardous to health, due to the high volume of harmful solvents utilized^[64]. LLE is one of the most frequently employed methods for analysing PAH in complex matrices (e.g. petroliferous wastewaters)^[65], despite the drawbacks of potential analyte loss from sorption to material surfaces, and a challenging phase separation^[66]. Additionally, the longevity of the sorption element is extended by the potential of the solvent elution of these analytes and thermodesorption in SBSE^[56]. Applying excessively high temperatures can significantly reduce the PDMS coating's shelf life. The ideal strategy involves solvent conditioning, but the solvent must be carefully chosen to prevent coating degradation^[57]. The most popular desorption solvents include acetonitrile, methanol^[67], combinations of these solvents and dichloromethane^[57], and mixes with water or aqueous buffers. The liquid desorption is accelerated by using mechanical shaking, increased temperature, or sonication. In addition, liquid desorption may be preferable to thermal desorption for minimizing PDMS phase contamination, offering additional attractive characteristics like cost-effectiveness, the chance for method improvement, and potential re-analysis^[67]. There may be drawbacks to employing SBSE, as there are other extraction methods. Optimizing the extraction parameters is necessary for proper method development, and the PDMS sorbent phase is more selective for nonpolar analytes^[58]. In SBSE, several factors impacting the extraction step should be assessed in order to facilitate the transfer of the analytes onto the extracting phase, including the extraction time and temperature, sample pH, the addition of an inert salt, stirring rate, and sample volume^[59]. To prevent PDMS-phase degradation and increase the lifetime of PDMS-coated stir bars, it is not advised to use too basic (pH > 9) or too acidic (pH < 2) conditions. In general, adding an inert salt to hydrophobic analytes does not increase extraction efficiency and may even decrease it. In contrast, adding inert salts causes the response for polar analytes to rise^[67]. Most applications for SBSE still come from the field of environmental sample extraction, which was its initial purpose. Although both volatile and semi-volatile organic molecules have been subjected to SBSE, this method is mostly utilised to identify semi-volatile low-

polarity pollutants, such as PAHs^[62]. There are several other methods to extract PAHs from aqueous solutions, including solid-phase extraction (SPE), soxhlet extraction^[68] (for PAHs in soils and sediments^[6]), continuous liquid-liquid extraction (CLLE), hollow fibre liquid-phase microextraction (HF-LPME)^[6], miniaturized homogeneous liquid-liquid extraction (MHLLE), cloud point extraction (CPE)^[60], liquid-phase microextraction (LPME), single-drop microextraction (SDME), solvent bar microextraction (SBME)^[69], and dispersive liquid-liquid microextraction (DLLME)^[36]. The SPE devices, however, have poor PAH sensitivity^[63], involve a number of processes, the extraction procedure may result in the loss of analytes^[70], and on natural waters and wastewaters, results in low recovery rates for highly lipophilic molecules such as hydrocarbons^[71].

The most sensitive, accurate, and precise approach is obtained by coupling SBSE (recommended for PAH extraction^[66]) to separation and detection systems and optimising the relevant parameters that affect both extraction and desorption^[62]. The PDMS-SBSE-HPLC-FLD approach is one of the most sensitive methods when compared to the established methods for PAHs analysis published in the literatures^[72].

7.3. Chromatographic techniques

It might be challenging to separate various chemicals from mixtures when their analytical properties are similar^[73]. Chromatographic techniques such as gas chromatography (GC), high-performance liquid chromatography (HPLC)^[36], capillary electrophoresis (CE)^[74], supercritical fluid chromatography (SFC), as well as onsite surface enhanced plasmon resonance (SERS)-based optical sensors and electrochemical sensors, have been developed^[35], when the employment of a separation procedure is typically necessary^[73]. The most used reference method for the analysis of PAHs is HPLC combined with two detectors: a fluorescence detector (FLD) and an ultraviolet (UV) detector^[26]. Additionally, GC with a mass spectrometer (MS) is also employed, but the HPLC-FLD-UV (more sensitive than the GC-MS method), allows detection limits that were 2 to 200 times lower for several PAHs^[26], and provides superior separation, strong resolution, and is non-destructive for this organic compounds^[75]. Therefore, high-performance liquid chromatography with fluorescence detection (HPLC-FLD) has often been used to evaluate PAHs^[76]. Due to their low volatilities, the propensity to disintegrate at high temperatures, and the potential to adsorb to the GC inlet and column, PAHs with molecular weights greater than 300 atomic mass units (amu) are always challenging to analyse with GC^[6]. However, a possible alternative to traditional analytical techniques

for the detection of these organic chemicals at trace levels in the water, is SBSE followed by gas chromatography coupled to tandem mass spectrometry (SBSE-GC-MS/MS)^[77].

7.4. Detection techniques

The study of PAHs contaminants in environmental samples may be effectively done using spectrofluorimetry because of its great sensitivity and selectivity^[78]. Fluorescence, using variable excitation and emission wavelengths, is one of the most employed detection systems for the analysis of PAHs in food, beverages, and water by HPLC^[35], since it is more selective and sensitive than UV detection^[36]. In order to learn more about the identity of the analyte, a fluorescence, and UV-Vis detector are frequently used in tandem for PAH analysis^[37]. The aromatic ring structures in the PAHs have intrinsic fluorescence properties^[79], and the strength of the emission varies on both the excitation and emission wavelengths, which gives rise to the selectivity of fluorescence spectroscopy. Because spectrofluorimetry has a high degree of selectivity, data collected in this method can be highly helpful in building multivariate calibration models that can identify several substances without any prior physical separation^[78]. Ultraviolet (UV) absorbance spectra for PAHs have a distinctive appearance. Each isomer has a distinct UV absorption spectrum because every ring structure has its own UV spectrum^[8]. In identifying PAHs, this is extremely helpful, because they are also fluorescent and, upon excitation, emit characteristic wavelengths of light^[80].

The mass spectrometer (MS) coupled with HPLC, is another important detector when several chemicals of interest are present, and it is especially helpful for identifying and characterizing trace polar components, providing structural information, and has been documented the utilization of HPLC-MS for the complete analysis of aromatic chemicals^[6].

Table 3 - Maximum excitation wavelength ($Ex\lambda_{max}$) and maximum emission ($Em\lambda_{max}$), in nanometers (nm)^[79], for the six different priority PAHs regulated by the applicable Portuguese legislation.

PAH	$Ex\lambda_{max}$ (nm)	$Em\lambda_{max}$ (nm)
Fluoranthene	284	464
Benzo[b]fluoranthene	254	440
Benzo[k]fluoranthene	248	436
Benzo[a]pyrene	263	408
Benzo[ghi]perylene	302	524
Indeno[1,2,3-cd]pyrene	239	566

When using a FLD and the PAH concentration is low, the photomultiplier tube (PMT) gain is effective for amplifying very weak signals, since its signal-amplifying properties increases with the voltage gain applied^[81].

8. Validation

The International Organization for Standardization (ISO), according to the NP EN ISO/IEC 17025:2018, defines that the validation is the verification by objective evidences that the method specified requirements are adequate for an intended use^[82]. This international standard also refers that laboratory-developed methods, non-standard methods, and standard methods that are utilised outside of their intended scope or otherwise altered must all be validated.

The results obtained after performing a test method are affected by systematic errors (bias or trueness), estimated by the difference between the mean value of many test results and the accepted reference^[83], and random errors (variance or precision) that are component of the measurement error which, in repeated measurements, varies unpredictably^[84].

Systematic error is described as an element of error that either stays constant or varies predictably during several studies of the same measurand. It cannot be decreased by performing more analyses with constant measurement conditions because it is independent of the number of measurements taken. Otherwise, random errors result from unpredictably changing influence quantities. Repeated observations of the measurand show differences because of these random effects. Although it cannot be eliminated, the random error of an analytical result can usually be diminished by making more observations^[85].

8.1. Selectivity

The ability of a method to identify specific analytes in mixtures or matrices without being influenced by other components with the same behaviour is known as analytical selectivity^[86]. The selectivity is evaluated by analysing chromatographic blanks (from a sample that is known to contain no analyte) in the expected time range of the analyte peak^[87]. It is especially crucial to check for interferents that, according to chemical principles, should respond to the test^[88].

8.2. Limit of detection (LOD) and limit of quantification (LOQ)

Determining the minimum concentration or, occasionally, amount of the analyte that can be detected, and determined with a reasonable degree of accuracy when employing a certain process, is one of the key objectives of analytical chemistry. The limit of detection and the limit of quantification, therefore, serve as common indicators of how well a certain trace analysis method performs^[89].

The limit of detection (LOD) is the minimum measured content, from which it is possible to detect the presence of the analyte with reasonable statistical certainty, and for chromatography methods, the noise-to-signal ratio for LOD should be 1:3^[87]. This analytical threshold corresponds to the smallest amount of analyte that can be detected in a sample but is not necessarily quantified as an exact value. A reading lower than the detection limit does not mean the absence of the analyte to be measured. It can only be stated that, with a defined probability, the concentration of the component in question will be below a certain value. In qualitative terms, the concept of detection limit corresponds to the minimum concentration that can be distinguished from the blank, that is, from a sample that contains the same matrix but does not contain the analyte^[90].

Samples with concentration levels close to or above the LOD are necessary for techniques such as chromatography, which depend on detecting a peak above the noise-to-signal ratio, and these can be made, for instance, by spiking a blank sample (e.g., in UPW) at low concentrations. Normally, providing an approximation of the LOD value is adequate for validation purposes, and three times the standard deviation (sd_0) approach is sufficient. In this case, sd_0 is determined according to the **Equation 1**^[86, 88].

$$sd_0 = \frac{sd_m}{\sqrt{n}} \quad (\text{Equation 1})$$

Where sd_m is the estimated standard deviation of m single results at very low concentration, and n is the number of replicate observations when reporting results. Hence, the calculated LOD is obtained by the **Equation 2**^[86].

$$LOD = 3 \times sd_0 \quad (\text{Equation 2})$$

Because the LOD was considered insufficient for quantitative analysis, the limit of quantification (LOQ) was created to provide additional statistical separation of the distributions of the true analyte signal and the blank measurement signal. Hence, the term "limit of quantification" can be used to refer to the lowest concentration or mass that a specific process can quantitatively analyse with a reasonable degree of confidence^[89].

In practice, LOQ usually corresponds to the lowest concentration calibration standard (excluding blank). This threshold, after being determined, must be tested to find out whether the accuracy and precision achieved are satisfactory. This test can be performed by passing, under conditions of intermediate precision, a series of internal standards, whose concentration is close to or equal to the quantification threshold. According to International Union of Pure and Applied Chemistry (IUPAC) recommendations, the coefficient of variation (CV) or relative standard deviation (RSD), that is the standard deviation divided by the average of the values found (for these standards), should not exceed 10%^[90], and the noise to signal ratio (e.g. in a chromatogram) should be 1:10^[87].

To get a reliable estimate of the standard deviation, there should be enough replicates. Generally, between 6 and 15 replicates are considered necessary, but 10 replicates are frequently advised in validation methods^[86].

8.3. Working range

The working range of an analytical method is the interval between the higher and lower levels that can be determined using the established method with verified precision, accuracy, and linearity^[87]. The LOQ limits the lower end of the working range, and concentrations at which significant aberrations in the analytical sensitivity are noted determine the upper end^[86]. The concentration range used for the linearity test will fall within this range^[87].

8.4. Analytical sensitivity and linearity

Analytical sensitivity is the difference in instrument response that results from a change in the quantity being measured (for instance, an analyte concentration)^[86], and is determined simultaneously with the linearity tests. It can be described as the slope of the linear regression calibration curve^[91], and is useful for testing whether an instrument is performing to a consistent and acceptable standard, and may be important for quality assurance of obtained analytical results^[88]. An analytical method is said to be linear if it can produce test results that are proportionate to the concentration of analytes within a specified range either immediately or through well-described mathematical adjustments^[91], and the residuals values (RE) are calculated with the linear regression model, according to **Equation 3**^[92].

$$RE(\%) = \frac{y_i - \hat{y}_i}{\hat{y}_i} \times 100 \quad (\text{Equation 3})$$

Where y_i and $\hat{y}_i$ represent the experimental and estimated response signal by the equation of the calibration curve, respectively.

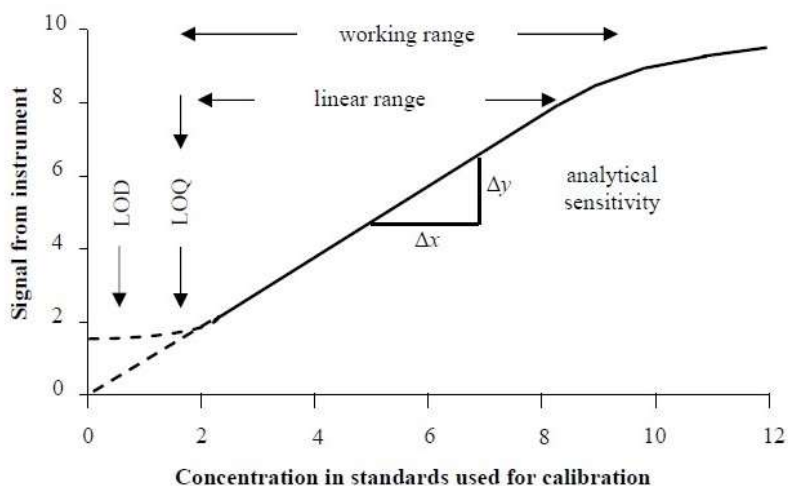


Figure 1 - Schematic example of a calibration curve, using an instrumental methodology, and the identification of the LOD, LOQ, working range, linear range and analytical sensitivity (figure adapted from Eurachem Guide, 2014)^[86].

The linearity can be assessed using a statistical model in accordance with the ISO 8466-1 and ISO 8466-2 standards. Therefore, from a set of ordered pairs, the linear calibration function (ISO 8466-1) and the non-linear calibration function (ISO 8466-2) are calculated, as well as the respective residual standard deviations, S_{y1} (**Equation 4**) and S_{y2} (**Equation 5**)^[90, 92-93].

$$S_{y1}^2 = \sqrt{\frac{\sum_{i=1}^N [y_i - (a + bx_i)]^2}{N-2}} \quad (\text{Equation 4})$$

$$S_{y2}^2 = \sqrt{\frac{\sum_{i=1}^N [y_i - (a + bx_i + cx_i^2)]^2}{N-3}} \quad (\text{Equation 5})$$

The difference in variances (DS^2) is calculated using the **Equation 6**^[92].

$$DS^2 = (N - 2) \times S_{y1}^2 - (N - 3) \times S_{y2}^2 \quad (\text{Equation 6})$$

Where N is the number of concentration levels, and the constants a, b and c refer to the constants of degree zero, one and two.

Furthermore, the variance of the non-linear calibration function S_{y2} is evaluated using a F-test, according to **Equation 7** and in order to verify significant differences^[92].

$$PG = \frac{DS^2}{S_{y2}^2} \quad (\text{Equation 7})$$

Then, by comparing the calculated PG (variances ratio) result and the corresponding tabulated value of the Fisher–Snedecor distribution (F), for a significance level of 1% and N-3 degrees of freedom, the following conditions must be assessed:

- i) If $PG \leq F$, the calibration function is linear, and the non-linear calibration function does not lead to a significant improved adjustment.
- ii) If $PG > F$, the calibration function is non-linear, and the working range should be adjusted to obtain a linear calibration function.

Hence, if the PG value is less than $F_{(1,N-3;0,01)}$, the calibration function is considered linear, and the working range is well adjusted^[92-93].

8.5. Trueness

Trueness is the proximity of agreement between a test result and the accepted reference value of the property being measured. The trueness is quantified in terms of "bias", with lower bias signifying more trueness^[88]. Comparing the mean of the findings from the candidate method with an appropriate reference value is a practical way to identify bias. There are three general strategies available: (a) reference material analysis and proficiency tests (PT); (b) recovery tests employing spiked samples; and (c) comparison with outcomes from another method. Bias studies should reflect the method scope, which may need analysing several sample types and/or analyte concentrations^[86].

Therefore, the bias can be expressed in absolute terms, according to **Equation 8**:

$$b = \bar{x} - x_{ref} \quad (\text{Equation 8})$$

Where b represents the bias, $\bar{x}$ is the mean of the results obtained and x_{ref} corresponds to the reference value.

The relative bias in percent is given by **Equation 9**:

$$b(\%) = \frac{\bar{x} - x_{ref}}{x_{ref}} \times 100 \quad (\text{Equation 9})$$

And the relative spike recovery is calculated according to **Equation 10**:

$$R(\%) = \frac{\bar{x}' - \bar{x}}{x_{spike}} \times 100 \quad (\text{Equation 10})$$

Where R is the relative spike recovery in percent, $\bar{x}'$ is the mean value of the spiked sample, $\bar{x}$ represents the mean of the experimental sample results, and x_{spike} is the added concentration^[86].

However, the relative recovery (Rec), sometimes known as the "apparent recovery", is also utilised in some analytical measurement sectors, and is defined by the ratio of the experimentally estimated mean sample results and the reference value, in the absence of analyte, applying the **Equation 11**^[86, 94].

$$Rec = \frac{\bar{x}}{x_{ref}} \quad (\text{Equation 11})$$

Where $\bar{x}$ is the mean concentration of the sample results experimental obtained and x_{ref} corresponds to the reference value.

For the estimation of the standard uncertainty associated with the trueness, considering the available recovery data of the reference value (u_{Rec}), of the reference material used, is applied the following **Equation 12**^[94]:

$$u_{Rec} = Rec \times \frac{\delta_{spike}}{\bar{x} \times \sqrt{n}} \quad (\text{Equation 12})$$

Where δ_{spike} is the obtained standard deviation of the spiked samples, and n is the number of considered observations.

Once the relative recovery, Rec, and its corresponding standard uncertainty, u_{Rec} , have been calculated under the different matrix and analyte conditions, it is necessary to assess whether Rec is significantly different from 1, by using a Student's t-test (t), as presented below in **Equation 13**^[85, 94].

$$t = \frac{|1 - R|}{u_{Rec}} \quad (\text{Equation 13})$$

The $t_{critical}$ value is obtained using a two-way Student's t-table (exemplified in **Attachment 1**), for the number of degrees of freedom associated with Rec and u_{Rec} , often to a 95% confidence level, if the respective number of degrees of freedom is known.

Therefore, for the significancy test, assessed by comparing the calculated t result and the corresponding $t_{critical}$ value, the following conditions must be evaluated:

- i) When $t \leq t_{critical}$, Rec is considered not to be significantly different from 1.
- ii) When $t > t_{critical}$, Rec is significantly different from 1.

When is necessary the results correction according to the calculated relative recovery ($t > t_{critical}$), the analyte concentration (c') is given by multiplying the initial estimate of the value of the quantity (c_i) by the inverse Rec factor, as shown in **Equation 14**^[94].

$$c' = c_i \times \frac{1}{Rec} \quad (\text{Equation 14})$$

Proficiency testing through interlaboratory comparisons (also known as external quality assessment)^[86], is used to assess individual laboratories performance for particular tests or measures and to monitor their ongoing performance^[95], is often used as a condition for laboratory accreditation^[96], and offers a generally available means to obtain information about laboratory bias^[95]. Since the estimated uncertainty should be consistent with the spread of results achieved by that laboratory over several proficiency test rounds, the laboratory's proficiency test results can be used to verify the evaluated uncertainty^[85].

For the performance calculation in proficiency testing, the following **Equation 15** should be used:

$$Z = \frac{X_{lab} - X_{ref}}{\delta} \quad (\text{Equation 15})$$

Where Z ("z-score") is the proficiency testing performance, X_{lab} is the laboratory test result, X_{ref} is the reference proficiency test value, and δ represents the unit of deviation, which can be the reference material standard deviation, the standard deviation of the mean of the laboratories in the interlaboratory test (SD), or the uncertainty of X_{ref} ^[96].

A common evaluation based on z-scores (Z), can be done according to the following scoring scale^[96]:

$$|Z| \leq 2 \quad (\text{Satisfactory})$$

$$2 < |Z| \leq 3 \quad (\text{Questionable})$$

$$|Z| > 3 \quad (\text{Unsatisfactory})$$

Therefore, a z-score between -2 and +2 is often considered as satisfactory in proficiency testing schemes, while a result above +3 or below -3 is regarded as unsatisfactory. Typically, a z score between +2 and +3 or -2 and -3 is considered as "Questionable." Even though various schemes may use relatively different definitions of the z-score, a z-score of 2 typically denotes a result that deviates by two standard deviations from the value assigned for the selected analyte^[97].

The recovery experiments employing spiked samples and the proficiency tests, are the most common strategies for measurement of this uncertainty component. According to EURACHEM/CITAC guide (QUAM^[85]), and ISO 11352:2012^[98], a minimum of six determinations or tests, are necessary to get a reliable estimative bases on the evaluation using mean recovery's or proficiency tests.

There are two components associated to the method and laboratory bias uncertainty (u_b): the discrepancy between observed and total analyte recovery, as well as the uncertainty surrounding the concentration of analyte added. The applicable matrix should be used in the recovery tests with at least six separate samples^[98].

The standard uncertainty estimated from recovery experiments for the method and laboratory bias (u_b), is calculated according to the **Equation 16**:

$$u_b = \sqrt{b_{rms}^2 + u_{add}^2} \quad (\text{Equation 16})$$

Where b_{rms} is the root mean square of the deviations from the recovery experiments, and u_{add} is the uncertainty in the concentration of the analyte added.

The uncertainty in the volume added and the uncertainty in the concentration of analyte added, form the uncertainty in the concentration of the analyte added (u_{add}).

The root mean square of the recovery experiment deviations (b_{rms}), is calculated using the **Equation 17**:

$$b_{rms} = \sqrt{\frac{\sum b_i^2}{n_\eta}} \quad (\text{Equation 17})$$

Where b_i is the deviation from the mean recovery (if results are corrected with this mean recovery) or from the complete recovery (100%), and n_η is the number of recovery experiments.

The uncertainty in the concentration of the analyte added (u_{add}), consists in the uncertainty of the volume added (u_v), and the uncertainty in the concentration of the solution added (u_{conc}), assessed using **Equation 18**.

$$u_{add} = \sqrt{u_v^2 + u_{conc}^2} \quad (\text{Equation 18})$$

The uncertainty of the concentration (u_{conc}) can be obtained from the certificate if the solution used in recovery tests is a certified reference material.

The uncertainty of the volume added (u_v) can frequently be estimated using data from volumetric labware manufacturers, and both systematic and random (repeatability) errors must be taken into consideration.

Therefore, the uncertainty component of the volume added (u_v) is given by **Equation 19**:

$$u_v = \sqrt{u_{v,b}^2 + u_{v,rep}^2} \quad (\text{Equation 19})$$

Where $u_{v,b}$ is the systematic uncertainty component of the volume added, and $u_{v,rep}$ is the random uncertainty component of the volume added (in repeatability conditions).

The standard uncertainty component for method and laboratory bias (u_b), is then calculated according to **Equation 16**.

8.6. Precision

Precision is the degree of similarity between independent test results, obtained under predetermined conditions^[88]. The standard deviation (or relative standard deviation), which is generated from results acquired by conducting replicate measurements on an appropriate material under predetermined conditions, is commonly used to represent the statistical factors that define the spread of results. Selecting the appropriate conditions is crucial for measuring measurement precision since they affect the sort of precision estimate that is produced. As precision is typically influenced by analyte concentration, it should be assessed across a range of concentrations. This could include concentrations at the boundaries of the measuring interval as well as a specific concentration of interest (like a regulatory limit)^[86]. The precision as RSD or CV percentage is calculated according to **Equation 20**.

$$RSD(\%) \text{ or } CV(\%) = \frac{\delta}{\bar{x}} \times 100 \quad (\text{Equation 20})$$

Where δ is the measured standard deviation and $\bar{x}$ represents the mean values, for a specific series of standards.

The uncertainty component for the within-laboratory reproducibility, u_{Rw} , (for a specific concentration and matrix) can be estimated from the standard deviation of these quality control (QC) results (e.g., as obtained from quality control charts) if stable QC samples that cover the entire analytical process (including all sample preparation steps) are regularly analysed under intermediate precision conditions (**Equation 21**). For the

estimation of this uncertainty component, a minimum of eight measurements are required^[98].

Therefore:

$$u_{RW} = S_{RW} \quad (\text{Equation 21})$$

Where S_{RW} is the standard deviation of the QC results.

It is important to analyse QC samples made up of several matrices and concentration levels, if the analytical method spans a wide concentration range or range of matrices, and the uncertainty component for the within-laboratory reproducibility (u_{RW}) varies with the concentration or matrix^[98].

The additional uncertainty component resulting from potential increased inhomogeneity of the analyte in the matrix, should also be considered if QC samples with an identical matrix to test samples are not available, and synthetic standard solutions (with a matrix that differs from that of routine samples) are used. This additional uncertainty brought on by inhomogeneity can be calculated, for instance, from range control charts employing samples from various matrices. However, since this uncertainty component covers only the repeatability component, it shall be combined with the uncertainty of the QC standard solutions results, to obtain a reliable estimate of the within-laboratory reproducibility (**Equation 22**)^[98].

$$u_{RW} = \sqrt{u_{RW,stand}^2 + u_{r,range}^2} \quad (\text{Equation 22})$$

Where $u_{RW,stand}$ is the uncertainty component of the results from the standard solution used as QC sample, and $u_{r,range}$ is the uncertainty component from the range control chart.

The uncertainty component from the range control chart ($u_{r,range}$), can be calculated from the mean range of a range control chart using the **Equation 23**^[98].

$$u_{r,range} = \frac{\bar{R}}{d_2} \quad (\text{Equation 23})$$

Where $\bar{R}$ is the mean range, and d_2 is dependent on the number of values from which the range is determined, according to the **Table 4**.

Table 4 - Factors for the calculation of the standard deviation from the mean range.

Range number of values	Factor d_2
2	1.128
3	1.693
4	2.059
5	2.326

The repeatability part of the uncertainty component for the within-laboratory reproducibility (u_{Rw}), is typically minimal if measurements are carried out at a concentration level significantly higher than the limit of quantification (LOQ)^[98].

8.7. Measurement uncertainty

Uncertainty is a range of values that can be properly assigned to the quantity being measured and is attributed to a measurement result^[86], characterising the dispersion of the quantity values being attributed to a measurand, based on the information used^[98].

In general, there are numerous components that make up measurement uncertainty. Some of these components can be identified by experimental standard deviations and evaluated based on the statistical distribution of the outcomes of a series of measurements. The other components are assessed using presumptive probability distributions based on experience or other data, and they can also be described by standard deviations^[85].

The assessment of the measurement uncertainty is based on the uncertainty component for the within-laboratory reproducibility (u_{Rw}), and the uncertainty component from method and laboratory bias (u_b)^[98]. After estimating these components of uncertainty (expressed as standard uncertainties), the combined standard uncertainty (u_c) is calculated according to **Equation 24**:

$$u_c = \sqrt{u_{Rw}^2 + u_b^2} \quad (\text{Equation 24})$$

However, the relevant contributions must add more than one third of the calculate combined standard uncertainty (u_c)^[98].

The expanded uncertainty U is calculated from a combined standard uncertainty (u_c) and a coverage factor (k) of 2 (corresponding to a symmetrical confidence interval of 95%), according to the **Equation 25**:

$$U = k \times u_C \quad (\text{Equation 25})$$

Each measurement result has a corresponding level of uncertainty. Typically, a full measurement result includes an indication of the uncertainty in the form $x \pm U$, where x is the measurement result and U is the expanded uncertainty.^[99]

8.8. Ruggedness (robustness)

The ruggedness or robustness of an analytical procedure is a measure to systematically evaluate the importance of small changes in method parameters. This is frequently employed for technique optimization and is particularly suitable for the rapid identification of significant effects^[100]. The approach can be used in situations where discrete impacts, such as matrix modifications or minor equipment configuration changes, have unpredictable consequences on the outcome^[85]. For its evaluation, the method can be tested under conditions of intermediate precision (e. g. varying the operating conditions within the laboratory, over many days and obtaining a significant number of results). If necessary, a detailed and careful analysis of the results in terms of precision of the method should then be carried out, providing valuable information about the behaviour of the methodology under different working conditions^[90].

8.9. Fitness for purpose

Users of the results anticipate being able to trust the data, and they depend on the laboratory to provide the best solution to the issue, or to provide a result that is appropriate for the task at hand. Understanding the user's needs is challenging for the laboratory. Prior to beginning any studies, an accredited laboratory must assess the needs of the user in accordance with ISO/IEC 17025 - *General requirements for the competence of testing and calibration laboratories*^[83].

Therefore, fitness for purpose refers to how closely a method's performance complies with the standards that describe the end-requirements user and are agreed upon by the analyst and the end-user of the data^[88], such as the validation requirement for the quantification limit, trueness, precision and the uncertainty in accordance with the regulatory limit of the applicable legislation.

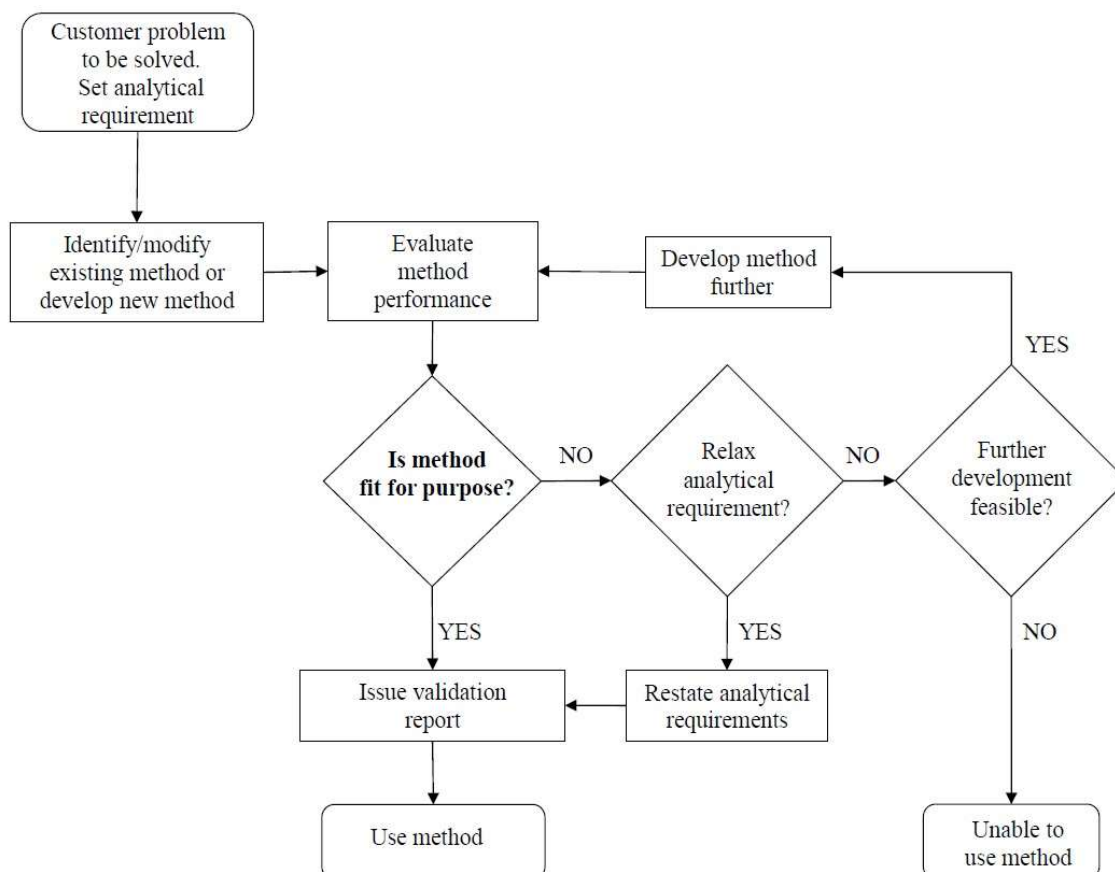


Figure 2 - Schematic example of a method validation process, from the customer issue through the laboratory assessment of whether the customer request can be fulfilled using a specific method (figure adapted from Eurachem Guide, 2014)^[86].

Despite of what existing analytical data may be available for the method, fitness for purpose will be determined by how the method performs when used by the analyst with the available equipment and facilities^[86].

8.10. Control charts

Internal quality control at the laboratory for chemical analysis entails a constant critical assessment of the laboratory's own analytical procedures and daily working practices. The control includes the entire analytical procedure, from the moment the sample enters the laboratory until the analytical result is produced. The usage of control charts is the most important instrument in this quality control^[83].

The laboratory runs control samples together with the test samples, and in a control chart, the control values are plotted, making it easy to show that the measurement technique operates within predetermined limits. No analytical results are presented if the control value is outside the limits, and corrective measures must be taken to identify the causes of errors and eliminate them^[83].

Theoretical spreads of the results around the mean value can be estimated using the normal distribution as a basis^[83]. Therefore, the probability of a point being more than 2 standard deviations (2s) from the mean value is approximately 5%, and more than 3 standard deviations (3s) is around 0.3% (**Figure 3**)^[96].

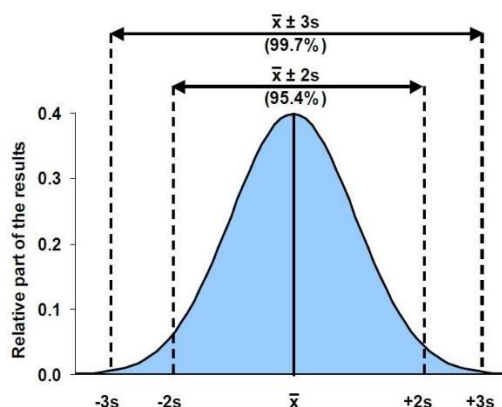


Figure 3 - Normal distribution curve illustrating the probability for an analytical result to be located within specified limits (figure adapted from NORDTEST – Technical Report 569, 2018)^[83].

Where $\bar{x}$ is the mean value of the analytical results, and s is the corresponding standard deviation.

The normal distribution curve, which describes the statistical properties of random variations, provided the basis for the control chart. The relation between the control chart equivalent (X-chart) and the normal distribution curve, is illustrated in **Figure 4**.

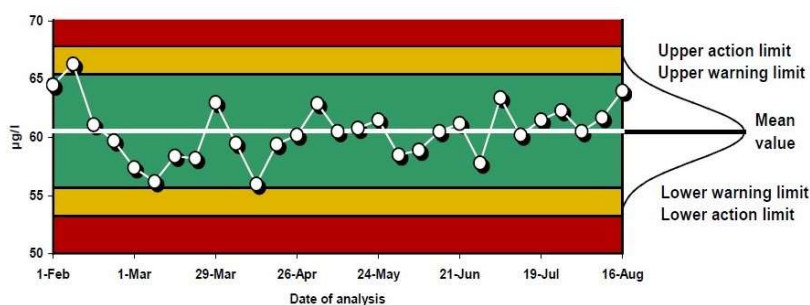


Figure 4 – X-chart example of the relation between the normal distribution curve and the control chart (figure adapted from NORDTEST – Technical Report 569, 2018)^[83].

A reference value or the mean value of the control values is represented by the central line (CL) in the control chart. The control chart typically contains four lines, plus the centre line. Two of them, referred to as the warning limits, are situated at a distance from the central line of two standard deviations ($CL \pm 2s$). Around 95% of the results should be within these limits (upper and lower warning limits), assuming that the results are normally distributed. Two additional lines (upper and lower action limits) are drawn in the control chart ($CL \pm 3s$) at a distance from the central line that is three times the standard

deviation. The 99.7% of the data that is typically distributed should fall between these lines, which are referred to as the action limits^[83]. The minimum number of replicates required for the construction of the control chart varies depending on the methodology but is normally between 6 and 15 measurements^[86].

8.10.1. X-charts

The X-charts are based on the distribution of control values around a true or expected value and have a central line, upper and lower warning limits, and action limits. A mean of multiple measurement results or a single result can be used to track the interaction of systematic and random effects for control values. The bias can be monitored by comparing the mean control value over time with the reference value while using a reference material (that is similar to a test sample) as the control sample^[83].

Control limits may be set according to the performance of the analytical method (statistical control limits), without considering the requirement on analytical quality, and this is the most common method to set the limits. Using the obtained control values, the mean value is estimated and is used to set the central line (**Figure 4**)^[83].

Therefore, the X-chart indicates how well the control values (mean values of several analyses or single values) are within control limits.

8.10.2. Range charts

The range chart's primary function is to indicate repeatability control, and the range is defined as the difference between the largest and lowest single result for two or more independent tests of the same sample and only contains positive upper limits (**Figure 5**)^[83].

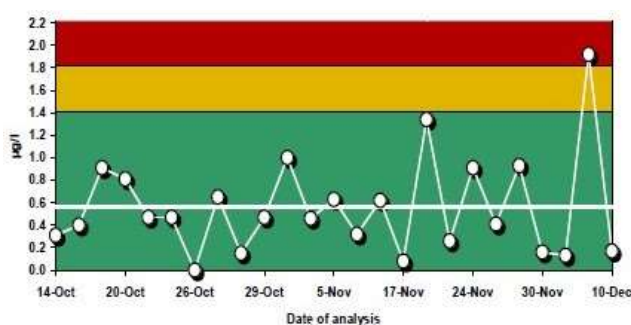


Figure 5 – Range chart example with standard deviation limit (green area), warning limit (yellow area) and action limit (red area) (figure adapted from NORDTEST – Technical Report 569, 2018)^[83].

The control limits are calculated from a standard deviation and can be set based on the performance of the method (statistical control limits), according to the **Table 5**.

Table 5 - Factors for calculation of central line, warning limits and action limits for the construction of range charts^[83].

Number of replicates	Standard deviation (s)	Warning limit WL	Action limit AL
	Mean range/d ₂	D _{WL} × s	D _{AL} × s
2	Mean range/1.128	2.834 × s	3.686 × s

8.10.3. Trends

On the control chart, systematic or non-random patterns might be an indication of minor deviations in the process mean or variability that might not be significant enough to be immediately manifested as points outside the control boundaries^[101].

There is typically at least one control value for each chart for each batch of analysis. In routine tasks, it's crucial to be vigilant if a control value deviates from the acceptable range, or if a certain pattern emerges in the control values over time (e.g., in x-charts). In the daily interpretation of quality control, there are three possible outcomes: (a) the method is in control; (b) the method is in control, but the long-term assessment reveals that the method is out of statistical control; and (c) the method is out of control^[83].

If the control value is inside the warning limits or if it is between the warning and action limits and the two preceding control values are within warning limits, the method is in control, and the analyst can report the results of the analysis^[83].

The method is in control but may be considered as out of statistical control if all control values are within the warning limits (is recommended a maximum of one out of the last three between warning and action limits), and if seven consecutive control values gradually increase or decrease^[101], or if 10 out of 11 control values in a row are located on the same side of the central line. The analyst can in this instance report the analytical results, but a potential issue may be emerging^[83].

If a control value is outside the action limits, or if it is between the warning and the action limit and at least two preceding control values (the rule is two out of three outcomes) are between those limits, then the method is out of control^[96]. No analytical findings can be presented in this situation, and reanalysis must be performed on all samples that have been examined, since the out-of-control value was discovered^[83].

One or more test rules must be broken for a process to be considered out-of-control, and any assignable sources of variation must be identified and addressed. These additional

test rules increase the control chart's capacity to identify more subtle changes in the undertaken process^[101].

9. Experimental

The reagents, standards, equipment, and material required for the implementation of this analytical method, as well as the experimental methodology used for this internship work, are detailed in this chapter.

9.1. Reagents and standards

The PAHs standard mix solution (PAH-Mix 3, product code DRE-L20950003AL in acetonitrile) with the analytes fluoranthene (50 µg/mL), benzo[b]fluoranthene (20 µg/mL), benzo[k]fluoranthene (20 µg/mL), benzo[a]pyrene (20 µg/mL), benzo[ghi]perylene (20 µg/mL) and indeno[1,2,3-cd]pyrene (40 µg/mL) were purchased from LGC Standards. The single PAH standard solutions with the concentration of 100 µg/mL for the analytes FLU (product code 14500-0820-100AN1,5), BBF (product code 14500-0120-100AN1,5), BKF (product code 14500-0140-100AN1,5), BAP (product code 14500-0230-100AN1,5), BPE (product code 14500-0210-100AN1,5) and IND (product code 14500-0880-100AN1,5), were purchased from NEOCHEMA. HPLC grade solvents acetonitrile, methanol, dichloromethane, and acetone were obtained from Fisher Scientific. The sodium thiosulfate pentahydrate, pro analysis (PA) used in water samples as chlorine inhibitor was supplied by Chem-Lab.

The stock solutions of PAHs with the concentration of 10 µg/L for BBF, BKF, BAP, and BPE, 20 µg/L for IND, and 25 µg/L for FLU were prepared in acetonitrile by dilution of 50 µL of PAHs standard mix solution (PAH-Mix 3) in a 100 mL volumetric flask with glass stopper and stored in a freezer at 5±3 °C. The working standards were prepared by the subsequent dilution of stock solutions, in ultrapure water (UPW) according to **Table 6**. The stock solutions with the concentration of 10 µg/L for a single PAH, were prepared in acetonitrile by dilution of 10 µL of the single PAH standard solution (100 µg/mL) in a 100 mL volumetric flask with a glass stopper and stored in a freezer at 5±3 °C. The single working standards solutions were prepared in ultrapure water (UPW) by dilution of 200 µL of the stock solution (10 µg/L) in a 200 mL volumetric flask with glass stopper.

The UPW (with 0.3% of acetonitrile) eluent solution, was prepared by dilution of 3 mL of acetonitrile in a 1000 mL volumetric flask.

Table 6 – PAHs stock solution volume needed to prepare the different levels of the calibration standards, according to the established final volume.

PAHs stock solution $\text{\text{¥}}$ (μL)	Final Volume (mL)	FLU (ng/L)	BBF (ng/L)	BKF (ng/L)	BAP (ng/L)	BPE (ng/L)	IND (ng/L)
75	500	-	-	1.5 (LOQ)	1.5 (LOQ)	-	-
75	250	7.5 (LOQ)	3 (LOQ)	3	3	-	6 (LOQ)
150	250	15	6	6	6	6 (LOQ)	12
200	200	25	10	10	10	10	20
300	200	37.5	15	15	15	15	30
1000	500	50	20	20	20	20	40
3000	1000	75	30	30	30	30	60
2000	500	100	40	40	40	40	80
1000	200	125	50	50	50	50	100
3000	500	150	60	60	60	60	120

$\text{\text{¥}}$ PAHs stock solution with a concentration range between 10-25 $\mu\text{g/L}$ for the considered analytes.

9.2. Instrumentation

The chromatographic analyses were performed using an Agilent 1100 Series HPLC system, equipped with an autosampler (G1329A), a degasser (G1322A), a quaternary pump (G1311A), a variable wavelength detector (G1314A), a programmable fluorescence detector (G1321A) and a column compartment (G1316A), fitted with a Inertsil ODS-P column (5 μm particle size; 250mm $\times$ 4.6mm I.D.) and a Inertsil ODS-P safeguard column (5 μm particle size; 10mm $\times$ 4.0mm I.D.).

The chromatographic data were collected and processed using the Agilent ChemStation Plus (Revision A.10.02) software.

The extraction was carried on a DLAB ten position magnetic hot plate stirrer, model MS-H-S10, and for the desorption was used a Thermolyne Cimarec magnetic stirrer, model S131120-33.

The eluent solutions were degasified in a J.P. Selecta ultrasonic cleaning bath, model Ultrasons-HD 3000867.

The UPW was acquired using a Millipore ultrapure water treatment system, model Milli-Q Academic, and the chemicals were weighed on a Mettler Toledo analytical balance, model AX204.

Digital thermometer from HANNA, model HI 98501 for controlling the sample's water bath extraction temperature, in 250 mL Duran beakers.

9.3. Materials

Preparation of standard solutions was performed in 100.0, 200.0, 250.0, 500.0, and 1000 mL, Class A, glass stoppered, Duran volumetric flasks, using 1.00, 2.00, 3.00, and 100 mL Hirschmann volumetric pipettes and 50.0; 100; 250; and 500 μ L Hamilton 1700 Series micro syringes.

The water sampling was accomplished in 500 mL amber glass bottles using polytetrafluoroethylene (PTFE) screw caps lined in aluminium.

For the sample's preparation, was used 1.00 and 100 mL Hirschmann volumetric pipettes, and a 100 mL amber glass bottles with PTFE screw caps.

The extraction was performed using 10 mm length stir bars coated with a 1 mm film thickness of PDMS (Twister SBSE), obtained from Gerstel, and handled with a magnetic tweezer.

Magnetic bar retriever and lint-free tissues for drying the stir bars (Twister SBSE).

The desorption was carried out in 2 mL amber glass vials, with 300 μ L conical inserts (autosampler compatible).

The conditioning of the stir bars was performed in 40 mL amber wide-mouth glass bottles, with PTFE screw caps.

For preparing the stirring bars conditioning solution, was used a 5 mL Socorex manual micropipette, model Acura 835.

9.4. Procedure

9.4.1. Samples and sampling

Drinking water, surface water, and groundwater samples were collected in 500 mL amber glass bottles, with approximately 40 mg of sodium thiosulfate pentahydrate, fully filled (without overflowing), using PTFE screw caps lined in aluminium and stored in the dark at 5 ± 3 °C before analysis. Within seven days of sample collection, all samples were extracted and analysed within 40 days of sample extraction. If the samples are not analysed after extraction, the 2 mL extract vials should be stored at 5 ± 3 °C.

9.4.2. Stir bar sorptive extraction (SBSE)

Before use, each stir bar was conditioned into a 40 mL amber wide-mouth glass bottle, containing 4 mL of dichloromethane-methanol mixture (1:1) and magnetically stirred for 5 min at 500 rpm. This procedure was repeated two times with fresh portions of the solvent mixture. Once conditioned, the stir bars were dried with lint-free tissue.

To avoid adsorption of PAHs upon glassware, 1 mL of methanol was placed into 100 mL amber glass bottles with PTFE screw caps, before adding 100 mL of water sample or standard solution (**Table 6**) using volumetric pipettes. Afterward, the solution was mixed thoroughly.

The amber glass bottles containing the previous solution were placed into a 250 mL beaker containing approximately 125 mL of UPW and positioned in a magnetic hot plate stirrer (brand DLAB, model MS-H-S10) at 65 ± 5 °C. After reaching the desired temperature of 65 ± 5 °C, the conditioned stir bars were added to the amber glass bottle containing the solution and stirred at the specified temperature, for 60 minutes at 500 rpm for PAHs extraction.

When the extraction time had elapsed, the stir bars were removed from the amber glass bottles using magnetic tweezers, dried on a lint-free tissue, and placed in 2 mL vials with a 300 µL conical glass insert containing 200 µL of acetonitrile. The PAHs desorption in acetonitrile was carried at room temperature, using a magnetic stirrer (brand Thermolyne Cimarec, model S131120-33) for 15 minutes at 1000 rpm.

Finished the desorption time, the stir bars were removed using a magnetic bar retriever and placed in 40 mL amber wide-mouth glass bottles, with PTFE screw caps. Subsequently, the stirring bars underwent the same conditioning process mentioned above, enabling their reuse in the extraction phase. The 2 mL vials containing the extract were then positioned in the autosampler of the HPLC-FLD.

9.4.3. High-performance liquid chromatography with fluorescence detector

As described above, the PAHs analysis was performed in an Agilent 1100 Series HPLC system equipped with an autosampler and a fluorescence detector. The analytes separations were conducted on a reversed-phase Inertsil ODS-P column (5µm particle size; 250mm×4.6mm I.D.) as stationary phase, and the degasified eluents solutions of acetonitrile and UPW (with 0.3% of acetonitrile) were employed as the mobile phase. All sample injections were held constant at 80 µL using a fixed volume injection loop. The flow rate was set at 1.5 mL/min and the column temperature was held at 28 °C. The isocratic elution program was set with 90% acetonitrile and 10% UPW (with 0.3% acetonitrile) for 22 minutes. Then, was increased to 100% acetonitrile to wash the column and within 2 minutes was back to 90% acetonitrile and 10% UPW (with 0.3% acetonitrile) and held for 2 minutes for reconditioning prior to the next run. Therefore, the

run time was about 22 minutes, with a total time of 26 minutes until the next injection. The details for the chromatographic separation conditions are described in **Table 7**, with a good resolution for the six PAHs.

Table 7 - Chromatographic separation conditions for the HPLC-FLD method.

Time (min)	UPW ^o (% v/v)	Acetonitrile (% v/v)	Column (°C)	Flow rate (mL/min)
0 - 22	10	90	28	1.5
22 - 24	0	100	28	1.5
24 - 26	10	90	28	1.5

^o UPW with 0.3% of acetonitrile.

To obtain improved sensitivity for the PAHs analysis, the PMT gain, the excitation and emission wavelength selection conditions for HPLC-FLD are shown in **Table 8**.

Table 8 – Time, excitation wavelength, emission wavelength and PMT gain program used for HPLC-FLD.

PAHs	Time (min)	Excitation (nm)	Emission (nm)	PMT gain
Fluoranthene	0.0	237	460	13
Benzo[b]fluoranthene	7.0	255	420	14
Benzo[k]fluoranthene				
Benzo[a]pyrene				
Benzo[ghi]perylene	15.5	255	420	18
Indeno[1,2,3-cd]pyrene	18.0	250	495	18

The analytes were identified by comparison of the retention time (RT) obtained for the six PAHs standard mixture, and the RT achieved by each single PAH standard.

Standard solutions underwent testing using identical conditions to the samples. Analysing the analyte peak heights at known concentrations facilitated the creation of calibration curves for each analyte, enabling the determination of the PAH concentration in the samples.

10. Results and Discussion

Application, selectivity, analytical sensitivity, linearity, working range, limit of quantification, limit of detection, trueness, precision, measurement uncertainty, and fitness for purpose, are typical performance characteristics required for an analytical method validation^[87-88, 90]. Hence, this validation study assessed all these criteria based on the experimental results from the SBSE-HPLC-FLD method. Further detailed discussion of these requirements will follow in subsequent chapters.

10.1. Sampling and storage

According to some authors, the water samples for PAHs analysis must be collected in amber glass containers^[38, 53], filled (without overflowing) at the sampling site and stoppers inserted to leave no air space^[38]. Chlorinated samples require the addition of 80 mg of sodium thiosulfate per litre of the sample, followed by thorough mixing before filling the sample vials^[53, 55, 102]. Accordingly, this study utilized 500 mL amber glass bottles (with 40 mg of sodium thiosulfate when needed) and followed the specified container-filling procedure.

From the moment of collection until extraction, all samples should be kept frozen or refrigerated at 4°C^[10, 103]. Samples, extracts, and standards must be stored in amber vials to prevent photolytic breakdown due to the light sensitivity of PAHs^[102], and the screw caps or bottle tops should be PTFE lined or wrapped in aluminium foil^[38], to avoid sample contaminations. Within seven days of sample collection, all samples must be extracted^[104], and they must all be fully analysed within 40 days of sample extraction^[102].

Therefore, the PTFE screw caps, and the glass bottleneck covered in aluminium foil, were chosen for PAHs water sampling. Samples were stored at 5 ± 3 °C, with a seven-day extraction period and sample analysis within 40 days, as chosen storage and handling conditions.

10.2. SBSE optimization

10.2.1. Extraction conditions

The analyte response may be suppressed by insufficient stir bar conditioning^[105]. Therefore, the stir bars were preconditioned in a vial containing 4 mL of a 1:1 mixture of dichloromethane / methanol, and stirred at 500 rpm for 5 minutes^[10], to purge the coating of organic impurities^[61]. This step was repeated two times to optimize the conditioning procedure.

In terms of the extraction step, the factors that have received the most research include extraction time, pH adjustment, the addition of an inert salt, the addition of an organic modifier, and stirring rate, followed by extraction temperature and sample volume^[61]. Certain factors, like sample pH adjustments or the introduction of inert salts, alter analytes or sample conditions, thus influencing the chemical equilibrium^[67]. Since the target PAHs are nonpolar^[62, 106], and non-ionisable substances^[107], pH has no impact on

their extraction. As a result, in this validation work, the impact of pH on PAHs extraction was not examined^[61, 107].

Regarding adding an inert salt, it's noteworthy that nonpolar PAHs in the solution are driven to the water's surface due to the salt's presence (termed the oil effect). This diminishes their interaction with the stir bar's PDMS coating and lowers analyte recovery^[106-107]. Overall, the addition of an inert salt to hydrophobic analytes has been noted to decrease extraction efficiency rather than increase it^[67], and for that reason, no salt was used. The sample volume is another important factor to be considered in the extraction step. Although higher sample volumes reduce extraction effectiveness, the increase in the mass of the analyte may raise the chromatographic response for SBSE. For the determination of PAHs in ambient waters, minor variations were seen in the 10 - 60 mL range^[67], and the selected sample volume can be between 10 mL and 250 mL when PDMS is the extraction agent^[108]. Therefore, was selected 100 mL of sample volume to maximize the FLD response to the analytes.

To reduce analyte adsorption to the glass walls, organic modifiers like methanol or acetonitrile are tested as additions during SBSE. However, the inclusion of such modifiers can also increase the solubility of the solutes in the aqueous phase and, as a result, reduce extraction efficiency. Usually, methanol is the selected organic modifier when using SBSE^[67], and has been demonstrated that 10% methanol addition (concerning the sample volume), enhanced the PAHs recoveries^[107]. Hence, in this experimental study, methanol was chosen. However, only 1 mL is added to a 100 mL sample volume to avoid disrupting the extraction efficiency.

Factors such as stirring rate accelerate the process and influence its kinetics. Consequently, the stirring rate is often investigated due to its potential to hasten extraction and enhance responses within a designated extraction time. This phenomenon is attributed to the reduced boundary layer between the stir-bar and the solution bulk. However, increasing the stirring speed might lead to physical damage to the extraction phase, as the stir bar directly contacts the sample vial's bottom. According to some authors, the stirring rate boosts reactions up to values in the 500–750 rpm range, but greater values have little to no impact^[67]. Hence, in this experiment, a speed rate of 500 rpm was chosen to optimize the extraction time without compromising the extraction phase.

The extraction temperature is thought to be a significant factor affecting the extraction effectiveness of the coated stir bars for PAHs^[56]. According to some authors, the extraction efficiency increases between 40°C and 60°C for these analytes^[56, 67], and in

some studies was observed an increase of extraction efficiency at temperatures up to 70 °C^[67, 72]. Hence, 65 ± 5 °C was selected as the extraction temperature for the target PAHs.

According to some authors, the extraction equilibrium between the target PAHs and the stir bars is reached after 50 - 60 minutes^[72, 109]. When using SBSE, an extraction time of 60 minutes allows for the polycyclic aromatic hydrocarbons to equilibrate (at 60 °C), and times longer than 60 minutes may lead to reduced signal areas^[56]. For this reason, an extraction time of 60 minutes was selected in the following experiments.

After the conditioning and extraction steps, to remove any undesired solvent mixture or water droplets, respectively, the stir bars were dried in a lint-free tissue^[105, 110], to prevent the PDMS coating damage.

10.2.2. Desorption conditions

Regarding the desorption step, the most studied variables include desorption solvent, acceptor phase volume, stirring rate, desorption time, and temperature^[10, 67, 74, 106-107, 110]. In this experimental work, liquid desorption was selected because is usually followed by HPLC-FLD^[10].

The volume of the acceptor phase must ensure that the coated stir-bars are completely submerged in the stripping solvent, to promote chemical desorption of the extracted solutes, and the solvents or mixes utilised in this process must be compatible with the PDMS polymer^[67, 107]. The most popular desorption solvents are acetonitrile, methanol, combinations of these solvents, or mixes with water or aqueous buffers^[67]. However, studies have shown that acetonitrile has the best desorption efficiency for the target PAHs^[56, 111], and enhances the chromatography peaks (sharp and symmetrical) when HPLC-FLD techniques are employed^[61]. Therefore, acetonitrile was selected and used as the desorption solvent.

Several authors used the liquid desorption into a vial incorporating a 250 µL glass insert (to reduce the solvent quantities)^[67, 110]. Using glass inserts, the common stripping solvent volume ranges from 100 to 200 µL^[67, 107]. Hence, was selected a 200 µL volume of acetonitrile^[10, 109-110], and a 300 µL conical glass insert, suitable for the complete immersion of the stir bar.

Experiments conducted at room temperature (20 to 25 °C) using acetonitrile as the solvent demonstrated that extending desorption time from 5 to 10 minutes led to increased peak areas. However, peak areas remained mostly constant between desorption times of 15 and 20 minutes^[106, 112]. Occasionally, mechanical agitation or shaking can be employed to reduce this duration for faster analysis^[105]. According to Hauser et al.^[109], at a temperature of 35 °C, the desorbed PAHs increased from 0 to 750 rpm as a result of the stir bars' orbital shaking accelerating. As a result, the method validation was performed with a desorption time of 15 minutes at 1000 rpm stirring speed, to enhance the desorption efficiency performed at room temperature.

All the selected experimental conditions for the SBSE conditioning, extraction, and desorption steps, are summed up in **Table 9**.

Table 9 – Summary of the selected experimental conditions in SBSE optimization.

SBSE	Solvents and volume	Stirring speed (rpm)	Temperature (°C)	Time (min)
Stir bars conditioning [°]	Methanol:Dichloromethane 4 mL, 1:1 (v/v)	500 rpm	20 – 25	5
Extraction conditions [°]	Methanol 1 mL	500 rpm	60 - 70	60
Desorption conditions [§]	Acetonitrile 0.2 mL	1000 rpm	20 – 25	15

[°] This procedure is repeated two times; [°] For a 100 mL sample volume; [§] Using 300 µL conical glass inserts.

10.3. HPLC-FLD method

In developing the HPLC-FLD method, a reversed-phase column was chosen along with acetonitrile and water as mobile phases, as they are commonly utilized for separating nonpolar polycyclic aromatic hydrocarbons^[24, 47]. A good resolution and selectivity for the six PAHs (see **Figure 6**), was achieved with an acetonitrile-water mobile-phase gradient (as summarized in **Table 7**), and the complete chromatographic separation was achieved within 22 minutes. All sample injections were held constant at 80 µL fixed volume injection, to obtain greater sensitivity for the PAHs determination^[36].

The PAHs intrinsic fluorescence properties, make the fluorescence detectors one of the most employed detection systems for the PAHs analysis in water matrices^[35]. The FLD offers the option of utilizing different excitation and emission wavelengths to enhance selectivity and sensitivity^[36]. This is especially valuable for detecting and quantifying emerging contaminants in aqueous samples with limited analyte volumes. For that reason, a FLD was chosen in this validation work. The FLD and PMT configuration was selected based on previously published studies in PAHs water samples analysis, using

HPLC-FLD^[61, 109, 113], and the excitation and emission wavelengths, as well the PMT gain programmes, are described in **Table 8**.

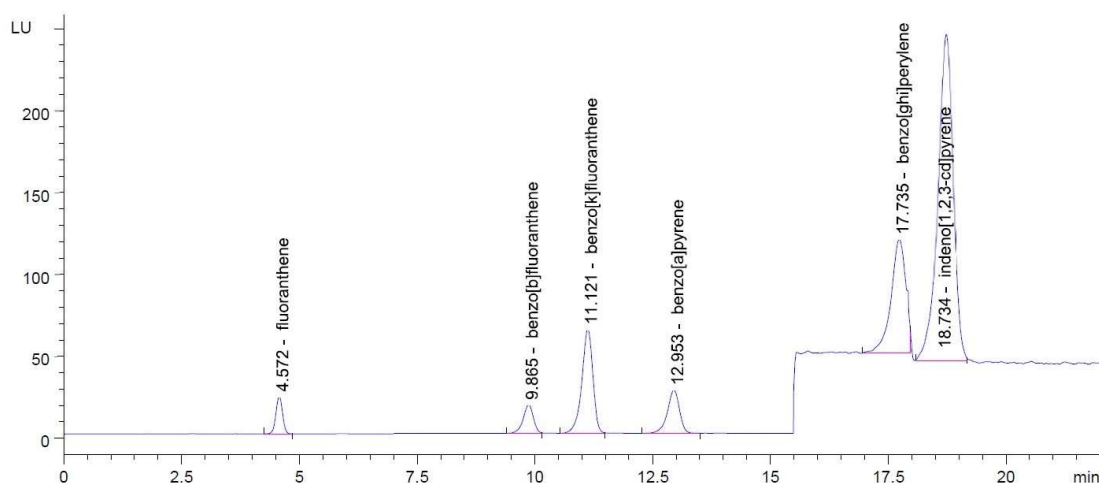


Figure 6 - Example of a SBSE-HPLC-FLD chromatogram for the target PAHs, obtained in this validation work.

PAHs were identified by comparing their retention times to the retention time obtained from the reference chromatogram of each individual analyte. The retention time window criterion for identification was set at three times the standard deviation of the retention times of standards injected on that day^[107]. In the separation and quantification, according to the selected HPLC-FLD conditions and calibration curves ($n = 12$), the following average retention times (min) were obtained: 4.6 (FLU), 9.8 (BBF), 11.1 (BKF), 12.9 (BAP), 17.6 (BPE) and 18.6 (IND). The RSD of retention time for each analyte was below 1.27% (obtained for fluoranthene), demonstrating the good repeatability achieved by the proposed procedure.

The performance of the HPLC-FLD method was evaluated by quality parameters (such as selectivity, quantification limits, detection limits, working range, analytical sensitivity, and linearity), that were determined using certified standard mix solutions. For each compound, the linearity of the calibration curve was obtained with a maximum of eight to ten calibration points (according to **Table 6** and exemplified in **Attachment 2**). This was accomplished by plotting analyte height versus concentration (with a minimum of 5 concentration levels)^[96], without ever rejecting the upper and lower standards that define the analyte working range. The observed linearity (correlation coefficient, r) ranged between 0.99941 and 0.99952 for all six PAH compounds, showing an excellent correlation. The calibration curves ($n = 12$) for the target PAHs were validated in accordance with the ISO 8466-1 and ISO 8466-2 standards, as exemplified in **Attachment 3**. The presented variation coefficients (using **Equation 20**) were less than

25% (the higher value was 24% obtained for benzo[ghi]perylene), and the residual values calculated with the linear regression model (**Equation 3**) remained lesser than 15%, indicating a good homogeneity of variances. The analytical sensitivity interval limits were evaluated considering the slope mean for each PAH, and the double of the obtained standard deviation.

Analyte quantification limits were determined to meet the fitness-for-purpose criteria specified in the Portuguese national water legislation (outlined in **Table 2**). In the case of drinking water, as per the applicable legislation (Decree-law nº 152/2017), the benzo[a]pyrene concentration should not exceed 10 ng/L, while the concentrations of the other four PAHs (benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, and indeno[1,2,3-cd]pyrene) should remain below 100 ng/L. Therefore, an acceptable approach is to set the quantification limits for the target PAHs at three-tenths of the legislated parametric value for each analyte (or sum of analytes)^[83]. However, the working range limits repeatability (n =10) was also evaluated to validate the experimental approach. In this assessment, all the RSD values remained less than 15.7% (obtained for benzo[k]fluoranthene LOQ) and all the relative biases (using **Equation 9**) were below 19.4% (attained with fluoranthene LOQ), indicating a good repeatability. Regarding the limits of detection, they were estimated according the **Equation 2**, considering the LOQ standard deviation obtained for each analyte in repeatability terms^[90].

In validation studies, the reference material used for calibration should not be employed to assess bias ^[86, 94]. Thus, in this work, were used different PAHs standard mix solutions batches, and all the evaluated quality parameters are summarized in **Table 10**.

Table 10 - Summary of the evaluated quality parameters results.

PAH	LOD (ng/L)	Working range (ng/L)	RT window (min)	Analytical sensitivity (average slope $\pm 2\delta$) ^a	r mean
Fluoranthene	1.1	7.5 - 150	4.4 – 4.8	0.18144 $\pm$ 0.04839	0.99946
Benzo[b]fluoranthene	0.5	3.0 - 60	9.7 – 10.0	0.34924 $\pm$ 0.10936	0.99949
Benzo[k]fluoranthene	0.7	1.5 -60	10.9 – 11.3	1.21698 $\pm$ 0.38391	0.99941
Benzo[a]pyrene	0.4	1.5 - 60	12.7 – 13.2	0.55542 $\pm$ 0.24122	0.99943
Benzo[ghi]perylene	1.8	6.0 -60	17.3 – 18.0	1.75324 $\pm$ 0.85760	0.99952
Indeno[1,2,3-cd]pyrene	1.7	6.0 - 120	18.2 – 19.1	1.82761 $\pm$ 0.73959	0.99944

^a The δ represents the standard deviation of the calibration curves slopes.

10.4. Trueness evaluation

As described above (chapter 8.5), trueness is the closeness of agreement between the average of an infinite number of replicates measured quantity values and a reference quantity value, and this assessment is expressed quantitatively in terms of “bias”^[85-86, 98]. The trueness evaluation in this experimental work was determined by recovery tests, which estimated the recovery of a known amount of analyte added to a previously analysed water sample and can be used as an indication of the expected level of bias.

When there are no significant analytical restrictions between water matrices, validation studies may include more than one water matrix^[114]. Therefore, the following validation studies for drinking water and groundwater were carried out in conjunction, apart from the surface water matrix.

In different water matrices, by measuring the relative spike recovery (according to **Equation 10**), was possible to assess the deviation from the mean recovery (b_i), using **Equation 8**. Considering ten to nineteen analytical results (a minimum of ten tests are required^[86]), was possible to calculate the root mean square of the deviations from the recovery experiments (b_{rms}), obtained with **Equation 17**. Yet, for trueness assessment, water samples were also spiked with analytes at both the LOQ and an intermediate quantification level (IQL), as specified in **Table 11**. This aimed to determine whether PAH recoveries could be influenced by the added quantity or the water matrix^[86].

Table 11 - Concentration levels used in spiked water samples for the six investigated PAHs.

Amount added	FLU (ng/L)	BBF (ng/L)	BKF (ng/L)	BAP (ng/L)	BPE (ng/L)	IND (ng/L)
LOQ	7.5	3	1.5	1.5	6	6
IQL	25	10	10	10	10	20

The experimental findings from drinking water and groundwater samples indicated a mean recovery of 97.7% at the LOQ, ranging from 90.3% (benzo[k]fluoranthene) to 109.9% (benzo[b]fluoranthene). When different quantities of reference material were introduced, the mean recovery for the six PAHs remained at 102.3%. In this scenario, the lowest and highest mean recovery values were seen with indeno[1,2,3-cd]pyrene (98.4%) and fluoranthene (107.7%), respectively. As a result, it was evident that for both matrices and various concentration levels, no matrix effects were observed. All recovery experiments at different concentration levels were below 110%, indicating the high trueness achieved through this experimental approach. The RSD of mean recoveries considering the two concentration levels, for the individual analytes, was under 14.2% (attained with fluoranthene), and the lowest value of 0.6% was accomplished by

indeno[1,2,3-cd]pyrene, demonstrating a good method robustness at different concentrations. Furthermore, the b_{rms} in the LOQ, for drinking water and groundwater samples, ranged between 0.0136 and 0.1134, obtained for fluoranthene and benzo[a]pyrene, respectively. At a higher concentration level, the determined b_{rms} oscillated from 0.0387 to 0.0820, achieved for benzo[b]fluoranthene and fluoranthene. Hence, the methodology's accuracy in both matrices was confirmed across various levels of spiked samples, with lower bias indicating higher trueness.

In terms of surface water matrix, the recovery experiments at the LOQ indicated notable loss in mean recovery for fluoranthene (63.3%), benzo[k]fluoranthene (66.5%), and indeno[1,2,3-cd]pyrene (65.4%) when compared to other water matrices. The highest recovery was observed for benzo[b]fluoranthene (98.8%), resulting in an overall mean recovery of 79.0% for all analytes. Increasing the added amount revealed a consistent pattern of lower recoveries in this water matrix, with indeno[1,2,3-cd]pyrene achieving 65.3%. Fluoranthene (97.6%) and benzo[a]pyrene (97.1%) exhibited the highest mean recoveries. Overall, the six PAHs achieved a mean recovery of 85.8% in this assessment. Thus, both at the LOQ and higher concentration levels, the mean recovery across all water matrices decreased from 99.9% and 102.3% (noted in drinking water and groundwater) to 79.0% and 85.8% (observed in surface water), respectively. The RSD of mean recoveries for individual analytes in surface water varied from 42.6% (fluoranthene) to 0.1% (indeno[1,2,3-cd]pyrene) at the two concentration levels. These divergent outcomes underscore the influence of water matrix and added quantities. For indeno[1,2,3-cd]pyrene, the matrix effect is evident (seen in the consistent RSD within the added range). In contrast, fluoranthene and benzo[k]fluoranthene displayed RSD values of 42.6% and 25.0% (between concentration levels), indicating a pronounced amount-added effect. At the higher concentration level, mean recoveries for these two analytes were 97.6% and 85.5%, respectively. Finally, the RSD between concentration levels for benzo[b]fluoranthene, benzo[a]pyrene, and benzo[ghi]perylene ranged from 8.2% (benzo[a]pyrene) to 12.9% (benzo[ghi]perylene). This observation confirms the absence of matrix or added amount effects for these specific PAHs. Notably, no matrix or added amount effects were observed in surface water samples for benzo[b]fluoranthene, benzo[a]pyrene, and benzo[ghi]perylene. For these PAHs, the mean recoveries between the LOQ and a higher concentration level, oscillated from 89.1% (benzo[a]pyrene) to 98.8% (for benzo[b]fluoranthene), and from 79.3% (benzo[ghi]perylene) to 97.1% (benzo[a]pyrene), respectively, and allow to assess the good trueness achieved for these PAHs. The RSD, in this case, was less than 13% between amounts added, demonstrating for the specified analytes, a good method

robustness. **Figure 7** illustrates these mean recoveries outcomes, for the investigated water matrices.

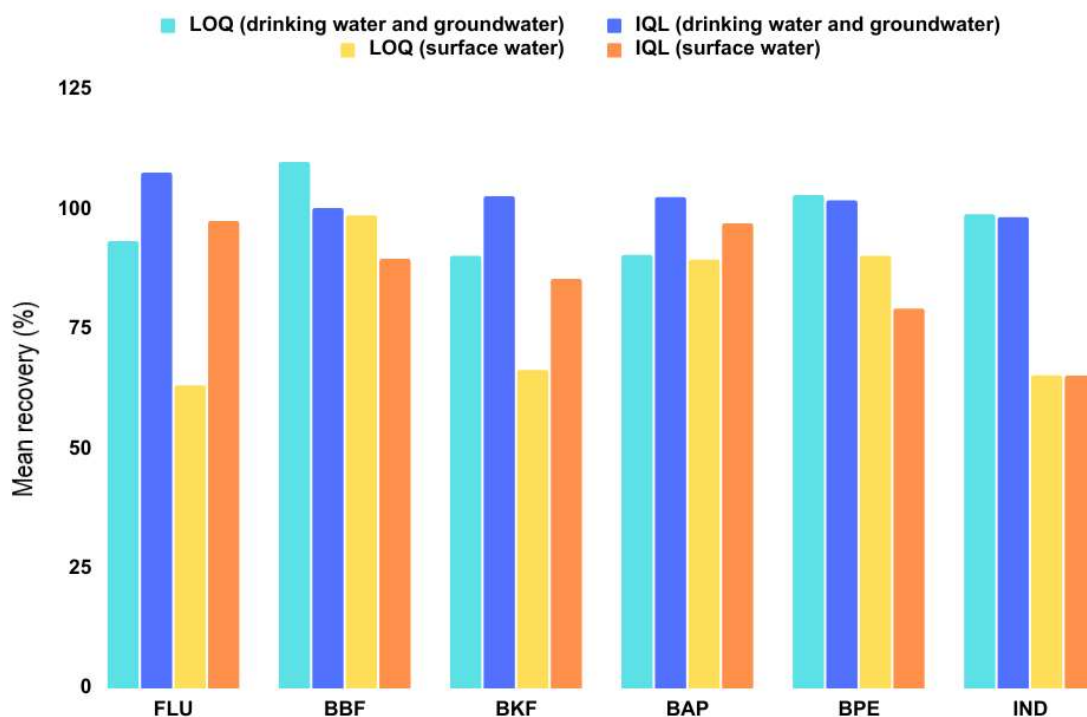


Figure 7 - Mean recoveries achieved for the six PAHs in the investigated water matrices, with the proposed method.

Furthermore, the b_{rms} in the LOQ, for surface water, varied from 0.0698 and 0.1593 acquired with benzo[a]pyrene and indeno[1,2,3-cd]pyrene, respectively. In a higher concentration level, the determined b_{rms} alternated from 0.0574 to 0.1474, achieved for fluoranthene and benzo[ghi]perylene. Despite the observed effects of matrix and added amount in surface water (shown in **Figure 8**), the methodology demonstrated strong bias performance for both matrices across varied spiked amounts.

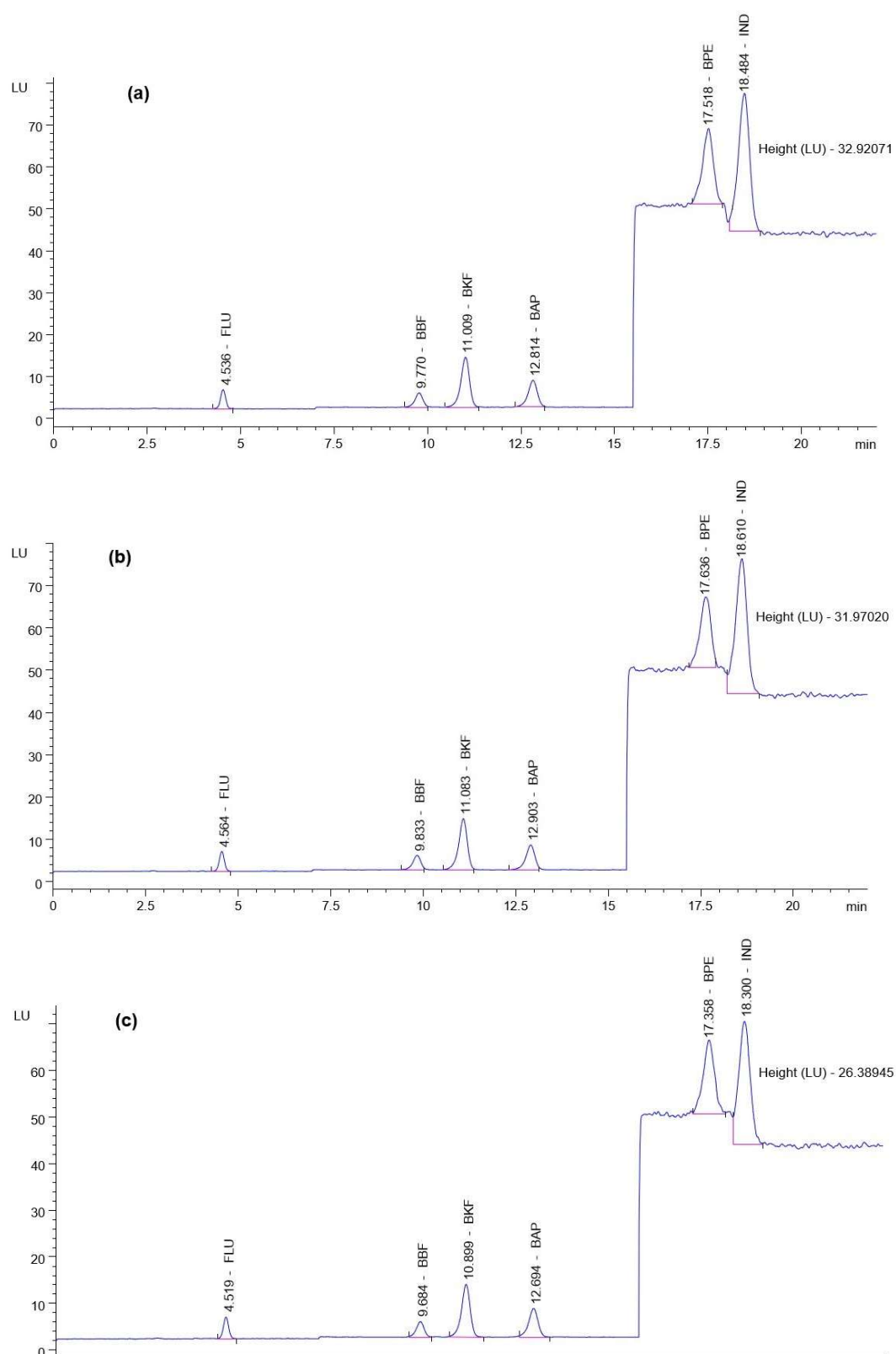


Figure 8 - Example of the water matrix effect observed in indeno[1,2,3-cd]pyrene peak height when adding an IQL amount. (a) QC sample: IQL standard prepared in UPW; (b) Recovery: IQL standard prepared in drinking water and groundwater; (c) Recovery: IQL standard prepared in surface water.

The matrix effect, possibly caused by higher total suspended solids and organic matter content^[40], makes interactions complex between higher molecular weight PAHs, such as indeno[1,2,3-cd]pyrene (shown in Table 1), and the PDMS stir bar adsorbent. It's crucial to account for the obtained mean recovery when reporting the LOQ. Concerning the added amount effect, higher analyte concentrations can induce interactions between PAHs and the adsorbent phase ^[115] resulting in improved mean recoveries, as observed with fluoranthene and benzo[k]fluoranthene. Consequently, depending on the validation's water matrix, it may be necessary to determine the LOQ based on actual experimental mean recovery outcomes. In some cases, reevaluating the initial target LOQ to a higher concentration level might be needed to address this concern, provided it aligns with the relevant parametric value as per applicable legislation. As a result, after conducting recovery tests on various water matrices, a Student's t-test was executed on spiked water samples with an added LOQ amount. This was prompted by the higher RSD value observed at this concentration level (at intermediate precision), in comparison to higher concentration levels. This significance test was assessed with **Equation 13**, by comparing the calculated t result and the corresponding $t_{critical}$ value, using a two-way Student's t-table, for the number of degrees of freedom associated with Rec and u_{Rec} , at a 95% confidence level, as described in **Table 12** and summarized in **Attachment 2**.

Table 12 – Student's t-test data achieved for the different water matrices.

PAH	Drinking water and groundwater					Surface water				
	df ^o	Rec [†]	u_{Rec} [‡]	t	$t_{critical}$	df ^o	Rec [†]	u_{Rec} [‡]	t	$t_{critical}$
Fluoranthene	9	0.9056	0.0041	23.027	2.262	12	0.8582	0.0242	5.863	2.179
Benzo[b]fluoranthene	9	1.0372	0.0146	2.543	2.262	12	0.9325	0.0257	2.631	2.179
Benzo[k]fluoranthene	9	0.7398	0.0214	12.148	2.262	18	0.5447	0.0169	27.011	2.101
Benzo[a]pyrene	9	0.9580	0.0218	1.930	2.262	18	0.9479	0.0156	3.341	2.101
Benzo[ghi]perylene	9	0.9286	0.0254	2.811	2.262	12	0.8137	0.0236	7.891	2.179
Indeno[1,2,3-cd]pyrene	9	0.8631	0.0154	10.668	2.262	12	0.5527	0.0254	17.605	2.179

^o The df stands for degree of freedom (n-1, where n represents the number of observations); [†] Rec represents the relative recovery; [‡] u_{Rec} is the standard uncertainty, considering the available relative recovery data of the reference value.

During this evaluation, it became evident that results correction was necessary for all analytes, except for benzo[a]pyrene in drinking water and groundwater matrices. This determination was based on relative recovery (Rec) calculated with **Equation 14**. For the determination of the standard uncertainty estimated from this recovery experiments (u_b), using **Equation 16**, the uncertainty in the concentration of the analyte added (u_{add}) is also necessary to be assessed (**Equation 18**). However, through careful selection of certified standards (e.g., as high-purity reference materials), and the volumetric equipment involved (e.g., the use of high-quality micro syringes) in fortifying the sample,

the contributions of the uncertainty in the concentration and volume added (u_{conc} and u_v , respectively) are negligible^[85, 94], so that according to **Equation 16**, u_{add} was not accounted. Moreover, the reference standards were continuously weighted before opening and after used for the intermediate solutions preparation, with a criterion of ($\pm$) 10 mg between consecutive uses (to evaluate the reference standard stability), reducing even more the u_{add} contribution. Consequently, in this experimental work, the evaluated u_b (for each water matrix) corresponds to calculated b_{rms} (**Equation 26**).

$$u_b = b_{\text{rms}} \quad (\text{Equation 26})$$

In this method validation no relevant systematic errors were identified, and all the obtained results for the trueness evaluation and measuring are summarized in **Table 13**, **Figure 9** and in the **Attachment 4**.

Table 13- Summary of obtained results for the trueness assessment.

PAH	Spiked level (ng/L)	Drinking water and groundwater			Surface water		
		MR [□] (%)	b_{rms}	u_b (%)	MR [□] (%)	b_{rms}	u_b (%)
Fluoranthene	7.5	93.4	0.0136	1.4	63.3	0.1177	11.8
	25	107.7	0.0820	8.2	97.6	0.0574	5.7
Benzo[b]fluoranthene	3	109.9	0.0423	4.2	98.8	0.0954	9.5
	10	100.3	0.0387	3.9	89.7	0.0833	8.3
Benzo[k]fluoranthene	1.5	90.3	0.0868	8.7	66.5	0.1312	13.1
	10	102.8	0.0588	5.9	85.5	0.0876	8.8
Benzo[a]pyrene	1.5	90.5	0.1134	11.3	89.5	0.0698	7.0
	10	102.6	0.0591	5.9	97.1	0.0606	6.1
Benzo[ghi]perylene	6	103.0	0.0821	8.2	90.3	0.1005	10.0
	10	101.9	0.0751	7.5	79.3	0.1474	14.7
Indeno[1,2,3-cd]pyrene	6	99.0	0.0555	5.5	65.4	0.1593	15.9
	20	98.4	0.0487	4.9	65.3	0.1458	14.6

□ Stands for mean recovery.

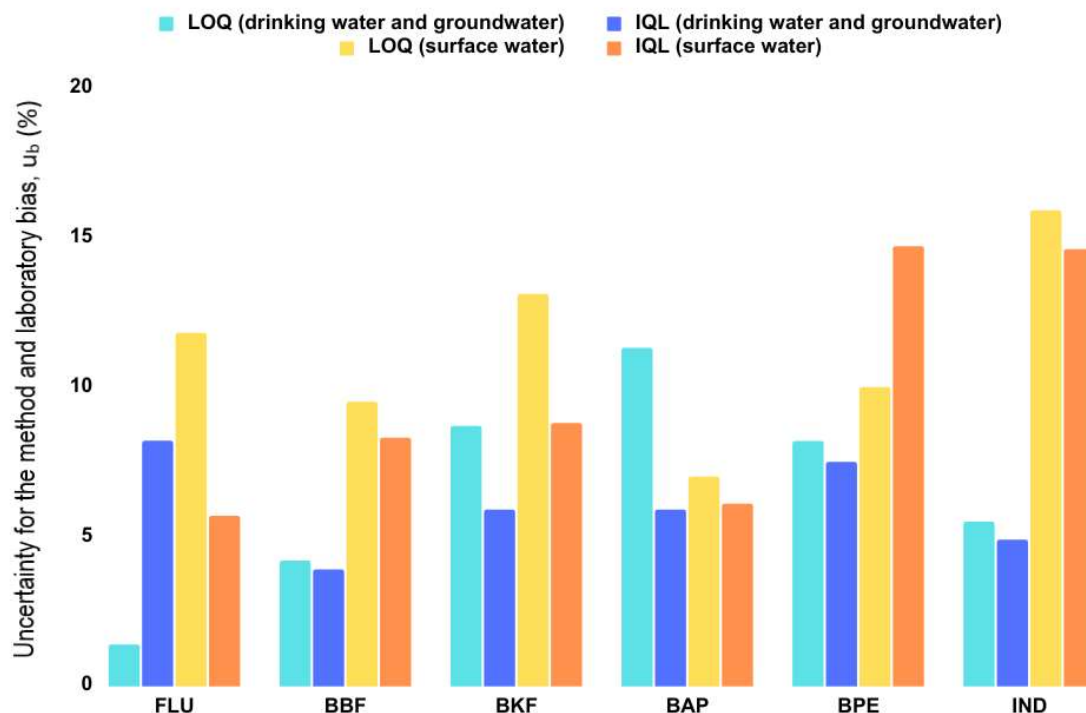


Figure 9 - Standard uncertainty estimated from the recovery experiments for the method and laboratory bias, in the different water matrices concentration levels.

Additionally, another strategy to acquired information about the method bias, is by the participation in proficiency testing schemes^[95]. PT schemes additionally confirm ongoing method performance and offer pertinent information for uncertainty assessment. ^[85]. Though, to obtain a reliable estimate, it is advised to conduct a minimum of six different trials, to determine an estimate of the bias from interlaboratory comparison results^[85, 98].

During this method validation phase, participation in a PT scheme was feasible after optimizing the SBSE-HPLC-FLD experimental conditions. The chosen PT (round 576, sample 7C) was sourced from LGC Standards (Aquacheck), and all results were satisfactory^[97], Excellent z-score values (all below one) were achieved for the six PAHs. **Figure 10** summarizes the PT scheme outcomes, confirming strong method performance and reproducibility for the analysed analytes.

Scheme: Water Chemistry (AQUACHECK)

7C - Polycyclic Aromatic Hydrocarbons (2 Spikes)

Analyte	Analyst	Method	Result	Units	Z Score	Assigned Value	SDPA	Number of results
Fluoranthene	EL	Other	36.5	ng/L	-0.42	38.1	3.81	43
Benzo(b)fluoranthene	EL	Other	20.55	ng/L	-0.52	21.68	2.168	56
Benzo(k)fluoranthene	EL	Other	17.16	ng/L	0.02	17.12	2.0	57
Benz(a)pyrene	EL	Other	9.38	ng/L	-0.63	10.01	1.001	58
Benzo(ghi)perylene	EL	Other	7.79	ng/L	-0.34	8.47	2.0	57
Indeno(1,2,3-cd)pyrene	EL	Other	9.11	ng/L	-0.69	10.48	2.0	58

Figure 10 - PT scheme results obtained for the six PAHs using this SBSE-HPLC-FLD methodology (figure adapted from Aquacheck laboratory individual report), where SDPA represents the standard deviation for proficiency assessment.

However, the acquired data cannot be used to assess the method bias, as only one satisfactory PT scheme was available for that purpose, and minimum of six satisfactory trials are required.

10.5. Precision assessment

As mentioned before (chapter 8.6), precision is the agreement between signals or measured quantity values, that were obtained through repeated measurements made on the same or comparable objects^[98], and is usually expressed by statistical parameters which describe the spread of results, as the standard deviation (SD) or the relative standard deviation (RSD)^[86].

During this method validation, the uncertainty component for within-laboratory reproducibility (u_{RW}), was determined from the standard deviation of quality control results. This involved combining the uncertainty component from routine QC sample RSD ($u_{RW,stand}$), and the uncertainty component from range control charts ($u_{r,range}$), established using mean range $\bar{R}$ from duplicates of proposed water matrices (**Equation 23**), to provide a consistent u_{RW} estimate based on **Equation 22**. Estimating both uncertainty factors demands at least eight measurements, with more measurements leading to higher estimation confidence^[98]. This validation employed between eight and twenty-six measurements for precision assessment.

In routine analysis, a 20% RSD for internal QC is common, and in certain cases, complex procedures like chromatography techniques may even require a 50% RSD ^[86]. During this validation procedure, a pre-established criterion of 25% RSD was chosen for internal quality control parameters (such as QC samples and water matrix duplicates), with every twentieth sample analysed being a QC sample.

The QC samples analysed under intermediate precision conditions (e.g., different days), and concentrated at the working range extremes with the highest RSD value, were used to assess $u_{RW,stand}$ for each PAH. The achieved results (from eighteen to twenty-one measurements), revealed benzo[k]fluoranthene and benzo[b]fluoranthene with the highest and lowest $u_{RW,stand}$, attained 16.9% and 6.8%, respectively. These results signify strong within-laboratory reproducibility, with an average RSD of 13.1% across the six PAHs. The additional uncertainty component due to inhomogeneity^[98], $u_{r,range}$, was estimated for the different water matrices from range control charts outcomes, through the mean range of eight quantifiable sample duplicates, spiked at different concentration levels (due analysed water absent of target analytes). For drinking water and groundwater, the obtained $u_{r,range}$ oscillated between 11.6% and 5.2%, achieved for indeno[1,2,3-cd]pyrene and benzo[ghi]perylene, respectively. The average $u_{r,range}$ value of 7.8%, considering all analytes, demonstrated the good repeatability achieved for this water matrices with this methodology.

Relative to the surface water, the assessed $u_{r,range}$ remained below 15.4% achieved for benzo[ghi]perylene, and the lowest value of 7.6% was accomplished by benzo[a]pyrene. The achieved $u_{r,range}$ mean of 10.2% contemplating all PAHs, discloses good repeatability in this water matrix, as through the investigated spiked water samples, the $u_{r,range}$ stayed below 15.4%, considering all the water matrices.

With both contributions evaluated ($u_{RW,stand}$ and $u_{r,range}$), the estimated uncertainty component for the within-laboratory reproducibility, u_{RW} , for drinking water and groundwater, ranged from 19.1% (for benzo[k]fluoranthene) to 9.1% (for benzo[b]fluoranthene), with an average result of 15.3% for the target PAHs. In surface water, the u_{RW} remained under 20.6% (achieved for benzo[ghi]perylene), and the mean value of this uncertainty component stayed at 16.7%.

Hence, the investigated water matrices have generally demonstrated, the good within-laboratory reproducibility achieved in different concentrations (above the LOQ for some PAHs), and no significant random errors were identified. All the precision assessment results are described in **Table 14, Attachment 5, and Figure 11**.

Table 14 – Summary of uncertainties achieved results to precision evaluation.

PAH	$u_{Rw,stand}$ (%)	Drinking water and groundwater		Surface water	
		$u_{r,range}$ (%)	u_{Rw} (%)	$u_{r,range}$ (%)	u_{Rw} (%)
Fluoranthene	15.2	8.7	17.5	9.8	18.1
Benzo[b]fluoranthene	6.8	6.0	9.1	8.7	11.0
Benzo[k]fluoranthene	16.9	8.9	19.1	9.6	19.4
Benzo[a]pyrene	11.5	6.2	13.1	7.6	13.8
Benzo[ghi]perylene	13.7	5.2	14.7	15.4	20.6
Indeno[1,2,3-cd]pyrene	14.2	11.6	18.3	10.0	17.4

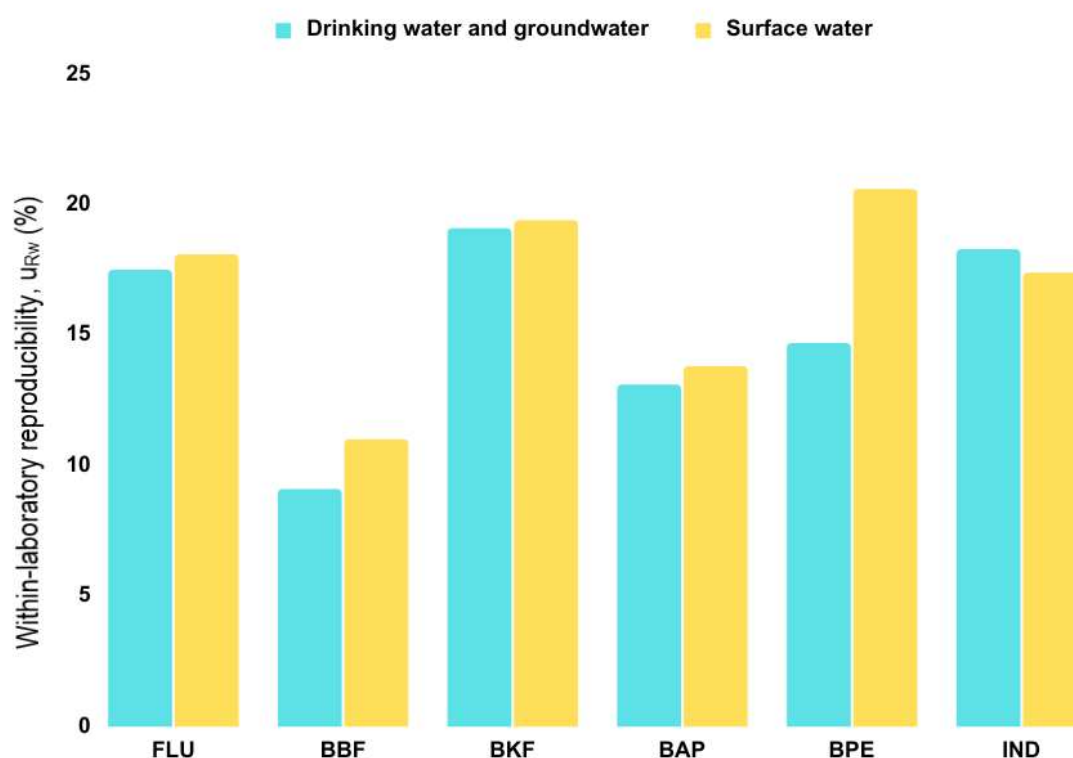


Figure 11 - Summarized within-laboratory reproducibility achieved with this validation method in different water matrices.

10.6. Measurement uncertainty

Measurement uncertainty can be interpreted as a quantitative estimate of accuracy, normally studied as two components: trueness and precision^[83, 86]. Accuracy is a measure of how closely a single result reflects the true value. Consequently, accuracy includes the systematic error and the random error that affects the outcomes, and a method that has a less random error and a smaller bias, is defined as more accurate^[86]. As no further uncertainty components than u_{Rw} and u_b are evaluated, the combined standard uncertainty, u_c , is calculated according to **Equation 24**.

Considering drinking water and groundwater matrices, the u_c values ranged between 10.0% and 19.2%, attained for benzo[b]fluoranthene and indeno[1,2,3-cd]pyrene, respectively. The average u_c result of 17.0% is representative of the good method accuracy when combining all the uncertainty components.

Regarding the surface water matrix, the accomplished u_c results were very similar comparing to other matrices, except for benzo[b]fluoranthene (14.6%), benzo[ghi]perylene (22.9%) and indeno[1,2,3-cd]pyrene (23.6%). For those PAHs, the highest values may result from a matrix effect such as increased dissolved organic matter^[107], diminishing the desired homogeneity between duplicates, and changing the stir bar analyte adsorption. Nonetheless, in this water matrix, the lowest result of 14.6% was assessed for benzo[b]fluoranthene. The average u_c of 20.3%, also reveals a good accuracy for this matrix, and considering all the target PAHs and water matrices, the calculated results stayed below the pre-establish value of 25%.

Therefore, for the investigated water matrices, was observed a significant contribution of random errors for the u_c assessment, comparing to systematic errors. However, this component of error varies in an unpredictable way and can diminish by increasing the number of observations^[85].

The expanded uncertainty, U , is necessary to provide an interval that can be expected to cover a significant portion of the distribution of values that can be reasonably attributed to the measurand^[85]. Hence, U was calculated using a coverage factor (k) of two, based on the desired 95% level of confidence^[85-86, 98], using **Equation 25**. The obtained results for the six PAHs in different water matrices are summarized in **Table 15** and illustrated in **Figure 12**.

Table 15 – PAHs combined and expanded uncertainties results accomplished in the investigated water matrices.

PAH	Drinking water and groundwater		Surface water	
	u_c (%)	U (%), $k=2$	u_c (%)	U (%), $k=2$
Fluoranthene	17.5	35.1	21.5	43.1
Benzo[b]fluoranthene	10.0	20.1	14.6	29.1
Benzo[k]fluoranthene	21.0	42.0	23.5	46.9
Benzo[a]pyrene	17.3	34.6	15.5	31.0
Benzo[ghi]perylene	16.8	33.7	22.9	45.9
Indeno[1,2,3-cd]pyrene	19.2	38.3	23.6	47.1

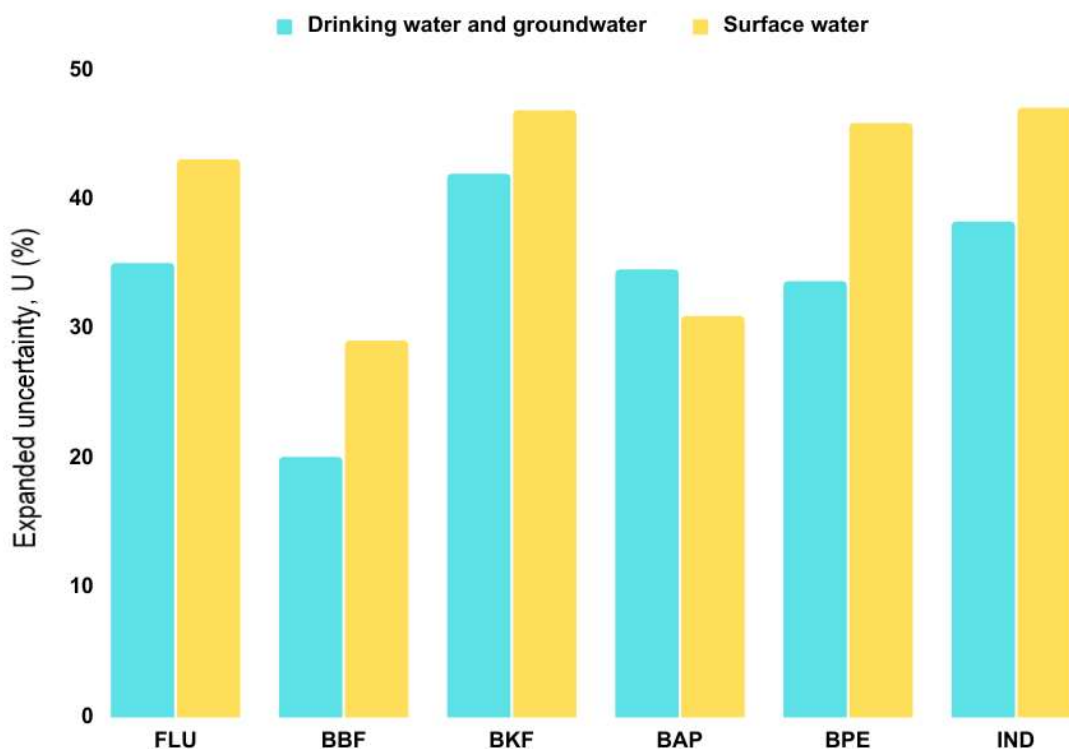


Figure 12 – Achieved expanded uncertainties results ($k=2$) for the investigated water matrices.

All the calculated expanded uncertainties, U , for a 95% level of confidence, were below 42.0% and 47.1%, obtained for drinking water and groundwater (with benzo[k]fluoranthene) and for surface water (by indeno[1,2,3-cd]pyrene), respectively.

These results are an essential element of the analytical requirement, to evaluate whether method is suitable for its intended purpose^[86], according the applicable Portuguese legislation (described in **Table 2**). Therefore, for all water matrices, due the fact that all the validation results (in trueness, precision, and uncertainty assessment), remained below the established requisites, is possible to confirm that the proposed method is in concordance with the mandatory legal requirements, for the analysis of the target PAHs.

10.7. Analytical performance: analysis of PAHs in water samples

Numerous studies have been conducted to determine PAHs in water samples, and a variety of analytical techniques have been used. For their identification, the most common are gas chromatography (GC) with a mass spectrophotometer (MS) or flame ionisation detector (FID), and high-performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection (FLD)^[26, 33, 35, 104]. Although both techniques are adequate for analysing PAHs in water samples, HPLC-FLD has a higher sensitivity and

a lower relative standard deviation percentage value^[26, 104], and for that reason, the most employed technique to evaluate these EOCs. The different extraction, desorption, chromatographic, and detection conditions, can be optimized to reduce time and solvents costs, diminish the quantification levels in smaller sample amounts, and increase the analytes mean recoveries, in several water matrices^[56, 61].

As PAHs are usually present in vestigial amounts in water samples^[6], is necessary to extract and concentrate the analytes so they can be identified and quantified^[56], to meet the stricter legislation requirements (e.g., uncertainty and ng/L quantification level). The SBSE, a microextraction technique, has acquired widespread acceptance as a highly effective sample preparation technique for enriching solutes from aqueous samples, and has been used extensively for PAHs analysis^[10, 64]. The complexity of the matrices involved can have a significant impact on SBSE efficiency, just as with many other sample preparation methods for trace analysis. For instance, significant amounts of dissolved or suspended inorganic or organic matter present in environmental matrices (e.g., surface water) can affect the extraction of the target compounds by PDMS or other phases, causing the extraction yield to significantly differ from sample to sample^[67]. This problem was identified during this research, as the obtained mean recoveries for the highest molecular PAH (indeno[1,2,3-cd]pyrene) in surface water, decreased at both concentration levels with 65.4% and 65.3%, in contrast with the good mean recoveries of 99.9% and 98.4% observed for cleaner waters such as drinking water and groundwater, at the LOQ and IQL, respectively. Fluoranthene and benzo[k]fluoranthene, also showed considerably low mean recoveries of 63.3% and 66.5%, respectively, in spiked surface water matrix when a small analyte amount (in the LOQ) was added, with less than 30.1% and 23.8% of total mean recoveries, comparing with both other matrices. Therefore, considering the achieved mean recoveries might be important to evaluate the sample turbidity factor and the method LOQ in more contaminated water matrices^[38], to predict and increase this methodology's performance and applicability. However, in this validation work, the SBSE-HPLC-FLD optimization steps, permitted generally good mean recoveries between 63.3% and 109.9%^[61, 106], considering all PAHs and water matrices.

The Student's t-test also revealed the necessity of results correction for all analytes (except for benzo[a]pyrene in drinking water and groundwater matrices), as the calculated t results are higher than the corresponding $t_{critical}$ values for the proposed water matrices. Consequently, the calculated method LOQ values (using **Equation 14**), were higher for most of the investigated PAHs (with benzo[b]fluoranthene exception), in comparison to the theoretical LOQ, as shown in **Table 16**.

Table 16- PAHs theoretical and method limits of quantification, according to the calculated relative recoveries and the Student's t-test conclusions, for the proposed water matrices.

PAH	Theoretical LOQ (ng/L)	Drinking water and groundwater		Surface water	
		Rec	Method LOQ (ng/L)	Rec	Method LOQ (ng/L)
Fluoranthene	7.5	0.9056	8.3	0.8582	8.7
Benzo[b]fluoranthene	3.0	1.0372	2.9	0.9325	3.2
Benzo[k]fluoranthene	1.5	0.7401	2.0	0.5447	2.8
Benzo[a]pyrene	1.5	n/a	1.5	0.9479	1.6
Benzo[ghi]perylene	6.0	0.9286	6.5	0.8137	7.4
Indeno[1,2,3-cd]pyrene	6.0	0.8361	7.2	0.5527	10.9

The good selectivity, sensibility, linearity ($R \geq 0.99941$), LOD (0.4 - 1.8, ng/L), method LOQ (1.5 – 10.9 ng/L), trueness ($u_b \leq 15.9\%$), precision ($u_{Rw} \leq 20.6\%$), expanded uncertainty ($U \leq 47.1\%$, $k = 2$), and the satisfactory PT scheme, are indicators of the good method performance and reproducibility, for PAHs quantification in drinking water, groundwater, and surface water. No trends were noticed in the obtained x-charts and range charts for the six PAHs, indicating that the method is statistically under control. This is documented by the control charts provided in **Attachment 6**.

Achieved the fitness for purpose^[86], the selected optimization conditions permitted a complete chromatographic separation within 22 minutes (26 minutes at ending post time), for a total volume of 39 mL of eluents spent per sample (according to **Table 7**), and the analysis of 20 samples per day. This allowed a reduced process time and/or eluents saving when compared to similar techniques for PAHs water analysis^[10, 61, 104, 116]. Furthermore, the SBSE technique is practically solvent-free and relatively simple to use^[117], and the stir bars reutilization after the conditioning step, diminishes the analytical procedure costs, enhancing the economic advantages.

11. Conclusion

A sensitive and accurate SBSE-HPLC-FLD method was optimized for the determination of priority pollutants PAHs (FLU, BBF, BKF, BAP, BPE and IND) in drinking water, groundwater, and surface water samples. The optimization of the SBSE step resulted in reduced use/disposal of solvents, and increased speed of the whole procedure, whereas the preference for HPLC-FLD permitted a speed-up separation and quantification of the target PAHs with high selectivity and sensibility. The improvements introduced in the whole method greatly enhanced its sensitivity allowing the determination of the low concentration levels involved in such samples, allowing the method to fulfill the detection limits concerning the respective parametric values, and the minimum performance characteristics of uncertainty of measurement, as required by the applicable Portuguese national legislation. Furthermore, this method validation is in accordance the fitness for purpose inside an accredited laboratory regarding the NP EN ISO 17025:2018, routinely applied for the analysis of PAHs in the proposed water matrices, meeting the internal (e.g., QC samples) and external (e.g., proficiency testing schemes) quality control requirements.

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Attachments

Attachment 1: Student's t-test

A) Drinking water and groundwater matrices

PAH	n	δ_{spike}	$\bar{x}$	Rec	u_{Rec}
Fluoranthene	10	0.1129	7.8848	0.9056	0.0041
Benzo[b]fluoranthene	10	0.1387	3.1115	1.0372	0.0146
Benzo[k]fluoranthene	10	0.1016	1.1097	0.7398	0.0214
Benzo[a]pyrene	10	0.1033	1.4370	0.9580	0.0218
Benzo[ghi]perylene	10	0.4820	5.5715	0.9286	0.0254
Indeno[1,2,3-cd]pyrene	10	0.2914	5.0168	0.8631	0.0154

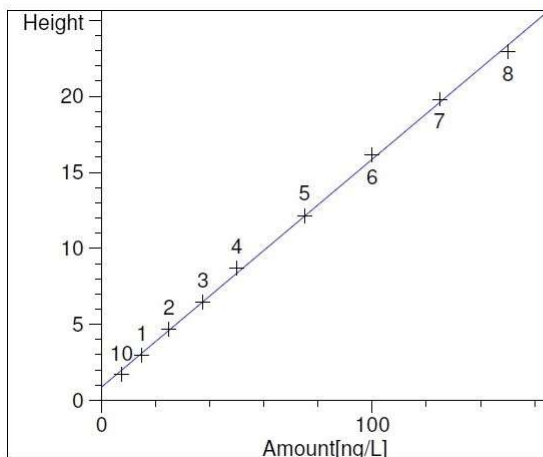
Where δ_{spike} is the standard deviation from n experimental outcomes, and $\bar{x}$ represents the mean concentration of the sample experimental results.

B) Surface water matrix

PAH	n	δ_{spike}	$\bar{x}$	Rec	u_{Rec}
Fluoranthene	13	0.6543	6.4361	0.8582	0.0242
Benzo[b]fluoranthene	13	0.2777	2.7974	0.9325	0.0257
Benzo[k]fluoranthene	19	0.1102	0.8171	0.5447	0.0169
Benzo[a]pyrene	19	0.1019	1.4219	0.9479	0.0156
Benzo[ghi]perylene	13	0.5108	4.8821	0.8137	0.0236
Indeno[1,2,3-cd]pyrene	13	0.5496	3.3164	0.5527	0.0254

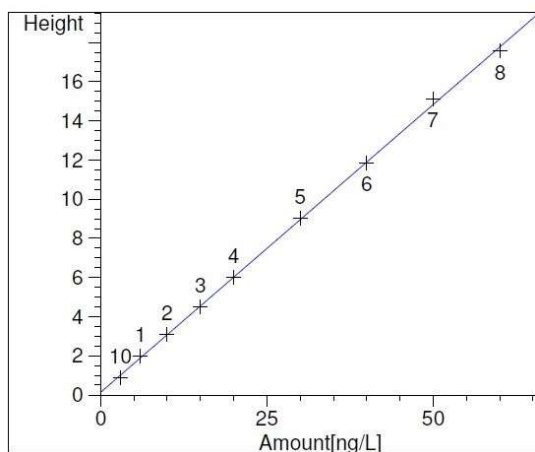
Where δ_{spike} is the standard deviation from n experimental outcomes, and $\bar{x}$ represents the mean concentration of the sample experimental results.

Attachment 2: PAHs calibration curves



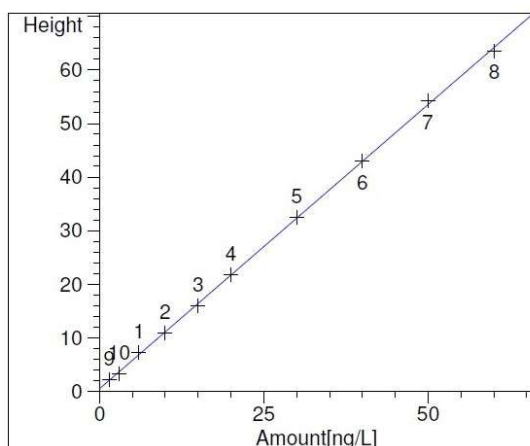
Fluoranthene at exp. RT: 4.572
 FLD1 A, Ex=237, Em=460, TT
 Correlation: 0.99943
 Residual Std. Dev.: 0.27543
 Formula: $y = mx + b$
 m: 1.50101e-1
 b: 8.60268e-1
 x: Amount[ng/L]
 y: Height

Working range: 7.5 - 150 [ng/L]



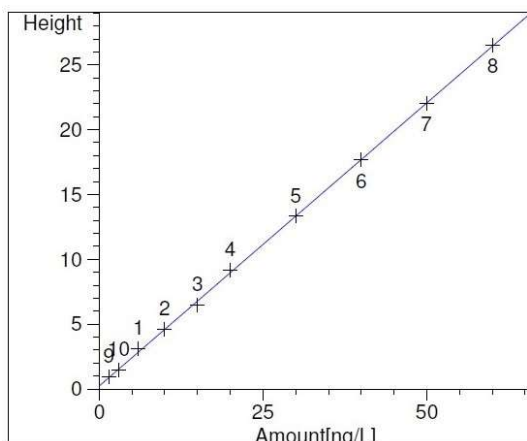
Benzo[b]fluoranthene at exp. RT: 9.865
 FLD1 A, Ex=237, Em=460, TT
 Correlation: 0.99973
 Residual Std. Dev.: 0.14724
 Formula: $y = mx + b$
 m: 2.94156e-1
 b: 1.39268e-1
 x: Amount[ng/L]
 y: Height

Working range: 3 - 60 [ng/L]



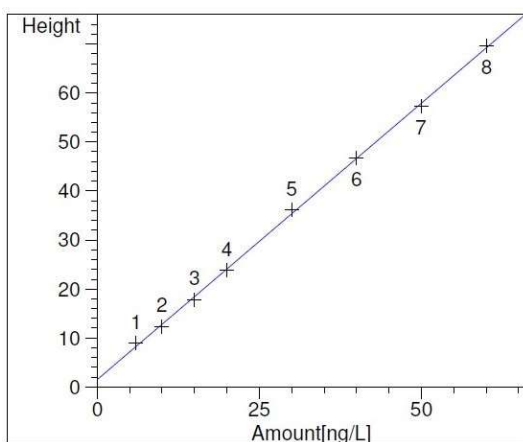
Benzo[k]fluoranthene at exp. RT: 11.121
 FLD1 A, Ex=237, Em=460, TT
 Correlation: 0.99985
 Residual Std. Dev.: 0.40002
 Formula: $y = mx + b$
 m: 1.06187
 b: 4.62906e-1
 x: Amount[ng/L]
 y: Height

Working range: 1.5 - 60 [ng/L]



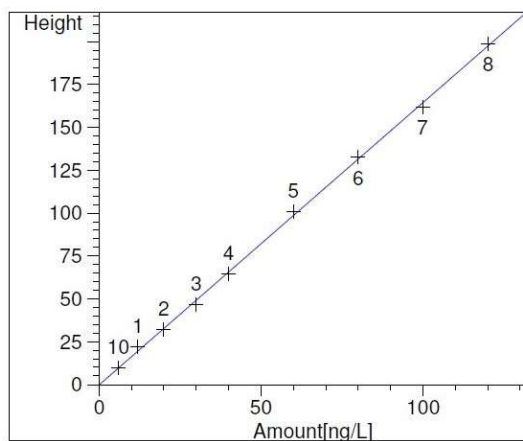
Benzo[a]pyrene at exp. RT: 12.953
 FLD1 A, Ex=237, Em=460, TT
 Correlation: 0.99984
 Residual Std. Dev.: 0.17176
 Formula: $y = mx + b$
 m: 4.37013e-1
 b: 2.33262e-1
 x: Amount [ng/L]
 y: Height

Working range: 1.5 - 60 [ng/L]



Benzo[ghi]perylene at exp. RT: 17.735
 FLD1 A, Ex=237, Em=460, TT
 Correlation: 0.99966
 Residual Std. Dev.: 0.62257
 Formula: $y = mx + b$
 m: 1.12916
 b: 1.51622
 x: Amount [ng/L]
 y: Height

Working range: 6 - 60 [ng/L]



Indeno[1,2,3-cd]pyrene at exp. RT: 18.734
 FLD1 A, Ex=237, Em=460, TT
 Correlation: 0.99958
 Residual Std. Dev.: 2.07018
 Formula: $y = mx + b$
 m: 1.64848
 b: -2.19120e-1
 x: Amount [ng/L]
 y: Height

Working range: 6 - 120 [ng/L]

Example of the six PAHs calibration curves achieved with this SBSE-HPLC-FLD method.

A) Fluoranthene

A scatter plot showing the relationship between Concentration (ng/L) on the x-axis and Height (cm) on the y-axis. The x-axis ranges from 0 to 160 with major ticks every 20 units. The y-axis ranges from 0 to 40 with major ticks every 5 units. There are 8 data points plotted as blue dots. A blue linear regression line is fitted to the data. The equation of the line is $y = 0.2199x + 0.3848$ and the coefficient of determination is $R^2 = 0.9986$.

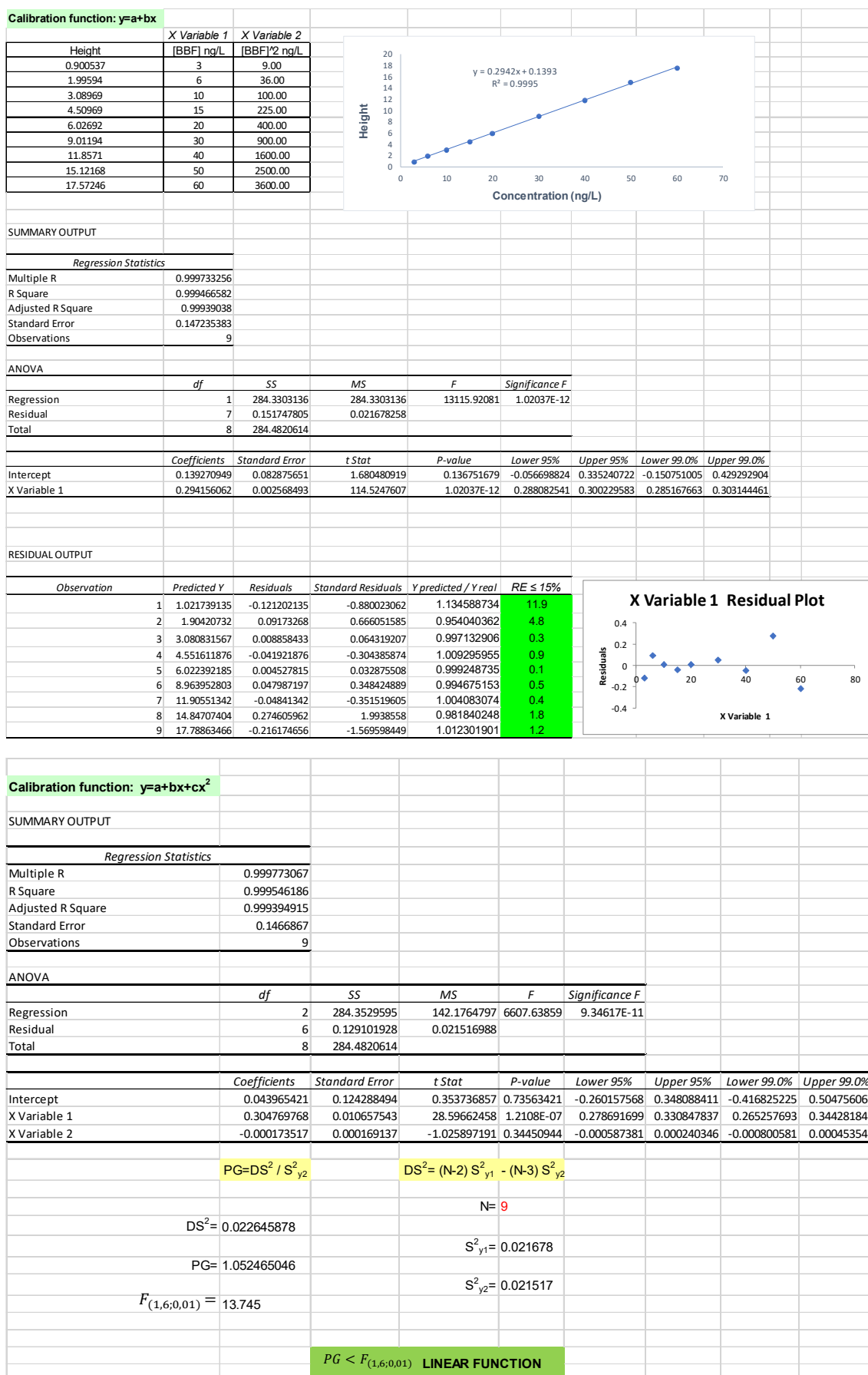
Concentration (ng/L)	Height (cm)
5	2.5
15	4.5
25	6.0
35	9.0
50	11.5
75	17.0
100	22.5
125	29.0
150	32.5

X Variable 1 Residual Plot

This residual plot shows the relationship between the residuals and X Variable 1. The x-axis represents X Variable 1, ranging from 0 to 200. The y-axis represents the residuals, ranging from -1 to 1. The data points show a clear upward trend, indicating a positive linear relationship between the variable and the residuals.

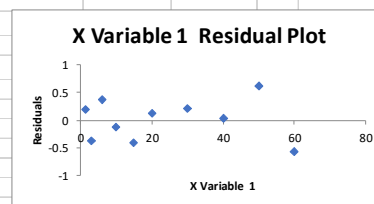
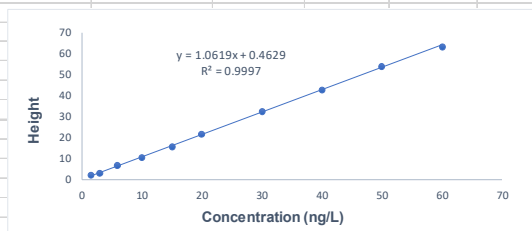
X Variable 1	Residuals
0	-0.1
10	-0.1
20	-0.2
30	-0.1
40	0.4
50	0.0
60	-0.2
70	-0.3
80	-0.4
90	-0.4
100	-0.4
110	-0.4
120	-0.4
130	-0.4
140	-0.4
150	-0.4
160	-0.4
170	-0.4
180	-0.4
190	-0.4
200	-0.4

B) Benzo[b]fluoranthene



C) Benzo[k]fluoranthene

Calibration function: y=a+bx																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
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D) Benzo[a]pyrene

Calibration function: $y=a+bx$		
	X Variable 1	X Variable 2
Height	[BAP] ng/L	[BAP] ² ng/L
0.953353	1.5	2.25
1.42729	3	9.00
3.11852	6	36.00
4.59508	10	100.00
6.45142	15	225.00
9.13025	20	400.00
13.32231	30	900.00
17.65859	40	1600.00
22.0578	50	2500.00
26.53461	60	3600.00

Scatter plot showing Height (Y-axis) versus Concentration (ng/L) (X-axis). The data points are fitted with a linear regression line. The equation of the line is $y = 0.4370x + 0.2333$ and the coefficient of determination is $R^2 = 0.9997$.

SUMMARY OUTPUT								
Regression Statistics								
Multiple R	0.99983854							
R Square	0.999677106							
Adjusted R Square	0.999636744							
Standard Error	0.171765151							
Observations	10							
ANOVA								
	df	SS	MS	F	Significance F			
Regression	1	730.7341438	730.7341438	24767.90594	2.97273E-15			
Residual	8	0.236026137	0.029503267					
Total	9	730.97017						
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 99.0%	Upper 99.0%
Intercept	0.233263984	0.085010336	2.743948484	0.025294379	0.037229796	0.429298171	-0.051978622	0.51850659
X Variable 1	0.437013092	0.002776833	157.3782257	2.97273E-15	0.430609703	0.443416481	0.427695741	0.446330443

RESIDUAL OUTPUT					
Observation	Predicted Y	Residuals	Standard Residuals	Y predicted / Y real	RE ≤ 15%
1	0.888783622	0.064569378	0.398719806	0.93227128	7.3
2	1.54430326	-0.11701326	-0.722563941	1.08198282	7.6
3	2.855342536	0.263177464	1.625136722	0.915608217	9.2
4	4.603394904	-0.008314904	-0.051345032	1.001809523	0.2
5	6.788460364	-0.337040364	-2.081244583	1.052242818	5.0
6	8.973525823	0.156724177	0.967781247	0.982834624	1.7
7	13.34365674	-0.021346743	-0.131817428	1.00160233	0.2
8	17.71378766	-0.055197663	-0.3408489	1.003125825	0.3
9	22.08391858	-0.026118583	-0.161283825	1.001184097	0.1
10	26.4540495	0.080560497	0.497465933	0.996963946	0.3

X Variable 1 Residual Plot

Residual plot showing X Variable 1 (X-axis) versus Residuals (Y-axis). The residuals are plotted against the predicted values, showing a random distribution around zero.

Calibration function: $y=a+bx+cx^2$							
SUMMARY OUTPUT							
Regression Statistics							
Multiple R	0.999847877						
R Square	0.999695777						
Adjusted R Square	0.999608857						
Standard Error	0.178236439						
Observations	10						
ANOVA							
	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>		
Regression	2	730.7477924	365.3738962	11501.2362	4.911E-13		
Residual	7	0.222377597	0.031768228				
Total	9	730.97017					
	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 99.0%</i>
Intercept	0.287095116	0.120525633	2.382025378	0.04873677	0.002097282	0.572092951	-0.134682323
X Variable 1	0.429868113	0.011275106	38.12541786	2.2222E-09	0.403206725	0.456529501	0.390411069
X Variable 2	0.000122098	0.000186278	0.655460611	0.53311293	-0.000318379	0.000562575	-0.000529779
	$PG=DS^2 / S_{y_2}^2$	$DS^2 = (N-2) S_{y_1}^2 - (N-3) S_{y_2}^2$					
		$N= 10$					
	$DS^2= 0.01364854$	$S_{y_1}^2 = 0.029503$					
	$PG= 0.429628612$	$S_{y_2}^2 = 0.031768$					
	$F_{(1,7;0,01)} = 12.246$						
$PG < F_{(1,7;0,01)}$ LINEAR FUNCTION							

E) Benzo[ghi]perylene

Calibration function: $y=a+bx$

	X Variable 1	X Variable 2
Height	[BPE] ng/L	[BPE] ² ng/L
8.9739	6	36.00
12.43435	10	100.00
17.89773	15	225.00
23.80044	20	400.00
36.19746	30	900.00
46.77465	40	1600.00
57.25004	50	2500.00
69.63772	60	3600.00

Scatter plot showing Height (Y-axis) versus Concentration (ng/L) (X-axis). The data points are fitted with a linear regression line. The equation of the line is $y = 1.1292x + 1.5162$ and the coefficient of determination is $R^2 = 0.9993$.

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999661258
R Square	0.999322631
Adjusted R Square	0.999209737
Standard Error	0.622568656
Observations	8

ANOVA

	df	SS	MS	F	Significance F
Regression	1	3430.886759	3430.886759	8851.805847	9.71484E-11
Residual	6	2.325550392	0.387591732		
Total	7	3433.21231			

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 99.0%	Upper 99.0%
Intercept	1.516220307	0.410541079	3.693224343	0.010169753	0.511662475	2.520778138	-0.005831193	3.038271806
X Variable 1	1.129162457	0.012001637	94.08403609	9.71484E-11	1.099795509	1.158529405	1.084667251	1.173657663

RESIDUAL OUTPUT

Observation	Predicted Y	Residuals	Standard Residuals	Y predicted / Y real	RE ≤ 15%
1	8.291195048	0.682704952	1.184456718	0.923923272	8.2
2	12.80784488	-0.373494876	-0.64799371	1.030037346	2.9
3	18.45365716	-0.55592716	-0.964504004	1.031061322	3.0
4	24.09946944	-0.299029445	-0.518800155	1.01256403	1.2
5	35.39109401	0.806365986	1.399002024	0.977723133	2.3
6	46.68271858	0.091931417	0.15949611	0.998034589	0.2
7	57.97434315	-0.724303152	-1.25662738	1.012651575	1.2
8	69.26596772	0.371752279	0.644970397	0.994661625	0.5

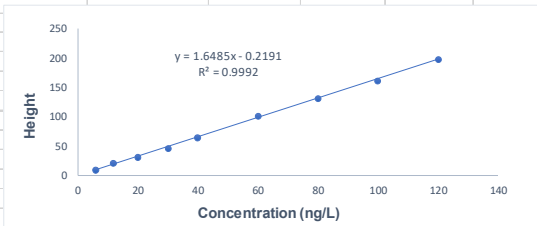
X Variable 1 Residual Plot

Residual plot for X Variable 1. The X-axis represents the concentration (ng/L) and the Y-axis represents the residuals. A single data point is plotted at approximately (6, 0.7).

Calibration function: $y=a+bx+cx^2$									
SUMMARY OUTPUT									
Regression Statistics									
Multiple R	0.999666578								
R Square	0.999333268								
Adjusted R Square	0.999066575								
Standard Error	0.67661402								
Observations	8								
ANOVA									
	df	SS	MS	F	Significance F				
Regression	2	3430.923277	1715.461639	3747.13229	1.14783E-08				
Residual	5	2.289032662	0.457806532						
Total	7	3433.21231							
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 99.0%	Upper 99.0%	
Intercept	1.695139589	0.774853799	2.18768959	0.08033028	-0.296685511	3.68696469	-1.429181719	4.819460897	
X Variable 1	1.112610542	0.060039333	18.53136077	8.42E-06	0.958274523	1.266946561	0.870523366	1.354697717	
X Variable 2	0.000255543	0.000904801	0.282430043	0.78893186	-0.002070322	0.002581408	-0.003392744	0.00390383	
	PG=DS ² / S ² _{y2}		DS ² = (N-2) S ² _{y1} - (N-3) S ² _{y2}						
	DS ² = 0.03651773		N= 8						
	PG= 0.079766729		S ² _{y1} = 0.387592						
	F _(1,5;0,01) = 16.258		S ² _{y2} = 0.457807						
PG < F _(1,5;0,01) LINEAR FUNCTION									

F) Indeno[1,2,3-cd]pyrene

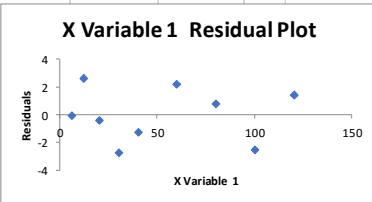
Calibration function: y=a+bx		
	X Variable 1	X Variable 2
Height	[IND] ng/L	[IND]^2 ng/L
9.63356	6	36.00
22.16668	12	144.00
32.31511	20	400.00
46.50819	30	900.00
64.48431	40	1600.00
100.86998	60	3600.00
132.44899	80	6400.00
162.05498	100	10000.00
199.03409	120	14400.00



SUMMARY OUTPUT								
Regression Statistics								
Multiple R	0.999580322							
R Square	0.999160821							
Adjusted R Square	0.999040938							
Standard Error	2.070178223							
Observations	9							
ANOVA								
	df	SS	MS	F	Significance F			
Regression	1	35718.57815	35718.57815	8334.483494	4.98386E-12			
Residual	7	29.99946514	4.285637877					
Total	8	35748.57761						
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 99.0%	Upper 99.0%
Intercept	-0.219120147	1.165259083	-0.188044144	0.856177104	-2.974520036	2.536279741	-4.296924847	3.858684552
X Variable 1	1.648478571	0.018056932	91.29339239	4.98386E-12	1.605780712	1.69117643	1.58528864	1.711668503

RESIDUAL OUTPUT					
Observation	Predicted Y	Residuals	Standard Residuals	Y predicted / Y real	RE ≤ 15%
1	9.67175128	-0.03819128	-0.019722068	1.003964399	0.4
2	19.56262271	2.604057293	1.344741391	0.882523802	13.3
3	32.75045128	-0.435341277	-0.224811273	1.013471756	1.3
4	49.23523699	-2.727046989	-1.408253563	1.058635844	5.5
5	65.72002227	-1.235712701	-0.638124983	1.019162998	1.9
6	98.68959413	2.180385875	1.125956461	0.978384194	2.2
7	131.6591655	0.789824451	0.407867228	0.994036765	0.6
8	164.628737	-2.573756974	-1.329094234	1.015881999	1.6
9	197.5983084	1.435781602	0.741441041	0.992786253	0.7

X Variable 1 Residual Plot



Calibration function: $y=a+bx+cx^2$								
SUMMARY OUTPUT								
Regression Statistics								
Multiple R	0.999595474							
R Square	0.999191112							
Adjusted R Square	0.998921483							
Standard Error	2.195320544							
Observations	9							
ANOVA								
	df	SS	MS	F	Significance F			
Regression	2	35719.66102	17859.83051	3705.7955	5.29255E-10			
Residual	6	28.91659376	4.819432293					
Total	8	35748.57761						
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 99.0%	Upper 99.0%
Intercept	0.439920122	1.860107868	0.236502479	0.82090994	-4.111599866	4.991440109	-6.456295912	7.336136155
X Variable 1	1.611781541	0.079750666	20.21025808	9.5333E-07	1.416638691	1.806924391	1.316111687	1.907451395
X Variable 2	0.000299969	0.000632829	0.474013267	0.6522476	-0.001248507	0.001848446	-0.002046198	0.002646137
	PG= $DS^2 / S^2_{y_2}$		$DS^2= (N-2) S^2_{y_1} - (N-3) S^2_{y_2}$					
	$DS^2= 1.082871384$		N= 9					
	PG= 0.224688577		$S^2_{y_1}= 4.285638$					
	$F_{(1,6;0,01)} = 13.745$		$S^2_{y_2}= 4.819432$					
		PG < $F_{(1,6;0,01)}$ LINEAR FUNCTION						

Attachment 4: Trueness data

A) Drinking water and groundwater matrices

PAH	Amount added (ng/L)	n	b (%)	Σbi^2	MR (%)	$b_{rms} = u_b$
Fluoranthene	7.5	10	5.1	0.0019	93.4	0.0136
Benzo[b]fluoranthene	3	10	3.7	0.0179	109.9	0.0423
Benzo[k]fluoranthene	1.5	10	-26.0	0.0754	90.3	0.0868
Benzo[a]pyrene	1.5	10	-4.2	0.1286	90.5	0.1134
Benzo[ghi]perylene	6	10	-7.1	0.0674	103.0	0.0821
Indeno[1,2,3-cd]pyrene	6	10	-16.4	0.0308	99.0	0.0555

PAH	Amount added (ng/L)	n	b (%)	Σbi^2	MR (%)	$b_{rms} = u_b$
Fluoranthene	25	10	-1.1	0.0673	107.7	0.0820
Benzo[b]fluoranthene	10	10	1.5	0.0149	100.3	0.0387
Benzo[k]fluoranthene	10	10	6.0	0.0345	102.8	0.0588
Benzo[a]pyrene	10	10	5.3	0.0349	102.6	0.0591
Benzo[ghi]perylene	10	10	-4.5	0.0565	101.9	0.0751
Indeno[1,2,3-cd]pyrene	20	10	5.1	0.0237	98.4	0.0487

Where b is the relative bias from n recovery experiments, Σbi^2 stands for the sum of the square deviation from the mean recovery (for all PAHs except for BAP) or the complete recovery (for BAP).

B) Surface water matrix

PAH	Amount added (ng/L)	n	b (%)	Σbi^2	MR (%)	$b_{rms} = u_b$
Fluoranthene	7.5	13	-14.2	0.1801	63.3	0.1177
Benzo[b]fluoranthene	3	13	-6.8	0.1184	98.8	0.0954
Benzo[k]fluoranthene	1.5	19	-45.5	0.3271	66.5	0.1312
Benzo[a]pyrene	1.5	19	-5.2	0.0925	89.5	0.0698
Benzo[ghi]perylene	6	13	-18.6	0.1313	90.3	0.1005
Indeno[1,2,3-cd]pyrene	6	13	-44.7	0.3299	65.4	0.1593

PAH	Amount added (ng/L)	n	b (%)	Σbi^2	MR (%)	$b_{rms} = u_b$
Fluoranthene	25	12	-9.3	0.0395	97.6	0.0574
Benzo[b]fluoranthene	10	12	-9.3	0.0834	89.7	0.0833
Benzo[k]fluoranthene	10	12	-11.6	0.0921	85.5	0.0876
Benzo[a]pyrene	10	12	1.2	0.0441	97.1	0.0606
Benzo[ghi]perylene	10	12	-26.7	0.2608	79.3	0.1474
Indeno[1,2,3-cd]pyrene	20	12	-29.1	0.2552	65.3	0.1458

Where b is the relative bias from n recovery experiments, Σbi^2 stands for the sum of the square deviation from the mean recovery (for all PAHs).

Attachment 5: Precision data

PAH	QC sample (ng/L)	n	Experimental Average (ng/L)	SD _{stand}	U _{Rw,stand} (%)
Fluoranthene	7.5	18	8.2	1.2445	15.2
Benzo[b]fluoranthene	3.0	20	3.1	0.2102	6.8
Benzo[k]fluoranthene	1.5	21	1.5	0.2502	16.9
Benzo[a]pyrene	1.5	21	1.4	0.1650	11.5
Benzo[ghi]perylene	6.0	19	6.0	0.8839	13.7
Indeno[1,2,3-cd]pyrene	6.0	20	6.6	0.9363	14.2

Where SD_{stand} is the standard deviation from n QC samples experimental results.

A) Drinking water and groundwater matrices

PAH	n	$\bar{R}$	U _{r,range} (%)	U _{Rw} (%)
Fluoranthene	8	0.0980	8.7	17.5
Benzo[b]fluoranthene	8	0.0681	6.0	9.1
Benzo[k]fluoranthene	8	0.1001	8.9	19.1
Benzo[a]pyrene	8	0.0694	6.2	13.1
Benzo[ghi]perylene	8	0.0588	5.2	14.7
Indeno[1,2,3-cd]pyrene	8	0.1314	11.6	18.3

Where $\bar{R}$ is the mean range.

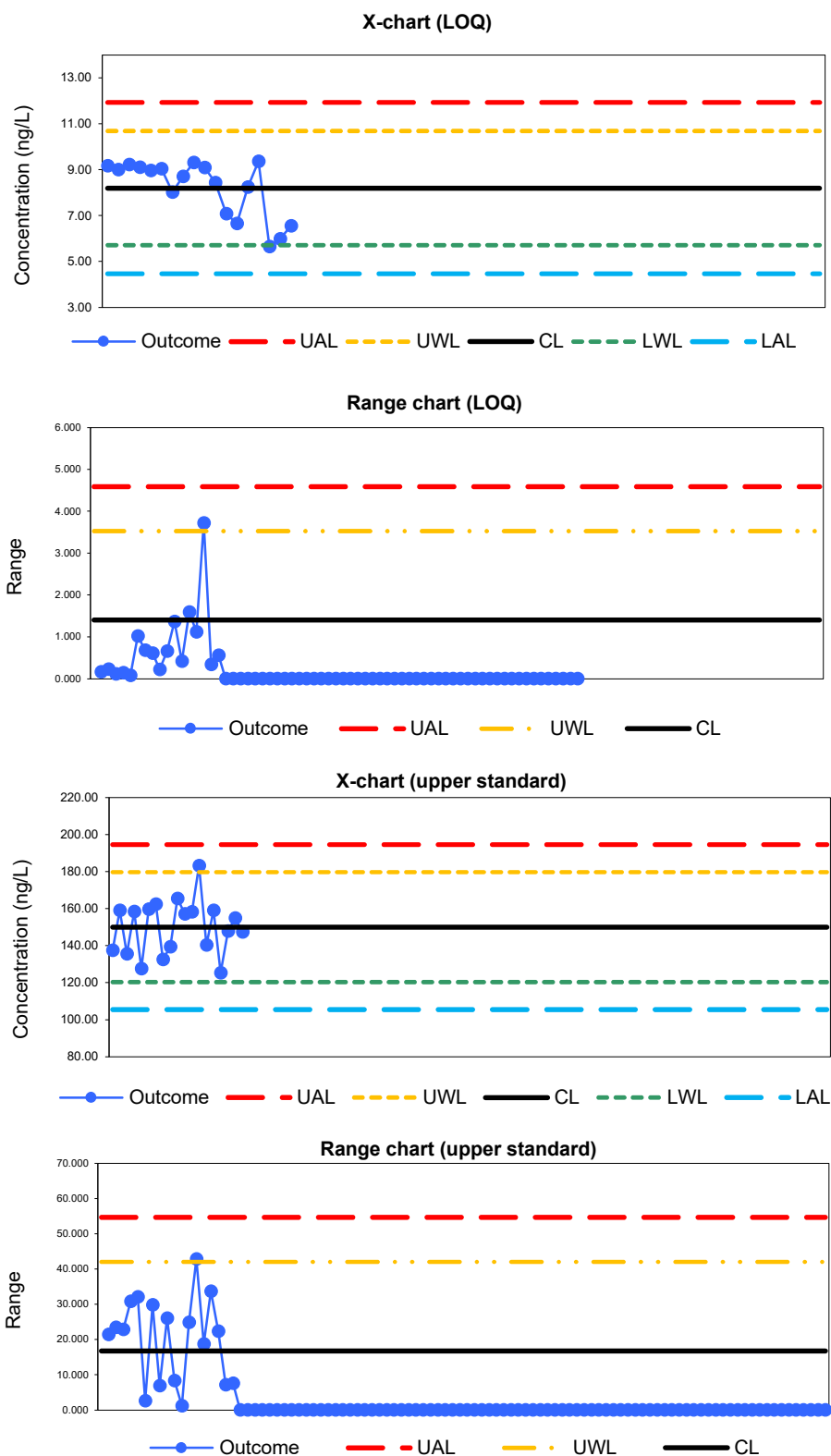
B) Surface water matrix

PAH	n	$\bar{R}$	U _{r,range} (%)	U _{Rw} (%)
Fluoranthene	8	0.1105	9.8	18.1
Benzo[b]fluoranthene	8	0.0976	8.7	11.0
Benzo[k]fluoranthene	8	0.1078	9.6	19.4
Benzo[a]pyrene	8	0.0857	7.6	13.8
Benzo[ghi]perylene	8	0.1732	15.4	20.6
Indeno[1,2,3-cd]pyrene	8	0.1124	10.0	17.4

Where $\bar{R}$ is the mean range.

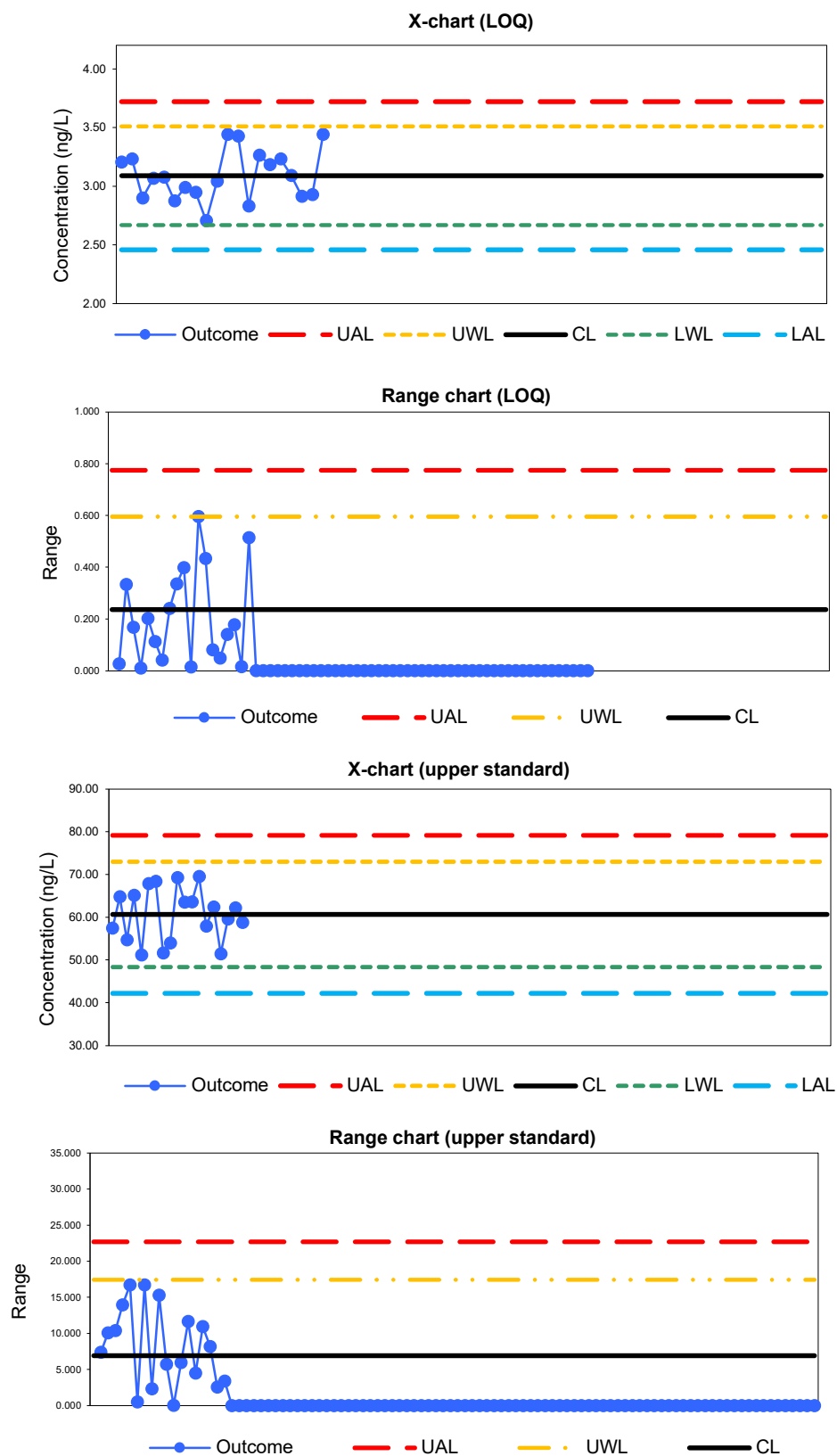
Attachment 6: Control charts

A) Fluoranthene



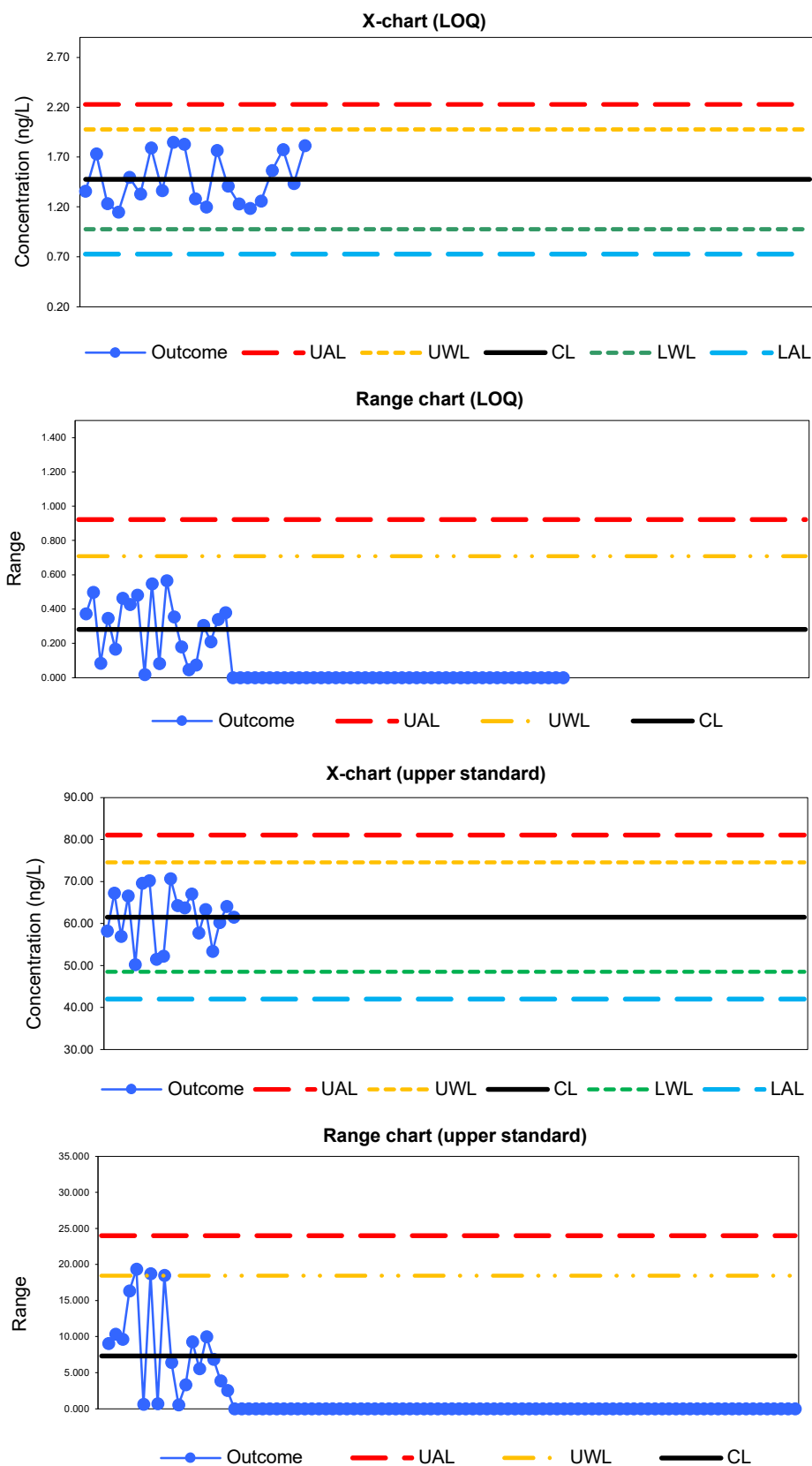
Where UAL and UWL are the upper action and warning limits, LAL and LWL represents the lower action and warning limits, and CL the central line, respectively.

B) Benzo[b]fluoranthene



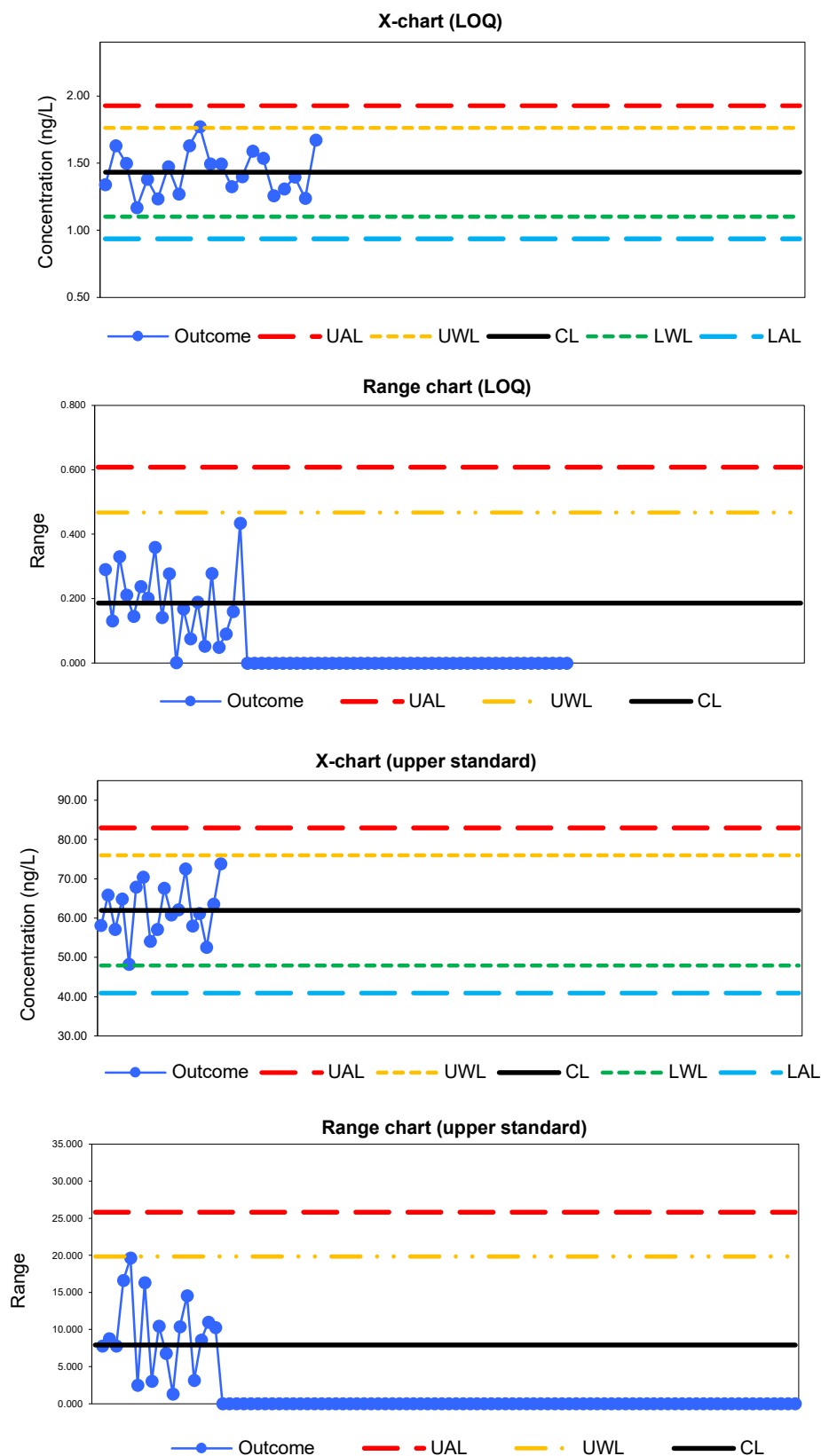
Where UAL and UWL are the upper action and warning limits, LAL and LWL represents the lower action and warning limits, and CL the central line, respectively.

C) Benzo[k]fluoranthene



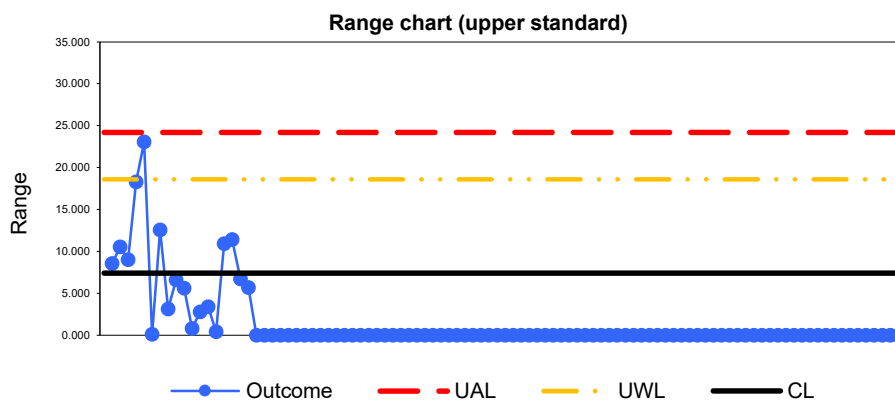
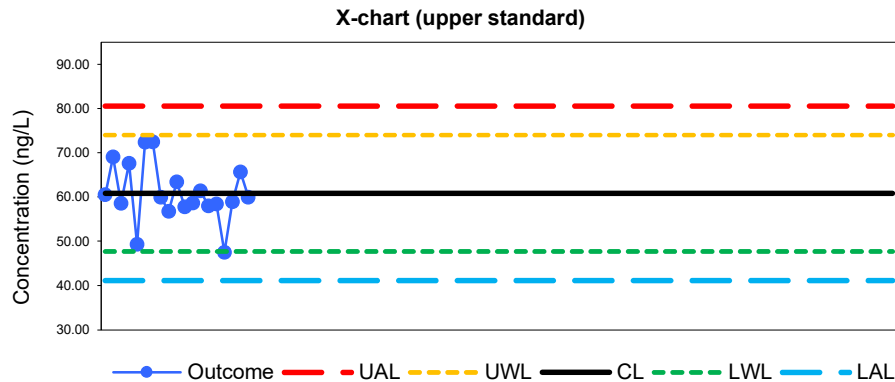
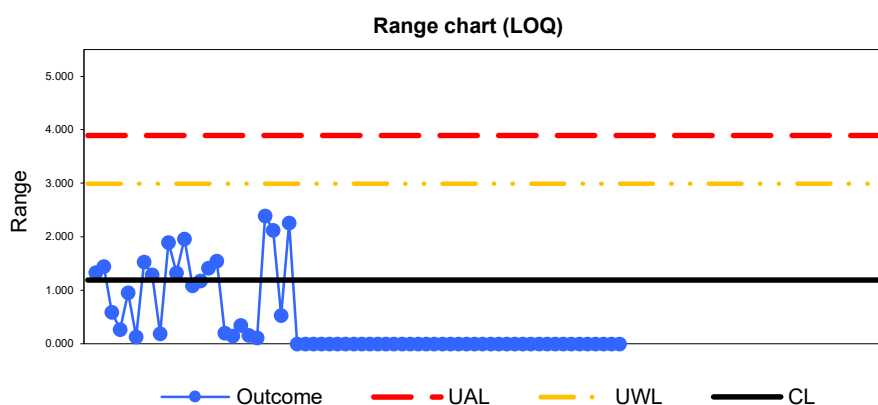
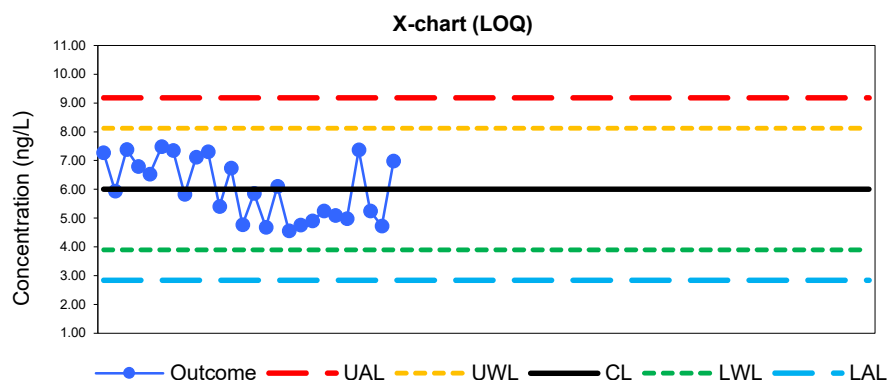
Where UAL and UWL are the upper action and warning limits, LAL and LWL represents the lower action and warning limits, and CL the central line, respectively.

D) Benzo[a]pyrene



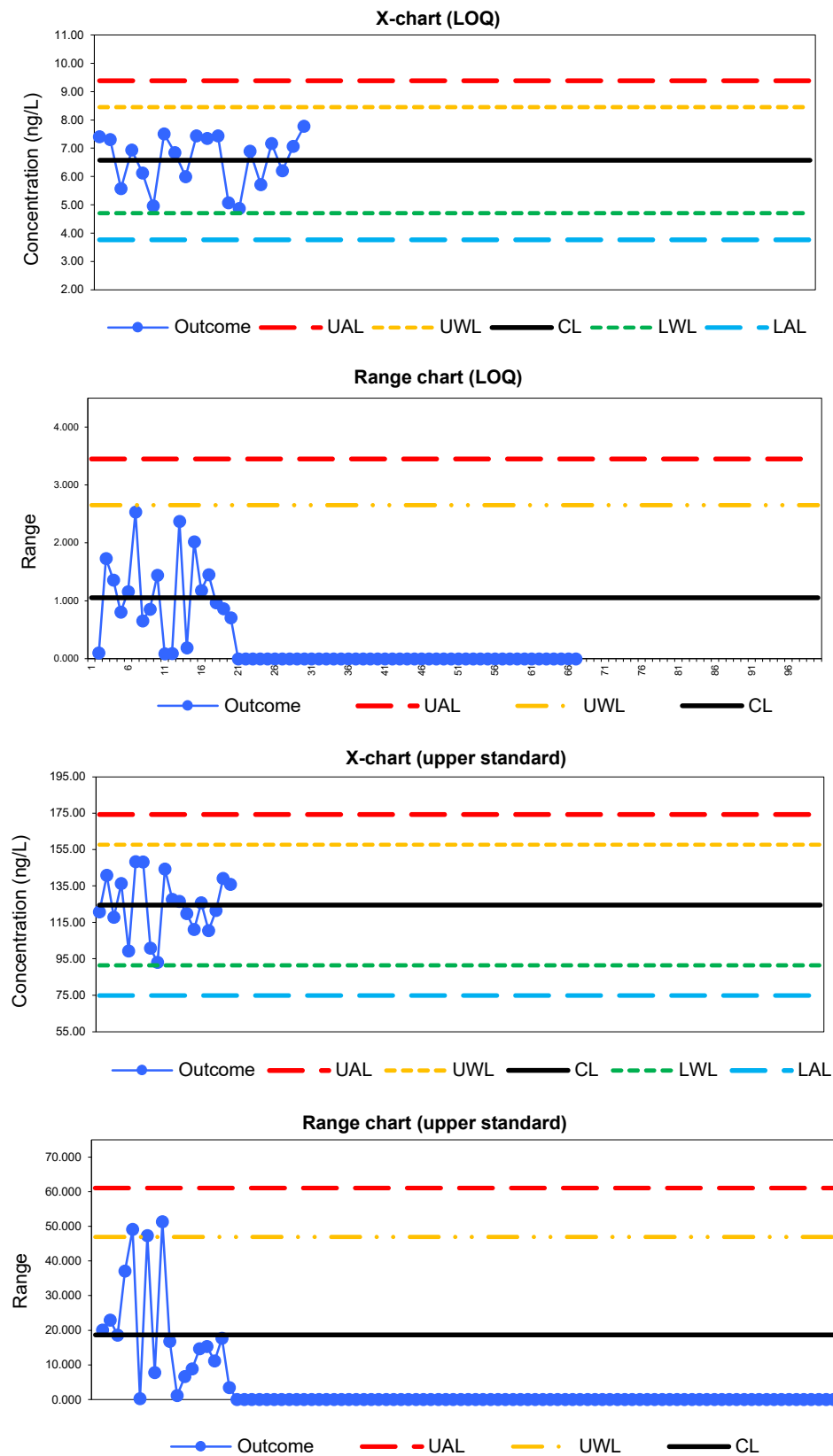
Where UAL and UWL are the upper action and warning limits, LAL and LWL represents the lower action and warning limits, and CL the central line, respectively.

E) Benzo[ghi]perylene



Where UAL and UWL are the upper action and warning limits, LAL and LWL represents the lower action and warning limits, and CL the central line, respectively.

F) Indeno[1,2,3-cd]pyrene



Where UAL and UWL are the upper action and warning limits, LAL and LWL represents the lower action and warning limits, and CL the central line, respectively.