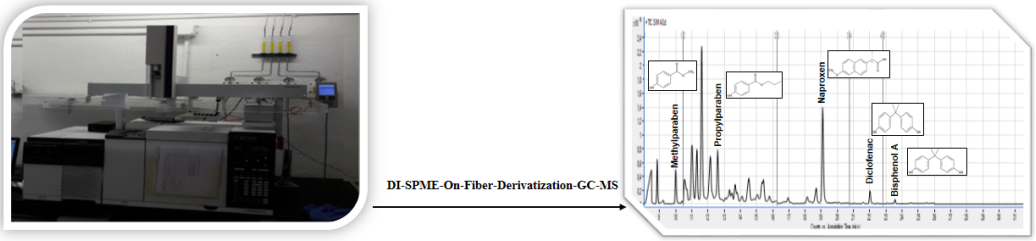


**DOCTORAL THESIS**

# PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN DIFFERENT TYPES OF SEWAGE SLUDGE

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PROGRAMA DE DOCTORADO EN QUÍMICA

TESIS DOCTORAL:

# Pharmaceuticals and personal care products in different types of sewage sludge

Presentada por Nereida Pérez Lemus
para optar al grado de
Doctora por la Universidad de Valladolid

Dirigida por:
Enrique Barrado Esteban
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Universidad de Valladolid

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Certifican que:

NEREIDA PÉREZ LEMUS ha realizado bajo su dirección el trabajo "Pharmaceuticals and personal care products in different types of sewage sludge", en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

Valladolid, a _____de_____de 2020

Dr. Enrique Barrado  Dr. Sara I. Pérez Elvira  Dr. Rebeca López Serna

The following publications have been included as part of this Doctoral Thesis:

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- N. Pérez-Lemus, R. López-Serna, S.I. Pérez-Elvira, E. Barrado, Sample pre-treatment and analytical methodology for the simultaneous determination of pharmaceuticals and personal care products in sewage sludge, Chemosphere. 258 (2020) 127273. https://doi.org/10.1016/j.chemosphere.2020.127273.

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## List of Abbreviations

| Nomenclature | |
|---|---|
| **AD** | Anaerobic digestion |
| **ANOVA** | Analysis of variance |
| **APCI** | Atmospheric pressure chemical ionization |
| **ASE** | Accelerated solvent extraction |
| **COD** | Chemical oxygen demand |
| **DI-SPME** | Direct immersion-solid-phase microextraction |
| **DS** | Digested sludge |
| **DSPE** | Dispersive solid-phase extraction |
| **DVB/CAR/PDMS** | Divinylbenzene/Carboxen/Polydimethylsiloxane |
| **ECD** | Electron capture detector |
| **ECDs** | Endocrine disrupting compounds |
| **ENMs** | Engineered nanomaterials |
| **EA** | Ethyl acetate |
| **EPA** | Environmental Protection Agency |
| **EPs** | Emerging pollutants |
| **ESI** | Electrospray ionization |
| **FDA** | Food and Drug Administration |
| **FLD** | Fluorescence detector |
| **FT-ICR** | Fourier transform ion cyclotron resonance |
| **GC** | Gas chromatography |
| **GC-MS/MS** | Gas chromatography-tandem mass spectrometry |
| **GF** | Glass fiber |
| **GPC** | Gel permeation chromatography |
| **HCl** | Hidrochloric acid |
| **HDPE** | High density polyethylene |
| **He** | Helium |
| **HPLC** | High performance liquid chromatography |
| **ILs** | Ionic liquids |
| **IS** | Internal standard |
| **ITH** | Intermediate thermal hydrolysis |
| **LC** | Liquid chromatography |
| **LC-MS/MS** | Liquid chromatography-tandem mass spectrometry |

**Nomenclature**

| | |
|---|---|
| **LLE** | Liquid-liquid extraction |
| **LOD** | Limit of detection |
| **LOQ** | Limit of quantification |
| **MAE** | Microwave-assisted extraction |
| **MeOH** | Methanol |
| **MLD** | Method limit of detection |
| **MLQ** | Method limit of quantification |
| **MS** | Mass spectrometry |
| **MSPD** | Matrix solid-phase dispersion |
| **MTBSTFA** | N-terc-Butyldimethylsilyl-N-methyltrifluoroacetamide |
| **NaCl** | Sodium Chloride |
| **NMs** | Nanomaterials |
| **NSAIDs** | Non-steroidal anti-inflammatory drugs |
| **PCPs** | Personal care and hygiene products |
| **PhACs** | Pharmaceuticals |
| **PHWE** | Pressurized hot water extraction |
| **PLE** | Pressurized liquid extraction |
| **PPCPs** | Pharmaceuticals and personal care products |
| **PSA** | Primary and secondary amine |
| **PTFE** | Polytetrafluroethylene |
| **QuEChERs** | Quick, Easy, Cheap, Effective, Rugged and Safe |
| **QqQ** | Triple quadrupole |
| **Q-TOF** | Quadrupole to time-of flight |
| **SALLE** | Salting-out liquid-liquid extraction |
| **SPE** | Solid-phase extraction |
| **SPME** | Solid-phase microextraction |
| **SRM** | Selecting reaction monitoring |
| **SUPRAs** | Supramolecular solvents |
| **TPs** | Transform products |
| **UHPLC** | Ultra-high-pressure liquid chromatography |
| **TH** | Thermal hydrolysis |
| **TS/N** | Signal-to-noise |
| **UAE** | Ultrasound-assisted extraction |

**Nomenclature**

| | |
|---|---|
| **USAEME** | Ultrasound-assisted emulsification microextraction |
| **UV** | Ultraviolet |
| **WWTPs** | Wastewater treatment plants |

**Summary**

Emerging pollutants (EPs), those for which there is no clear and specific legislation, constitute a large group of chemical compounds used in human and animal health. It includes pharmaceutical compounds and personal care products (PPCPs); medicines, clean-up products, cosmetics, fragrances and hormones both natural and synthetic. These compounds have been off the radar of environmental science, which is more concerned with apolar, toxic, persistent and bioaccumulative pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorophenyls (PCBs) and dioxins. However, the development of new methods of analysis has made it possible to warn of the presence of these EPs, mainly due to the aggravation of the problems of storage and disposal of sludge from the Wastewater Treatment Plants (WWTPs) due to the increase in the volume of treated water and, consequently, the volume of sludge to be managed.
The trend in urban waste management is towards recycling, with special emphasis on agricultural use as a fertilizer or soil amendment. However, the presence of PPCPs has raised enormous concern about the possible adverse effects on humans and wildlife, since despite their low concentrations in the environment (ng $L^{-1}$ or µg $L^{-1}$), there is evidence that at these levels they can produce serious damage to humans and ecosystems. It has been demonstrated that the purification procedures are not completely effective since the main objective of the WWTPs is the elimination of contaminants above mg $L^{-1}$. In fact, many of these compounds have physical-chemical properties that favour their adsorption to waste sludge that is used as agricultural soil fertilizer to minimize the use of chemical fertilizers and improve soil quality. Studies in several WWTPs determined an increase in the discharge of pharmaceutical products and pesticides, among other compounds, into our waters and finally into rivers.

The research developed in this Doctoral Thesis began with a review of the different analytical approaches for the determination of EPs, including PPCPs, in environmental matrices. Sample preparation techniques and instrumental methods proposed to evaluate PPCPs in sewage sludge were reviewed. Three main steps were examined: extraction, clean-up, and analysis. Sample preparation is critical as the compounds of interest are typically found at low concentrations in such complex matrices.

In view of this, an analytical method was developed and validated for the simultaneous determination of 14 PPCPs in sewage sludge. The optimal experimental conditions for sample pre-treatment were established. As a result, microwave-assisted extraction (MAE) was combined with an in-situ clean-up stage and a filtration step. A combination of MilliQ®/MeOH 95:5 (v/v) water adjusted to pH 9 proved to be the optimal solvent mixture for the extraction. The instrumental part of the method presents an important novelty based on a fully automated sample preparation for the analysis of PPCPs. It consisted of a Direct Immersion Solid Phase MicroExtraction followed by On-fiber Derivatization coupled to Gas Chromatography – Mass Spectrometry (DI-SPME-On-fiber derivatization - GC-MS).

The analytical method has been validated following international validation guidelines with excellent results in terms of accuracy (precision and veracity), sensitivity (limits of detection and quantification), selectivity, linearity and robustness. This analytical method is an ecological alternative for many routine analysis laboratories worldwide. In addition,

the method was applied to different samples generated in both thermal hydrolysis (TH) and anaerobic digestion (AD) pilot scale plants. In thermal hydrolysed samples, the highest concentration values corresponded to salicylic acid (1,000 ng $g^{-1}$). However, the highest concentrations of the contaminants of interest after AD corresponded to naproxen (9,355 ng $g^{-1}$). In the case of implementing a TH stage prior to digestion, both salicylic acid (10,045 ng $g^{-1}$) and triclosan (762 ng $g^{-1}$) showed the highest concentrations in the TH influent. On the other hand, salicylic acid (4,267 ng $g^{-1}$) and triclosan (417 ng $g^{-1}$) were also the contaminants with the highest concentrations after AD.

Finally, the last chapter of the Doctoral Thesis corresponds to a developed method for the simultaneous determination of 60 PPCPs (e.g., antibiotics, analgesic/non-steroidal anti-inflammatory drugs, hormones, lipid regulators, hormones, among others) in dewatered digested sludge samples. Sample pre-treatment consisted of ultrasound-assisted extraction (UAE) was combined with an in-situ clean-up stage and a filtration step. A combination of MilliQ® water/MeOH 95:5 (v/v) water adjusted to pH 9 was used as solvent mixture for the extraction. Instrumental part of the method consisted of an online Solid Phase Extraction (SPE) coupled Ultra-High-Pressure Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS). Excellent results were observed in terms of limit of detection and quantification for 31 compounds of interest. In these sludge samples, the highest concentrations corresponded to enrofloxacin (12,875 ng $g^{-1}$), 4-hydroxybenzoic acid (4,027 ng $g^{-1}$), sulfamethoxazole (1,267 ng $g^{-1}$), and clofibrate (1,090 ng $g^{-1}$).

**Resumen**

Los contaminantes emergentes (CEs), aquellos para los que no existe una legislación clara y específica, constituyen un amplio grupo de compuestos químicos empleados en la salud humana y animal. Incluye compuestos farmacéuticos y productos de cuidado personal (PPCPs): medicamentos, productos de limpieza, cosméticos, fragancias y hormonas tanto naturales como sintéticas. Estos compuestos han estado fuera del radar de la ciencia medioambiental, más dedicada a contaminantes apolares, tóxicos, persistentes y bioacumulables como los hidrocarburos aromáticos policíclicos (PAHs), los policlorofenilos (PCBs) y las dioxinas. No obstante, el desarrollo de nuevos métodos de análisis ha permitido alertar de la presencia de estos CEs**,** debido fundamentalmente al agravamiento de los problemas de almacenamiento y eliminación de lodos de las EDARs por el incremento de volumen de agua depurada y, en consecuencia, el volumen de lodos a gestionar.

La tendencia de la gestión de los residuos urbanos es el reciclado, habiéndose potenciado especialmente su valorización agrícola como abono o enmienda del suelo. Sin embargo, la presencia de PPCPs, ha suscitado una enorme preocupación por los posibles efectos adversos para los seres humanos y la fauna silvestre, ya que a pesar de sus bajas concentraciones en el medio ambiente (ng $L^{-1}$ o µg $L^{-1}$), existen evidencias de que a estos niveles pueden producir daños serios en los seres humanos y en los ecosistemas. Está demostrado que los procedimientos de depuración no son completamente efectivos ya que el objetivo principal de las EDARs es la eliminación de contaminantes por encima del mg $L^{-1}$. De hecho, muchas de estos compuestos presentan propiedades físico-químicas que favorecen su adsorción a los fangos de desecho que son utilizados como fertilizantes de suelos agrícolas para minimizar el empleo de fertilizantes químicos y mejorar la calidad de los suelos. Estudios en diversas EDARs, determinaron un aumento de vertidos de productos farmacéuticos y plaguicidas, entre otros compuestos, a nuestras aguas y finalmente a los ríos.

La investigación desarrollada en esta Tesis comenzó con una revisión de los diferentes enfoques analíticos para la determinación de CEs incluyendo PPCPs, en matrices ambientales. Se revisaron las técnicas de preparación de la muestra y los métodos instrumentales propuestos para evaluar los PPCPs en los lodos residuales. Se examinaron tres pasos principales: extracción, limpieza y análisis, siendo fundamental la preparación de las muestras, ya que los compuestos de interés se encuentran normalmente a bajas concentraciones en matrices tan complejas.

A la vista de todo ello, se ha desarrollado y validado un método analítico para la determinación simultánea de 14 PPCPs en los lodos de depuración. Se establecieron las condiciones experimentales óptimas del pre-tratamiento de la muestra. Como resultado de ello, se combinó la extracción asistida por microondas (MAE) con una etapa de limpieza in-situ y una etapa de filtración. Una combinación de agua MilliQ®/MeOH 95:5 (v/v) ajustada a pH 9 resultó ser la mezcla de disolventes óptima para la extracción. La parte instrumental del método presenta una importante novedad basada en una preparación de la muestra totalmente automatizada para el análisis de los PPCPs. Consistió en una microextracción en fase sólida por inmersión directa, seguida de una derivatización en fibra, acoplada en línea a la cromatografía de gases-espectrometría de masas (DI-SPME-On-fiber derivatización - GC-MS).

El método analítico ha sido validado siguiendo guías internacionales de validación con la obtención de excelentes resultados en términos de exactitud (precisión y veracidad), sensibilidad (límites de detección y cuantificación), selectividad, linealidad y robustez.

Este método analítico supone una alternativa ecológica para muchos laboratorios de análisis de rutina en todo el mundo. Además, el método se aplicó a diferentes muestras generadas en plantas a escala piloto tanto de hidrólisis térmica (TH) como de digestión anaeróbica (AD). En muestras hidrolizadas térmicamente, los valores más altos de concentración correspondieron al ácido salicílico (1000 ng $g^{-1}$). Sin embargo, las concentraciones más altas de los contaminantes de interés después de la AD correspondieron al naproxeno (9355 ng $g^{-1}$). En el caso de añadir una etapa de TH previa la digestión, tanto el ácido salicílico (10 045 ng $g^{-1}$) como el triclosan (762 ng $g^{-1}$) presentaron las concentraciones más elevadas en el influente de la TH. Por otro lado, el ácido salicílico (4267 ng $g^{-1}$) y el triclosan (417 ng $g^{-1}$) también fueron los contaminantes con las mayores concentraciones después de la AD.

Para finalizar, el último capítulo de la Tesis Doctoral corresponde a un método desarrollado para el análisis de un número considerable de PPCPs (antibióticos, anti-inflamatorios no esteroideos, hormonas, reguladores de lípidos, hormonas, entre otros) en muestras de lodo digerido deshidratado. La parte instrumental del método consiste en una extracción en fase sólida (SPE) en línea acoplada a cromatografía líquida de ultra alta presión-espectrometría de masas en tándem (UHPLC-MS/MS). Se observaron excelentes resultados en cuanto al límite de detección y cuantificación para 31 de los PPCPs iniciales. En estas muestras de lodo, las concentraciones más altas correspondían a enrofloxacina (12.875 ng $g^{-1}$), ácido 4-hidroxibenzoico (4.027 ng $g^{-1}$), sulfametoxazol (1.267 ng $g^{-1}$) y clofibrato (1.090 ng $g^{-1}$).

# Objectives

This Doctoral Thesis is part of the Research Project called "**Sludge thermal hydrolysis: Efficient integration of water, energy and agriculture**", which studies the use of sludge from urban Wastewater Treatment Plants (WWTPs) as a possible fertilizer to replace chemical fertilizer as a better environmental alternative.

The applicability of WWTP sludge as a fertilizer lies in a treatment line that achieves the required quality. To this end, a subject of growing interest, which is specifically addressed in this project, is the fact that there are numerous organic contaminants commonly used both in homes and in public places, which have not been considered in the design and operation of treatment processes such as personal care products, pharmaceuticals, paints, hydrocarbons, among others. In addition, these organic contaminants may cause adverse effects on wildlife and humans.

Therefore, the study of the so-called emerging contaminants has become a priority objective for the World Health Organization (WHO) and the Environmental Protection Agency (EPA). It is necessary to develop analytical methods that are highly selective and with high sensitivity in order to achieve an optimal determination of these compounds.

In line with the above, the objectives set out in this thesis are the following:

1. To undertake a review of the analytical methodologies employed for the determination of pharmaceuticals and personal care products (PPCPs) in sewage sludge samples.

2. To identify and quantify the emerging contaminants of interest such as PPCPs in sewage sludge samples, developing different physical and chemical techniques for their analysis.

3. To improve the methods of analysis. For the routine application of the methods of analysis, it is necessary the improvement of experimental parameters (e.g., amount of solid sample, solvent volume, amount of reagents, extraction times, among others). For this purpose, different experiments were carried out to determine the most influential experimental parameters and to achieve their optimal value.

4. To develop and validate the analytical procedures for the analysis of the PPCPs in thickened mixed sludge, using the technique of gas chromatography-mass spectrometry (GC-MS), emphasizing both sample preparation and analysis of the emerging contaminants of interest.

5. To evaluate the quality of different types of sludge from two indoor pilot scale reactors run at Department of Chemical Engineering and Environmental Technology of the University of Valladolid (Spain): thermally pre-treated mixed sludge and digested sludge by means of the identification and quantification of PPCPs applying the methodology developed.

6. To develop an alternative analytical procedure for the analysis of PPCPs in dewatered digested sludge, using the technique of ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), emphasizing both sample preparation and analysis of the target PPCPs.

# Chapter I. State of the art review

## 1. Introduction

Emerging pollutants (EPs) are a great concern because of their detrimental effects on the health of human beings as well as aquatic and terrestrial life [1]. EPs include pharmaceuticals and personal care products (PPCPs) whose presence in the environment has not been yet regulated as stated in Directive 2013/39/EU on priority substances in the field of water policy [2].

PPCPs represent a large number of chemicals used in daily life including medicines, cosmetic and personal hygiene products. The active ingredients of PPCPs are products such as non-steroidal drugs like analgesics, antibiotics, antiepileptics, β-blockers, blood-lipid regulators, antiretroviral drugs and steroid drugs (hormones). As an example, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed pain medications. NSAIDs are used for the treatment of osteoarthritis, rheumatoid arthritis, inflammation, fever and sever and chronic pain and therefore improve quality of daily life [3]. Personal care products include cosmetic and personal hygiene products such as antimicrobials, fragrances, UV filters, and surfactants, among others. For instance, endocrine disrupting compounds such as parabens, are widely used as preservatives in PPCPs because their toxicity levels are theoretically low [4]. These drugs (active ingredients and preservatives), excreted in the environment via urine, feces, wastewater, sewage sludge and manure [5–6], are known to be persistent, bio-active and bio-accumulative as they are cleared at a faster rate than that of their natural degradation. These agents can pose a threat to drinking water supplies [7] and may be a health risk due to their estrogen activity and effects on the endocrine system [1,4,8,9]. PPCPs have been detected in water bodies throughout the world, even in Antarctic waters [10]. Moreover, in Europe, the rate of increase in the consumption and production of PPCPs has grown markedly in the last 20 years. Several studies examining the impacts of a wastewater treatment plant (WWTP) in Spain have shown that PPCPs contribute to water toxicity in a greater measure than traditional priority pollutants [11]. Conventional WWTPs are not designed to remove organic micropollutants. In fact, effluents from such plants are now considered to be a major point source of endocrine disrupting compounds and PPCPs in the receiving environment. For this same reason, PPCPs are commonly found in sewage sludge, as the residue left behind after the treatment of wastewater from various sources, including homes, industrial plants, and medical facilities [12]. The sewage sludge generated is often employed in agricultural and forestry activities, mainly due to its capacity to fertilize soils and the low economic impact of this practice [13], which leads to their spread in the environment.

In the past few years, numerous procedures for the determination of these emerging contaminants have been developed for use on the sewage sludge solid matrix. From an analytical perspective, sewage sludge (i.e., primary, secondary, digested sludge, compost) is challenging because of the complex nature of its matrix. In addition, its characteristics vary depending on the inputs to the WWTP.

In this study, the latest trends in methodology for the determination of PPCPs in sewage sludge are reviewed in detail. Focusing on the past six years after the last review published in 2012 (used as a reference for the present review) [14], 273 papers were identified, 67 of which deal explicitly with the determination of PPCPs in sewage sludge samples. A couple of recent general reviews have considered emerging contaminants in sludge samples [15] and aquatic ecosystems [16]. Martín-Pozo et al. recently provided a general overview of methodologies used to determine emerging contaminants in sewage sludge [15]. Here we present a holistic collection and critical review of all methodologies

described to date that have been used for the determination of PPCPs throughout in sewage sludge. In effect, 85% of the literature gathered in this compilation has never been analyzed or discussed before.

The present article focuses on both current sample preparation procedures and instrumental analysis techniques including an assessment of the impact and efficiency of each stage and technique on several validation parameters. In addition, we discuss possible analytical perspectives for the future and provide novel information on the use of miniaturized and automated techniques as well as green chemistry approaches.

## 2. Analysis of sewage sludge samples

Studies worldwide have observed the presence of PPCPs in several environmental matrices. Concentrations of some PPCPs such as diclofenac (NSAID), propranolol (anti-hypertension agent), triclosan (broad-spectrum antibacterial agent), triclocarban (antibacterial agent), and miconazole (azole antifungal agent) are commonly observed in the sewage sludge of most WWTPs. For instance, in Brazil, diclofenac has been found at concentrations of 25 to 60 ng $g^{-1}$, propranolol at 61.2 to 94.3 ng $g^{-1}$, triclosan at 2086 to 5466 ng $g^{-1}$ and miconazole at 313 to 515 ng $g^{-1}$ [93]. In India, propranolol has been detected in samples at concentrations of 46 to 54 ng $g^{-1}$, triclocarban at a mean concentration of 11.125 ng $g^{-1}$ and miconazole averaged a concentration of 250 ng $g^{-1}$ [59]. In France, diclofenac, triclosan and miconazole have been found at concentrations around 24 ng $g^{-1}$, 824 and 63 ng $g^{-1}$, respectively, and propranolol was observed at levels between 82 and 849 ng $g^{-1}$ [100].

Sewage sludge is a complex matrix. It is not uniform in composition and concentrations of organic contaminants depend on the nature of inputs to the WWTP. Further, sludge contains substances that could interfere when trying to determine analytes of interest. Such interference may impact the whole analytical process, from sample preparation to instrumental detection. Thus, it is necessary to first remove these from samples using clean-up procedures.

**Table 1.1** and **1.2** (as part of the publication by **Pérez-Lemus et al., (2019)** contained in **Appendix II**) present a summary of the references reviewed here. All types of sludge (i.e., primary, secondary, digested, and compost) were subjected to similar analytical approaches which roughly consisted of a sample pre-treatment followed by an instrumental analysis. The different methods used are described in the following sections.

Despite similar analytical protocols (extraction, clean-up and analysis), differences did exist in terms of the quantity of sample treated or the amount of solvent in each matrix. Some of the studies reviewed used different amounts of sample and extraction solvent for different types of sludge with ultrasound as the extraction technique: Kopperi et al. [37] used 0.05 g of sample and 6 mL of solvent (acetonitrile) in composted sludge samples; Abril et al. [58] used 1 g of sample and 3 mL of solvent (methanol: acetic acid (1:1)) in digested sludge samples; Shafrir [49] used 2 g of sample and 10 mL of solvent (methanol: water (1:1) in secondary sludge samples; Lonappan et al. [31] used 0.5 g of sample and 20 mL of solvent (methanol) in primary sludge samples; and Yan et al. [40] used 2 g of sample and 10 mL of solvent (methanol/citric acid/$Na_2$EDTA (2:1:1)) in dewatered sludge samples.

Further, sample quantities and solvents also varied for different extraction techniques on the same type of sludge. Examples for digested sludge are 0.1 g [43], 1.5 g [76] or 3g [64] and 6 mL of methanol:water (1:1) [43], 22 mL of hexane: dichloromethane (1:1) [76] or 20 mL of methanol:water (1:1)) [64] used in ultrasound [43], pressurized liquid [76], or microwave [64] extraction procedures, respectively.

The matrices associated with each type of sludge differ because their characteristics vary as the sludge goes through several treatment stages. For instance, major changes are produced by thickening, dewatering and digestion. In thickening and dewatering treatments, total solid (dry solids) concentrations increase and the volume of sludge is reduced. Following digestion treatment, the load of total solids is reduced (via the reduction of volatile suspended solids). Several sludge matrices should be, therefore, treated separately and their analysis should be viewed as a challenge to be addressed in future work.

### 2.1. Sample pre-treatment

The sampling of different types of sludge is particularly important to assess the distribution of PPCPs along the sludge line. According to Tables 1 and 2, sampling sludge locations within WWTPs depends on the type of sewage sludge sample required for the subsequent analysis. In the literature reviewed, a large number of studies preferred sampling sewage sludge [92,100] (suspension with a dry solids content of 3 to 4 % weight arising from the purification of wastewaters). Some authors sample the sludge after the final dewatering step to obtain a representative bulk product [22,78]. Other researchers carry out their sampling after the anaerobic digestion step in which some of the organic matter is removed [43,64]. However, few publications considered sampling in primary and secondary tanks [42,77].

Representative sludge samples can be collected from the WWTP sludge line. Sample volumes in the studies reviewed differed, e.g.: 1 L samples were collected weekly over a period of four weeks by Schoeman et al. [53]; random grab samples were pooled to provide a sample weighing about 500 g by Gago-Ferrero et al. [34]; and five grab samples collected daily were pooled to give a single sample (approximately 2 L) of sludge per day over three consecutive days by Jelic et at. [74].

The materials used for sample collection also differed. Thus, one report describes the collection of solid pasty sludge using a metal bucket and the collection of liquid sludge using a sample probe. Thereafter, the samples were packed in glass bottles with a wide-mouth PTFE stopper [100]. Other materials such as 1L clear Schott bottles [53] or antimicrobial plastic bags after sewage sludge dewatering [34] were also utilized for sample collection. These samples were then transported to the laboratory where they were frozen and lyophilized [53, 59, 88] or dried in air to room temperature [50], and passed through a 2 mm Ø sieve and homogenized [50] or were macerated in a glass mortar for some minutes [93]. Finally, the lyophilized samples were stored at −20 °C [65] until their analysis.

Sample preparation takes up most of the analysis time. It usually includes a process of extraction followed by a clean-up step. A variety of techniques have been used to extract PPCPs from sewage sludge samples in the last 6 years. Besides traditional approaches such as Soxhlet [20,21] and ultrasound [28,34], other methods based on microwave [62,65] or pressurized liquid [72,74] are gaining popularity. Most extraction techniques are not sufficiently selective and clean-up procedures are also needed after extraction.

**Figures 1.1** and **1.2** show each of the extraction and clean-up techniques used, respectively, over the last 6 years (reviewed here) compared with the previous five-year period.

### 2.1.1. Extraction

Solvent extraction of solid samples, commonly known as solid–liquid extraction, is one of the oldest techniques of solid sample preparation. This technique serves to remove and separate compounds of interest from insoluble high-molecular-weight fractions and other compounds that could interfere with subsequent steps of the analytical process [17]. Soxhlet is a reference extraction technique that belongs to that group. Some authors prefer this extraction procedure because of some advantages. For example, samples are repeatedly brought into contact with fresh portions of extractant, which facilitates displacement of the transfer equilibrium. In addition, filtration is not necessary after leaching, which increases sample yield. Further, several simultaneous extractions can be performed in parallel because of the low cost of basic equipment [18]. However, Soxhlet also has some shortcomings: it is time consuming, labor intensive and requires the use of large volumes of organic solvents (300-500 mL) and large samples (10-30 g). These features go against some of the main objectives of so-called "green chemistry" such as sustainable development and being environmentally friendly. Recent modifications have tried to bring the Soxhlet technique closer to these objectives. Hence, a technical version designated automated Soxhlet extraction was developed as a more competitive extraction technique. This was initially implemented with the commercial equipment Soxtec® System HT, which provided fundamental savings in time and extractant volume [19]. Automated Soxhlet extraction (Soxtec) uses a combination of reflux boiling and Soxhlet extraction in two extraction steps boiling and rinsing, followed by solvent recovery. Despite such developments, Soxtec does not improve on the scarce versatility of the conventional Soxhlet device. Only 7% of the reports reviewed here have employed the Soxhlet technique [20,21,22,23,24] (Table 1.1) as also observed in the previous review published in 2012 [14]. Despite the development of Soxtec, the publications mentioned above used Soxhlet as the extraction technique. **Figure 1.1** summarizes all the information analyzed.

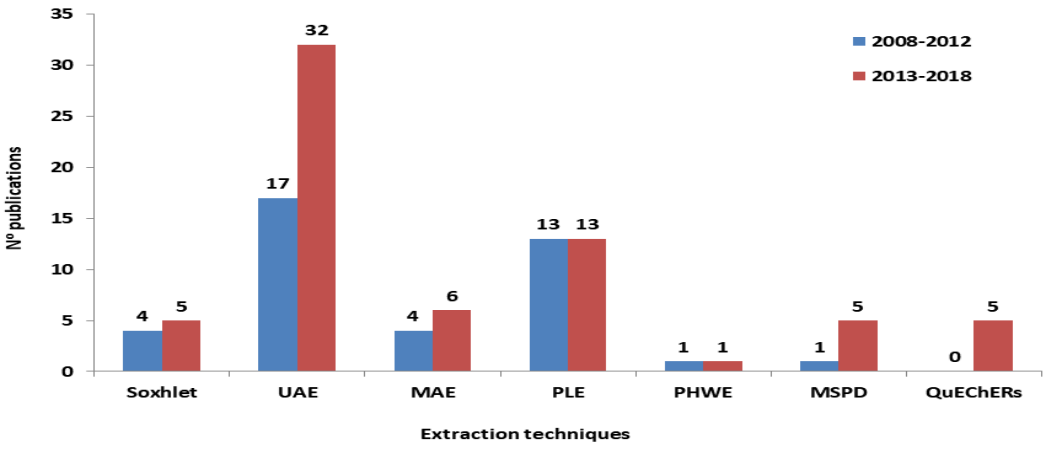


**Fig. 1.1. Extraction techniques for PPCPs in sewage sludge**

Ultrasound-assisted extraction (UAE) is an alternative to Soxhlet extraction for solid matrices and has been widely used in PPCP procedures. Some of the latest examples are described in three of the reports reviewed here [28,53,54]. The cavitation of UAE reduces the extraction time in comparison with Soxhlet but, in contrast, it is less reproducible. This cavitation process consists of bubble formation, growth and implosion occurring during the propagation of an ultrasound wave in a liquid medium [25]. The principle of ultrasound cavitation is described in a diagram included in the publication [142].

The solvent is chosen based on physical criteria such as viscosity, surface tension and vapor pressure. All these parameters will affect the acoustic cavitation phenomenon [26]. Sonication extraction is faster than Soxhlet extraction (30–60 min per sample) but filtration is required after extraction. UAE is an environment-friendly technique in that it is energy- and time saving. Compared to Soxhlet, less solvent is required and the extraction time is shorter. Hence, using ultrasound, extractions can be completed in minutes, simplifying manipulation and work-up, and employing just a fraction of the energy usually required for a traditional extraction method such as Soxhlet [27]. As mentioned earlier, many studies in the last six years have examined this extraction technique (**Figure 1.1**).

A more modern technique used to determine PPCPs in sewage sludge is microwave-assisted extraction (MAE). This approach uses microwave energy to directly heat the solvent to extract compounds of interest, thus accelerating the speed of extraction. The benefit of MAE is the use of small amounts of solvent compared to Soxhlet and sonication extraction (30 mL in MAE versus 300–500 mL for Soxhlet extraction) which enables the control of extraction parameters such as time, power or temperature [60]. In addition, this green technique offers protection for thermo-labile constituents. However, as UAE, MAE also has its shortcomings: a filtration step is required after extraction, and organic solvents and a subsequent extract cleaning-up step are needed. Further, the equipment for MAE is relatively expensive. Thus, probably because of all these downfalls, only a small number of studies addressing MAE have been reported in the literature reviewed [55, 61-65] over the last 6 years (Table 1.2). However, the number of studies reviewed is still higher compared to the previous review [14], which only mentioned four references [66-69].

Another extraction method is pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE). This is a fully automatic technology which uses low volumes of liquid extractants such as hexane, ethanol and acetone at high pressure (usually up to 200 bar) and temperature (usually up to 200 ºC) without reaching the critical point to recover those target analytes with short extraction times [70]. PLE has proven very effective for extracting target analytes. However, extracts usually contain a complex matrix as well. Thus, a clean-up procedure is often needed after extraction to remove interferences. Solid phase extraction (SPE) with a great variety of sorbents has been the most common clean-up technique when PPCPs are the target analytes [13,71-81]. However, gel permeation chromatography (GPC) has also been used to purify organic pollutants [35]. PLE has many advantages over traditional extraction techniques as efficient ways of increasing automation, shortening the extraction time and reducing the amount of organic solvents. PLE usually entails extraction times of around 15 minutes per sample and uses between 15-40 mL of solvent. In addition, the instrumentation allows for extraction in an unattended operation. It is regarded as reasonably easy and exhaustive, offering quantitative recoveries with little spare time spent on method development [70]. All these attractive features have meant that many of the works reviewed used PLE to extract PPCPs from sewage sludge. Some of the most relevant examples are [13,55,71,80,81]. The number of recent publications is comparable to those reported [82-84] (Table 1.2) in the previous review published in 2012 [14] (**Figure 1.1**).

An even more environmentally-friendly technique is pressurized hot water extraction (PHWE). This technique uses pressurized water as an extraction fluid at elevated temperature. Water has several positive features such as easy access, safety and can be recovery or disposed of with minimal environmental concerns [85]. Temperature is the most important parameter to optimize in this technique as it affects extraction efficiency and selectivity. Elevated temperatures provide certain advantages such as high diffusion, low viscosity and surface tension [85]. The best features of PHWE are the use of small amounts of organic solvents [86] and its low cost. In the future, this green extraction technique is expected to help manipulate large sample sizes for industrial applications. Despite these commented advantages, only two references of the use of PHWE as the extraction technique was found in the last 6-years reviewed [87,144] along with one more [88] in the previous five-year period [14].

Recently some authors have replaced the more traditional extraction techniques such as UAE or Soxhlet and also MAE or PLE with novel methodologies including MSPD (matrix solid-phase dispersion) or QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). These approaches have as their main goal to improve the method's sensitivity and selectivity as isolation and purification are combined in a single step. The main sources of error of most analytical methodologies are avoided. Main benefits are the short time required for sample preparation and their efficiency in cleaning-up the extract [93, 101].

MSPD for the extraction of PPCPs in sewage sludge was introduced in 1989 and applied to the extraction of solid, semisolid or viscous samples. It consists of homogenizing the sample with a dispersing agent (abrasive solid) onto a solid support, allowing the disruption of the sample and the extraction of target analytes by means of a suitable elution solvent [89]. The great interest in MSPD may be attributed to the advantages it offers and its simplicity and flexibility which have contributed to its choice over more classical sample preparation methods [90]. MSPDE is rapid, scarcely manual-intensive and eco-compatible. After extraction, depending on the nature of target analytes and the instrumentation used for their detection, a clean-up procedure may or not be needed. This

technique and PLE have sometimes been employed together as the solvents used at high pressures and temperatures increase analyte recoveries when interactions of the analytes with the solid matrix are really strong. The method's selectivity is related to the elution solvent utilized and the nature of the sorbent materials. Lipophilic sorbent materials such as $C_{18}$-bonded silica or $C_8$-bonded silica are employed in numerous applications, although the latter is used less frequently [90]. The solvents chosen for elution depend on the nature of the solid material. Organic solvent mixtures are mainly used, however, hot water offers excellent results in certain applications (mostly in PLE procedures). MSPD extraction has several benefits such as reduced amounts of solvents and sample, short extraction times, low cost and good performance at room temperature and atmospheric pressure with acceptable yields and selectivity. The technique is suitable for a great variety of analytes and environmental matrices due to its flexibility and versatility. Some reports exist in the literature [91-95] (Table 1.2) for the last 6 years. In contrast, only one study was found in the previous period from 2008 to 2012 [96]. This indicates a large increase in the use of this technique.

Finally, one of the most novel techniques employed to determine PPCPs in environmental matrices is QuEChERS. This procedure offers benefits such as the use of a small content of organic solvents, scarcely time consuming, good recoveries and high selectivity. It mainly consists of two steps, salting-out liquid-liquid extraction (SALLE) and dispersive solid-phase extraction (DSPE) for extract clean-up [97].

QuEChERS encompasses both extraction and clean-up steps for complex environmental matrices. This reduces sample preparation to approximately 20 minutes. The technique uses less solvent than ASE (usually up to 10 mL), and entails minimal times and costs. Some reports of QuEChERS applications exist in the literature reviewed here [98-102] (Table 1.2) but no studies addressed this issue in the five-year period before 2012 [14].

Overall, as depicted in **Figure 1.1**, UAE emerges as the most popular extraction technique (49%), followed by PLE (19%) and MAE (9%). Thus, the trend observed until 2012 reviewed by [14] has been maintained in the last six years. Nonetheless, UAE seems to have lately experienced a boost, most probably because of its simplicity and high performance as well as affordability and availability at most of laboratories around the world.

### 2.1.2. Clean- up

Most extraction techniques for PPCPs in sewage sludge are not sufficiently selective and a clean-up step is usually subsequently necessary. Some of the most common interfering constituents of sludge are compounds such as lipids and substances added to sewage sludge during processing such as surfactants and polymer colloids, among others. Although interference can occur at any stage of the analytical process, instrumental analysis based on liquid chromatography interphase to mass spectrometry by electrospray ionization is especially sensitive to matrix effects [55].

$C_{18}$ is a clean-up agent commonly used to remove interfering lipids and lipophilic compounds in extracts contained in organic solvents. PSA (primary and secondary amine) has also proved effective for the removal of acidic interferences such as humic and fulvic acids (main components of compost) among others [55]. $C_{18}$ and PSA (primary and secondary amine) are examples of some clean-up agents commonly used in dispersive solid-phase extraction (d-SPE) [102]. Thus, the choice of sorbent must be adequate to retain interferences present in each particulate sludge matrix. Deficiencies in the

extraction process have been also attributed to the presence of co-extracted matrix components [34].

Solid-phase extraction (SPE) is the most popular technique for the clean-up of PPCPs after extraction from sewage sludge, and from environmental samples in general [28,30,54,78]. This procedure is quick and simple to operate and can be easily automated and coupled to instrumental techniques such as liquid chromatography (LC) [103].

There are three general extraction mechanisms used in SPE: polar, non-polar and ion exchange. More than half of the works found in the literature during the last 6 years have employed reverse-phase SPE (63%). The retention mechanism is the interaction of non-polar groups of the analytes of interest and the non-polar functional groups on the sorbent (Van der Waals forces) [104]. In many cases, extraction was performed in a polar solvent [13,24,39-43,45-48,50,52,56,59,62,64,74,75,77,80,81]. Mixed-mode SPE is an extraction approach involving sorbents which are designed to exhibit two or more primary interactions for analyte retention. Most mixed-mode sorbents include hydrophobic functional groups in combination with ion-exchange functional groups. In some cases, Oasis MCX (Mixed-Mode Cation-eXchange) has been used for the clean-up of extracts containing acidic pharmaceuticals [39,62,64]. Oasis MAX (Mixed-Mode Anion-eXchange) has been also used in other cases [62,64]. However, the reverse phase sorbent patented in Oasis HLB (Hydrophilic-Lipophilic Balanced) has been the preferred option over the last six years [13,24,29,30,32,39,40,42,48-50,56,59,64,74,75,77,80,81]. It is a universal polymer reversed-phase sorbent that was developed for the extraction of a wide range of acidic, basic and neutral compounds from various matrices. Another type of adsorbent is based on $C_{18}$-silica and used to adsorb analytes of even weak hydrophobicity from aqueous solutions [43,52]. In the 1990s, a miniaturized variation of SPE emerged as a solid-phase microextraction technique (SPME). This method involves an alternative preconcentration technique to LLE or SPE. It consists of a silica fiber coated with a thin layer of an extractant polymer, which can be placed in the head space (HS-SPME) or subjected to direct immersion (DI-SPME) in solid, liquid or gaseous samples. As the fiber is desorbed in the injection port of a gas chromatography system, the use of solvents is eliminated and possible losses of analytes and contamination of the samples are reduced. [28,57] are examples found in the literature reviewed here.

Gel permeation chromatography (GPC), also known as size-exclusion chromatography (SEC), is a method in which component separation is based on differences in molecular weight or size. It requires short analysis times and small volumes of mobile phases. It has been widely employed to isolate and analyze biomacromolecular substances such as sugar, peptides, proteins, rubbers, and others, on the basis of their size. GPS has been also applied to PPCPs, usually in combination with other clean-up techniques. In particular, [35] made use of GPC along with a silica gel column to clean up 153 pharmaceuticals, herbicides, antioxidants, intermediates, organic solvents and chemical raw materials. Three studies reviewed by [14] for the period 2008 to 2012 included GPC and normal-phase SPE used together as the clean-up procedure [106-108].

Liquid–liquid extraction (LLE) is an effective separation method for compounds having different solubility in two immiscible liquids. These two liquids are generally water, with or without additives, and a nonpolar organic solvent. Polar compounds prefer the aqueous layer while nonpolar compounds are extracted into the organic layer. In salting-out systems, water-miscible solvents have been investigated for the extraction or concentration of analytes that cannot be extracted by conventional LLE methods. This salting-out often occurs at high salt concentrations [109]. However, LLE extracts are not particularly clean in comparison with other more intensive sample preparation

procedures. The first applications of this technique to PPCPs in sludge were reported by [54,63].

Overall, the vast majority of publications, 60% of the reports reviewed here, chose SPE as the clean-up approach, as shown in **Figure 1.2**. Only isolated examples of other techniques have been found such as florisil [51], silica [90] or $MgSO_4$ [98].

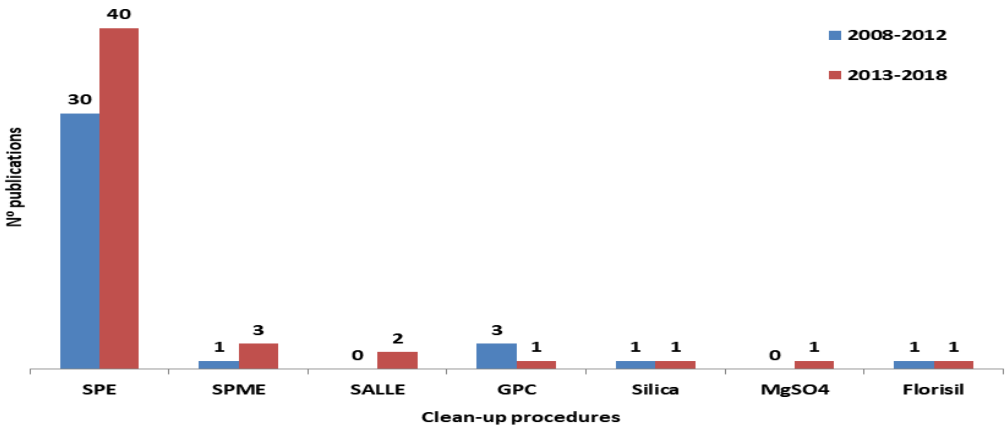


**Fig.1.2. Clean- up techniques for PPCPs in sewage sludge**

## 2.2. Instrumental analysis

Instrumental analysis for PPCPs in sewage sludge is basically based on chromatographic separation coupled to mass spectrometry. PPCPs are mostly polar compounds with limitations of volatility and/or thermal stability for their analysis by gas chromatography (GC) [28]. Nonetheless, these limitations have been overcome by derivatization processes such as acylation (acetylation), alkylation [33] and silylation [28,37,50,65]. GC is a relatively inexpensive instrumental technology which enables this kind of analysis to be carried out by a wide range of laboratories around the world, including those in developing countries [20,53]. Overall, 25% of the reports reviewed chose GC-based on instrumental techniques. In comparison to the period reviewed by [14], there seems to have been a decline in the popularity of GC **(Figure 1.3)**. Most GC approaches are coupled to mass spectrometry (MS) detection in both a single and tandem (MS/MS) modality. Other detection approaches were found coupled to GC such as electron capture detector (ECD) [22]. Triple quadrupole (QqQ) is the most common analyzer mainly used in selected reaction monitoring (SRM) mode for quantitative analysis [51,76]. However, some examples of target analysis in high resolution by quadrupole to time-of flight (Q-TOF) couplings have been also found in the literature [37,53,79]. As pointed out in the previous section, SPME is a pre-treatment technique which allows automation when coupled to GC and was employed by [28] and [129] for the analysis of 12 PPCPs and 8 macrocyclic musk fragrances in sewage sludge respectively. This constitutes the only examples of pre-treatment coupling to instrumental analysis in our realm.

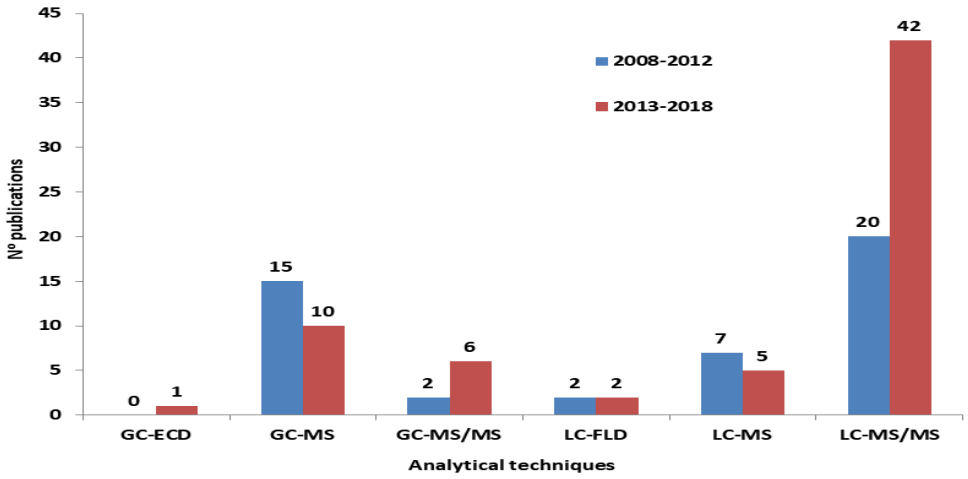


**Fig.1.3. Instrumental analysis techniques for PPCPs in sewage sludge**

However, despite the above, LC-based on instrumental analysis has become the most popular technique **(Figure 1.3)** in the determination of PPCPs in environmental matrices including sewage sludge. This is probably because of its higher versatility as a larger spectrum of compounds can be readily analyzed with no prior derivatization or alike. Again, mass spectrometry is the preferred detection option, but some examples (2) of coupling to fluorescence detection have been also found [61,99]. This repeats the scenario as in the period reviewed by [14] where a single example of this coupling was cited [110]. In contrast, ultraviolet (UV) detection cited years ago [111] is no longer an interesting option. Within MS modalities, MS/MS was found to have the greatest applicability, in particular using QqQ in SRM mode for target analysis. Hence, 63% of the LC works reviewed fit this classification. Nevertheless, interest in the use of other tandem combinations such as Q-TOF has been recently sparked due to improvements in the dynamic range and sensitivity of TOF. In addition, TOF analyzers offer a high resolution capacity. This ensures high selectivity and reduces the probability of false positive results. In addition, they open the possibility of qualitative analysis of un-known compounds, which is not readily available in QqQ. Electrospray ionization (ESI) is the most commonly used ionization approach as it allows mild ionization of the target analyte and molecular ions usually remain un-fragmented [47,75,100]. Nonetheless, apolar compounds might undergo poor ionization by ESI, and atmospheric pressure chemical ionization (APCI) is then recommended as in [31,49]. Weak acids and bases such as formic acid and ammonium acetate are usually used as mobile phase modifiers when working at +ESI and –ESI respectively. Moderate acidic (~3) and basic pHs (~8) are provided by formic acid and ammonium acetate respectively. In this regard, a larger number of PPCPs contain basic functional groups (such as amines) with pKa values above pH 3 rather than acidic functional groups (such as alcohols) with pKa values below pH 8. Therefore, PPCPs are more prone to be positively ionized and are more efficiently analyzed by +ESI rather than -ESI.

Within LC, fast chromatography has emerged as an improved modality over high performance liquid chromatography (HPLC). The ultra-high version (UHPLC) was

introduced under the trade mark UPLC™ in 2004 and triggered many advances in instrumentation and column technology, which have led to a significant increase in resolution, speed and sensitivity. Column efficiency increases with reduction of stationary phase particle size (usually <1.7µm) and mobile phase delivery is done at <15,000 psi (about 1000 bar) [112]. Separations are mostly completed in less than 10 min and some even in under 2 min [32,62,72]. UHPLC often provides narrow peaks (in few seconds or even less) offering a high-speed detection response (> 100Hz) [112].

Over these past 6 years, out of 47 of the applications using LC, 14 were fast chromatography. This in comparison to the previous 5-year period reviewed by [14], in which only 8% of studies examined this kind of liquid chromatography, reveals a clear upward trend in the use of UHPLC likely attributable to its many benefits mentioned. Overall, as depicted in **Figure 3**, LC has been the most popular instrumental technique (73%) for the determination of PPCPs in environmental matrices including sewage sludge. Hence, the trend observed up until 2012 and reviewed by [14] has been maintained over the last six years.

## 2.3. Currents trends and future perspectives in the determination of PPCPs in sewage sludge

The concept of "green chemistry", otherwise known as sustainable chemistry, was introduced 20 years ago and refers to the design of chemicals and processes that reduce and eliminate the use or generation of hazardous substances. When applying and proposing new methods and processes of analysis, sustainability should be considered a necessary characteristic. By automatizing a technique, the use of resources, including time, usually becomes more efficient. In addition, human error and analyst exposure to hazards are minimized [113]. Besides automation, miniaturization in analytical chemistry has also become a dominant trend recently replacing traditional sample preparation. The goal is to provide high extraction efficiencies in short times and minimize the amount of sample and so reduce the consumption of reagents and solvents. In addition, after automation and miniaturization, many sample preparation methodologies are susceptible to being incorporated into instrumental analysis systems such as GC or LC [113]. Hence, in the early 2000s, a research group developed simple procedures based on SPME or USAEME (ultrasound-assisted emulsification-microextraction) for the analysis of allergenic fragrances, synthetic musks, phthalates and preservatives in water samples [114-116]. While the use of miniaturized and automatized methodologies for the determination of PPCPs in water matrices is a reality [117,118], the reports reviewed here barely show the use of miniaturization techniques for the determination of the contaminants of interest in sewage sludge. Only two studies found in the literature offer an analytical method for the determination PPCPs and PCPs in sewage sludge by DI-SPME-On-fiber derivatization-GC-MS [28] and HS-SPME-GC-MS [129] respectively. Interest in microextraction processes has been renewed due to the incorporation of new materials, either as suitable substitutes for conventional halogenated organic solvents or other types of toxic reagents [113]. At present sufficient technology already exists for research groups to develop miniaturized and automatized analytical methods for the determination of PPCPs in sewage sludge.

Additionally, there are concerns in the scientific community over the presence of transformation products (TPs). Many of these TPs have shown to be as pernicious as the parent PPCPs they come from. Clear efforts are currently focusing on the identification in environmental water samples of metabolites and other TPs generated over the PPCP

life cycle, such as during treatment processes in WWTPs [28,36]. However, there is no evidence in the literature yet of this trend in relation to sewage sludge.

Many PPCPs consist of chiral molecules and each enantiomer usually exerts different toxicity according to its biological properties [119]. Hence, reports exist of the determination of chiral pharmaceuticals by chiral LC-MS/MS [64,120] in sewage sludge samples. Nonetheless, much more work is still needed in this area.

Future perspectives related to the development of new sample preparation methods differ depending on the type of the pollutant. There is increasing interest in nanotechnology in important sectors of science and technology such as engineering, medicine or agriculture, among others. Nanotechnology is making progress in technologies for protecting the environment too. However, nanotechnology's unique characteristics can lead to unforeseen environmental problems [121]. In parallel, the use of novel solid and liquid phase materials has increased in the last years including nanomaterials (NMs), ionic liquids (ILs) or supramolecular solvents (SUPRAS) used in the analysis of environmental samples. Engineered nanomaterials (ENMs) are materials or chemical substances with particle sizes between 1 to 100 nanometers in at least one dimension [122]. There is great interest in innovations produced in the industrial, commercial and medical sectors due to the physical and chemical properties of these materials. However, some of their properties (chemical reactivity, surface area and particle size) pose a risk to health and the environment [123]. Some works have described applications of nanoadsorbents in environmental water samples [124,125]. In the near future, NMs could be applied to sewage sludge samples. SUPRAS are nanostructured liquids generated from compounds with both hydrophilic and lipophilic properties (amphiphiles) [126]. SUPRAS have been employed for the extraction and preconcentration of emerging pollutants in environmental water samples [127]. However, there are still no reports of applications of SUPRAS to sewage sludge. ILs are salts whose ions are poorly coordinated, which makes these solvents liquid at temperatures below 100°C, or even at room temperature (room temperature ionic liquids) [128]. One publication reports on the determination of musk fragrances in sewage sludge based on IL-HS-SPME followed by GC-MS/MS [129].

## 3. Data processing

Environmental sample matrices are complex and their analysis and subsequent data processing are extremely difficult. For many years, a traditional approach offering reliable rapid identification and quantification of target compounds has been used [130]. In total, 98% of the reports reviewed employed target analysis to determine PPCPs in sewage sludge samples. However, target analysis has the drawback that only a limited number of compounds can be determined and many pollutants present are ignored [131].

A comprehensive picture can be obtained by non-target analysis which does not require "a priori" selection of contaminants. This approach is able to detect any analyte present above the MDL. In addition, retrospective analysis is possible [131]. Anthropogenic compounds such as pharmaceuticals and personal care products, flame retardants, plasticizers, polymer additives and other well-known persistent organic pollutants can be identified using this approach. Suspect screening is a non-target analysis. Both suspect and non-target analysis are based on the power and development of high-resolution mass spectrometric instruments. These techniques serve to acquire full scan spectra and allow a retrospective analysis of emerging contaminants after the data has been acquired, while

providing two essential factors for non-target analysis: accurate-mass and high-resolution [131]. The most common MS analyzers used for this purpose, such as Orbitrap or the Fourier transform ion cyclotron resonance (FT-ICR) device, can be linked to different ionization sources (ESI, APPI and APCI) and different separation techniques (GC, LC and GCxGC) depending on the class of compounds to be examined [128]. However, in the past 6 years, only one study has used this method to determine emerging contaminants in sewage sludge. This study [37] described a non-targeted approach based on GCxGC-TOFMS. In contrast, numerous reports exist of a non-target approach for the determination of these contaminants in wastewater; some examples being [132,133].

Target methods are usually quantitatively more powerful as they show a greater sensitivity and dynamic range than untargeted methods. Regardless, analyte quantification is usually performed through the use of authentic chemical standards and the construction of calibration curves. Calibration curves are used to understand the instrumental response to an analyte and to predict its concentration in a sample. Over the past six years, the calibration methods reported in the literature to determine PPCPs have been based on approaches including an internal standard, standard additions, matrix matched or external standard. The choice of a specific calibration method depends on a number of factors such as affordability, matrix complexity, and number of samples, among others. External standard calibration has been one of the most commonly used calibration approaches among the reports reviewed here. This approach is inexpensive as well as quick and easy to set up. On the downside, it is greatly affected by the stability of the chromatographic detector system and the presence of chromatographic interferences in the sample. Some of the publications reviewed make use of this quantification approach [53,75,77,95] (Table 1.1 and 1.2). When matrix problems are suspected, a more reliable calibration may be obtained via matrix-matched calibration. This may make up for matrix effects although it does not eliminate the underlying cause because the effect intensity may differ from one matrix or sample to another, and can be also affected by the matrix concentration. In fact, matrix-matched calibration is a particular type of external calibration in which the calibration standards are prepared using a simulated sample that initially does not contain the analyte. Of the reports reviewed, 22% chose matrix-matched as calibration method (Tables 1.1 and 1.2), which represents an increase in comparison with the period reviewed by [14], in which only 6% of the publications selected the matrix-matched method [134-136]. Another calibration alternative is based on standard addition. This method is more accurate and precise and overcomes more matrix effects than external and matrix-matched calibration approaches, as it uses the sample itself to build the calibration curve. However, it entails the preparation of a different calibration curve per sample. It is therefore labor intensive, time-consuming, and requires large sample amounts, which is usually in disagreement with green chemistry principles. Overall, 14% of the publications reviewed here used this calibration method (Tables 1.1 and 1.2). In contrast, previous publications reviewed by [14] reported this calibration approach less frequently (9%). Finally, an internal standard (IS) is a reference species with similar physicochemical properties and similar analytical behavior to the compounds of interest not expected to be found in the samples. This calibration method is not as useful for GC and HPLC methods involving non-MS detectors unless the internal standards can be separated from target compounds chromatographically. The advantage of this calibration method is that fluctuations are monitored in every sample. It assumes that the behavior of the IS is identical to that of the analyte. Thus, the selection of a suitable IS is mandatory. The use of internal standard calibration approaches has experienced a boom in the last few years. In effect, 47% of the reports reviewed selected this procedure (Tables 1.1 and 1.2) versus 4% reported in the prior review [14]. In

particular, the use of stable-isotope-labeled analogues of the analytes has become popular because of its efficiency and reliability to compensate for any alteration in the signal due to casualties across the whole analytical process. However, for highly multi-component applications, it requires a significant economic investment, unaffordable for many laboratories.

**Figure 1.4** depicts the frequency of each calibration method used in the reports reviewed. The use of isotopically labeled analogues in internal standard calibrations has been the most popular choice.

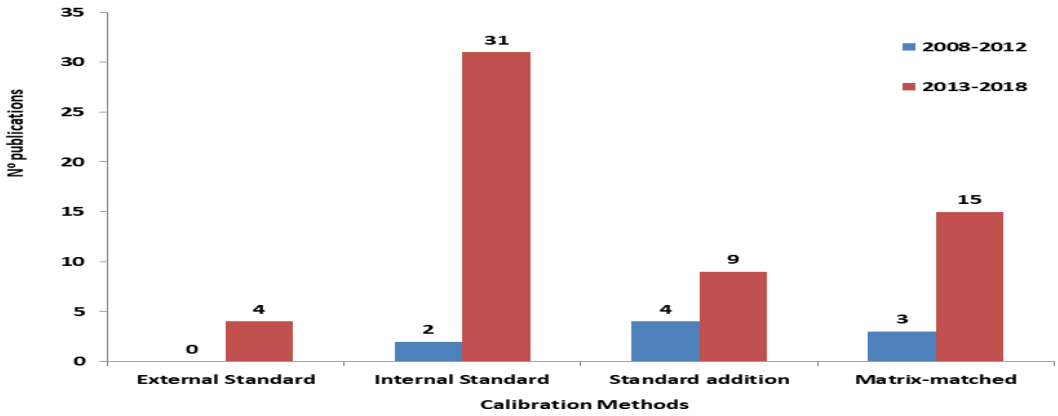


**Fig. 1.4. Calibration methods used in the quantificaction of PPCPs in sewage sludge**

## 4. Validation

The purpose of validation of an analytical procedure is to confirm that the analytical method used for experimental tests is suitable for that purpose. Method validation was established in analytical laboratories in the late 1970s, recognizing its importance in obtaining standard methods. The United States Food and Drug Administration (FDA) [137] and Eurachem [138] have published guidelines for methods validation.

To a large extent, the reliability and capacity of analytical methods have improved to a large extent as a result of recent technical advances [139]. The main validation parameters provided in the publications are (Tables 1.1 and 1.2):

a. Accuracy is the closeness of agreement between test results across the specified range and an accepted reference value. In our particular case, it is expressed as the percentage recovery of each analyte after the whole analytical protocol (absolute recovery). Some authors also provide improved recovery rates after adjusting for method deficiencies when applying an internal standard calibration approach (relative recovery). The reports reviewed showed considerably high analyte relative recoveries. Thus, 35 out of 67 publications showed percentages higher than 70% and 22 out of 67 publications obtained values below 70%. In contrast, 17 out of 47 publications were found for the five years before 2012 with percentages higher than 70% and 13 studies with values below 70%.

b. Precision is the closeness in agreement between individual results obtained for a repeatedly applied procedure on a homogeneous sample, comprising repeatability and intermediate precision. In our particular case, method repeatability is usually expressed as the standard deviation, relative standard deviation or coefficient of variation. Overall, 72% of the reports reviewed cited values below 20%. In comparison, for the period reviewed by [14], 23 out of 47 publications reported values below 20%.
c. Limits of detection (LOD) and limits of quantification (LOQ) can be directly obtained from the linearity test in the validation protocol. Hence, the lowest amount of analyte that can be detected under the stated experimental conditions is the LOD, while LOQ is the lowest amount of analyte that can be quantitatively determined with precision and accuracy under the stated experimental conditions. Among the publications included in the present review, 35% obtained LOQs below 100 ng $g^{-1}$. Additionally, 16 and 22 out of 67 publications obtained LOQs below 50 and 10 ng $g^{-1}$, respectively. These figures reflect the improvement in signal to noise ratios of current analytical methodologies produced over the last few years. Effectively, LOQs levels were commonly reported as LODs in studies conducted before 2012.
d. The matrix effect is attributable to components of the sample matrix that co-elute with the compound(s) of interest and interfere with the ionization process in the mass spectrometer. This may cause ionization suppression or enhancement and negatively affect method accuracy. It is usually expressed as the percentage of signal suppression, and consequently negative values are interpreted as signal enhancement. In most cases, signal suppressions were measured. In contrast to that observed in the review of 2012 [14], strong effects of signal suppression were described including values from 14 to 100% [140] or higher than 30% [141].
In one study [34], 148 pharmaceuticals and illicit drugs were analyzed in sewage sludge and the matrix effect assessed. For 12 out of the 148 target compounds, a signal enhancement in the range -11 to -90% was reported, and for 136 target compounds, signal suppression was reported in the range 3-92%.
e. The dynamic range is closely related to the response of the instrumental detector, and describes the concentration span, in orders of magnitude, over which the method provides a response proportional to the concentration of a given compound. Accordingly, linearity ranges of 3 orders of magnitude are usually reported for single quadrupole [28] and TOF [100] MS detectors, and of 5 orders of magnitude for triple quadrupole [102] MS detectors.

**Tables 1.1 and 1.2** summarize the validation values cited in the 67 reports reviewed for the determination of PPCPs in sewage sludge samples from 2012 to the present.

## 5. Impacts of sewage sludge analytical procedures on validation parameters

Each stage in the analytical procedure (extraction, clean-up, instrumental analysis, etc.) may to some extent have an effect on the validation parameters examined.

Extraction and clean-up steps are thought to be the main contributors to absolute recovery [55]. In the literature reviewed, various studies have addressed the determination of PPCPs both in sewage sludge and sewage. In many of those cases, methodology was common for both matrices but an extraction step was added at the beginning of the protocol for the sludge samples. For instance, Křesinová et al. [72] used PLE followed by

SPE with ENVI C18-DSK SPE disk and LC-ToFMS for the determination of PPCPs in sludge. The same methodology was employed when these PPCPs were determined in water samples, but a PLE extraction step did not precede the protocol. This extra step for the solid samples led to lower absolute recoveries for most of the compounds, indicating how extraction influences method accuracy. Accordingly, amitriptyline, 2-chloroprothioxanthen-9-one and melitracene carbinol rendered recoveries of 97.6%, 96.7%, 88.1%, respectively. These percentages decreased to 92.8%, 89.5 % and 86.8%, for the same compounds in solid samples [72]. Additionally, López-Serna et al. [28] showed how dramatic the impact of the extraction step can be on the accuracy. These authors employed a fully automated method based on online extraction by DI-SPME followed by on-fiber derivatization coupled to GC-MS for sewage samples. In sewage sludge samples, UAE preceded the sewage methodology. The absolute recoveries reported in this paper for compounds such as ibuprofen, salicylic acid and diclofenac were 77.77%, 21.43%, and 83.07%, respectively, in sewage samples. However, in sludge, these recoveries dropped to 18.18% for ibuprofen, 17.92% for salicylic acid, and 65.89% for diclofenac. Among the different extraction techniques discussed in the present paper, UAE, MAE and PLE seem the most popular. PLE is considered to be much more effective at extracting analytes from solid samples than UAE or MAE, leading to higher real recoveries. However, PLE is also described to extract more components of the matrix along with the analytes of interest. This means the associated matrix effect diminishes the given absolute recovery rate [70]. Nonetheless, PLE and MAE are usually shown to be slightly more efficient than UAE for extracting PPCPs from sludge as observed by Dorival-García et al. [55]. For instance, Gao et al. [77] tested a method based on PLE-SPE-LC-MS/MS and the absolute recoveries reported for compounds such as sulfamethoxazole, tetracycline and oxytetracycline in sludge samples were 78%, 54%, and 52%, respectively. Similarly, Shafrir et al. [49] used a method based on UAE-SPE-LC-MS/MS and reported absolute recoveries such as 17%, 22%, and 17% for sulfamethoxazole, tetracycline and oxytetracycline, respectively. Gago-Ferrero et al. [34] developed a method that combined UAE and LC-MS/MS, and absolute recoveries reported in this paper for compounds such as propranolol, diclofenac and sulfamethoxazole in sewage sludge samples were 53%, 27%, and 63%, respectively. In contrast, Eyser et al. [73] made use of a method based on PLE followed by LC-MS/MS and reported recoveries of up to 96% for propranolol, 85% for diclofenac, and 33% for sulfamethoxazole in sewage sludge samples.

The presence of the analytes of interest along with matrix components in the sample influences every step of the analysis. GC combined with EI ionization MS operating in SIM mode did not cause any apparent matrix effect during the determination of PPCPs in sewage sludge [50]. In LC, the matrix effect differs when it is interphased with MS by ESI or APCI. Lonappan et al. [31] compared the use of LC-ESI-MS/MS and LDTD-APCI-MS/MS to quantify diclofenac in wastewater sludge samples. These authors reported that matrix effects due to interactions between diclofenac and co-extracted compounds could cause signal suppression in the ESI source. In fact, competition for ionization could exert signal enhancement or suppression phenomena [50] [73]. However, they reported that matrix interferences in LDTD-APCI-MS/MS did not significantly affect the signal [31]. Additionally, Luque-Muñoz et al. [54] used UHPLC-MS/MS in their instrumental analysis and reported matrix effect values such as -25% for propylparaben or -37% for benzophenone-6. However, Abril et al. [58] reported matrix effects of -79% for propylparaben and -81% for benzophenone-6 for HPLC-MS/MS. This lesser matrix effect might be attributed to the better resolution capacity of UHPLC. While in conventional HPLC, analytes could co-elute with the matrix compounds, in UHPLC

they may reach the detector at different retention times. Sample preparation usually includes a clean-up step that partially removes interferences from the matrix [73]. SPE has been the preferred method among those examined here due to its simplicity and the use of small volume of organic solvents. However, these clean-up procedures might have marked performance deficiencies in multi-residue-methods [73]. Oasis HLB SPE cartridges are based on a co-polymer which is very efficient at recovering a wide range of compounds in environmental matrices. Nonetheless, it is not highly selective and matrix interferences may not be successfully reduced [62]. Petrie et al. [62] observed that Oasis MCX and MAX reduced matrix suppression more satisfactorily. These authors reported matrix suppression values of 59.2% for diclofenac, 88.6% for naproxen and 80.0% for ibuprofen using MCX SPE [62]. Other authors such as Gago- Ferrero et al. [33] reported matrix enhancement values for the same pollutants: -18% for diclofenac, -36% for naproxen and -43% for ibuprofen without the use of any clean-up step. After comparing examples from the literature for sewage samples, we found that Klančar et al. [143] employed Strata X cartridges for SPE combined with LC-MS/MS and reported matrix effect values of 83% for naproxen, 79% for propranolol and 96% for tramadol. These matrix effect rates are substantially higher than those observed by Petrie at al. [62] who used Oasis HLB-based SPE followed by LC-MS/MS and reported percentages of around 30%, 57% and 62% respectively for the same compounds.

Precision (expressed as repeatability) is usually affected by the number of stages included in the analytical procedure. A strategy to achieve good precision has been to automatize some of the method stages (e.g., PLE, SMPE) to minimize the human error impact. In the literature, two fully automated methods DI-SPME – on fiber derivatization-GC-MS [28] and HS-SPME-GC-MS [129] have been used to determine PPCPs and PCPs in sewage sludge, respectively. López-Serna et al. [28] reported satisfactory intra-day repeatability (expressed as %RSD) values such as 0.87% for propylparaben, 1.59% for naproxen and 2.99% for triclosan, among others. Vallecillos et al. [129] also reported good intra-day repeatability results such as 1% for exaltone, 8% for muscone, and 9% for exaltolide, among others. However, SPME fibers used for a large number of samples might lead to significant carry over effects. López-Serna et al. [28] reported carry over rates of up to 10% and 13% for diclofenac and triclosan, respectively.

Sensitivity is mainly affected by the instrumental analysis technique employed [28]. In the revised literature, different groups have examined the use of similar methods with different detectors such as FL [61], Q-MS [13], QqQ-MS [62], or QToF-MS [72] for the determination of PPCPs in sludge samples. For instance, Morales-Toledo et al. [61] developed a method based on MAE and SPE combined with UHPLC-FLD for the determination of pharmaceuticals in sludge samples, and reported method LODs for naproxen and ibuprofen below 86.5 ng $g^{-1}$. Much lower LODs were observed by Petrie et al. [62] for a similar method based on MAE and SPE followed by UHPLC-MS/MS. In particular, they reported method LODs of 0.07 ng $g^{-1}$ for ibuprofen and 0.60 ng $g^{-1}$ for naproxen. Among the analyzers used in mass spectrometry, QqQ has usually provided lower LODs than QToF. Hence, Peysson et al. [100] made use of a method based on QuEChERs followed by UPLC-QToF and reported LODs as low as 17 ng $g^{-1}$ for sulfamethoxazole and 3 ng $g^{-1}$ for propranolol, among others. Even lower limits of 0.6 ng $g^{-1}$ and 0.3 ng $g^{-1}$ respectively for the same compounds were reported by Cerqueira et al. [101] for a similar pre-treatment method followed by UHPLC-QqQ-MS. The use of GC usually leads to higher LODs in comparison to LC, even when the detection method is MS. This is usually attributed to incomplete derivatization of the non-volatile PPCPs and/or a poorer ionization rate of the resulting substance. UHPLC provides narrower

chromatographic peaks than conventional HPLC. Accordingly, the same area will offer a greater height, which entails an increase in signal intensity, and so sensitivity. For instance, Gago-Ferrero et al. [34] achieved LOQs of 4.1 ng $g^{-1}$ for diclofenac and 9.8 ng $g^{-1}$ for salicylic acid by applying a method based on UAE and UHPLC-MS/MS. In contrast, Boix et al. [38] reported lower limits (eg., 63 ng $g^{-1}$ for diclofenac and 35 ng $g^{-1}$ for salicylic acid) using a similar method but with HPLC as the chromatographic stage.

Selectivity and throughput (multiresiduality) are usually improved following the same pattern as sensitivity. Thus, the probability of providing false negatives or positives is decreased when a MS detector is used, especially if in a tandem configuration (QqQ or QToF). Gago-Ferrero et al. [34] used LC-MS/MS as the instrumental analysis technique for the simultaneous determination of 148 pharmaceuticals and illicit drugs in sewage sludge. Similarly, Peysson et al. [100] used LC-ToF/MS to determine 136 pharmaceuticals and hormones in sewage sludge. In contrast, Morales-Toledo et al. [61] only determined four substances (acetylsalicylic acid, ibuprofen, naproxen and gemfibrozil) in sludge samples by LC-FLD.

Differences in linearity range have been reported depending on the instrumental detector. Hence, for instance methods including QqQ usually attain 5 orders of magnitude [102]. However, up to 3 orders are reported for QToF-based methods [100].

Regardless of these factors, through the use of quantification approaches such as internal standard with isotope dilution, standard addition or matrix-matched techniques most technical deficiencies during extraction, clean-up, instrumental analysis, etc. may be circumvented, compensated and corrected. This means that a partial, non-optimal method developed for the pre-treatment and instrumental stages might still be sufficient to achieve a methodology capable of fulfilling analytical requirements, provided sensitivity is appropriate and the quantification approach is powerful.

## 6. Conclusions

The studies reviewed here examining the determination of PPCPs in sewage sludge consider a wide variety of emerging pollutants in environmental matrices. The most frequently investigated PPCPs belong to the class of pharmaceutical products. In effect, 49 out of the 67 reports reviewed focused on the detection and quantification of pharmaceuticals in sewage sludge.

In some studies, traditional sample pre-treatment techniques such as Soxhlet were replaced with more modern techniques such as MAE or PLE, or alternative techniques like QuEChERS or MSPD. However, UAE emerged as the most popular extraction technique for determining PPCPs in sewage sludge reported in almost half of the publications. This method provides safe, fast and easy sample preparation. It also makes use of small sample sizes and amounts of solvents. Usually after the extraction step, a clean-up protocol is needed as extraction is never completely selective. For this purpose, SPE was the technique most frequently used on pollutants after their extraction from environmental samples. For the determination of PPCPs in sewage sludge, LC and GC coupled to MS were the techniques of choice. Among the LC procedures, several studies chose UHPLC over HPLC because of its better resolution and shorter run times as well as its lesser demands in terms of solvent and sample quantities.

In recent years, novel solid and liquid phase materials and miniaturization and automation of the analytical techniques are becoming a dominant trend as they eliminate the limitations of current analysis technologies. Minimizing sample size decreases the consumption of expensive and toxic reagents and solvents, thus fulfilling the principles of green chemistry.

Most reported studies employed a target analysis to determine PPCPs in sewage sludge samples. Only one of the studies reviewed applied a non-target quantification method. Thus, a challenge to be addressed in the near future might be the individual treatment of each sludge-associated matrix. A boost in non-targeted approaches is expected for the determination of PPCPs in sewage sludge, as occurred for their analysis in aqueous matrices.

Finally, this review reports improved validation parameters in comparison with previously reviewed periods, especially regarding precision and sensitivity. This is mostly attributed to developments in analytical instrumentation.

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# Chapter II. Establishment of optimal experimental conditions for sample pre-treatment

## 1. Introduction

The term *"emerging pollutant*" has begun to have great relevance in recent years and corresponds to those compounds of diverse origin and chemical nature whose presence and consequences in the environment have gone unnoticed until relatively recently [1].

Pharmaceuticals and personal care products (PPCPs) are a group of emerging contaminants that include a great variety of chemical substances that differ in their structure, function and properties [2]. PPCPs include a wide range of chemical compounds such as pharmaceuticals (PhACs), personal care and hygiene products (PCPs), surfactants, flame retardants, endocrine disrupting compounds (EDCs) or industrial additives.

For several years, diverse studies have confirmed the presence of PPCPs in various environmental matrices at concentrations to capable of causing adverse effects on the ecosystems and human health [3]. Principal discharges of PPCPs into the environment depend on the industrial and agricultural waste, pharmaceutical industry, accidental bills and principally urban wastewater after incomplete adsorption being excreted by the urine and feces [4].

Despite the potential risk posed by PPCPs, there are not currently regulations governing the discharge limits of these compounds present in wastewater when they are discharged into rivers or the sea, with the exception of two compounds such as nonylphenol and octylphenol. However, in recent years, some measures have started to be taken to limit the discharge of certain compounds. In January 2012, the European Commission made a proposal [5] to change **Directive 2000/60/EEC** with regard to priority substances in the field of water policy where the maximum allowable concentrations in surface water corresponding to three PPCPs such as 17α-ethinyl estradiol, 17 β-estradiol and diclofenac. In countries such as Canada, the use of triclosan in consumer products has been prohibited due to its endocrine disrupting nature and relevant associations such as the United States Environmental Protection Agency (EPA) have requested the banning of this compound in consumer products.

## 2. Characterization of the selected compounds

In this Doctoral Thesis, a total of 14 PPCPs belonging to diverse categories (i.e., pharmaceuticals, endocrine disruptors, preservatives and fungicides) were initially selected as target analytes. The selection criteria were based on their high use in daily life, ubiquity in aquatic environments and/or recognized toxicity. The most significant physical-chemical properties are reported in **Table A1** (as **Appendix I**). These compounds of interest are classified in different groups:

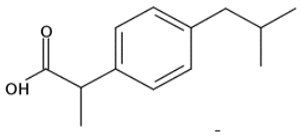
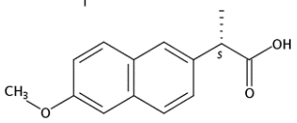
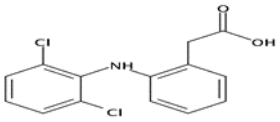
### 2.1. Pharmaceuticals

Pharmaceuticals are the emerging contaminants that have attracted the most interest since the 1990s [2]. In fact, the most widely prescribed drugs in medicine are analgesics/anti-inflammatories such as ibuprofen and diclofenac.

- Analgesics/anti-inflammatories: Anti-inflammatories are used to prevent or reduce inflammation of tissues as well as to reduce certain ailments associated

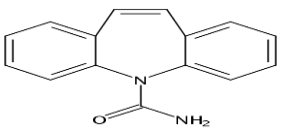
with flu processes due to their analgesic nature. In addition, they are compounds of acidic character since they present in their structure a carboxyl group as it can be observed in the compounds shown in **Table 2.1**.

**Table 2.1. Analgesics /anti-inflammatories compounds**

| Compound Name | Molecular Formula | Molecular Weight (g/mol) | Chemical Structure |
|---|---|---|---|
| Ibuprofen (IBP) | $C_{13}H_{18}O_2$ | 206.3 |  |
| Naproxen (NPX) | $C_{14}H_{14}O_3$ | 230.3 |  |
| Diclofenac (DCF) | $C_{14}H_{10}Cl_2NO_2Na$ | 318.1 |  |

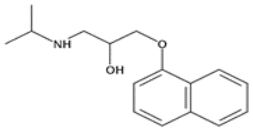
- Neuropharmaceuticals: These compounds are used in the treatment of diseases affecting the nervous system. Carbamazepine (**Table 2.2**) is a widely consumed compound due to its antiepileptic characteristics. However, its recalcitrant character makes it difficult for its elimination during biological wastewater treatments.

**Table 2.2. Neuropharmaceutical compound**

| Compounds name | Molecular Formula | Molecular Weight (g/mol) | Chemical Structure |
|---|---|---|---|
| Carbamazepine (CBZ) | $C_{15}H_{12}N_2O$ | 236.3 |  |

- Antihypertensives: These compounds are frequently used since high blood pressure is the most common cardiovascular disease in the world. Propranolol (**Table 2.3**) is a β-blocker present in municipal water effluents reaching levels above 0.017 μg/L.

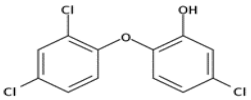
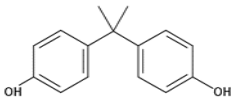
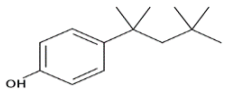
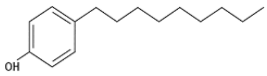
**Table 2.3. Anti-hypertensive compound**

| Compound Name | Molecular Formula | Molecular Weight (g/mol) | Chemical Structure |
|---|---|---|---|
| Propranolol (PNL) | $C_{16}H_{21}NO_2$ | 259.3 |  |

## 2.2. Endocrine disrupting compounds

Endocrine Disrupting Compounds are chemicals that have the ability to alter the hormonal system of humans and animals, causing adverse health effects [6]. In this case, some compounds were selected such as bisphenol A (used in the manufacture of plastics), triclosan (a component used as an antibacterial in toothpaste) and two compounds used in cleaning products such as 4-tert-octylphenol and 4-nonylphenol (**Table 2.4)**.

**Table 2.4. Endocrine disrupting compounds**

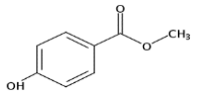
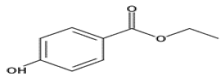
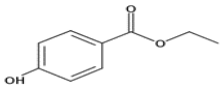
| Compound Name | Molecular Formula | Molecular Weight (g/mol) | Chemical Structure |
|---|---|---|---|
| Triclosan (TCS) | $C_{12}H_7O_2Cl_3$ | 289.5 |  |
| Bisphenol A (BPA) | $C_{15}H_{16}O_2$ | 228.3 |  |
| 4-tert-octylphanol (OP) | $C_{14}H_{22}O$ | 206.0 |  |
| 4-nonylphenol (NP) | $C_{15}H_{24}O$ | 220.3 |  |

## 2.3. Parabens

Parabens are currently preservatives and widely used in cosmetics and pharmaceuticals but also in food and industrial products. These compounds and their salts are mainly used for their bactericidal and fungicidal properties. They are considered ideal preservatives

because they have a wide anti-microbial activity and are stable with pH variation [7-8]. The parabens studied correspond to methylparaben, ethylparaben and propylparaben (**Table 2.5**).

**Table 2.5. Parabens compounds**

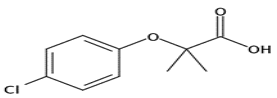
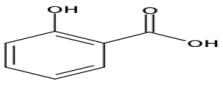
| Compound Name | Molecular Formula | Molecular Weight (g/mol) | Chemical Structure |
|---|---|---|---|
| Methylparaben (MP) | $C_8H_8O_3$ | 152.2 |  |
| Ethylparaben (EP) | $C_9H_{10}O_3$ | 166.2 |  |
| Propylparaben (PP) | $C_{10}H_{12}O_3$ | 180.2 |  |

Others compounds of interesting are:

- Clofibric acid (**Table 2.6**) is a metabolite of the drug clofibrate. It has a role such as anticholesteremic drug, antilipemic drug, antineoplastic agent, marine xenobiotic metabolite, and herbicide.

- Salicylic acid is a conjugate acid of a salicylate. It has direct activity as an anti-inflammatory agent and acts as a topical antibacterial agent due to its ability to promote exfoliation (**Table 2.6**).

**Table 2.6. Other compounds selected**

| Compound Name | Molecular Formula | Molecular Weight (g/mol) | Chemical Structure |
|---|---|---|---|
| Clofibric acid (CA) | $C_{10}H_{11}O_3Cl$ | 214.6 |  |
| Salicylic acid (SA) | $C_7H_6O_3$ | 138.1 |  |

## 3. Material and methods

### 3.1. Standards and reagents

All PPCPs standards were of high purity grade (> 95%, Sigma-Aldrich, Madrid, Spain) and were acquired as neutral non-solvated molecules, except for diclofenac (sodium salt). PPCPs acquired are summarized in **Table A1** (as **Appendix I**).

Individual stock solutions at 1,000 mg $L^{-1}$ for all PPCPs standards were prepared in methanol (MeOH). From them, a stock solution with all the target compounds was prepared in MeOH at 20 mg $L^{-1}$. Fresh serial dilutions (2, 0.5, 0.05, 0.005) mg $L^{-1}$ in acetone were subsequently prepared from it when need them. All solutions were stored at -20 °C in darkness.

High purity solvents, i.e., LC-MS Chromasolv® Ethyl Acetate (EA) grade from Fluka (Madrid, Spain), SupraSolv® GC-MS MeOH grade by Merck Millipore (Madrid, Spain), Sodium Chloride (NaCl) and Hidrochloric acid (HCl) with 37% purity were supplied by Panreac (Barcelona, Spain). Aluminium oxide by Sigma-Aldrich (Tres Cantos, Madrid, Spain). Acetone ($C_3H_6O$), with 99% purity, was supplied by Cofarcas (Burgos, Spain). N-terc-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with a purity greater than 99% was obtained from Sigma-Aldrich (Tres Cantos, Madrid, Spain). The Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibres were acquired from Supelco (Tres Cantos, Madrid, Spain). All aqueous solutions were prepared in deionized water with a resistivity not less than 18 MΩ cm. Helium (He) with 99.999% purity was acquired from Abelló Linde S.A. (Alcalá de Henares, Madrid, Spain).

### 3.2. Sludge sampling and preservation

Sewage sludge samples were collected from a Wastewater Treatment Plant (WWTP) in Valladolid (Spain). This WWTP serves a population of 344,600 inhabitants. The wastewater treatment consists of a primary purification step (primary sludge) followed by a biological treatment consisting of a conventional active sludge process (secondary sludge). The mixture of the generated sludge is treated in a thickening step that reduces the volume of the sludge by concentrating or partially eliminated water. The WWTP of Valladolid treats approximately 101,000 $m^3$ $day^{-1}$ of wastewater. It generates around 9,600 $m^3d^{-1}$ of biogas by digesting 2,500 $m^3d^{-1}$ of sludge. The resulting thickened mixed sewage sludge is used as fertilizer in substitution of chemical alternatives, as recommended the European Commission [9].

The collection and preparation of samples consisted of the following steps:

1. *Sampling*. Grab samples of thickened mixed sludge were randomly collected and combined to provide a final sample of approximately 25 kg. The samples were collected in high density polyethylene (HDPE) drums with polypropylene screw caps. Then, they were properly sealed and taken to the laboratory under conditions of refrigeration and darkness.

2. *Centrifugation*. Immediately after arrival to the lab, 200 mL of the homogenized sewage sludge were centrifuged at 10,000 rpm for 10 min in a Thermo Sorvall

Legend RT+ Refrigerated Benchtop Centrifuge (Madrid, Spain). The solid phase was then collected and stored in the dark at -20 ºC.

3. *Freeze-Drying*. After two days of congelation, an amount around ~25-30 g of solid phase was freeze-dried and stored at -20 ºC in darkness until analysis.

### 3.3. Sample pre-treatment

A total of 14 PPCPs were initially chosen for analysis in sewage sludge samples. Sample preparation involves most of the analysis time and usually contains an extraction process followed by a clean-up step [10]. In this Doctoral Thesis, an establishment of experimental conditions was developed to find out the optimal experimental conditions for sample pre-treatment. A large number of samples were prepared and analyzed in different sequences over a long period of time.

The developed sample pre-treatment provided an environmentally friendly procedure following the principles of green chemistry. Some characteristics presented in this pre-treatment were a reduction in the volume of solvent used and shorter sample preparation time, among others.

The initial analytical methodology consisted of taking an exact amount of freeze-dried sewage sludge (~ 0.8 g) and spiking it with 600 µL of a freshly made solution containing all the analytes at 2 mg $L^{-1}$ in acetone.

#### 3.3.1. Extraction

**a. Extraction technique**

Two extraction techniques such as **Ultrasound Asssisted Extraction (UAE)** and **Microwave Assisted Extraction (MAE)** were compared. Both techniques used are not sufficiently selective and a clean-up stage is needed after extraction [10].

UAE is a widely used technique to analyze PPCPs in solid matrices as in [11-13]. It has certain advantages that make it an environmentally friendly technique such as shorter extraction time (minutes) and smaller solvent volume.

On the other hand, more modern extraction techniques such as MAE have also been used for the analysis of these compounds in solid matrices such as the case of this work here presented and other examples reported as [14-16]. MAE, as UAE, also presents advantages such as short extraction time (min) and small amounts of solvent comparable to ultrasound technique.

- **<u>Ultrasound extraction</u>**

1) An exactly known amount (~0.8 g) of freeze-dried sludge was weighed into a polypropylene centrifuge tube (50 mL) and spiked with 600 µL of a freshly made solution containing all the analytes at 2 mg $L^{-1}$ in acetone. Then, it was kept in contact overnight in the extraction hood to allow solvent evaporation. 2) Considering the final volume needed for the instrumental analysis, 12.0 mL of an extraction solution (MilliQ® water at pH 9) was added to the tube. After reviewing related literature [10], MilliQ® water at pH 9 and MilliQ® water/MeOH, 95:5 (v/v) at pH 9 were considered as tentative extraction solvents. Subsequently, an in-situ clean-up stage was performed by adding 100.0 mg of activated alumina ($Al_2O_3$) or silica gel ($SiO_2$) at 100 ºC during 48 h. The centrifuge tube was then vortex-stirred for 1 min and the extraction was carried out for 30 min at room

temperature in a JP Selecta Univeba ultrasound bath of 50 W and 60 Hz (Barcelona, Spain). 3) The extract was centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a 25-mL glass beaker. 4) Subsequently, 12.0 mL of the extraction solvent was added again and a new extraction cycle was carried out. 5) The total volume of supernatant collected was measured (20-22 mL) and a saturation with 36% NaCl (weight/volume) was performed. Variations to the described 12+12 mL volume combinations for the extraction solvent were not tested as they were not expected to significantly influence the extraction performance. Then, the pH was measured by a Crison pH-Meter Basic 20 and adjusted to 3 by adding a few drops of diluted solutions of HCl (10%, 1% and/or 0.1%) as needed. 6). The total supernatant volume was vacuum filtered and 17.0 mL was collected in a 20.0 mL SPME glass vial. The resulting solution was analysed by online direct immersion solid phase microextraction followed by on-fiber derivatization, online coupled to gas chromatography - mass spectrometry (DI-SPME - on-fiber derivatization – GC-MS). **Table 2.7** shows two different samples using UAE and MAE.

**Table 2.7. Comparison of two extraction techniques**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| 1' | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | MAE (1 cycle) | $SiO_2$/100 | Vacuum |
| 7 | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | UAE (2 cycle) | $SiO_2$/100 | Vacuum |

- **Microwave extraction (1-cycle)**

1) An exactly known amount (~0.8 g) of freeze-dried sludge was weighed into a microwave equipment vessel and spiked it with 600 µL of a freshly made solution containing all the analytes at 2 mg $L^{-1}$ in acetone. Then, it was kept in contact overnight in the extraction hood to allow solvent evaporation. 2) Considering the final volume needed for the instrumental analysis, 24.0 mL of the extraction solution (MilliQ® water at pH 9) were added. Consequently, an in-situ clean-up stage was performed by adding 100.0 mg of activated $SiO_2$ at 100 ºC during 48 h. Up to 12 samples were able to be prepared simultaneously. The vessel was then vortex-stirred for 1 min and the extraction was carried out in a computer-controlled microwave heater with fibre optic temperature registration (Milestone START-D Microwave Digestion System). The extraction process, which was carried out at 110 ºC and 500 W, lasted 60 minutes in total (10 minutes until reaching a temperature of 110 ºC, 30 minutes of extraction and 20 minutes of cooling). After microwave irradiation, the vessels were cooled off by an air current (<45 °C). 3) The resulting extract was, then, centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a 25-mL glass beaker. 4) The total volume of supernatant collected was measured (20-22 mL). Then, a saturation with 36% NaCl (weight/volume) was performed and the pH was adjusted to 3 by adding a few drops of diluted solutions of HCl (10%, 1% and/or 0.1%) as needed. 5). Total supernatant volume was vacuum filtered and 17.0 mL was collected in a 20.0 mL SPME glass vial. The resulting solution was analysed by

online DI-SPME - on-fiber derivatization – GC-MS. **Table 2.8** indicates two samples prepared to know the aggressiveness of extraction techniques.

**Table 2.8. Comparison of the aggressiveness of extraction techniques**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| X | - | 600 | MilliQ® water at pH 9 | 24.0 | MAE (1 cycle) | - | Vacuum |
| X' | - | 600 | MilliQ® water at pH 9 | 24.0 | UAE (2 cycle) | - | Vacuum |

- **Microwave extraction (2-cycles)**

1) An exactly known amount (~0.8 g) of freeze-dried sludge was weighed into a microwave equipment vessel and spiked it with 600 µL of a freshly made solution containing all the analytes at 2 mg $L^{-1}$ in acetone. Then, it was kept in contact overnight in the extraction hood to allow solvent evaporation. 2) Considering the final volume needed for the instrumental analysis, 12.0 mL of the extraction solution (MilliQ® water at pH 9) were added. Up to 12 samples were able to be prepared simultaneously. The vessel was then vortex-stirred for 1 min and the extraction was carried out in a computer-controlled microwave heater with fibre optic temperature registration (Milestone START-D Microwave Digestion System). The extraction process, which was carried out at 110 ºC and 500 W, lasted 60 minutes in total (10 minutes until reaching a temperature of 110 ºC, 30 minutes of extraction and 20 minutes of cooling). After microwave irradiation, the vessels were cooled off by an air current (<45 ºC). 3) The resulting extract was, then, centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a 25-mL glass beaker. 4) Subsequently, 12.0 mL of the extraction solvent was added again and a new extraction cycle was carried out. 5) The total volume of supernatant collected was measured (20-22 mL). Then, a saturation with 36% NaCl (weight/volume) was performed. Variations to the described 12+12 mL volume combinations for the extraction solvent were not tested as they were not expected to significantly influence the extraction performance. Then, the pH was measured by a Crison pH-Meter Basic 20 and adjusted to 3 by adding a few drops of diluted solutions of HCl (10%, 1% and/or 0.1%) as needed. 6) The total supernatant volume was vacuum filtered and 17.0 mL was collected in a 20.0 mL SPME glass vial. The resulting solution was analysed by online DI-SPME - on-fiber derivatization – GC-MS. **Table 2.9** shows some samples prepared for evaluation of different cycles used in MAE.

**Table 2.9. Comparison of different cycles of MAE**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| 1 | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | MAE (1 cycle) | - | Vacuum |
| A | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | MAE (2 cycle) | - | Vacuum |

### b. Extraction solvent

Two solvents were tested for the extraction step as MilliQ® water at pH 9 and MilliQ® water/MeOH, 95:5 (v/v) at pH 9. These solvents were chosen for their ability to extract PPCPs from these solid samples. **Table 2.10** indicates a comparison between two extraction solvents.

**Table 2.10. Comparison of different extraction solvents**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| d | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | MAE (1 cycle) | $SiO_2$/500 | Vacuum |
| e | 0.8 | 600 | 5% MeOH / pH 9 MilliQ® water | 24.0 | MAE (1 cycle) | $SiO_2$/500 | Vacuum |

## 3.3.2. Clean-up stage

The extraction stage included an extract clean-up to increase extraction efficiency, sensitivity and minimization or elimination of interferences that may affect the determination of compounds of interest [10].

### a. In-situ or non in-situ clean-up

In-situ and non in-situ clean-up stage were studied. In-situ clean-up was described in the section 3.3.1 for ultrasonic and microwave extraction. On the other hand, the non in-situ extraction consisted of carrying out the clean-up stage after the UAE or MAE extraction. Once the extraction was made, the extract was collected into a plastic centrifuge tube (50 mL) and 100 or 500 mg of activated $Al_2O_3$ were added and centrifuged at 10,000 rpm for 10 min. Subsequently, steps 5 and 6 were carried out. **Table 2.11** indicates some samples prepared for evaluation of in-situ or non in-situ clean-up.

**Table 2.11. Comparison of different extraction solvents**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| I | 0.8 | 600 | 5% MeOH / pH 9 MilliQ® water | 24.0 | MAE (1 cycle) | $Al_2O_3$/100 (In-situ) | Syringe |
| IV | 0.8 | 600 | 5% MeOH / pH 9 MilliQ® water | 24.0 | MAE (1 cycle) | $Al_2O_3$/100 (Non in-situ) | Syringe |

### b. Types of clean-up agents

Several types of agents were selected to carry out the clean-up stage. An adequate choice of clean-up agent is needed to achieve the retention of interferences present in each solid matrix. The types of agents used are actually known as hexane, $Al_2O_3$ and $SiO_2$. Hexane is a solvent that has many drawbacks as it is easily flammable, very harmful and dangerous to the environment. On the contrary, $Al_2O_3$, $SiO_2$ behave more respectfully towards the environment.

In order to select the clean-up agent, 500.0 mg of two adsorbents such as activated $Al_2O_3$ and silica gel or 5 mL of hexane were individually tested, by adding them along the sample and the extraction solvent. Afterwards, the extraction was carried out as discussed in the previous **section 3.3.1. Table 2.12** indicates samples prepared for evaluation of type of clean-up agent adecuated.

**Table 2.12. Comparison of different types of agents**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| d | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | MAE (1 cycle) | $SiO_2$/500 | Vacuum |
| f | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | MAE (1 cycle) | $Al_2O_3$/500 | Vacuum |
| g | 0.8 | 600 | MilliQ® water at pH 9 | 24.0 | MAE (1 cycle) | Hexane/5 mL | Vacuum |

### c. Amount of clean-up agent

In order to know the most appropriate amount of cleaning agent to achieve the best possible result, the best clean-up agent for sample preparation was previously known (activated alumina). Several samples were then prepared using different amounts of activated $Al_2O_3$ (i.e., 100.0, 500.0 and 1000.0) mg. The steps used were described in the

section corresponds to microwave extraction with some differences showed in **Table 2.13**.

**Table 2.13. Comparison of different amounts of agent**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| I | 0.8 | 600 | 5% MeOH / pH 9 MilliQ® water | 24.0 | MAE (1 cycle) | $Al_2O_3$/100 | Syringe |
| II | 0.8 | 600 | 5% MeOH / pH 9 MilliQ® water | 24.0 | MAE (1 cycle) | $Al_2O_3$/500 | Syringe |
| III | 0.8 | 600 | 5% MeOH / pH 9 MilliQ® water | 24.0 | MAE (1 cycle) | $Al_2O_3$/1000 | Syringe |

### 3.3.3. Filtration

Two extract filtration modes were assessed. When possible along the whole analytical method, glass material was selected over any kind of plastic in containers and utensils. This preference was extended to the filtration steps too. Hence, 0.7-µm GF syringe filtration (2.5 cm diam.) was compared to 0.7-µm GF membrane vacuum filtration. Filtration corresponds to the last stage of sample preparation and different samples were prepared using both filtration methods. An example of samples prepared are shown in the following table (**Table 2.14)**

**Table 2.14. Comparison of extract filtration modes**

| Sample | Freeze-dried sludge mass (g) | SS' stock solution (µL) | Extraction solvent | Extraction solvent volume (mL) | Extraction technique | Clean-up adsorbent/mass (mg) | Filtration |
|---|---|---|---|---|---|---|---|
| VI | 0.8 | 600 | 5% MeOH / pH 9MilliQ® water | 24.0 | UAE (2 cycles) | $Al_2O_3$/100 | Syringe |
| VII | 0.8 | 600 | 5% MeOH / pH 9MilliQ® water | 24.0 | UAE (2 cycles) | $Al_2O_3$/100 | Vacuum |

Analysis of target compounds was based on automatized DI-SPME, followed by on-fiber derivatization, coupled to GC (Agilent 7890B) detected by MS (Agilent 5977A). This method was based on another one published elsewhere [17]. However, important upgrades were implemented. All the material used and upgrades applied have been comented on the following chapter of the Doctoral Thesis.

The total analysis time for each injection was 31.6 min. Target compounds were recorded in five acquisition windows along the run time. Acquisition stopped at 26 min. Data acquisition and evaluation were performed by Agilent Technology Mass Hunter B.07.03.2129 software. **Table 2.15** shows the primary ions (**in black**) and two secondary ions monitored for each compound.

**Table 2.15. MS parameters for the target compounds**

| Analyte | Chemical name | Adquisition window | $t_R$ (min) | SIM ions, m/z | | |
|---|---|---|---|---|---|---|
| 1 | Methylparaben | 1 | 10.46 | **209.1** | 210.1 | 135.1 |
| 2 | Clofibric acid | 2 | 11.44 | **143.1** | 271.1 | 185.1 |
| 3 | Ethylparaben | | 11.50 | **223.1** | 224.1 | 151.1 |
| 4 | Ibuprofen | | 12.10 | **263.2** | 264.2 | 117.1 |
| 5 | 4-tert-octylphenol | | 12.64 | **249** | 250 | 320 |
| 6 | Propylparaben | | 13.21 | **237.2** | 238.2 | 151.1 |
| 7 | Salicylic acid | | 13.81 | **309.2** | 310.2 | 195.1 |
| 8 | 4-nonylphenol | | 14.21 | **263.1** | 305.1 | 264.1 |
| 9 | Propranolol | 3 | 19.31 | **144.1** | 215.2 | 316.2 |
| 10 | Naproxen | | 19.78 | **287.2** | 185.1 | 288.2 |
| 11 | Triclosan | | 20.51 | **347** | 345 | 200 |
| 12 | Carbamazepine | 4 | 22.13 | **193** | 194 | 293 |
| 13 | Diclofenac | | 22.57 | **352.1** | 214.1 | 354.1 |
| 14 | Bisphenol A | 5 | 24.08 | **441** | 207 | 442 |

Here is an example of a chromatogram obtained of the sample b prepared (**Fig. 2.1**), identifying the compounds of interest.

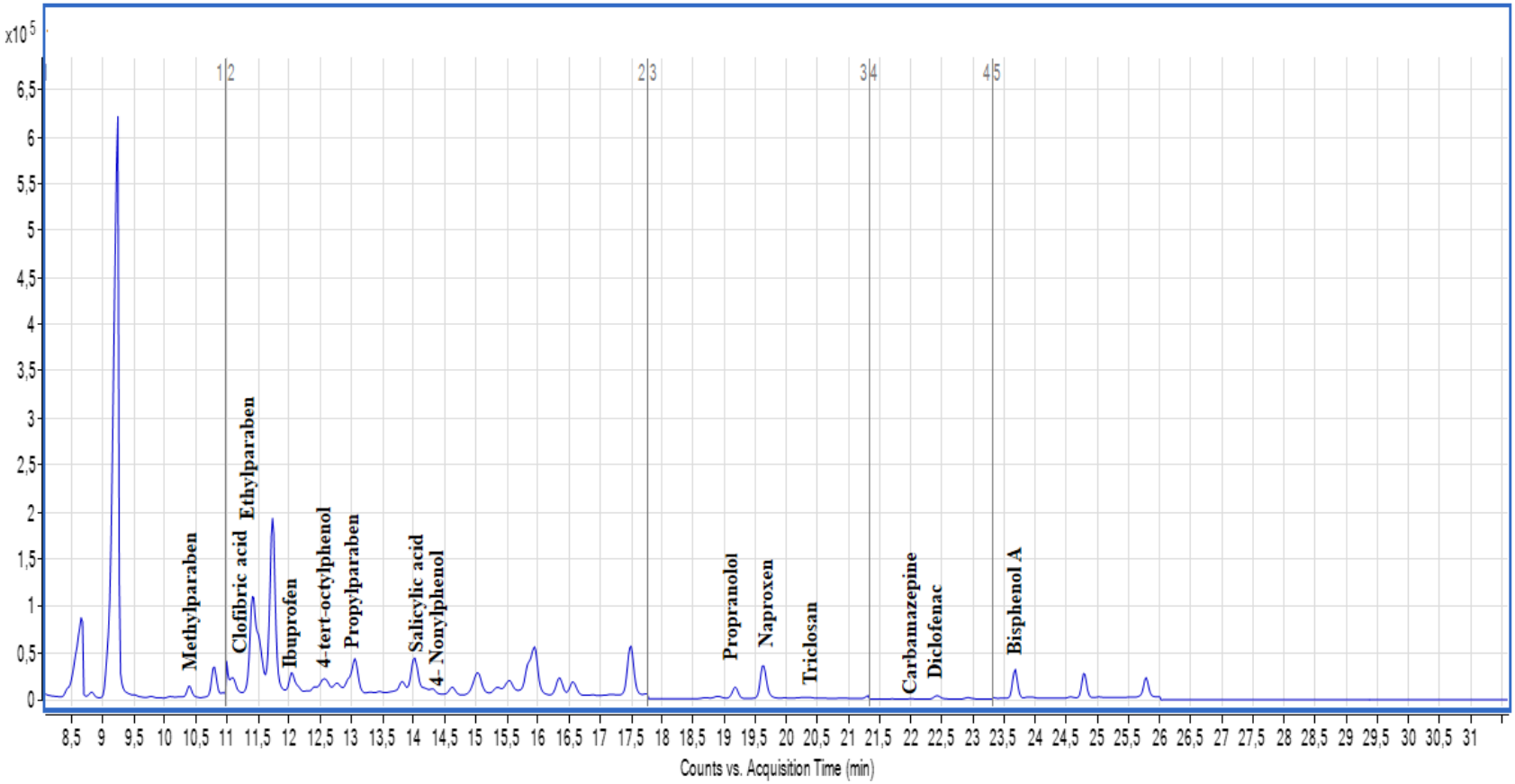



**Fig. 2.1. Chromatogram from a thickened-mixed sludge sample after applying of optimal experimental conditions**

## 4. Results and discussion

Several operational parameters were evaluated and optimized to achieve the best analytical conditions for all PPCPs. For this purpose, 0.8-g freeze-dried samples were spiked with 600 µL of a freshly made solution containing all the analytes at 2 mg $L^{-1}$ in acetone, i.e., at a spiking concentration of 1500 ng $g^{-1}$.

Some of the target parameters, i.e., extraction solvent, extraction technique, type and amount of adsorbent and filtration method, among others were discontinuous. Therefore, some precautions were taken into consideration during the experimental design.

Optimized sensitivity was the proposed goal for the method development. Thus, total signal-to-noise (TS/N), i.e., the sum of the individual S/N ratio for each target compound, was selected as the response variable during the statistical study in order to get a compromise among the performance of all the compounds.

The distribution of the optimizing parameters along the method phases is shown in **Fig. 2.2**, and were as follows (1) solvent extraction, (2) extraction technique and number of cycles of the extraction technique, (3) type of adsorbent and amount used in the clean-up stage, (4) most suitable filtration method. The influence of each parameter was evaluated in triplicate. Total method sensitivity, based on TS/N for all target analytes, was the criterion selected as mentioned to achieve an optimum multicomponent method.

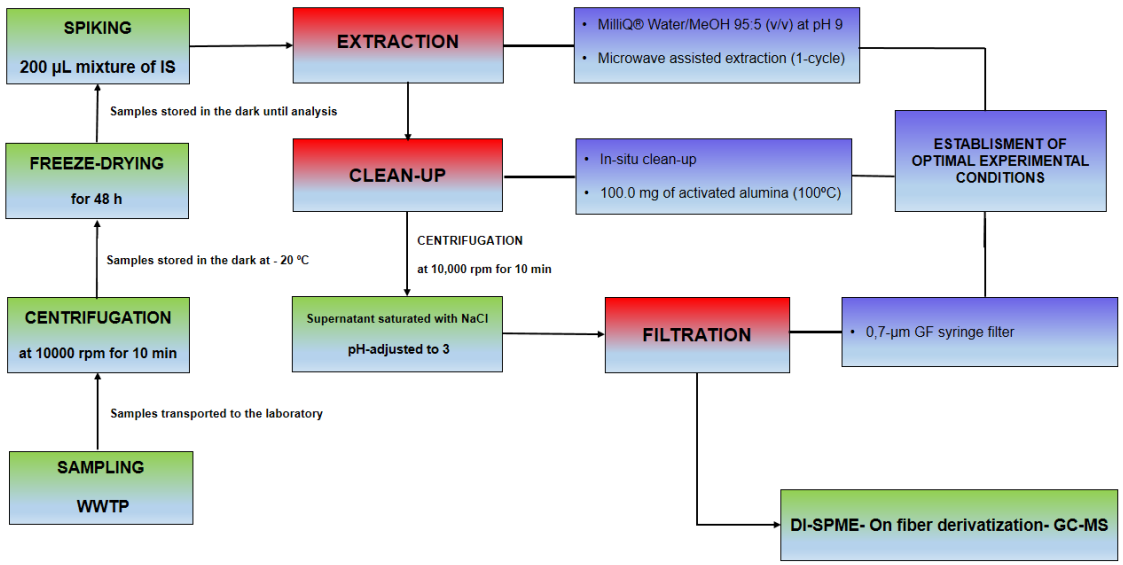



**Fig. 2.2. Distribution of the optimizing factors within the pre-treatment stages during the PPCPs analysis**

### 4.1. Extraction technique

The performance of UAE and MAE were compared. MAE offers benefits such as automation and shorter extraction in comparison with UAE [10].

A 2% improvement in TS/N was achieved by applying the MAE technique over UAE (**Table 2.16**). **Table 2.17** also reported a 41% improvement in TS/N applying the MAE technique over UAE. Therefore, MAE is better extraction technique than UAE.

**Table 2.16. Extraction techniques**

| **Sample** | 7 | 1' | 5 | A |
|---|---|---|---|---|
| **Extraction techhnique** | UAE | MAE | UAE | MAE |
| **Number of cycles** | 2 | 1 | 2 | 2 |
| **TS/N** | 3140 | **3207** | 1662 | **2344** |

Additionally, a study of the aggressiveness of the extraction technique was carried out. In this case, an 89% improvement in TS/N was achieved by applying the MAE technique over UAE (**Table 17**). Therefore, MAE is a more aggressive extraction technique than UAE.

**Table 2.17. Agressiveness of the extraction technique**

| **Sample** | X | X' |
|---|---|---|
| **Extraction technique** | UAE | MAE |
| **Number of cycles** | 2 | 1 |
| **TS/N** | 35899 | **67887** |

In addition, effectiveness of a solid-liquid extraction for a given extraction volume, usually improves by dividing it into several extraction cycles. On the other hand, the experimental error may increase. Hence, the TS/N was evaluated after 1 vs 2 MAE cycles were carried out. Extraction solvent volume varied from 12.0 mL per cycle to 24 mL in 2 and 1 MAE cycle performances, respectively.

**Table 2.18. Number of MAE extraction cycles**

| **Sample** | b | e |
|---|---|---|
| **Extraction technique** | MAE | MAE |
| **Number of cycles** | 2 | 1 |
| **TS/N** | 588 | **1116** |

The results reported that a 1 MAE cycle rendered a 90% improvement in TS/N compared to 2 MAE cycles (**Table 2.18**). Therefore, a decrease in the analysis time and number of cycles was justified.

### 4.2. Extraction solvent

Two solvents such as MilliQ® water at pH 9 and MilliQ® water/MeOH, 95:5 (v/v) at pH 9 were tested. These solvents were examined for their ability for the extraction of PPCPs in sewage sludge samples. The TS/N results showed that MilliQ® water /MeOH, 95:5 (v/v) at pH 9 reported a 9% improvement in TS/N compared to MilliQ® water at pH 9 (**Table 2.19**). Therefore, MilliQ® water/MeOH, 95:5 (v/v) at pH 9 was selected as the extraction solvent.

**Table 2.19. Extraction solvents**

| **Sample** | 1 | B |
|---|---|---|
| **Extraction solvent** | MilliQ® water at pH 9 | MilliQ® water/MeOH at pH 9 (95:5) |
| **TS/N** | 1936 | **2119** |

### 4.3. Clean-up stage

Some preliminary tests were focused on the assessment of the clean-up type. More specifically, in-situ and non in-situ clean-up were compared. The results showed that in-situ clean-up provided with a 58% improvement in TS/N over non in-situ clean-up (**Table 2.20**). Therefore, the choice of an in-situ clean-up was justified.

**Table 2.20. In-situ and non in-situ clean-up**

| **Sample** | 1 | 4 |
|---|---|---|
| **Clean-up** | In-situ | Non in-situ |
| **TS/N** | **4825** | 3046 |

In order to select the clean-up agent, 100.0 mg of different adsorbents such as activated $Al_2O_3$ (at 100 ºC for 48 hours), activated silica gel ($SiO_2$ at 100 ºC for 48 hours) and 5 mL of hexane were individually tested, by adding them along the sample and the extraction solvent. Afterwards, the extraction was carried out as discussed in the previous **section 2.3**. Activated $Al_2O_3$ obtained a 93% and 171% improvement in TS/N compared to activated $SiO_2$ and hexane, respectively (**Table 21**). Therefore, activated $Al_2O_3$ was chosen as the best clean-up adsorbent.

**Table 2.21. Different clean-up agents**

| **Sample** | f | g | h |
|---|---|---|---|
| **Clean-up agent** | $Al_2O_3$ | $SiO_2$ | Hexane |
| **TS/N** | **1365** | 705 | 503 |

Once selected the clean-up agent, three different amounts (100.0, 500.0 and 1000.0) mg of activated $Al_2O_3$ were tested for the in-situ clean-up task. The best results were observed for 100.0 mg activated $Al_2O_3$. In fact, a 94% and 171% improvement were reported in TS/N compared to 500.0 mg and 1000.0 mg, respectively (**Table 2.22**).

**Table 2.22. Different amounts of clean-up agent**

| **Sample** | I | II | III |
|---|---|---|---|
| **Amount of agent** | $Al_2O_3$/100 | $Al_2O_3$/500 | $Al_2O_3$/1000 |
| **TS/N** | **1365** | 705 | 503 |

### 4.4. Filtration

Two extract filtration modes were assessed. Hence, 0.7-µm GF syringe filtration (2.5 cm diam.) was compared to 0.7-µm GF membrane vacuum filtration. The TS/N results showed that syringe filtration obtained a 32% improvement in TS/N compared to the vacuum filtration, most probably due to the elimination of sample transferences (**Table 2.23**). Syringe filtration is a faster and more suitable approach for small volumes.

**Table 2.23. Filtration modes**

| **Sample** | VI | VII |
|---|---|---|
| **Filtration mode** | Syringe filter | Vacuum |
| **TS/N** | **5189** | 3929 |

The results obtained for the TS/N during the optimization are collected and depicted in **Fig.2.3.**

After the optimization, four of the initial PPCPs of interest (propranolol, 4-tert-octhylphenol, 4-nonylphenol and carbamazepine) proved to be inadequate for their analysis by online DI-SPME – on-Fiber Derivatization – GC-MS as they presented a very low TS/N ratio (< 5.00) even at the optimized method conditions. Therefore, they were ruled out and the final method included 10 PPCPs and is described in **chapter 3**.

## 5. Conclusions

Several groups of pharmaceuticals (analgesics, anti-inflammatories, neuropharmaceuticals, antihypertensives, and lipid regulators), endocrine disruptor compounds and some preservatives were the subject of the establishment of optimal experimental conditions for sample pre-treatment. The optimal experimental conditions included a 1-cycle MAE combined with an in-situ clean-up stage using 100.0 mg of activated $Al_2O_3$ for the reduction or elimination of interferences associated with this type of environmental matrices. A mixture of MilliQ® water/MeOH 95:5 (v/v) at pH 9 turned out being the best performing extraction solvent. Futhermore, a filtration step prior to the sample analysis was required. The instrumental part of the method consisted of an online DI-SPME-on fiber derivatization-GC-MS. The resulting environmentally friendly methodology decreased the use of expendable material (small amounts of reagents, reusable SPME fiber and derivatizing agent, among others). In addition, this fully automatized methodology was fast and analyst convenient to determinate PPCPs in sewage sludge.

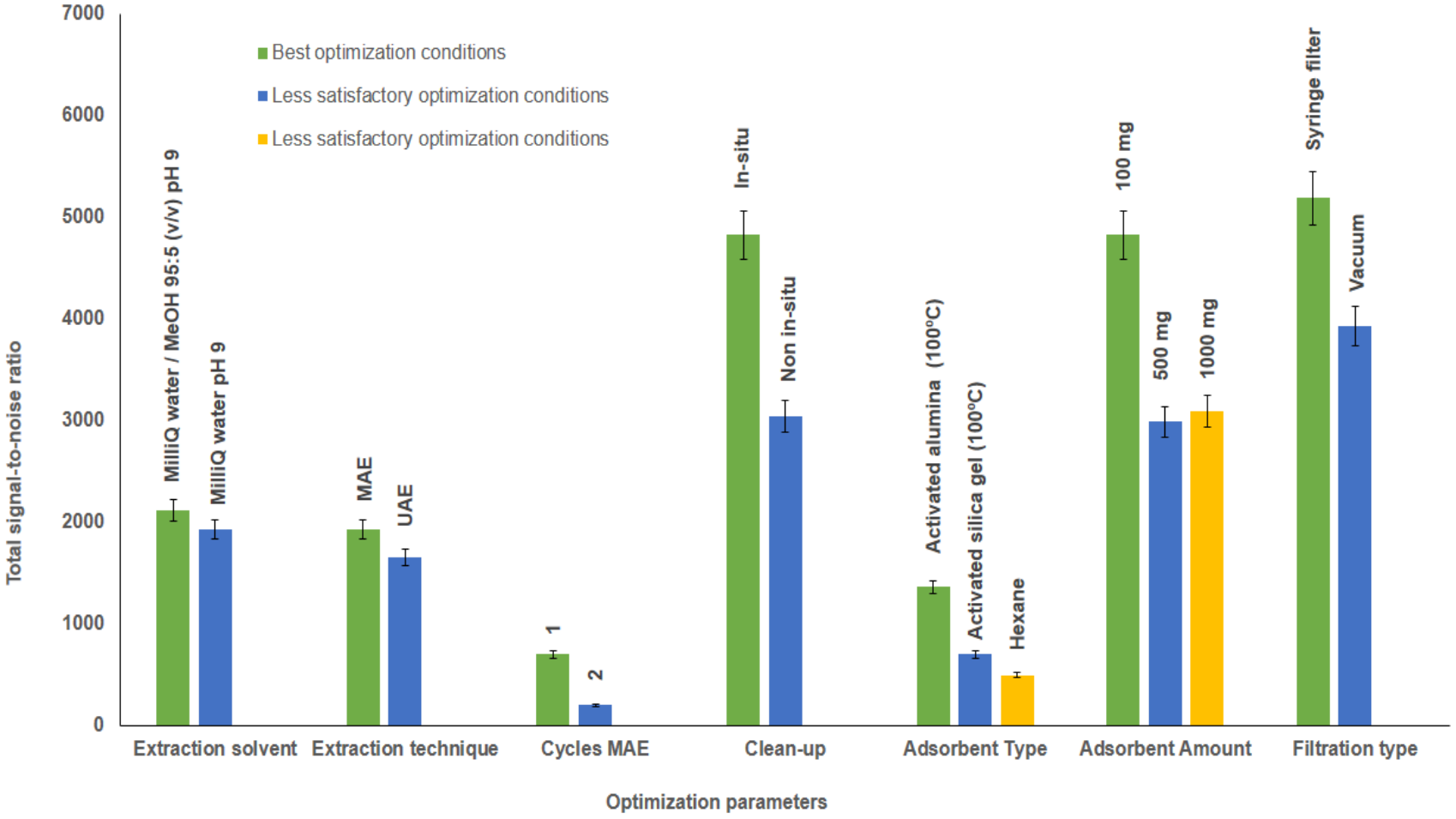



**Fig.2.3. Optimal experimental conditions of the sample pre-treatment parameters**

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# Chapter III. Validation of the analytical methodology

## 1. Introduction

Chemical pollution is one of the most important problems that impact on our planet. It is a cyclical process that affects all types of environment (air, water and soil) as well as living beings, both emitters and receivers of pollutants [1].
The World Health Organization, the Environmental Protection Agency and the European Commission are the main bodies dedicated to the protection of public and environmental health. Within their priority lines of research, the study of the so-called "emerging pollutants" (EPs), compounds of different origin and chemical nature, can be outstanding. Their presence and consequences on the environment have gone unnoticed until recently [2]. In accordance with the Directive 2013/39/EU, EPs are those that are not included in the systematic monitoring programmes of the European Union at present. However, they present a significant risk because of a continuous exposure can cause potentially adverse effects because of the bioaccumulation such as endocrine disruption or chronic toxicity even though their concentrations in the environment are relatively low, range from ng $L^{-1}$ to μg $L^{-1}$ [3].

A wide variety medicines, cosmetics, fragrances, clean-up products and synthetic or natural hormones are considered as EPs. These pharmaceutical and personal care products constitute a heterogeneous group with large differences in structure, function and properties [4]. Pharmaceuticals have attracted the most interest and have been the subject of the most in-depth studies, particularly in the 1990s.

One of the essential problems is that Wastewater Treatment Plants (WWTPs) are not capable of removing many EPs since they were designed to eliminate organic matter and nutrients in concentrations higher than mg $L^{-1}$ [5]. Therefore, these contaminants are present in surface water and groundwater as well as in drinking water. In addition, the primary degradation of some of them in WWTPs or environment can produce even more persistent and dangerous products [6]. Thus, soils that are fertilised with sewage sludge might end up accumulating PPCPs and the underlying aquifers become contaminated.

In recent years, a large number of methodologies have been developed for the determination of EPs in solid matrices as sewage sludge. Traditional sample preparation is being replaced by miniaturized and automated techniques. In addition, some sample preparation methodologies can be directly incorporated into liquid chromatography (LC) or gas chromatography (GC) [7]. In the 1990s, Pawliszyn and colleagues developed a miniaturized solid phase extraction technique known as Solid-Phase Microextraction (SPME) [8]. This sample preparation technique is fast, simple, effective and can be coupled to GC or LC. The static procedure "fiber SPME" is the most common format and presents great popularity due to advantages such as simplicity of operation, solvent-free nature, moderately short extraction time, complete automation and simple coupling with chromatography [9]. However, the analysis of polar compounds in environmental samples has not been so much explored with it, particularly when the pre-treatment of the sample is followed by GC. This is probably due to the fact that a derivatization step is necessary for the analysis of non-volatile and/or thermolabile compounds.

This study aimed to contribute to the detection and quantification of 10 PPCPs in sewage sludge samples thanks to development and optimization of an analytical methodology with a fully automated analysis method based on online DI-SPME-On-fiber

derivatization-GC-MS. To the best of the authors' knowledge, only another scientific paper has been found suggesting the use of this technique for the analysis of PPCPs in sludge samples [10].Thanks to automatized sludge extraction, matrix in-situ clean-up and isotope dilution quantification approach, the resulting methodology here presented stands out for its robustness, short time consumption and environmental and analyst safety.

## 2. Material and methods

### 2.1. Standards and reagents

The standards for all PPCPs (**Table A1** as **Appendix I**) were of high purity grade (> 95%, Sigma-Aldrich, Madrid, Spain) as neutral non-solvated molecules, except for diclofenac (sodium salt).

Ten internal standards, such as the isotopically labelled rac-ibuprofen-d3, rac-naproxen-d3, propyl-d7-paraben, salicylic acid-d4, triclosan-d3, diclofenac-d4, methylparaben-d4, ethylparaben-d5, clofibric acid-d4 and bisphenol A-d8 (LGC Standards, Barcelona, Spain) (**Table A1**), were used.

Individual stock solutions at 1,000 mg $L^{-1}$ for both PPCPs standards and isotopically labelled internal standards were prepared in methanol (MeOH). From them, a stock solution with all the analytes was prepared in MeOH at 20 mg $L^{-1}$. Fresh serial dilutions (2, 0.5, 0.05, 0.005) mg $L^{-1}$ in acetone were subsequently prepared from it when need them. A mixture of isotopically labelled internal standards in MeOH and their corresponding serial dilutions in acetone (2, 0.5, 0.05, 0.005) mg $L^{-1}$ were also prepared. All solutions were stored at -20 °C in darkness.

High purity solvents, i.e., LC-MS Chromasolv® Ethyl Acetate (EA) grade from Fluka (Madrid, Spain), SupraSolv® GC-MS MeOH grade by Merck Millipore (Madrid, Spain), Sodium Chloride (NaCl) and Hidrochloric acid (HCl) with 37% purity were supplied by Panreac (Barcelona, Spain). Aluminium oxide by Sigma-Aldrich (Tres Cantos, Madrid, Spain). Acetone ($C_3H_6O$), with 99% purity, was supplied by Cofarcas (Burgos, Spain). N-terc-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with a purity greater than 99% was obtained from Sigma-Aldrich (Tres Cantos, Madrid, Spain). The Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibres were acquired from Supelco (Tres Cantos, Madrid, Spain). All aqueous solutions were prepared in deionized water with a resistivity not less than 18 MΩ cm. Helium (He) with 99.999% purity was acquired from Abelló Linde S.A. (Alcalá de Henares, Madrid, Spain).

### 2.2. Sample preservation and pre-treatment

Sewage sludge samples were collected from a WWTP in Valladolid (Spain). The characteristics of the WWTP were mentioned in the sampling step of the previous chapter. The thickened mixed sewage sludge, used as fertilizer in substitution of chemical alternatives [11], was collected and prepared as mentioned in the sampling, centrifugation and freeze-drying steps of the previous chapter.

Once the sample was prepared, the proposed method for sludge analysis consisted of the following stages:

i. *Spiking*. An exact amount of freeze-dried sewage sludge (~ 0.8 g) was placed in a vessel and spiked with 200 µL of a solution at 2 mg $L^{-1}$ in acetone containing a mixture of all isotopically labelled internal standards and homogenized. Then, it was kept in contact overnight in the extraction hood to allow solvent evaporation and internal standard fixation. Sample size was chosen by recommendations found in the literature for similar matrixes [7].

ii. *Pre-treatment for desorption of the analytes to aqueous phase*. The sample underwent, then, microwave assisted extraction (MAE) in a Milestone START-D Microwave Digestion System (Madrid, Spain) at 110 ºC during 30 minutes to facilitate the desorption of the analytes. Twenty-four millilitres of a MilliQ® water/MeOH mixture, 95:5 (v/v) at pH 9 were used as extracting solvent. At this pH, all the target compounds were supposed to be as negative ions **(Table A1),** increasing their affinity for the liquid phase. Subsequently, 100.0 mg of activated alumina ($Al_2O_3$ at 100 ºC for 48 hours) were added for matrix in-situ clean-up.

iii. *After MAE centrifugation*. The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was collected (20-22 mL) with a glass pipette and transferred to a 25-mL glass beaker. The total of supernatant was saturated with ~7.5 g NaCl (solubility in water at 25 ºC is 359 g $L^{-1}$) at 36% (weight/volume) to increase the ionic strength. The resulting sample was also pH-adjusted to 3 with HCl, in order to increase the analyte lipophilia by shifting their acid-base equilibrium into neutral molecules. Finally, the extract was filtered through a 0.7-µm glass fiber (GF) syringe filter and 17.0 mL of the filtrate was collected in a 20.0 mL SPME glass vial.

### 2.3. Analysis by GC-MS

Analysis of target compounds was based on automatized direct immersion solid phase microextraction (DI-SPME), online followed by on-fiber derivatization, coupled to gas chromatography (Agilent 7890B) detected by mass spectrometry (Agilent 5977A) (GC-MS). This method was based on another one published elsewhere [9]. However, important upgrades were implemented. Hence, 90 min sample extraction at a penetration depth of 60 mm, 45 min derivatization step at a penetration depth of 45 mm, orbital agitation at 350 rpm with a stirring regime of 6s on/20s off were implemented to increase SPME fiber life time **(Table A2)**. In fact, these adjustments extended average fiber lifespan beyond 80 injections and up to 130 injections with no signs of performance deterioration, which entails a 62% lifespan increase.with no signs of performance deterioration. A DVB/CAR/PDMS SPME fiber was utilized for the analysis. Chromatographic separation was achieved on a capillary HP-5MS GC column (30 m length, 0.25 mm i.d., 0.25 µm film thickness) with He as carrier gas at a constant flow rate of 1.2 mL $min^{-1}$. Injector temperature was set at 250 ºC, while the GC oven temperature increased from 70 ºC (held for 3 min during fiber desorption) to 150 ºC at 50 ºC $min^{-1}$, to 220 ºC at 5 ºC $min^{-1}$ and finally to 300 ºC (held for 5 min) at 10 ºC $min^{-1}$. The total analysis time for each injection was 31.6 min. Mass detection was obtained in electron impact ionization mode (70 eV) with selected ion monitoring (SIM) and a

filament delay of 8 min. The GC–MS interface, ion source and quadrupole temperatures were set at 280, 230 and 150 °C, respectively.

Target compounds were recorded in six acquisition windows along the run time. Acquisition stopped at 26 min. Data acquisition and evaluation were performed by Agilent Technology Mass Hunter B.07.03.2129 software. **Table 3.1** shows the primary ions (in black) and two secondary ions monitored for each compound.

**Table 3.1: MS parameters for the final target compounds**

| Analyte | Chemical name | Adquisition window | [a]$t_R$ (min) | [b]SIM ions, m/z | | |
|---|---|---|---|---|---|---|
| 1 | Methylparaben | 1 | 9.531 | **209.1** | 210.1 | 135.1 |
| 2 | Clofibric acid | | 10.449 | **143.1** | 271.1 | 185.1 |
| 3 | Ethylparaben | 2 | 10.536 | **223.1** | 224.1 | 151.1 |
| 4 | Ibuprofen | | 11.059 | **263.2** | 264.2 | 117.1 |
| 5 | Propylparaben | 3 | 12.062 | **237.2** | 238.2 | 151.1 |
| 6 | Salicylic acid | | 12.760 | **309.2** | 310.2 | 195.1 |
| 7 | Naproxen | 4 | 18.508 | **287.2** | 185.1 | 288.2 |
| 8 | Triclosan | | 19.311 | **347.0** | 345.0 | 200.0 |
| 9 | Diclofenac | 5 | 21.571 | **352.1** | 214.1 | 354.1 |
| 10 | Bisphenol A | 6 | 23.096 | **441.0** | 207.0 | 442.0 |

[a]$t_{R:}$ retention time
[b]SIM: selected ion monitoring

**Fig. 3.1 and Fig. 3.2** show representative SIM chromatograms obtained from hydrolysed and anaerobically digested thickened-mixed sludge, respectively.

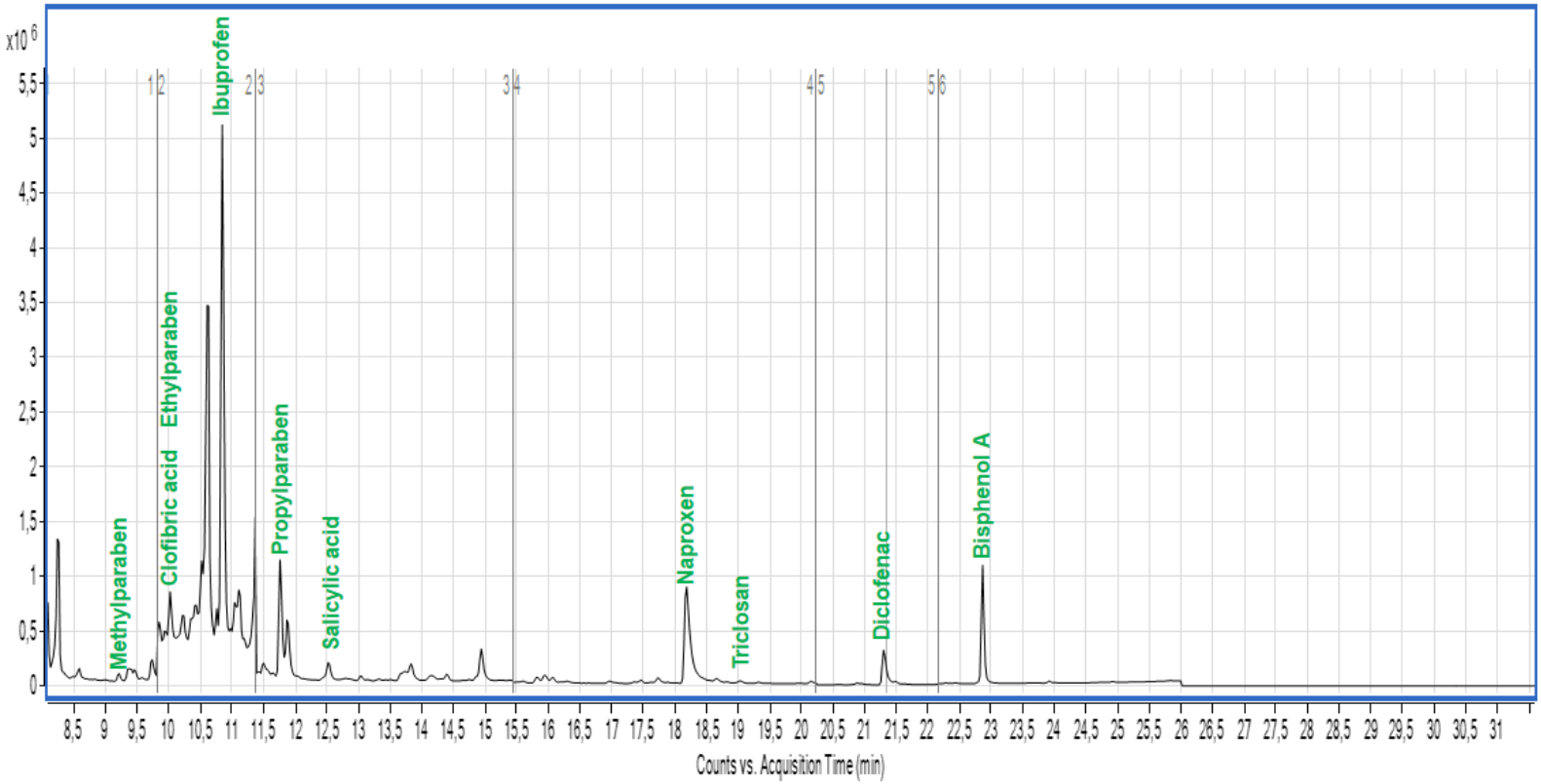



**Fig.3.1. Chromatogram from a hydrolysed thickened-mixed sludge sample after applying the optimal experimental conditions**

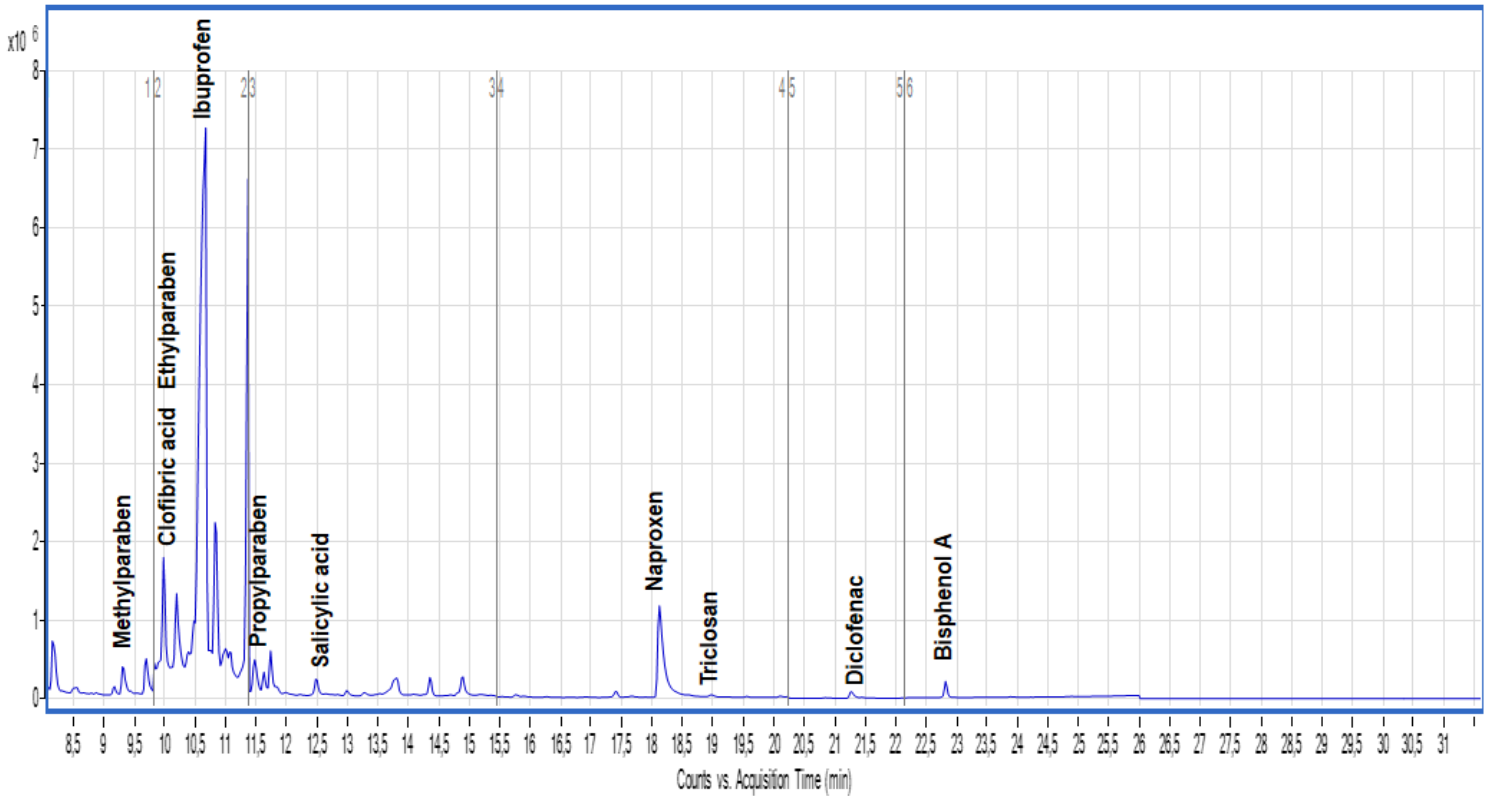



**Fig.3.2. Chromatogram from an anaerobically-digested thickened-mixed sludge sample after applying after applying the optimal experimental conditions**

## 3. Validation of the developed method and applications

### 3.1. Method validation

Several regulatory bodies have published guidelines for method validation. Methodologies for the analysis of PPCPs in sewage sludge have not followed a homogenous criterion. Hence, Dorival-García et al. [12] and Luque-Muñoz et al. [13] followed the American Food and Drug Administration [14] and Azzouz et al. [15] and Peysson et al. [16] selected the International Association of Official Analytical Chemists [17].
Authors such as Cristale and Lacorte [18] and Evans et al. [19] as well as this present study used as a reference for the method development and validation a directive executed by the European Union [20], concerning products of animal origin due to the absence of specific guidelines.

The following validation parameters were determined for the 10 PPCPs that showed sufficient sensitivity as explained in the previous section (methylparaben, ethylparaben, clofibric acid, ibuprofen, propylparaben, salicylic acid, naproxen, triclosan, diclofenac and bisphenol A) in thickened mixed sludge. Each test was performed in triplicate (n = 3) and spiked at two significant concentration levels of 1000 ng $g^{-1}$ and 1500 ng $g^{-1}$ with the optimal experimental conditions and average results are shown in **Tables 3.2 and 3.3.**

1. Accuracy: In our specific case, it was expressed as absolute recoveries (%). They were calculated by comparing the peak areas obtained from spiked samples employing the optimized method with the peak areas of direct injections (2µL) of equivalent amounts of the standards in ethyl acetate solutions. Quantification method was based on an isotope dilution (10 isotopically labelled analogues) calibration curve. It was prepared with MilliQ® water samples saturated in NaCl at pH 3 adjusted and filtered through 0.7-µm. These samples were spiked at different levels of concentration and 10 internal standards (isotopic analogues to 10 of the target analytes) were also added. Observed absolute recoveries were below 70% for all target compounds **(Table 3.2)** in sewage sludge. These absolute recoveries were very similar to those reported in other studies for the analysis of sewage sludge samples [21, 22]. Nonetheless, these deficiencies were properly corrected by the isotopic dilution quantification approach. In fact, relative recoveries for all target compounds, which were calculated as the ratio between the absolute recoveries for each compound and the recoveries of their corresponding internal standard, were obtained in the range 86-108% **(Table 3.2)**.

2. Matrix effect: It refers to the impact the components of the sample matrix may exert on the analysis of the analytes of interest. More specifically, it is mainly due to the fact that co-eluting matrix elements may hamper the ionization process of the analytes in the mass spectrometer [7]. Matrix effect is typically expressed as the percentage of signal suppression. In our particular case, to determine the matrix effect associated to sewage sludge samples, empty glass vials were similarly spiked as the validation samples and underwent the same optimized methodology. The matrix effect corresponds to the differences between the areas obtained in the samples with and without matrix. The results reported in the sewage sludge **(Table 3.2)** were close to 100% in signal suppression for many of the compounds like in other reported studies [23]. However, these deficiencies

were included within the accuracy of the method discussed above and corrected by the use of isotope dilution quantification. That showed that the clean-up and automation here proposed not only reduced drastically the analysis time, analyst exposure and disposable material consumption, but also maintained the efficiency of the conventional methods in terms of matrix effect.

3. Precision: It refers to method repeatability and was expressed as the relative standard deviation (%RSD) of the area observed for analogous samples prepared in triplicate with the optimized method. The analyses were performed in the same day (intra-day) as well as in different days (inter-day). The overall method repeatability was acceptable for the sludge samples. The %RSD values were lower than 10% for most of the compounds when the analyses were performed in the same day (intra-day precision). In addition, the %RSD values in different days (inter-day precision) were lower than 21% for most the compounds **(Table 3.2)**. These results reported a precision similar to previous methodologies for sludge samples [21, 22, 24].

4. Method limits of detection (MLDs) and quantification (MLQs) were experimentally calculated as the concentration providing a signal-to-noise ratio of 3 and 10, respectively, for each target analyte in each matrix. MLDs were lower than 20 ng $g^{-1}$ and MLQs lower than 65 ng $g^{-1}$ for most of the target compounds in sludge samples **(Table 3.2)**. They were considered acceptable for trace analysis of target compounds in this type of matrix. In addition, these values were similar to, or even lower than, values reported in analogous multicomponent methods based on GC-MS [10, 22] and even LC-MS/MS [25].

5. Instrumental carry over: An irrelevant carryover effect was observed during the instrumental analysis despite the reuse of the derivatizing agent and SPME fiber for a considerable number the samples (~100). MilliQ® water samples saturated in NaCl and pH 3 adjusted and filtered through 0.7-µm (blanks) were run under the optimized instrumental method right after spiked sludge samples at different levels of concentration. The peak areas from both the blanks and the spiked samples were then compared. Most of the blanks contained less than 4% of the previous signal from the sludge samples **(Table 3.3)**. Therefore, the carryover effect was considered insignificant and desorption and fiber conditioning were adequately validated. This constituted an important achievement over related methodologies such us [10].

6. Dynamic range: The quantification method was based on a matrix-matched approach, the samples were prepared in the matrix and run the same best-conditions method. Eight-point calibration curves were built by spiking equal sludge samples covering the range from 31.5 to 2500 ng $g^{-1}$, for all target compounds. The calibration curves reported **(Table 3.3)** corresponded to linear equations with correlation coefficients ($R^2$) above 0.99 within the indicated concentration range. Up to 3 orders of magnitude were observed. Linearity ranges up to 3 [10] and 2 [21] orders of magnitude have been reported elsewhere. On the other hand, the lack-of-fit test were performed. Eight-point calibration curves were also built by spiking equivalent sludge samples covering the range from 31.5 to 2500 ng $g^{-1}$, for all target compounds. Two-point calibration curves were prepared in triplicate (n=3). The calibration curves obtained **(Table 3.4)**

corresponded to linear equations for some compounds (e.g. methylparaben, propylparaben diclofenac) and two and three degree polynomial equations for compounds such as (ibuprofen and salicylic acid) and (clofibric acid, ethylparaben, naproxen, triclosan and diclofenac), respectively. All of them, with correlation coefficients ($R^2$) above 0.99 within the indicated concentration range.

**Table 3.2: Accuracy, Matrix effect and Precision for Thickened Mixed Sludge**

| | Accuracy | | Matrix effect | Precision | | | |
|---|---|---|---|---|---|---|---|
| **Chemical Name** | **Absolute recovery (%)** | **Relative recovery (%)** | **Signal Supression (%)** | **Intraday (%RSD)** | **Interday (%RSD)** | **MLD (ng $g^{-1}$)** | **MLQ (ng $g^{-1}$)** |
| **Methylparaben** | 56.38 | 99.13 | 32.85 | 5.23 | 21.32 | 10.90 | 28.06 |
| **Clofibric acid** | 32.74 | 86.76 | 94.63 | 8.91 | 32.93 | 13.07 | 61.75 |
| **Ethylparaben** | 48.89 | 98.47 | 52.89 | 24.36 | 34.79 | 31.33 | 104.43 |
| **Ibuprofen** | 48.07 | 100.41 | 77.61 | 19.12 | 19.59 | 9.08 | 30.26 |
| **Propylparaben** | 36.45 | 99.97 | 97.58 | 11.93 | 5.48 | 19.39 | 64.64 |
| **Salicylic acid** | 15.16 | 101.38 | 98.96 | 6.93 | 19.14 | 3.14 | 10.48 |
| **Naproxen** | 64.43 | 102.05 | 95.85 | 0.73 | 6.00 | 1.71 | 5.69 |
| **Triclosan** | 1.45 | 107.98 | 84.15 | 5.71 | 3.57 | 16.11 | 53.69 |
| **Diclofenac** | 43.72 | 106.25 | 99.00 | 3.94 | 0.94 | 10.75 | 35.83 |
| **Bisphenol A** | 10.37 | 102.00 | 98.76 | 12.99 | 16.24 | 30.22 | 100.72 |

**Table 3.3: Dynamic range and Carry over for Thickened Mixed Sludge**

| | Dynamic range | | | Carry over (%) |
|---|---|---|---|---|
| **Chemical name** | **Equation** | **$R^2$** | **Linear range (ng $g^{-1}$)** | **After spiked SS samples** |
| **Methylparaben** | y = 981.51x + 46926 | 0.9959 | MLQ-991 | 3.08 |
| **Clofibric acid** | y = 1098.5x + 2596.8 | 0.9961 | MLQ-991 | 0.60 |
| **Ethylparaben** | y = 339.53x + 8299.9 | 0.9966 | MLQ-961 | 0.07 |
| **Ibuprofen** | y = 1107x + 49495 | 0.9958 | MLQ-971 | 0.33 |
| **Propylparaben** | y = 1067.3x + 9560.9 | 0.9965 | MLQ-991 | 0.54 |
| **Salicylic acid** | y = 1837.5x – 88983 | 0.9967 | MLQ-991 | 3.81 |
| **Naproxen** | y = 1003.9x – 3847.9 | 0.9950 | MLQ-981 | 0.08 |
| **Triclosan** | y = 483.81x – 6821.3 | 0.9970 | MLQ-991 | 22.64 |
| **Diclofenac** | y = 307.62x – 8708.5 | 0.9957 | MLQ-923 | 0.09 |
| **Bisphenol A** | y = 828.44x + 16549 | 0.9969 | MLQ-2466 | 12.92 |

**Table 3.4: Lack-of-fit test for Thickened Mixed Sludge**

| | Dynamic range | | |
|---|---|---|---|
| **Chemical name** | **Equation** | **$R^2$** | **Linear range (ng $g^{-1}$)** |
| **Methylparaben** | $y = 734.703x + 10376$ | 0.9943 | LOQ-2472 |
| **Clofibric acid** | $y = -0.0002x^3 + 1.0253x^2 - 408.12x + 208015$ | 0.9988 | LOQ-1284 |
| **Ethylparaben** | $y = -0.0005x^3 + 1.4727x^2 - 204.09x + 34108$ | 0.9965 | LOQ-1225 |
| **Ibuprofen** | $y = -0.0087x^2 + 51.026x - 403.93$ | 0.9997 | LOQ-613 |
| **Propylparaben** | $y = 1199,2x - 14797$ | 0.9987 | LOQ-2491 |
| **Salicylic acid** | $y = -0.0091x^2 + 52.303x - 620.04$ | 0,9972 | LOQ-1276 |
| **Naproxen** | $y = -0.0007x^3 + 2.7041x^2 + 151.05x + 111625$ | 0.9979 | LOQ-1245 |
| **Triclosan** | $y = -0.0002x^3 + 0.6685x^2 + 149.07x + 126795$ | 0.9991 | LOQ-1850 |
| **Diclofenac** | $y = 198.32x - 7711,4$ | 0.9974 | LOQ-2373 |
| **Bisphenol A** | $y = 1E-06x^3 - 0.0138x^2 + 62.155x - 4284$ | 0.9989 | LOQ-1446 |

A calibration curve **(fig. 3.3)**, generated by matrix-matched, corresponding to **diclofenac** (analgesic/anti-inflammatory), is shown below.

The equation of the adjusted model, which describes the relationship between the **Response** (counts) and the **Concentration** (ng $g^{-1}$), is as follows:

$$\text{Response (counts)} = -7711{,}4 + 198.32 * \text{Concentration (ng } g^{-1})$$

The P-value in the Analysis of Variance (ANOVA) was less than 0.05. Hence, there was a significant statistical relationship between Response (counts) and Concentration (ng $g^{-1}$) with a 95.0% confidence level. Furthermore, the R-Square statistic parameter indicated that the adjusted model explains 99.7448% of the variability in Response (counts). The correlation coefficient was equal to 0.998723, indicating a relatively strong relationship among the variables.

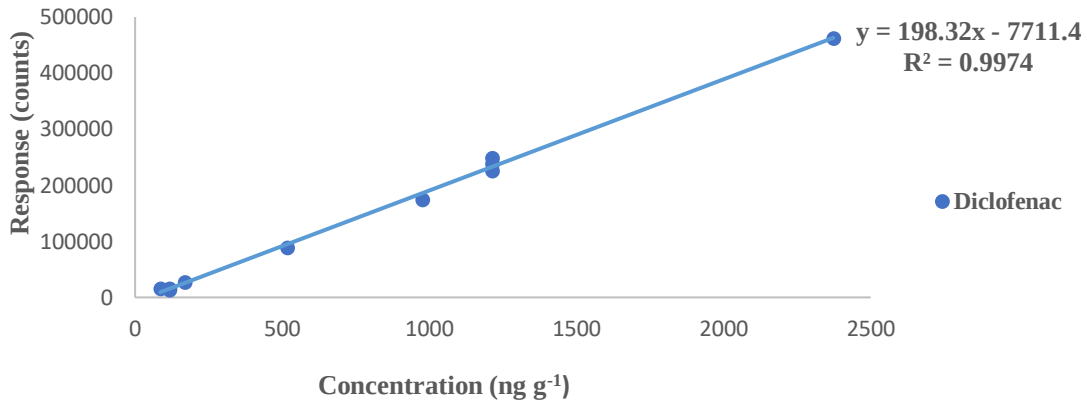



**Fig. 3.3. Calibration curve based on matrix-matched quantification approach for diclofenac**

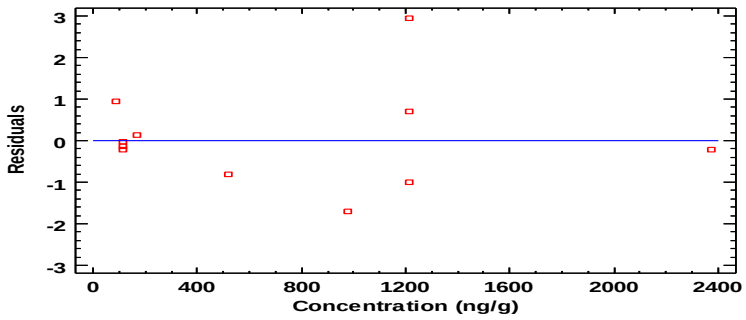



**Fig. 3.4. Residuals graphic for diclofenac**

**Table 3.5. Coefficients of the adjusted model**

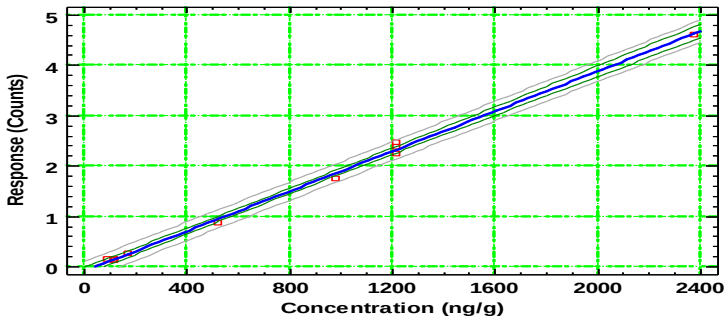
| | *Least-squares* | *Standard* | *Statistic* | |
|---|---|---|---|---|
| Parameter | Estimated | Error | T | P-value |
| Intercept | **-7711,28** | 3395,92 | -2.27075 | 0.0493 |
| Slope | **198.315** | 3.34392 | 59.3061 | 0.0000 |

**Table 3.6. ANOVA**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 2.1096E11 | 1 | 2.1096E11 | 3517,22 | **0.0000** |
| Residue | 5.39813E8 | 9 | 5.99792E7 | | |
| Total (Corr.) | 2.115E11 | 10 | | | |

The **Lack-of-fit test** is designed to determine whether the selected model is adequate to describe the observed data, whether a more complicated model should be used. The test was performed comparing the variability of the residues in the current model with the variability between observations made in repeated values of the independent variable X. Two calibration curves are shown in the **figures 3.5** and **3.8** and the rest of calibration curves are reported in **Appendix I.**

In the case of **figure 3.5**, the P-value for the lack of adjustment in the ANOVA (**Table 3.7**) is higher than or equal to 0.05, the model seemed to be adequate with a 95.0% confidence level.




**Diclofenac**

**Fig. 3.5. Graphic of the adjusted model for diclofenac**

**Table 3.7. ANOVA with lack of adjustment**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 2.1096E11 | 1 | 2.1096E11 | 3517,22 | 0.0000 |
| Residue | 5.39813E8 | 9 | 5.99792E7 | | |
| Lack of adjustment | 2.7663E8 | 5 | 5.53261E7 | 0.84 | **0.5829** |
| Pure error | 2.63183E8 | 4 | 6.57957E7 | | |
| Total (Corr.) | 2.115E11 | 10 | | | |

In addition, **figure 3.6** also shows calibration curve of **naproxen** (analgesis-anti-inflammatory).

The equation of the adjusted model, which describes the relationship between the **Response** (counts) and the **Concentration** (ng $g^{-1}$), is as follows

Response (counts) = -72778,4 + 2439,93*Concentration (ng $g^{-1}$)

The P-value in ANOVA (**Table 3.9**) was less than 0.05. Hence, there was a significant statistical relationship between Response (counts) and Concentration (ng $g^{-1}$) with a 95.0% confidence level. Furthermore, the R-Square statistic parameter indicated that the adjusted model explains 99.1853% of the variability in Response (counts). The correlation coefficient was equal to 0.998723, indicating a relatively strong relationship among the variables.

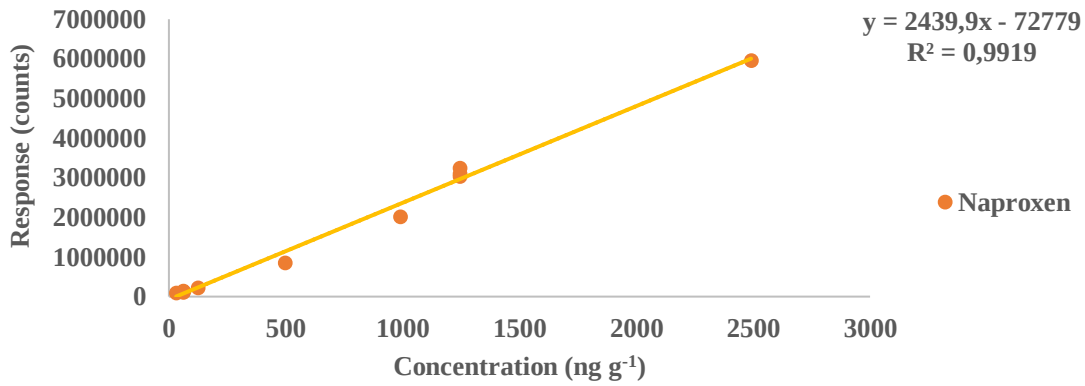



**Fig. 3.6. Calibration curve based on matrix-matched quantification approach for naproxen**

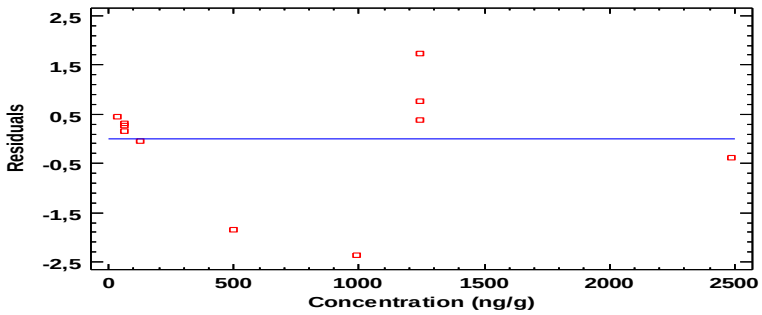



**Fig. 3.7. Residuals graphic for naproxen**

**Table 3.8. Coefficients of the adjusted model**

| | *Least-squares* | *Standard* | *Statistic* | |
|---|---|---|---|---|
| Parameter | Estimated | Error | T | P-value |
| Intercept | **-72778,4** | 77348,1 | -0.94092 | 0.3713 |
| Slope | **2439,93** | 73.7086 | 33.1024 | 0.0000 |

**Table 3.9. ANOVA**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 3.69886E13 | 1 | 3.69886E13 | 1095,77 | **0.0000** |
| Residue | 3.03804E11 | 9 | 3.37559E10 | | |
| Total (Corr.) | 3.72924E13 | 10 | | | |

In the case of **figure 3.8**, the P-value for the lack of adjustment in the ANOVA (**Table 3.10**) showed that is lower than or equal to 0.05 and the model did not seem to be adequate with a 95.0% confidence level.

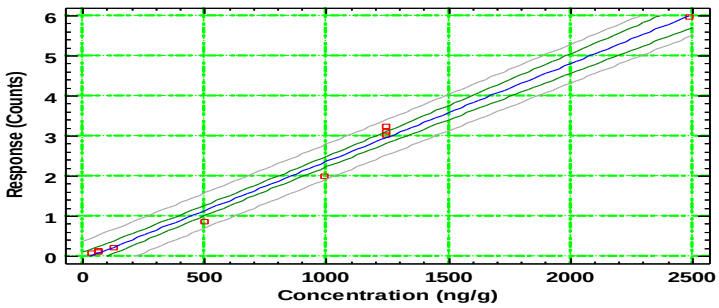



**Fig. 3.8. Graphic of the adjusted model for naproxen**

**Table 3.10. ANOVA with lack of adjustment**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 3.69886E13 | 1 | 3.69886E13 | 1095,77 | 0.0000 |
| Residue | 3.03804E11 | 9 | 3.37559E10 | | |
| Lack of adjustment | 2.82525E11 | 5 | 5.6505E10 | 10.62 | **0.0200** |
| Pure error | 2.12785E10 | 4 | 5.31963E9 | | |
| Total (Corr.) | 3.72924E13 | 10 | | | |

In the case of **degree 3 polynomial regression**, the results obtained are shown below:

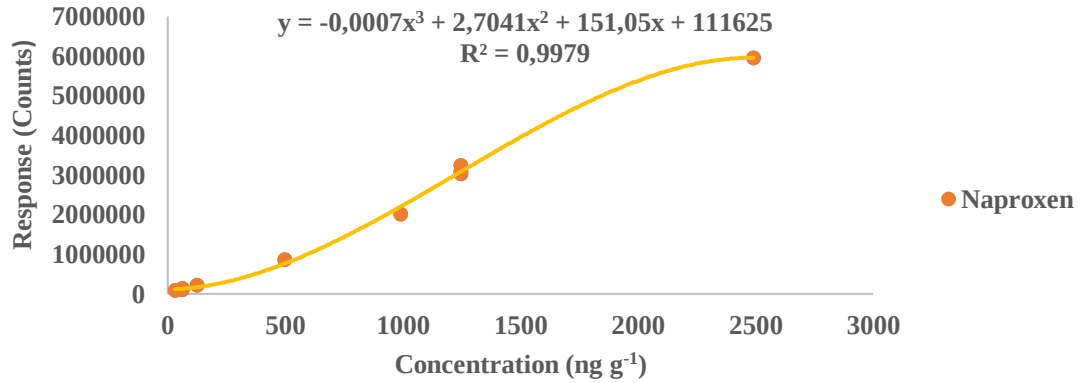



**Fig. 3.9. Polynomial curve based on matrix-matched quantification approach for naproxen**

The equation of the adjusted model, which describes the relationship between the **Response** (counts) and the **Concentration** (ng $g^{-1}$), is as follows:

Response (counts) = 111625 + 151.05*Concentration (ng $g^{-1}$) + 27041*Concentration (ng $g^{-1}$)^2 - 0.0007*Concentration (ng $g^{-1}$)^3

The P-value in ANOVA (**Table 3.12**) was less than 0.05. Hence, there was a significant statistical relationship between Response (counts) and Concentration (ng $g^{-1}$) with a 95.0% confidence level. Furthermore, the R-Square statistic parameter indicated that the adjusted model explains 99.7941% of the variability in Response (counts). The correlation coefficient was equal to 0.997059, indicating a relatively strong relationship among the variables.

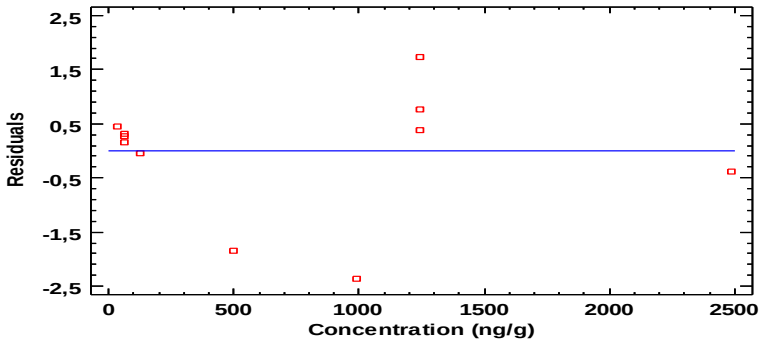



**Fig. 3.10. Residuals graphic for naproxen**

**Table 3.11. Coefficients of the adjusted model**

| *Parameter* | *Estimated* | *Error Standard* | *Statistic T* | *Value-P* |
|---|---|---|---|---|
| CONSTANT | 111625 | 64391.2 | 1.73355 | 0.1266 |
| Concentration (ng $g^{-1}$) | 151.054 | 513.676 | 0.294065 | 0.7772 |
| Concentration (ng $g^{-1}$)^2 | 2.70405 | 0.595221 | 4.54293 | 0.0027 |
| Concentration (ng $g^{-1}$)^3 | -0.000731407 | 0.000160854 | -4.54701 | 0.0026 |

**Table 3.12. ANOVA**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 3.72157E13 | 3 | 1.24052E13 | 1130.97 | **0.0000** |
| Residue | 7.67805E10 | 7 | 1.09686E10 | | |
| Total (Corr.) | 3.72924E13 | 10 | | | |

In the case of **figure 3.11**, the P-value for the lack of adjustment in the ANOVA (**Table 3.13**) is higher than or equal to 0.05, the model seemed to be adequate for data observed with a 95.0% confidence level.

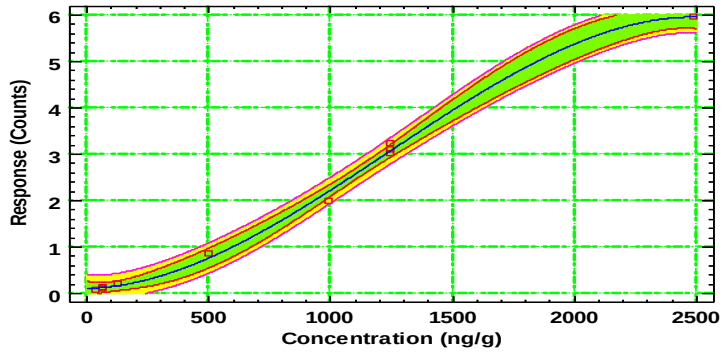



**Fig. 3.11. Lack-of-fit test for naproxen**

**Table 3.13. ANOVA with lack of adjustment**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 3.72157E13 | 3 | 1.24052E13 | 1130,97 | 0.0000 |
| Residue | 7.67805E10 | 7 | 1.09686E10 | | |
| Lack of adjustment | 5.5502E10 | 3 | 1.85007E10 | 3.48 | **0.1299** |
| Pure error | 2.12785E10 | 4 | 5.31963E9 | | |
| Total (Corr.) | 3.72924E13 | 10 | | | |

In summary, the method developed has been successfully validated for 10 PPCPs with different physical-chemical properties. Quantification based on matrix-matched approach and the results reported a linear regression for compounds such as methylparaben and diclofenac. However, other pollutants needed a polynomial regression with degree 2 or 3 as the case of ibuprofen and naproxen, among others.

On the other hand, revising the results achieved, it was decided to use the internal standard method over calibration curve for the analysis of different types of sludge collected from the WTTP in Valladolid. The use of internal standard means that the standard is in the same matrix as the analyte. The interferences present, wheter positive or negative, due to the matrix will affect the standards in the same way as they affect the analyte. The drawback is that they are really expensive and in some cases it is not possible get them. Fortunately, ten internal standards, such as the isotopically labelled rac-ibuprofen-d3, rac-

naproxen-d3, propyl-d7-paraben, salicylic acid-d4, triclosan-d3, diclofenac-d4, methylparaben-d4, ethylparaben-d5, clofibric acid-d4 and bisphenol A-d8 were used for different types of sludge.

### 3.2. Analysis of sludge samples

The proposed method was successfully applied to samples of different types of sludge from two indoor pilot scale reactors run at the Department of Chemical Engineering and Environmental Technology of the University of Valladolid (Spain): thermally pre-treated mixed sludge and digested sludge. The experimental devices to treat the sludge were: a 2-L thermal hydrolysis (TH) treatment plant treating thickened mixed sludge at 180 ºC during 30 min, and a 5-L continuous anaerobic digester operating in mesophilic conditions. Both reactors were daily supplied with thickened mixed sludge from the WWTP in Valladolid, whose details were described in the previous chapter. TH is a pre-treatment that reduces the viscosity of the sludge, increases its organic load and improves both the dehydratability and degradability of the treated sludge [26]. Anaerobic digestion (AD) decomposes organic matter with the aid of different microorganisms and the final product includes added-value products such as biogas (60-70%) and biomass that could be used as fertilizer [27].
One litre samples were grabbed in high density polyethylene (HDPE) drums for the inlets of both reactors at the beginning of the experiments. The same amount of sample was grabbed for the outlets after 2 and 24 hours of treatment of TH and AD, respectively. All samples were promptly centrifuged and the solid phase was stored at -20 ºC and darkness until analysis.

The results, which are displayed in **Table 3.14**, showed a significant degradation (range in 33-90%) of most of the compounds of interest during the TH process. However, the concentration of some PPCPs (propylparaben and bisphenol A) increased slightly after this treatment. Similarly, some concentrations remarkably decreased after the AD **(Table 3.15)**. This was the case of salicylic acid (99.9%), triclosan (48%), diclofenac (22%) and bisphenol A (32%). In contrast, clofibric acid, propylparaben and naproxen increased their concentrations after AD. An explanation for these augmentation events could be related to compound adsorption phenomena onto the solid residue during the sludge treatment. In addition, non-monitored pro-drugs and metabolites such as glucuronides could easily turn into the target analytes after the tested processes [28, 29]. Authors such as Boix et al., [30] also reported similar increases in the studied contaminants after urban sewage sludge anaerobic digestion.

A significant correlation between lipophilicity and the persistence of pharmaceutical residues was observed by [31] during anaerobic digestion. This mentioned correlation was also observed for naproxen in the present study.

Regardless, observed concentrations for the inlet and outlet sewages sludge samples were in the ng $g^{-1}$ level, in all cases. In particular, the compounds of interest were found at concentrations between <MLQ-8,332 ng $g^{-1}$ in the inlets. Ranges of 15-1,675 ng $g^{-1}$ and <MLQ-9,355 ng $g^{-1}$ were determined for TH and AD outlets, respectively.

## 4. Conclusions

An analytical method for the determination of PPCPs in urban sewage sludge has been designed, improved and validated, which can be used in routine analysis laboratories around the world. The instrumental analysis was based on an online DI-SPME-on fiber derivatization-GC-MS. The resulting environmentally friendly methodology decreased the use of expendable material (small amounts of reagents, reusable SPME fiber and derivatizing agent, …) and was successfully validated for 10 PPCPs (methylparaben, clofibric acid, ethylparaben, ibuprofen, propylparaben, salicylic acid, naproxen, triclosan, diclofenac and bisphenol A), with MLDs and MLQs below 30 ng $g^{-1}$ and 100 ng $g^{-1}$, respectively. The quantification method consisted of a matrix-matched approach. The calibration curves obtained from the lack-of-fit tests performed for the compounds of interest reported linear equations for certain compounds such as diclofenac and grade 2 or even grade 3 polynomial equations for compounds such as ibuprofen and naproxen, respectively. The linear range was obtained in the range from MLQ to 2,491 for all target analytes.

Real samples from both TH and AD pilot scale plants were analyzed. Some PPCPs such as methylparaben, clofibric acid, propylparaben and diclofenac were found at concentrations below 100 ng $g^{-1}$ (d.w.) in thermal hydrolysed samples. In contrast, another as salicylic acid presented a concentration above 1,000 ng $g^{-1}$ (d.w.) for the same matrix. A different scenario was observed after AD treatment. Some PPCPs such as methylparaben, clofibric acid, ethylparaben, ibuprofen, salicylic acid and bisphenol A were found at concentrations below 50 ng $g^{-1}$ (d.w). However, naproxen presented a concentration above 9,355 ng $g^{-1}$ for the same matrix.

**Table 3.14: Average concentrations of PPCPs after thermal hydrolysis treatment at 180ºC for 30 min**

| | **Methylparaben** | **Clofibric acid** | **Ethylparaben** | **Ibuprofen** | **Propylparaben** |
|---|---|---|---|---|---|
| | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** |
| **TM_In** | 108 (12) | 150 (12) | 150(10) | 425 (3) | 52 (6) |
| **TM_Out** | 73 (17) | 15 (33) | 138 (19) | 125 (19) | 72 (11) |

| | **Salicylic acid** | **Naproxen** | **Triclosan** | **Diclofenac** | **Bisphenol A** |
|---|---|---|---|---|---|
| | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** |
| **TM_In** | 2,500 (4) | 175 (7) | 2300 (21) | 158 (14) | 875 (13) |
| **TM_Out** | 1,675 (18) | 107 (21) | 945 (8) | 37 (22) | 957 (13) |

**TM_In: Thickened-Mixed Sludge Influent to thermal hydrolysis**
**TM_Out: Hydrolysed Thickened-Mixed Sludge Effluent after thermal hydrolysis**

**Table 3.15: Average concentrations of PPCPs in sewage sludge after anaerobic digestion**

| | **Methylparaben** | **Clofibric acid** | **Ethylparaben** | **Ibuprofen** | **Propylparaben** |
|---|---|---|---|---|---|
| | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** |
| **TM_In** | < MLQ (15) | 51 (33) | < MLQ (4) | 105 (8) | 113 (14) |
| **TM_Out** | < MLQ (17) | 61 (11) | < MLQ (21) | 48 (13) | 216 (11) |

| | **Salicylic acid** | **Naproxen** | **Triclosan** | **Diclofenac** | **Bisphenol A** |
|---|---|---|---|---|---|
| | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** |
| **TM_In** | 2,695 (2) | 8,332 (6) | 172 (13) | 250 (7) | 31 (21) |
| **TM_Out** | < MLQ (11) | 9,355 (1) | 90 (10) | 194 (7) | 21 (28) |

**TM_In: Thickened-Mixed Sludge Influent to anaerobic digestion**
**TM_Out: Thickened-Mixed Sludge Effluent after anaerobic digestion**

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# Chapter IV. Applications to dewatered digested sludge samples

## 1. Introduction

Several biological treatment possibilities can be adopted in wastewater treatment plants (WWTPs) for sludge stabilization, such as composting, anaerobic and aerobic digestion [1]. All these processes produce stable biosolids and value-added products such as compost and bioenergy (i.e. methane) with limited environmental impact [1-4].

Anaerobic digestion (AD) is a biological process in which microorganisms break down the organic matter in absence of oxygen, and gasses such as methane (over 60%), carbon dioxide and hydrogen sulphide are generated [5].

AD has the capacity to significantly reduce final solids, as well as destroying the majority of the pathogenic microorganisms in the sludge. Apart from these favourable aspects, AD presents a limitation: it is a relatively slow process due to the complex matrices as it requires a longer retention time in the hydrolysis stage and a bigger volume of bioreactor [1]. Taking into account that around 50% of the operation costs in a WWTP are related with the processing of sludge [5,6], the optimization of the AD is a key aspect for WWTPs economics. And thermal hydrolysis appears as a possible solution to enhance this limitation.

The thermal hydrolysis process (TH) is a pre-treatment to the anaerobic digestion process (AD) of organic matter and has been successfully employed around the world for over 20 years [7]. This pre-treatment achieves a reduction in sludge viscosity and an increase in dissolved organic matter, thus enhancing its subsequent removal by AD. It also reduces the volume of treated sludge to be disposed and transport and processing costs [8].

The most effective TH process consists of heating the organic matter under high pressure followed by sudden decompression of the pressurized material (steam explosion effect). This treatment breaks down the cell structure, which results in improved AD performance (increased biogas production from a more biodegradable waste) [8]. TH also sterilizes the waste and the destruction of pathogens or pasteurization allows to obtain a high quality sludge that can be used as fertilizer in substitution of chemicals [9]. Sludge concentration and a proper integration of the TH process are key aspects to achieve energetically self-sufficiency [10,11]. The pre-treatment of sludge with TH presents beneficial effects on sludge stabilization. Some researchers have used TH process with other pre-treatment technologies to achieve a high degree of efficiency, including thermal-$H_2O_2$ and thermal-alkaline [12]. Most recent research apply for innovative TH application in the sludge line, using either an intermediate thermal hydrolysis (ITH) between two AD stages [13] or a TH process after AD stage and before dewatering [14]. The use of ITH has accomplished a better performance of organic matter removal and biogas production, an enhanced dewaterability of the digestate, and an increase in the the degradation of recalcitrant chemical oxygen demand (COD) during the second AD stage [15].

Apart from those proven benefits of biogas increase, sludge reduction and hygienization, there is another important aspect to take into account, related to expected new regulations on emerging contaminants content for biosolids. AD of sludge seems to be partially effective for the elimination of pharmaceutically active compounds. Some studies exhibit that biocides (such as triclosan and triclocarban) and surfantants (such as sodium alkylsulfates) also reduced their concentration after AD [5].

On the other hand, authors as Chen et al. [16] reported that the persistence of trace organic pollutants of sludge causes some concerns for AD. For instance, methanogens are also extremely susceptible to trace organic pollutants (i.e., chlorophenols, halogenated

aliphatic and N-substituted aromatic compounds). In addition, a few anaerobic co-metabolic pathways generate some really toxic pollutants (e.g., nonylphenol, estradiol, etc.) with important effects for anaerobic sludge digestion [17]. In the case of secondary sewage sludge, AD is becoming gradually challenging due to the execution of severe rules on nitrogen limits, the removal of primary sedimentation units and longer sludge ages, among others [18,19].

The present chapter focuses on both sludge sample preparation and analysis of some micropollutants of interest. The concentration of those compounds present in sludge samples is measured in a process when TH is performed as inter-treatment between two AD stages, and compared with only two AD stages.

## 2. Materials and methods

### 2.1. Standards and reagents

All PPCPs standards were of high purity grade (> 95%, Sigma-Aldrich, Madrid, Spain) and were acquired as neutral non-solvated molecules, except for diclofenac (sodium salt). PPCPs acquired are summarized in **Table A1 (as Appendix I).**

Ten internal standards, such as the isotopically labelled rac-ibuprofen-d3, rac-naproxen-d3, propyl-d7-paraben, salicylic acid-d4, triclosan-d3, diclofenac-d4, methylparaben-d4, ethylparaben-d5, clofibric acid-d4 and bisphenol A-d8 (LGC Standards, Barcelona, Spain) **(Table A1)** were used.

Individual stock solutions at 1,000 mg $L^{-1}$ for isotopically labelled internal standards were prepared in methanol (MeOH). From them, a mixture of isotopically labelled internal standards in MeOH at 20 mg $L^{-1}$ and their corresponding serial dilutions in acetone (2, 0.5, 0.05, 0.005) mg $L^{-1}$ were prepared. All solutions were stored at -20 °C in darkness.

High purity solvents, i.e., LC-MS Chromasolv® Ethyl Acetate (EA) grade from Fluka (Madrid, Spain), SupraSolv® GC-MS MeOH grade by Merck Millipore (Madrid, Spain), Sodium Chloride (NaCl) and Hidrochloric acid (HCl) with 37% purity were supplied by Panreac (Barcelona, Spain). Aluminium oxide by Sigma-Aldrich (Tres Cantos, Madrid, Spain). Acetone ($C_3H_6O$), with 99% purity, was supplied by Cofarcas (Burgos, Spain). N-terc-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with a purity greater than 99% was obtained from Sigma-Aldrich (Tres Cantos, Madrid, Spain). The Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibres were acquired from Supelco (Tres Cantos, Madrid, Spain). All aqueous solutions were prepared in deionized water with a resistivity not less than 18 MΩ cm. Helium (He) with 99.999% purity was acquired from Abelló Linde S.A. (Alcalá de Henares, Madrid, Spain).

### 2.2. Sludge sampling

As in previous chapters, sewage sludge samples were collected from a WWTP in Valladolid (Spain). In this case, the wastewater treatment consists of an activated sludge biological process in which organic matter is descomposed. The surplus biological sludge generated during aerobic treatment is mixed with primary sludge and fed to anaerobic digestion. Digestion reduces the total mass of solids, destroys pathogens, and makes it

easier to dewater or dry the sludge. Digested sludge (DS) is called biosolids, having the appearance and characteristics of a rich potting soil.

DS is dewatered before disposal. Dewatered sludge still contains a significant amount of water (as much as 80%) but, even with that moisture content, sludge no longer behaves as a liquid and can be handled as a solid material. This is the sludge used in this study.

### 2.3. Pilot plants for thermal pre-treatment and anaerobic digestion

DS was thermally hydrolysed in a pilot plant to assess the effect of a thermal treatment on emerging contaminants removal. The lab-scale TH plant used consisted of a stainless-steel cylindrical batch reactor with a working volume of 1 L (2 L total volume) coupled to a steam boiler and a 35L stainless-steel flash tank. Sludge was manually fed to the reactor through a feeding cone and a ball valve, and then a saturated steam (16 bar) was supplied from the boiler and regulated with a control valve to achieve a temperature of 170 ºC in the reactor. Every batch was maintained for 30 min before a sudden decompression of the sludge to atmospheric pressure (steam explosion effect), while the process vapours were released. These operating conditions were based on previous studies [20], which reported optimal conditions for sludge pre-treatment of 160 – 180 ºC for 20 to 40 min and a sudden decompression to atmospheric pressure.

Anaerobic digestion in the laboratory was performed in continuously-fed digesters (20 L working volume) built in polyethylene and coated with an electric resistance to maintain mesophilic conditions (35 ºC). Sludge was fed with peristaltic pumps to achieve a residence time of 20 d, and the biogas flow rate was measured by liquid displacement at atmospheric pressure. Two digesters were operated: one fed with dewatered sludge (CONTROL digester), and another one fed with thermally pre-treated sludge (TH digester).

### 2.4. Sample preservation and pre-treatment

Sample collection and pre-treatment consisted of the following steps:

I. *Sampling collection and preservation*. Grab samples of dewatered digested sludge were randomly collected and combined to provide a final sample of approximately 25 kg. The samples were collected in high density polyethylene (HDPE) drums with polypropylene screw caps. Then, they were properly sealed and taken to the laboratory under conditions of refrigeration and darkness.

II. *Freeze-Drying*. After two days of refrigeration, an amount around ~25-30 g of solid phase was freeze-dried and stored at -20 ºC in darkness until analysis.

III. *Spiking*. An exact amount of freeze-dried sewage sludge (~ 0.8 g) was placed in a vessel and spiked with 200 µL of a solution at 2 mg $L^{-1}$ in acetone containing a mixture of all isotopically labelled internal standards and homogenized. Then, it was kept in contact overnight in the extraction hood to allow solvent evaporation and internal standard fixation. Sample size was chosen by recommendations found in the literature for similar matrixes [21].

IV. *Pre-treatment for desorption of the analytes to aqueous phase*. The sample underwent, then, microwave assisted extraction (MAE) in a Milestone START-D Microwave Digestion System (Madrid, Spain) at 110 ºC during 30 minutes to facilitate the desorption of the analytes. Twenty-four millilitres of a MilliQ® water/MeOH mixture, 95:5 (v/v) at pH 9 were used as extracting solvent. At this pH, all the target compounds were supposed to be as negative ions, increasing their affinity for the liquid phase. Subsequently, 100.0 mg of activated alumina ($Al_2O_3$ at 100 ºC for 48 hours) were added for matrix in-situ clean-up.

V. *After MAE centrifugation*. The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was collected (20-22 mL) with a glass pipette and transferred to a 25-mL glass beaker. The total of supernatant was saturated with ~7.5 g NaCl (solubility in water at 25 °C is 359 g $L^{-1}$) at 36% (weight/volume) to increase the ionic strength. The resulting sample was also pH-adjusted to 3 with HCl, in order to increase the analyte lipophilia by shifting their acid-base equilibrium into neutral molecules. Finally, the extract was filtered through a 0.7-µm glass fiber (GF) syringe filter and 17.0 mL of the filtrate was collected in a 20.0 mL SPME glass vial.

### 2.5. Analysis by GC-MS

Analysis of target compounds was based on automatized direct immersion solid phase microextraction (DI-SPME), online followed by on-fiber derivatization, coupled to gas chromatography (Agilent 7890B) detected by mass spectrometry (Agilent 5977A) (GC-MS). This method was based on another one published elsewhere [18]. However, important upgrades were implemented. Hence, 90 min sample extraction at a penetration depth of 60 mm, 45 min derivatization step at a penetration depth of 45 mm, orbital agitation at 350 rpm with a stirring regime of 6s on/20s off were implemented to increase SPME fiber life time **(Table A2)**. In fact, these adjustments extended average fiber lifespan beyond 80 injections and up to 130 injections with no signs of performance deterioration, which entails a 62% lifespan increase.with no signs of performance deterioration with no signs of performance deterioration. A DVB/CAR/PDMS SPME fiber was utilized for the analysis. Chromatographic separation was achieved on a capillary HP-5MS GC column (30 m length, 0.25 mm i.d., 0.25 µm film thickness) with He as carrier gas at a constant flow rate of 1.2 mL $min^{-1}$. Injector temperature was set at 250 ºC, while the GC oven temperature increased from 70 ºC (held for 3 min during fiber desorption) to 150 ºC at 50 ºC $min^{-1}$, to 220 ºC at 5 ºC $min^{-1}$ and finally to 300 ºC (held for 5 min) at 10 ºC $min^{-1}$. The total analysis time for each injection was 31.6 min. Mass detection was obtained in electron impact ionization mode (70 eV) with selected ion monitoring (SIM) and a filament delay of 8 min. The GC–MS interface, ion source and quadrupole temperatures were set at 280, 230 and 150 °C, respectively.

10 target compounds were recorded in six acquisition windows along the run time. Acquisition stopped at 26 min. Data acquisition and evaluation were performed by Agilent Technology Mass Hunter B.07.03.2129 software. The primary ions (in black) and two secondary ions monitored for each compound are reported in the following table (**Table 4.1**).

**Table 4.1: MS parameters for the final target compounds and internal standards**

| Analyte | [a]IS | Chemical name | Adquisition window | [b]$t_R$ (min) | [c]SIM ions, m/z | | |
|---|---|---|---|---|---|---|---|
| 1 | | Methylparaben | 1 | 9.531 | **209.1** | 210.1 | 135.1 |
| | 1 | Methylparaben-d4 | | 9.524 | **213.2** | 214.2 | 139.1 |
| 2 | | Clofibric acid | 2 | 10.449 | **143.1** | 271.1 | 185.1 |
| | 2 | Clofibric acid-d4 | | 10.427 | **143.1** | 275.1 | 75.1 |
| 3 | | Ethylparaben | | 10.536 | **223.1** | 224.1 | 151.1 |
| | 3 | Ethylparaben-d5 | | 10.463 | **228.2** | 229.2 | 230.2 |
| 4 | | Ibuprofen | | 11.059 | **263.2** | 264.2 | 117.1 |
| | 4 | rac Ibuprofen-d3 | | 11.074 | **266.2** | 267.2 | 164.2 |
| 5 | | Propylparaben | 3 | 12.062 | **237.2** | 238.2 | 151.1 |
| | 5 | Propylparaben-d7 | | 11.989 | **244.2** | 245.2 | 152.1 |
| 6 | | Salicylic acid | | 12.760 | **309.2** | 310.2 | 195.1 |
| | 6 | Salicylic acid-d4 | | 12.751 | **313.2** | 314.2 | 312.2 |
| 7 | | Naproxen | 4 | 18.508 | **287.2** | 185.1 | 288.2 |
| | 7 | rac Naproxen-d3 | | 18.459 | **290.2** | 188.1 | 207.1 |
| 8 | | Triclosan | | 19.311 | **347.0** | 345.0 | 200.0 |
| | 8 | Triclosan-d3 | | 19.309 | **350.0** | 348.0 | 200.0 |
| 9 | | Diclofenac | 5 | 21.571 | **352.1** | 214.1 | 354.1 |
| | 9 | Diclofenac-d4 | | 21.528 | **356.1** | 218.1 | 158.1 |
| 10 | | Bisphenol A | 6 | 23.096 | **441.0** | 207.0 | 442.0 |
| | 10 | Bisphenol A-d8 | | 23.036 | **449.4** | 211.2 | 450.4 |

[a]IS: internal standard
[b]$t_R$: retention time
[c]SIM: selected ion monitoring

### 2.6. Quantification method

Quantification was based on an internal standard approach. Five-point calibration curves were built by spiking equal sludge samples covering the range from 59 to 47,059 ng $L^{-1}$, for all target compounds. Three-point calibration curves were prepared in triplicate (n=3). The calibration curves obtained for the most compounds were linear, with correlation coefficients ($R^2$) above 0.99 for all target compounds (**Table 4.2**).

**Table 4.2: Lack-of-fit test for dewatered digested sludge**

| | Dynamic range | | |
|---|---|---|---|
| **Chemical name** | **Equation** | **$R^2$** | **Linear range (ng $g^{-1}$)** |
| **Methylparaben** | $y = 1.0632x + 0.0076$ | 0.9996 | LOQ-1412 |
| **Clofibric acid** | $y = 2.2394x + 2.5362$ | 0.9992 | LOQ-1369 |
| **Ethylparaben** | $y = 1.4967x + 0.0427$ | 0.9983 | LOQ-1412 |
| **Ibuprofen** | $y = -0.397x^2 + 1.4682x + 0.0432$ | 0.9906 | LOQ-538 |
| **Propylparaben** | $y = -0.2193x^2 + 1.1837x + 0.0043$ | 0.9991 | LOQ-857 |
| **Salicylic acid** | $y = 1.4737x + 0.0374$ | 0.9987 | LOQ-1397 |
| **Naproxen** | $y = 0.507x – 0.0017$ | 0.9965 | LOQ-1412 |
| **Triclosan** | $y = 0.4612x + 0.0962$ | 0.9974 | LOQ-1412 |
| **Diclofenac** | $y = 0.6196x + 0.013$ | 0.9905 | LOQ-1313 |
| **Bisphenol A** | $y = 0.8685x – 0.0038$ | 0.9985 | LOQ-1398 |

A calibration curve **(fig. 4.1)**, generated by internal standard, corresponding to methylparaben (preservative) is shown below.

The equation of the adjusted model, which describes the relationship between the **Response** (counts) and the **Concentration** (ng $L^{-1}$), is as follows:

$$\text{Response (counts)} = -0.00760258 + 1.06313*\text{Concentration (ng } L^{-1})$$

The P-value in the Analysis of Variance (ANOVA) was less than 0.05. Hence, there was a significant statistical relationship between Response (counts) and Concentration (ng $L^{-1}$) with a 95.0% confidence level. Furthermore, the R-Square statistic parameter indicated that the adjusted model explains 99.9627% of the variability in Response (counts). The correlation coefficient was equal to 0.999814, indicating a relatively strong relationship among the variables.

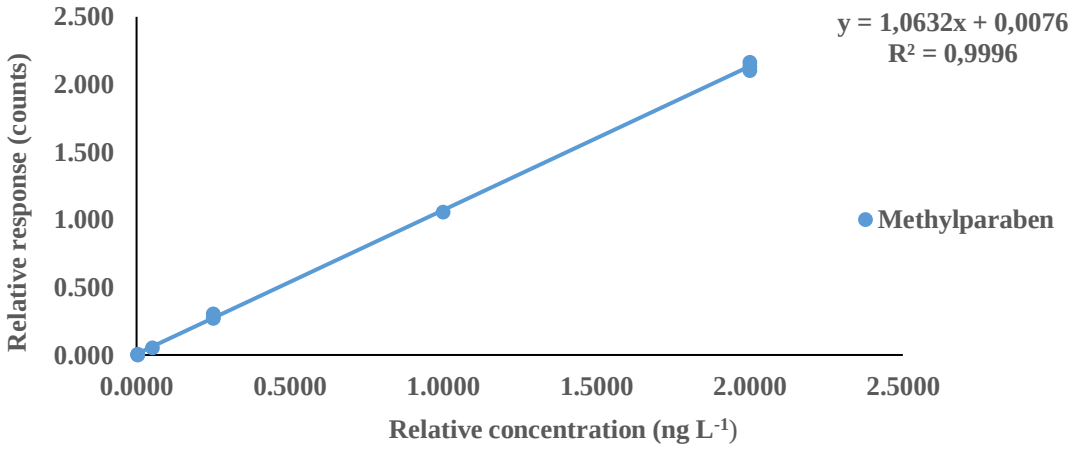


**Fig.4.1. Calibration curve based on internal standard quantification approach for methylparaben**

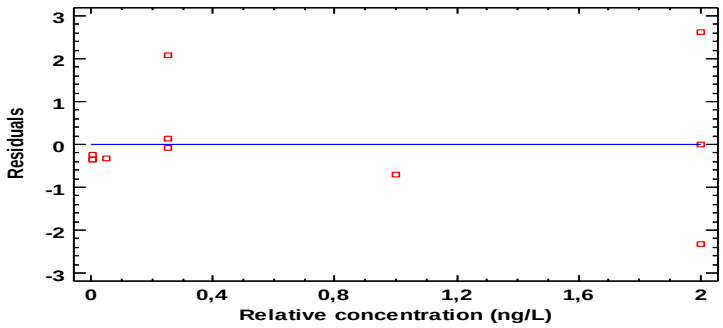


**Fig.4.2. Residuals for methylparaben**

In the case of **figure 4.3,** the P-value for the lack of adjustment in ANOVA (**Table 4.3**) is also higher than or equal to 0.05 and the model seemed to be adequate for data observed with a 95.0% confidence level.

**4.3. Coefficients of adjusted model**

| | *Least-squares* | *Standard* | *Statistic* | |
|---|---|---|---|---|
| *Parameter* | *Estimated* | *Error* | *T* | *P-value* |
| Intercept | **0.00760258** | 0.00749483 | 1.01438 | 0.3369 |
| Slope | **1.06313** | 0.0068444 | 155.328 | 0,0000 |

**Table 4.4. ANOVA**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *P-value* |
|---|---|---|---|---|---|
| Model | 8.64461 | 1 | 8.64461 | 24126,84 | **0.0000** |
| Residue | 0.00322469 | 9 | 0.000358299 | | |
| Total (Corr.) | 8.64784 | 10 | | | |

The **Lack-of-fit test** is designed to determine whether the selected model is adequate to describe the observed data, whether a more complicated model should be used. The test was performed comparing the variability of the residues in the current model with the variability between observations made in repeated values of the independent variable X. Two generated calibration curves are shown in the following **figures 4.3** and **4.6** and the rest of calibration curves are reported in **Appendix I.**

In the case of **figure 4.3**, the P-value for the lack of adjustment in the Analysis of Variance (ANOVA) (**Table 4.5**) showed that is higher than or equal to 0.05 and the model seemed to be adequate for data observed with a 95.0% confidence level.

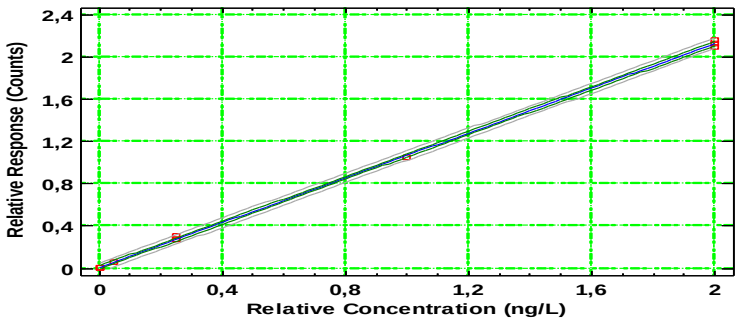


**Fig. 4.3. Lack-of-fit test for methylparaben**

**Table 4.5. ANOVA with lack of adjustment**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 8.64461 | 1 | 8.64461 | 24126,84 | 0.0000 |
| Residue | 0.00322469 | 9 | 0.000358299 | | |
| Lack of adjustment | 0.000650688 | 3 | 0.000216896 | 0.51 | **0.6925** |
| Pure error | 0.002574 | 6 | 0.000429 | | |
| Total (Corr.) | 8.64784 | 10 | | | |

In the case of **fig. 4.4** shows calibration curve generated by a target analyte as **salicylic acid** (analgesic/anti-inflammatory).

The equation of the adjusted model, which describes the relationship between the **Response** (counts) and the **Concentration** (ng $L^{-1}$), is as follows:

$$\text{Response (counts)} = -0.03739 + 1.47377*\text{Concentration (ng } L^{-1})$$

The P-value in the ANOVA was less than 0.05. Hence, there was a significant statistical relationship between Response (counts) and Concentration (ng $L^{-1}$) with a 95.0% confidence level. Furthermore, the R-Square statistic parameter indicated that the adjusted model explains 99.8718% of the variability in Response (counts). The correlation coefficient was equal to 0.999359, indicating a relatively strong relationship among the variables.

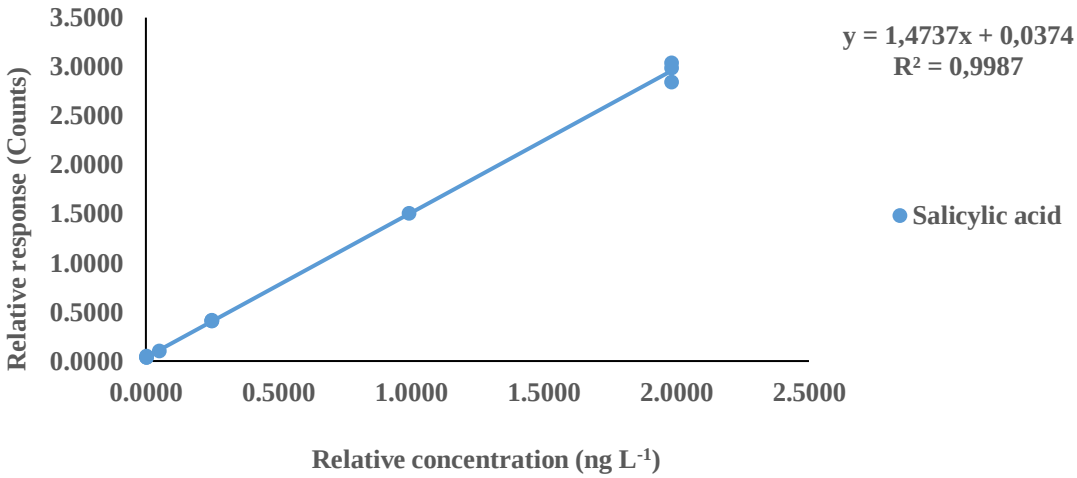


**Fig. 4.4. Calibration curve based on internal standard quantification apparoach for salicylic acid**

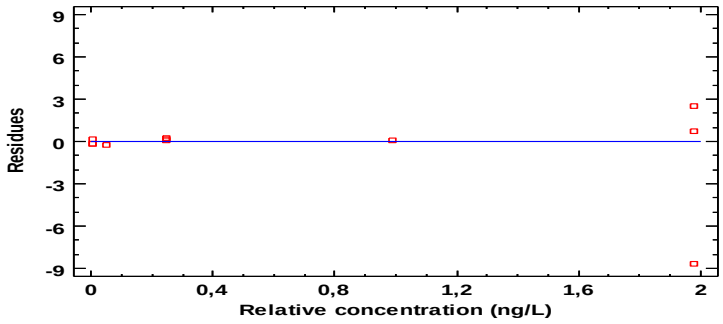


**Fig.4.5. Residuals for salicylic acid**

## 4.6. Coefficients of adjusted model

| | *Least-squares* | *Standard* | *Statistic* | |
|---|---|---|---|---|
| *Parameter* | *Estimated* | *Error* | *T* | *P-value* |
| Intercept | **0.03739** | 0.0190801 | 1.95963 | 0.0817 |
| Slope | **1.47377** | 0.0176002 | 83.7357 | 0.0000 |

**Table 4.7. ANOVA**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *P-value* |
|---|---|---|---|---|---|
| Model | 16.2801 | 1 | 16.2801 | 7011.67 | **0.0000** |
| Residue | 0.0208968 | 9 | 0.00232186 | | |
| Total (Corr.) | 16.301 | 10 | | | |

In the case of **figure 4.4**, the P-value for the lack of adjustment in the ANOVA (**Table 4.8)** showed that is higher than or equal to 0.05 and the model seemed to be adequate with a 95.0% confidence level.

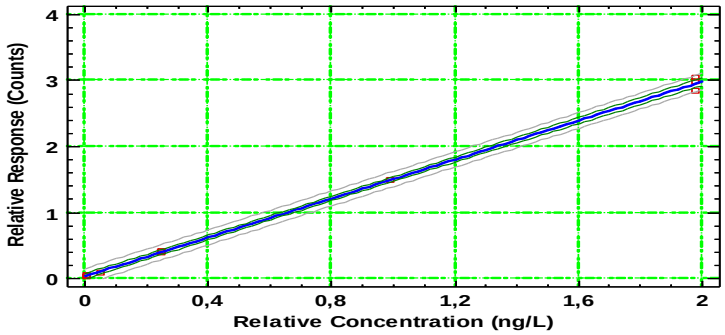


**Fig. 4.6. Lack-of-fit for salicylic acid**

**Table 4.8. ANOVA with lack of adjustment**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 16.2801 | 1 | 16.2801 | 7011,67 | 0.0000 |
| Residue | 0.0208968 | 9 | 0.00232186 | | |
| Lack of adjustment | 0.000321663 | 3 | 0.000107221 | 0.03 | **0.9918** |
| Pure error | 0.0205751 | 6 | 0.00342918 | | |
| Total (Corr.) | 16.301 | 10 | | | |

### 3. Results and discussion

As described in Material and Methods, dewatered sludge was sampled from the WWTP and treated by thermal hydrolysis and digestion. One digester was fed with dewatered sludge (CONTROL digester), and the other one with thermally pretreated sludge (TH digester).

The analytical method for the determination of PPCPs was used to evaluate the occurrence of the compounds of interest in the different types of sludge samples, named according to the following table:

**Table 4.9. Types of sludge used to evaluate the occurrence of the compounds of interest**

| Nomenclature | Type of sludge | Process |
|---|---|---|
| DW_In | Dewatered sludge | CONTROL-digester inlet |
| DW_Out-1 | Dewatered digested sludge-1 | CONTROL digester outlet (measurement 1) |
| DW_Out-2 | Dewatered digested sludge-1 | Control digester outlet (measurement 2) |
| TH_In | Dewatered hydrolysed sludge | TH-digester inlet |
| TH_Out | Dewatered hydrolysed digested sludge | TH-digester outlet |

Each analysis was carried out in triplicate (n = 3). **Fig. 4.7** and **4.8** show the chromatograms obtained for samples of both CONTROL and TH digesters, therefore corresponding to digested effluents of dewatered sludge and hydrolysed sludge, respectively.

Average results of the measured compounds are reported in **Tables 4.10** and **4.11**. **Table 4.10** presents the results for the CONTROL digester fed with dewatered sludge, whose effluent was sampled in two moments (DW_Out-1 and 2). And Table 4.10 presents the results for the digester fed with dewatered and hydrolysed sludge (TH-digester).

When analysing the values for the CONTROL digester, both measurements 1 and 2 exhibit a similar behabiour. It can be observed that there is none or scarce removal for most of the target emerging micropollutants. Only ibuprofen and propylparaben presented a removal around 60%, while for others such as methylparaben, ethyhylparaben, diclofenac and naproxen the removal was in the range 10-30%. The rest of compounds (clofibric acid, salicylic acid, triclosan and bisphenol A) exhibited an increase in their concentration during AD.

An explication for this observed augmentation episodes could be associated to compound adsorption phenomena onto the solid residue during the sludge treatment. Additionally, non-monitored pro-drugs and some metabolites such as glucuronides might easily turn into the target compounds after the tested processes [19, 22]. Some authors as Boix et al., [23] also showed analogous increases in the considered contaminants after urban sewage sludge anaerobic digestion

Regarding the TH-digester, which is the digester fed with dewatered and hydrolysed sludge, the results displayed in **Table 4.11**, report a relevant degradation (from 31 to 76% removal) of most of the compounds of interest during a TH pre-treatment followed by an AD process. This was the case of diclofenac (76%), triclosan (45%), salicylic acid (57%) and propylparaben (34%). Only ibuprofen and bisphenol A increased slightly their concentration in the AD process.

Taking into account the results obtained, it can be stated that TH pre-treatment allowed to reach higher degradation percentages compared to AD process without TH pre-treatment. Some authors as Díaz et al. [15] also reported that TH process improved the elimination of emerging pollutants and the use of TH as intertreatment between two AD stages presented the maximum removal efficiency of organic matter, solids and nitrogen.

Regarding emerging pollutants, an enhancement of their total removal from 50% in conventional AD to 80% in TH processes was observed.

Finally, regarding the observed concentrations for the inlet and outlet, it is important to mention that it was expressed in ng $g^{-1}$ level, in all cases. Specifically, the compounds of interest were found at concentrations between <MLQ-10,045 ng $g^{-1}$ in the inlets. Ranges of <MLQ-4,267 ng $g^{-1}$ and <MLQ-962 ng $g^{-1}$ were determined for TH and AD outlets, respectively.

## 4. Conclusions

An improved method for the determination of PPCPs at low levels was satisfactorily applied in different types of sludge samples. The quantification method consisted of an internal standard approach. Most of the calibration curves obtained for target compounds were linear. Only ibuprofen and propylparaben reported calibrations curves based on degree two polynomial regression. For both ibuprofen and propylparaben, the linear range was obtained in the range from LOQ to 538 and 857, respectively, being lower than for the rest of the compounds.
In dewatered digested sludge samples, the main compounds present to the highest values were triclosan (702 ng $g^{-1}$), and bisphenol A (761 ng $g^{-1}$). Only ibuprofen and propylparaben presented a significant removal (around 60%), while for others such as methylparaben, ethyhylparaben, diclofenac and naproxen the removal scarce (10-30%).

In the case of implementing a thermal hydrolysis step prior to the digestion, the analytical method was also successfully applied to determinate PPCPs. In influent, the highest values were obtained for compounds such as salicylic acid (10,045 ng $g^{-1}$) and triclosan (762 ng $g^{-1}$). A relevant degradation (from 31 to 76% removal) occurred of most of the compounds of interest during a TH pre-treatment followed by an AD process. In the AD effluent, the highest concentrations were obtained for salicylic acid and triclosan (4,267 ng $g^{-1}$ and 417 ng $g^{-1}$, respectively). The rest of the pollutants were found at concentration below 200 ng $g^{-1}$. The maximum removal efficiencies were detected for pharmaceuticals such as diclofenac (76%) and salicylic acid (57%). The lowest removals were obtained for propylparaben (34%) and methylparaben (31%).

From the results obtained, it can be concluded that the thermal pre-treatment of dewatered sludge from digestion improved the removal of many target compounds evaluated.

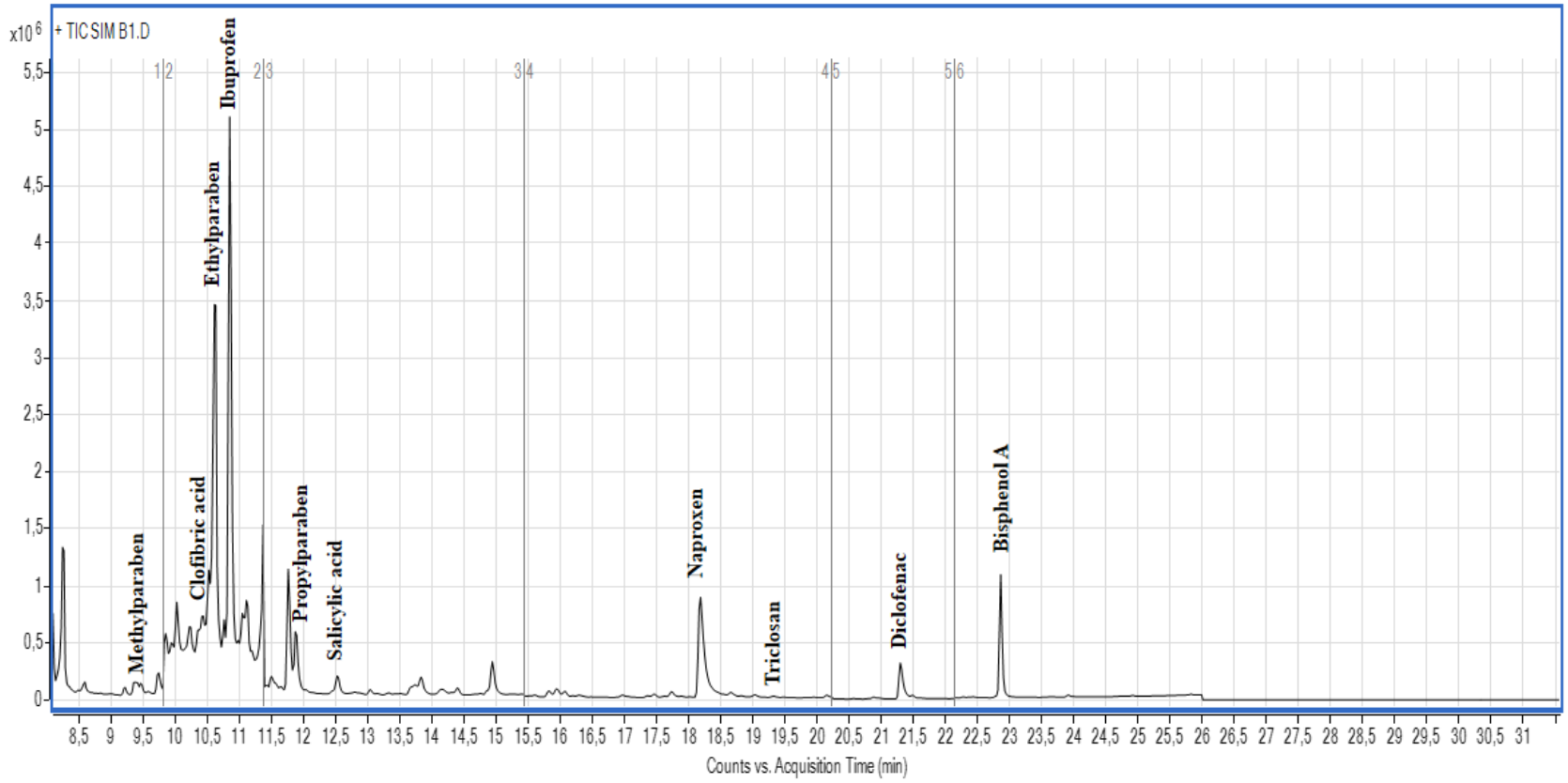


**Fig. 4.7. Chromatogram obtained for dewatered digested sludge (DW_Out)**

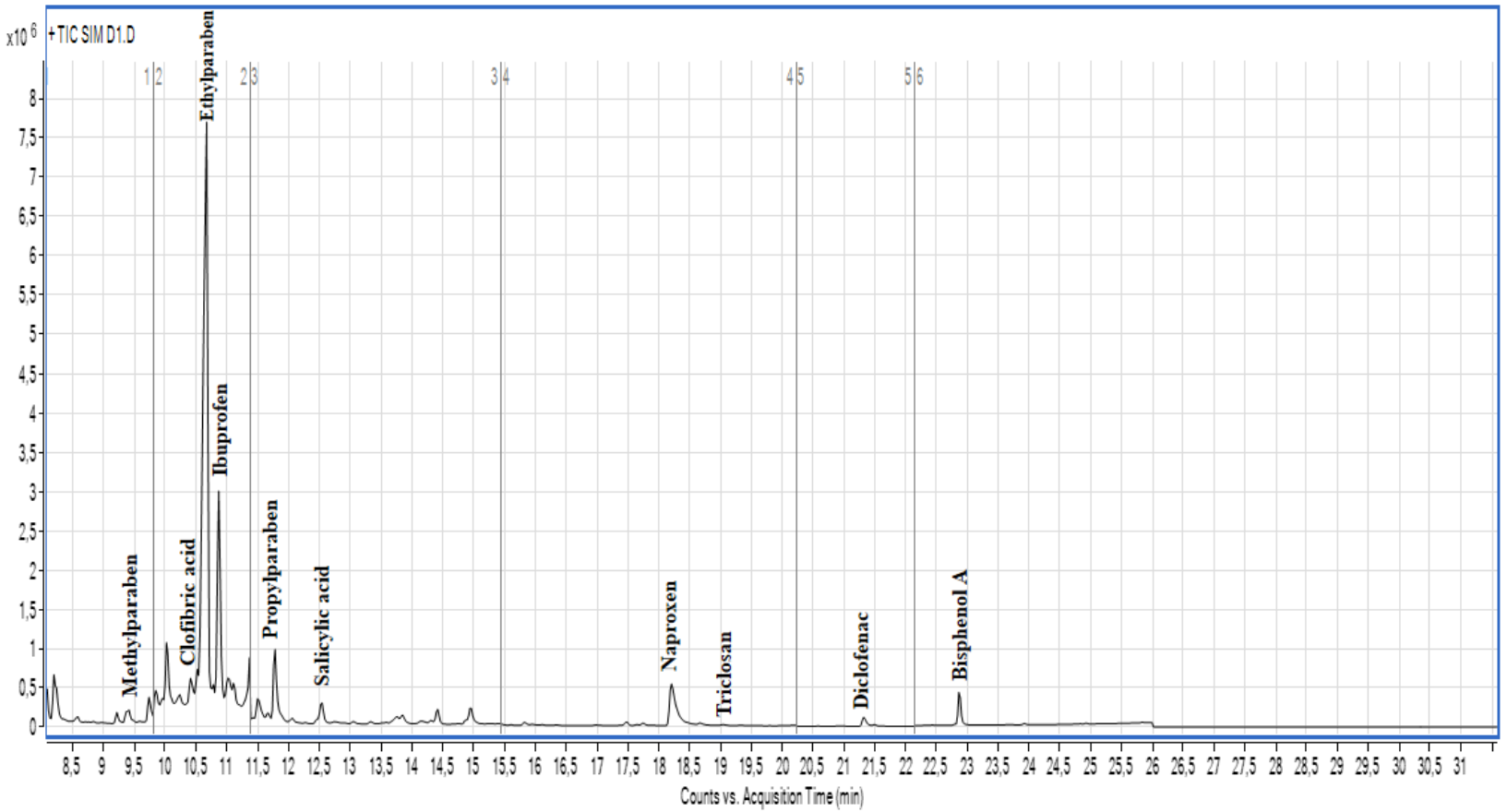


**Fig. 4.8. Chromatogram obtained for dewatered hydrolysed digested sludge (TH_Out)**

**Table 4.10: Average concentrations of PPCPs for dewatered sludge inlet and outlet of anaerobic digestion (CONTROL-digester)**

| | Methylparaben | Clofibric acid | Ethylparaben | Ibuprofen | Propylparaben |
|---|---|---|---|---|---|
| | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) |
| **DW_In** | 220 (6) | 51(5) | 18 (0.4) | 139 (29) | 180 (14) |
| **DW_Out-1** | 157 (1) | 72 (15) | 16 (13) | 58 (13) | 74 (2) |
| **DW_Out-2** | 194 (11) | 61 (18) | 15 (15) | 72 (15) | 96 (16) |

| | Salicylic acid | Naproxen | Triclosan | Diclofenac | Bisphenol A |
|---|---|---|---|---|---|
| | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) | Concentration ng $g^{-1}$ (%RSD) |
| **DW_In** | 283 (14) | 44 (23) | 702 (11) | 64 (14) | 761 (15) |
| **DW_Out-1** | 333 (24) | 37 (11) | 921 (15) | 46 (7) | 1,114 (19) |
| **DW_Out-2** | 401 (20) | 36 (3) | 828 (22) | 46 (10) | 1,212 (15) |

**DW_In: Dewatered sludge Influent to anaerobic digestion**
**DW_Out-1: Dewatered sludge Effluent after anaerobic digestion (measurement 1)**
**DW_Out-2: Dewatered sludge Effluent after anaerobic digestion (measurement 2)**

**Table 4.11: Average concentrations of PPCPs for dewatered and hydrolysed (170ºC-30minutes) sludge inlet and outlet of anaerobic digestion (TH-digester)**

| | **Methylparaben** | **Clofibric acid** | **Ethylparaben** | **Ibuprofen** | **Propylparaben** |
|---|---|---|---|---|---|
| | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** |
| **TH_In** | 108 (12) | <LOQ (5) | <LOQ (5) | 31 (6) | 104 (34) |
| **TH_Out** | 74 (17) | <LOQ (4) | <LOQ (2) | 49 (18) | 69 (5) |

| | **Salicylic acid** | **Naproxen** | **Triclosan** | **Diclofenac** | **Bisphenol A** |
|---|---|---|---|---|---|
| | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** | **Concentration ng $g^{-1}$ (%RSD)** |
| **TH_In** | 10045 (15) | 2 (2) | 762 (11) | 158 (14) | 148 (5) |
| **TH_Out** | 4267 (4) | 2 (4) | 417 (21) | 37 (22) | 191 (13) |

**TH_In: Dewatered and Hydrolysed sludge Influent to anaerobic digestion**
**TH_Out: Dewatered and Hydrolysed sludge Effluent after anaerobic digestion**

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# Chapter V. Analysis of sludge samples by LC-MS/MS

## 1. Introduction

Pharmaceuticals and personal care products (PPCPs) are a group of the compounds known as emerging organic pollutants, which include several classes of organic chemicals [1]. Pharmaceuticals are mainly used to treat human and animal diseases, and personal care products are specially used to get a better quality of daily life [2].

For several years, diverse studies have confirmed the presence of PPCPs in various environmental matrices at concentrations able to cause adverse effects on the ecosystems and human health [3]. Principal discharges of PPCPs into the environment depend on the industrial and agricultural waste, pharmaceutical industry, accidental spills and, principally, urban wastewater after incomplete adsorption being excreted by the urine and faeces [4]. Nowadays, the average amount of sewage sludge produced per person per day on the European continent is valued at 90 g d.w [5]. The generated biosolids are predominantly reused in agriculture as soil improvement. In spite of the obvious benefits related to the recycling of nutrients and organic matter, it constitutes an additional route of entry of organic pollutants into the environment. Subsequently, an exhaustive report of the presence of PPCPs in sewage sludge is essential to obtain a complete description of these emerging contaminants in the environment and to carry out a reliable risk assessment [6]. The use of analgesic and anti-inflammatory drugs is really high in countries of southern Europe as Spain [7] and ibuprofen and salicylic acid are compounds with the highest concentrations found in wastewater and sludge [8].

On the other hand, analytical methodologies developed for the determination of PPCPs in environmental matrices have had a great boom in recent years. These compounds present a wide range of psychochemical properties and include different polar and non-volatile substances. Pérez-Lemus et al. [9] and Primel et al. [10] reported that the instrumental analysis has been managed by techniques as Gas Chromatography coupled to Mass Spectrometry (GC-MS), which is a common technique in routine analysis laboratories all over the world, involving developing countries [11]. However, Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) is the main technique used for the analysis of PPCPs in environmental matrices due to its selectivity, specificity and versatility [12, 13]. However, this analysis technique is really costly and many laboratories worldwide cannot afford this type of instrumentation.

The main goal of this chapter was to develop a multi-residue method for the simultaneous determination of a significant number of PPCPs in sewage sludge. The target analytes belonged to diverse therapeutic groups of pharmaceuticals (antibiotics, analgesics/anti-inflammatories, hormones, lipid regulators, psychiatric and cardiovascular drugs) and personal care groups (preservatives, anti-parasitics, surfactants, plasticizers and antimicrobials). Target compounds were extracted by ultrasound-assisted extraction (UAE) using a mixture of MilliQ® water/methanol (MeOH) mixture, 95:5 (v:v) adjusted to pH 9. A filtration step through 0.45 µm prior to the sample analysis was carried out.The extracts were cleaned up by online solid-phase extraction (SPE), which was coupled to ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The resulting methodology was tested by the trace determination of 60 PPCPs in sewage sludge samples, standing out short time consumption and environmental and analyst safety. Finally, the limitations encountered in applying this methodology were also discussed.

## 2. Materials and methods

### 2.1. Standards and reagents

All PPCPs standards were of high purity grade (> 95%, Sigma-Aldrich, Madrid, Spain) and were acquired as neutral non-solvated molecules, except for diclofenac (sodium salt) and atorvastatin (calcium salt). PPCPs acquired are summarized in **Table A3** (as **Appendix I**).

Sixteen internal standards, such as the isotopically labelled enrofloxacin-d5, sulfadiazine-d4, sulfadimidine-d4, sulfamethoxazole-d4, ciprofloxacin-d8, danofloxacin-d3, rac-ibuprofen-d3, rac-naproxen-d3, propyl-d7-paraben, salicylic acid-d4, triclosan-d3, diclofenac-d4, methylparaben-d4, ethylparaben-d5, clofibric acid-d4 and bisphenol A-d8 (LGC Standards, Barcelona, Spain) **(Table A3** as **Appendix I)**, were used.

Individual stock solutions at 1,000 mg $L^{-1}$ for both PPCPs standards were prepared in methanol (MeOH), MeOH/$H_2O$ (1:1) and 0.2% HCl MeOH/$H_2O$ (1:1). Isotopically labelled internal standards were prepared in MeOH. From them, a stock solution with all the analytes was prepared in MeOH at 20 mg $L^{-1}$. Fresh serial dilutions (2, 0.5, 0.05, 0.005) mg $L^{-1}$ in methanol were subsequently prepared from it when need them. A mixture of isotopically labelled internal standards in MeOH and their corresponding serial dilutions in methanol (2, 0.5, 0.05, 0.005) mg $L^{-1}$ were also prepared. All solutions were stored at -20 °C in darkness.

High purity solvents, i.e., LC-MS grade MeOH, LC-MS grade acetonitrile (ACN) and formic acid (FA) 98% by Labbox (Madrid, Spain), Ethylenediaminetetraacetic acid (EDTA) and Aluminium oxide from Sigma-Aldrich (Tres Cantos, Madrid, Spain), Hidrochloric acid (HCl) with 37% purity and Sodium Hidroxide (NaOH) with 98% purity and were supplied by Panreac (Barcelona, Spain). All aqueous solutions were prepared in deionized water with a resistivity not less than 18 MΩ cm. Nitrogen 99.999% ($N_2$) was obtained from Abelló Linde S.A. (Alcalá de Henares, Madrid, Spain).

### 2.2. Sample preservation and pre-treatment

As in previous chapters, sewage sludge samples were collected from a Wastewater Treatment Plant (WWTP) in Valladolid (Spain). In particular, samples consisted of dewatered digested sludge. Valladolid WWTP operation conditions were described in the previous chapter.

Sample collection and pre-treatment consisted of the following steps:

I. *Sampling*. Grab samples of dewatered digested sludge were randomly collected and combined to provide a final sample of approximately 25 kg. The samples were collected in high density polyethylene (HDPE) drums with polypropylene screw caps. Then, they were properly sealed and taken to the laboratory under conditions of refrigeration and darkness.

II. *Freeze-Drying*. After two days of refrigeration, an amount around ~25-30 g of solid phase was freeze-dried and stored at -20 ºC in darkness until analysis.

III. *Spiking*. An exact amount of freeze-dried sewage sludge (~ 0.3 g) was placed in a vessel and spiked with 200 µL of a solution at 2 mg $L^{-1}$ in MeOH containing a

mixture of all isotopically labelled internal standards and homogenized. Then, it was kept in contact overnight in the extraction hood to allow solvent evaporation and internal standard fixation.

IV. *Pre-treatment for desorption of the analytes to aqueous phase*. The sample underwent, then, UAE in a Sonorex Digitec ultrasonic bath of 160W and 35 kHz (Navarra, Spain) during 30 minutes at room temperature to facilitate the desorption of the analytes. Twelve millilitres of a MilliQ® water/MeOH mixture, 95:5 (v/v) at pH 9 were used as extracting solvent. Subsequently, 100.0 mg of activated alumina ($Al_2O_3$ at 100 ºC for 48 hours) were added for matrix in-situ clean-up.

V. *After UAE centrifugation.* The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was collected (5 mL) with a glass pipette and transferred to a 25-mL glass beaker. Subsequently, twelve millilitres of the extraction solvent was added again and a new extraction cycle was carried out. The volume of supernatant was collected (5 mL) with a glass pipette and the supernatant was pooled together. Subsequently, the extract was adjusted to 3 by adding as few drops of diluted solutions of HCl (1%, 0.1% and/or 0.01%) as needed and filtered through a 0.7-µm glass fiber (GF) syringe filter. Then, 1.0 mL of the filtrate was collected and filtered again through a 0.45-µm polytetrafluoroethylene (PTFE) syringe filter and additioned to a 2.0 mL vial.

### 2.3. Analysis by LC-MS/MS

The instrumental part of the method presented a substancial novelty with respect to fully automated sample preparation for the analysis of PPCPs based on Direct Immersion Solid Phase MicroExtraction followed by On-fiber Derivatization coupled to Gas Chromatography – Mass Spectrometry (DI-SPME-On-fiber derivatization - GC-MS) Instrumental analysis consisted of automatized SPE coupled to UHPLC and detected by MS/MS (online SPE-UHPLC-MS/MS). Instrumental analysis was performed by a Sciex Exion UHPLC system connected to a Sciex 6500+ triple-quadrupole mass spectrometer from Sciex equipped with an electrospray ionization (ESI) source operated in both positive and negative mode in the same run. Chromatographic separation was achieved by a Phenomenex reversed-phase column Kinetex EVO C18 (2.1 mm × 50 mm, particle size 1.7 µm), which was temperature-controlled at 40°C along the entire chromatogram. The gradient run at 500 µL $min^{-1}$ with 0.1% FA (v/v) in water and 0.1% FA in MeOH as mobile phases starting with 5% of the organic phase for 1 minute and then increasing to 100% in 2 min, held at 100% for 3 min, and finally returning to the initial conditions, which were kept for 4 min. The total run time for each injection was 10 min and injection volume was set at 10 µL. Mass spectrometry acquisition was performed in selected-reaction monitoring (SRM) mode, recording the transitions between the precursor ion and the two most abundant product ions for each target analyte, thus achieving four identification points per compound (2002/657/EC) [14]. **Table A13 (**as **Appendix I)** shows the specific details for each SRM transition. In addition, ESI operational settings were: capillary voltage, 4500 V; capillary temperature, 400 °C; both gas 1 and 2, 45 psi.

Data acquisition and evaluation were performed by SciexOS software. **Fig. 5.1** shows a representative chromatogram obtained from a dewatered digested sludge sample.

## 3. Results and discussion

The injection volume was optimized to achieve high resolution and reproducibility in chromatographic analysis. It had a significant effect on the peak shape and retention time. Experiences with 100, 200 and 300 µL were carried out but they did not provide lower method limits of detection (MLDs) and quantification (MLQs) than the experiences completed with 50 µL, whereas calibration curves acquired were not so linear, with $R^2$ below 0.99 within the concentration range indicated for more compounds of interest. Therefore, 50 µL was selecteded as the final injection volume.

Quantification method was based on peak areas and performed by both internal standard and matrix-matched approaches in dewatered digested sludge. Seven-point calibration curves were generated by spiking sludge aliquots covering the range from 2 to 16,800 ng $g^{-1}$, for all target compounds. Three of the calibration levels were prepared in duplicate (n=2). Linearity was rated by the linear correlation coefficient ($R^2$). Calibration curves were linear for almost all the compounds, with $R^2$ above 0.99 within the concentration range indicated in **Table 5.1**.

After assessing the data, some of the initial PPCPs of interest proved to be inadequate for their analysis by online SPE – UHPLC-MS/MS as they showed a very weak or even no response whatever. Among these PPCPs, some hormones (progesterone, estrone, β-estradiol, 17-α-ethinylestradiol), antibiotics (doxycycline, tetracycline, oxytetracycline, among others) and surfactants (4-tert-octylphenol and 4-nonylphenol) were included. In addition, other initial PPCPs **(Table A13)** such as bisphenol A did not provide an adequate peak for their analysis by LC. However, this compound was successfully analysed by DI-SPME-on fiber derivatization-GC-MS, as demonstrated in previous chapters. Therefore, these compounds mentioned above were excluded from the online SPE – UHPLC-MS/MS.

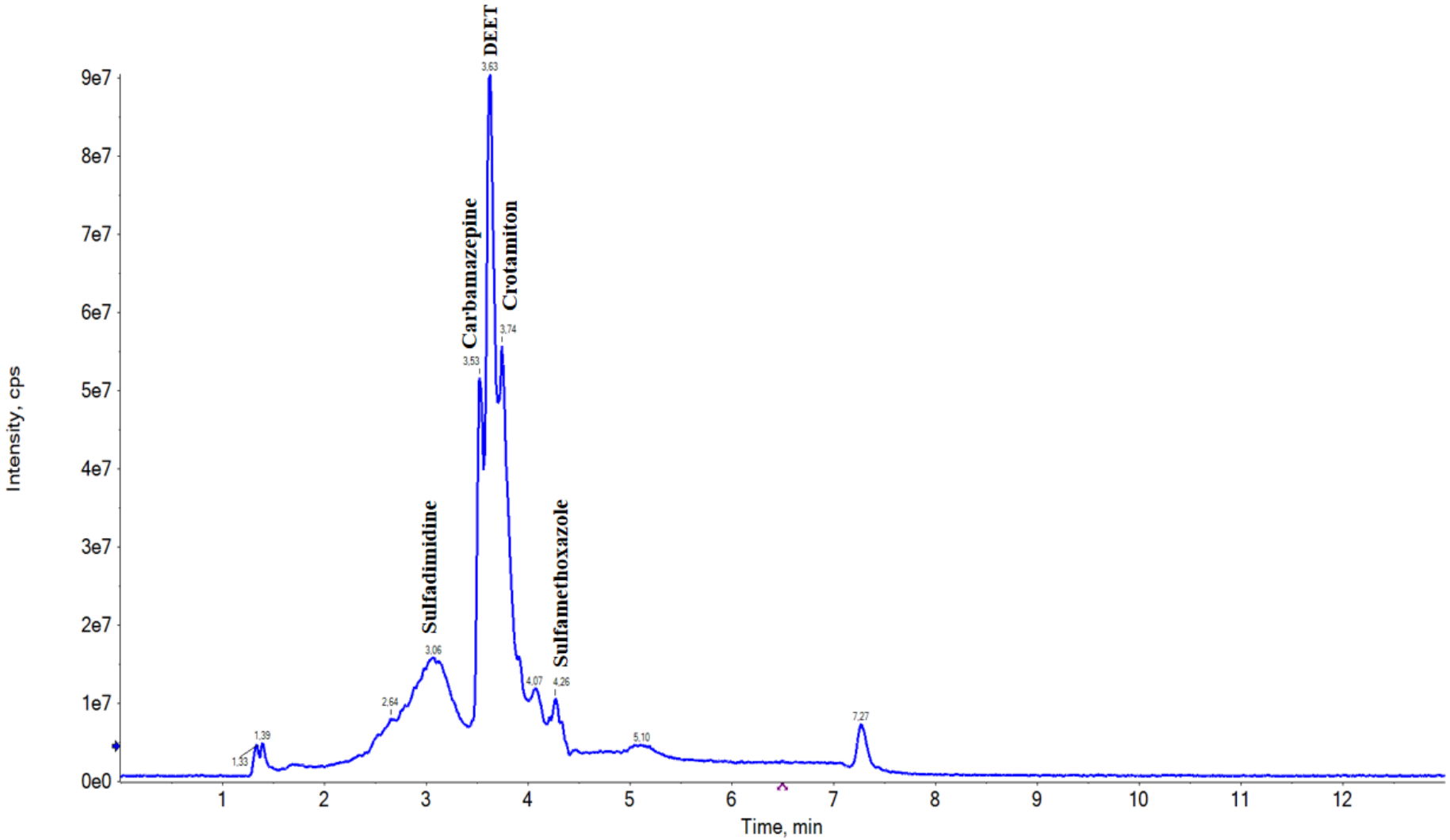


**Fig.5.1. Chromatogram from a dewatered digested sludge sample after applying of a 50 µL injection volume**

**Table 5.1: Dynamic range and precision for dewatered digested sludge**

| | Dynamic range (ng $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| **Chemical name** | **Equation** | **$R^2$** | **Linear range (ng $g^{-1}$)** | **[a][ ] ng $g^{-1}$ (d.w) (%RSD)** | **MLD (ng $g^{-1}$)** | **MLQ (ng $g^{-1}$)** |
| Penicillin G | y = 203.57x – 3015.2 | 0.9971 | LOQ-13130 | 87 (29) | 36.84 | 122.81 |
| Levofloxacin | y = 456.5x – 1693.7 | 0.9917 | LOQ-13760 | 792 (20) | 31.6 | 103.9 |
| Sulfadimidine | y = 0.9865x + 0.0252 | 0.9999 | LOQ-13478 | 800 (15) | 0.71 | 2.4 |
| Sulfadiazine | y = 0.078x + 0.0235 | 0.9999 | LOQ-13263 | 373 (23) | 51.1 | 170.3 |
| Sulfamethoxazole | y = 1.0068x + 0.0249 | 0.9999 | LOQ-12942 | 1,267 (8) | 1.4 | 4.7 |
| Sulfamethizole | y = 15089x - 337599 | 0.9943 | LOQ-13476 | 22 (29) | 2.3 | 7.7 |
| Sulfathiazole | y = 8327.4x - 4E+06 | 0.9949 | LOQ-13930 | 482 (5) | 20.0 | 66.5 |
| Tylosin | y = 383.91x - 42137 | 0.9967 | LOQ-11388 | 138 (30) | 58.9 | 196.0 |
| Clarithromycin | y = 1504.4x - 85605 | 0.9971 | LOQ-13218 | 76 (16) | 14.1 | 46.9 |
| Apramycin | y = 9966.8x - 76854 | 0.9957 | LOQ-14155 | 21 (21) | 0.7 | 5.3 |

**C**ontinued **(Table 5.1)**

| | Dynamic range (ng $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| **Chemical name** | **Equation** | **$R^2$** | **Linear range (ng $g^{-1}$)** | **[a][ ] ng $g^{-1}$ (d.w) (%RSD)** | **MLD (ng $g^{-1}$)** | **MLQ (ng $g^{-1}$)** |
| Tiamulin | y = 2000.7x - 8298.2 | 0.9994 | LOQ-10550 | 25 (30) | 9.3 | 31.0 |
| Florfenicol | y = 1418.4x + 4E+06 | 0.9946 | LOQ-14100 | 444 (7) | 15.4 | 51.3 |
| Trimethoprim | y = 20142x + 928089 | 0.9985 | LOQ-13309 | <LOQ (21) | 5.9 | 19.7 |
| Metronidazole | y = 15414x - 349454 | 0.9975 | LOQ-13309 | 26 (2) | 1.2 | 3.7 |
| Dexametasone | y = 948.68x - 35057 | 0.9978 | LOQ-13318 | 66 (34) | 18.8 | 62.7 |
| Diclofenac | $y = -0.0311x^2 + 0.6734x + 0.0397$ | 0.9991 | LOQ-6169 | 160 (7) | 42.7 | 147.0 |
| Naproxen | y = 0.5842x + 0.0475 | 0.9999 | LOQ-13286 | 5 (3) | 2.0 | 6.8 |
| Ibuprofen | y = 0.9728x + 0.1984 | 0.9949 | LOQ-12705 | 52 (34) | 52.7 | 175.8 |
| Salicylic acid | y = 1.4967x + 0.2884 | 0.9998 | LOQ-13174 | 34 (12) | 10.5 | 34.9 |
| Acetaminophen | y = 14206x – 2505.8 | 0.9886 | LOQ-13797 | 440 (17) | 20.6 | 68.7 |
| Clofibric acid | y = 1.2254x + 0.0977 | 0.9995 | LOQ-12893 | <LOQ (31) | 0.6 | 2.2 |
| Clofibrate | y = 115.57x + 28.94 | 0.9964 | LOQ-14623 | 1,090(21) | 99.2 | 331.0 |

Continued **(Table 5.1)**

| | Dynamic range (ng $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| **Chemical name** | **Equation** | **$R^2$** | **Linear range (ng $g^{-1}$)** | **[a][] ng $g^{-1}$ (d.w) (%RSD)** | **MLD (ng $g^{-1}$)** | **MLQ (ng $g^{-1}$)** |
| Gemfibrozil | y = 80.663x -897.8 | 0.9931 | LOQ-13648 | 305 (5) | 24.8 | 83.1 |
| Propranolol | y = 1345.2x - 11665 | 0.999 | LOQ-11680 | 80 (11) | 32.9 | 110.0 |
| Atenolol | $y = 0.1958x^2 + 2657.1x - 77.919$ | 0.9986 | LOQ-6758 | 20 (17) | 18.6 | 61.8 |
| Carbamazepine | $y = -4.076x^2 + 138922x + 9E+06$ | 0.999 | LOQ-13445 | <LOQ (7) | 0.6 | 2.0 |
| Fenbendazol | y = 959.31x -2308.7 | 0.9982 | LOQ-13241 | 97 (8) | 32.1 | 107.0 |
| Iohexol | $y = 0.055x^2 + 587.79x – 6312.9$ | 0.9980 | LOQ-6591 | 116 (30) | 188.0 | 626.0 |
| DEET | $y = -1.2216x^2 + 77971x + 8E+06$ | 0.9969 | LOQ-13124 | <LOQ (0.9) | 0.6 | 2.1 |
| Atrazine | y = 53698x + 610438 | 0.9975 | LOQ-13192 | <LOQ (15) | 0.9 | 3.0 |
| Methylparaben | y = 0.6075x + 0.2702 | 0.9921 | LOQ-13779 | 173 (14) | 48.4 | 161.2 |
| Ethylparaben | y = 1.3485x + 0.0082 | 0.9996 | LOQ-13433 | 2 (2) | 6.8 | 22.5 |
| Propylparaben | y = 0.1786x + 0.0062 | 0.9998 | LOQ-13757 | <LOQ (15) | 5.4 | 17.9 |
| 4-hydroxybenzoic acid | $y = -0.1591x^2 + 8214.2 + 4E+06$ | 0.9906 | LOQ-16767 | 4,027 (15) | 28.2 | 94.0 |

Continued **(Table 5.1)**

| | Dynamic range | | | | | |
|---|---|---|---|---|---|---|
| **Chemical name** | **Equation** | **$R^2$** | **Linear range (ng $g^{-1}$)** | **[1][ ] ng $g^{-1}$ (d.w) (%RSD)** | **[b]MLD (ng $g^{-1}$)** | **[c]MLQ (ng $g^{-1}$)** |
| Caffeine | y = 9122.5x - 992546 | 0.9956 | LOQ-13592 | 149 (11) | 21.3 | 71.1 |
| Crotamiton | y = 38310x - 942604 | 0.9982 | LOQ-13076 | 27 (7) | 1.1 | 3.73 |
| Enrofloxacin | y = 0.2501x – 0.0227 | 0.9877 | LOQ-13861 | 12,875 (14) | 6.54 | 21.8 |
| Ofloxacin | y = 518.53x + 50648 | 0.9895 | LOQ-14195 | 818 (16) | 43.4 | 144.8 |
| Erythromycin | y = 33.13x - 7156.8 | 0.9827 | LOQ-13376 | 363 (30) | 28.9 | 96.5 |
| Atorvastatin | $y = -0.0436x^2 + 511.52x - 505129$ | 0.9855 | LOQ-2729 | 991 (13) | 48.7 | 162.0 |

[1] []: Average concentration in dewatered digested sludge

The calibration curves, generated by both internal standard and matrix-matched, corresponding to sulfadiazine and trimethoprim, are shown below.

**Fig. 5.2** shows the calibration curve generated by **sulfadiazine** (antibiotic).

The equation of the adjusted model, which describes the relationship between the **Response** (counts) and the **Concentration** (ng $g^{-1}$), is as follows:

Response (counts) = 0.0235427 + 0.0779934*Concentration (ng $g^{-1}$)

The P-value in the Analysis of Variance (ANOVA) **(Table 5.3)** was less than 0.05. Hence, there was a significant statistical relationship between Response (counts) and Concentration (ng $g^{-1}$) with a 95.0% confidence level. Furthermore, the R-Square statistic parameter indicates that the adjusted model explains 99.8248% of the variability in Response (counts). The correlation coefficient is equal to 0.999124, indicating a relatively strong relationship among the variables.

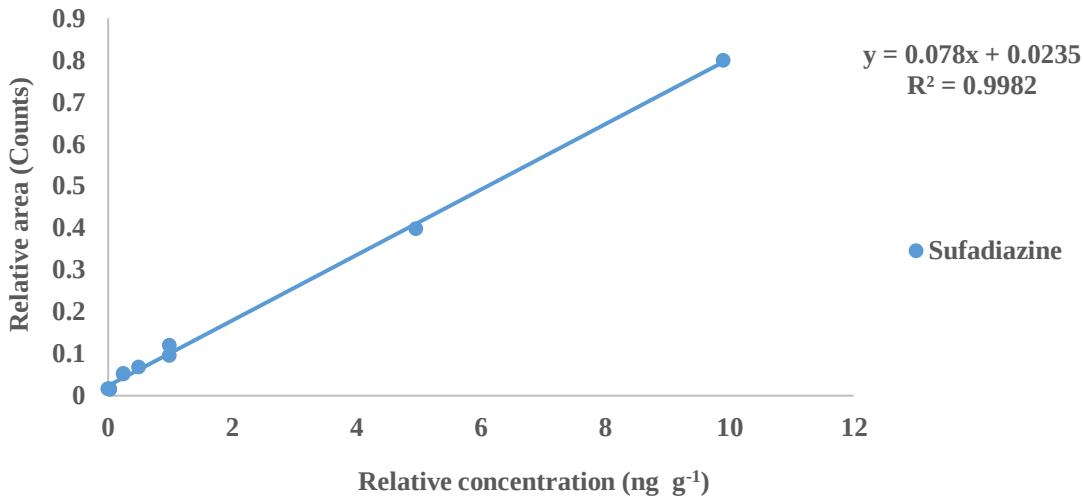


**Fig 5.2. Calibration curve based on internal standard quantification approach for sulfadiazine**

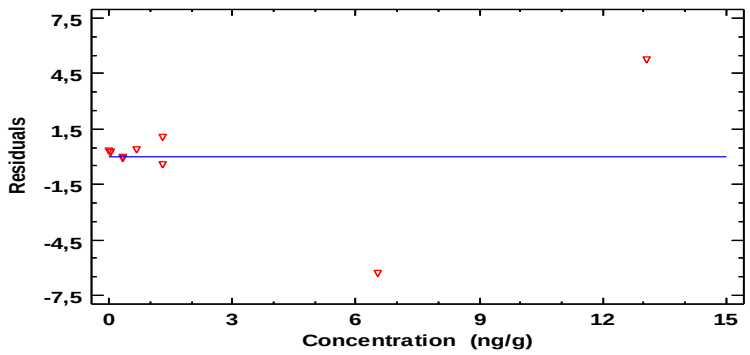


**Fig.5.3. Residuals for sulfadiazine**

**Table 5.2. Coefficients of adjusted model**

| | *Least-squares* | *Standard* | *Statistic* | |
|---|---|---|---|---|
| *Parameter* | *Estimated* | *Error* | *T* | *P-value* |
| Intercept | **0.0235427** | 0.00408284 | 5.76626 | 0.0004 |
| Slope | **0.0779934** | 0.00115525 | 67.5124 | 0,0000 |

**Table 5.3. ANOVA**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *P-value* |
|---|---|---|---|---|---|
| Model | 0.565422 | 1 | 0.565422 | 4557.92 | **0.0000** |
| Residue | 0.00099242 | 8 | 0.000124053 | | |
| Total (Corr.) | 0.566414 | 9 | | | |

The **Lack-of-fit test** is designed to determine whether the selected model is adequate to describe the observed data, whether a more complicated model should be used. The test was performed comparing the variability of the residues in the current model with the variability between observations made in repeated values of the independent variable X. Two calibration curves are shown in the following **figures 5.4** (sulfadiazine) and **5.7** (trimethoprim) and others calibration curves are reported in **Appendix I.**

In the case of **figure 5.4**, the P-value for the lack of adjustment in the Analysis of Variance (ANOVA) (**Table 5.4**) showed that is higher than or equal to 0.05 and the model seemed to be adequate for data observed with a 95.0% confidence level.

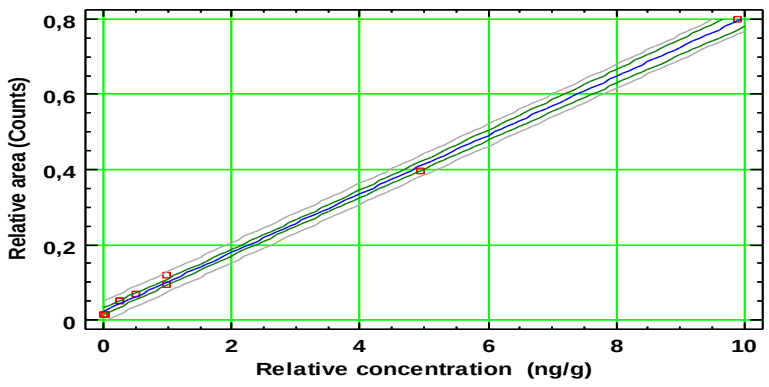


**Fig 5.4. Lack-of-fit test for sulfadiazine**

**Table 5.4. ANOVA with lack of adjustment**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 0.565422 | 1 | 0.565422 | 4557,92 | 0.0000 |
| Residue | 0.00099242 | 8 | 0.000124053 | | |
| Lack of adjustment | 0.000692548 | 5 | 0.00013851 | 1.39 | **0.4192** |
| Pure error | 0.000299872 | 3 | 0.0000999573 | | |
| Total (Corr.) | 0.566414 | 9 | | | |

In addition, **fig. 5.5** also shows a calibration curve of trimethoprim (antibiotic).

The equation of the adjusted model, which describes the relationship between the **Response** (counts) and the **Concentration** (ng $g^{-1}$), is as follows:

Response (counts) = 928089 + 20141,7*Concentration (ng $g^{-1}$)

In this case, the P-value in the ANOVA **(Table 5.6)** was also less than 0.05. Hence, there was a significant statistical relationship between Response (counts) and Concentration (ng $g^{-1}$) with a 95.0% confidence level. Furthermore, the R-Square statistic parameter indicated that the adjusted model explains 99.8457% of the variability in Response (counts). The correlation coefficient was equal to 0.999228, indicating a relatively strong relationship among the variables.

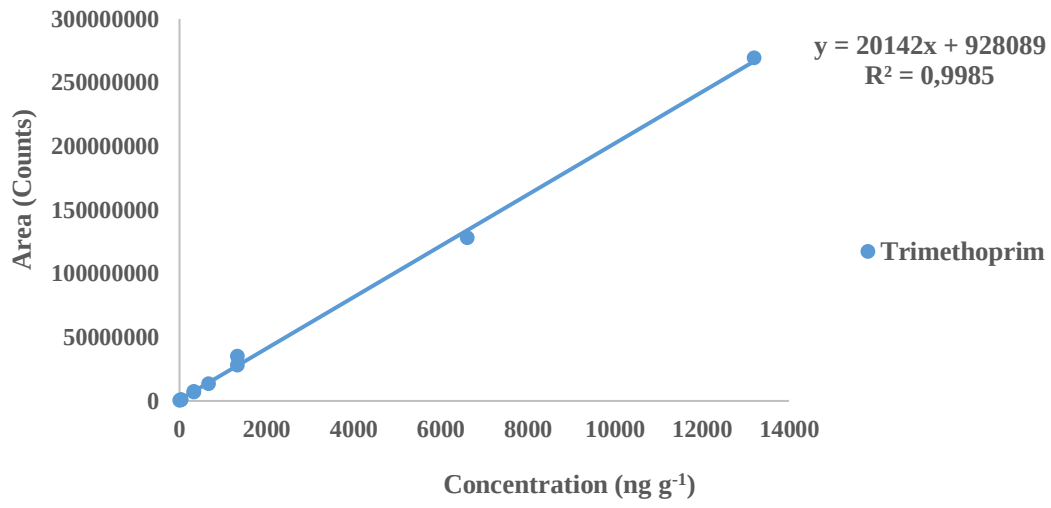


**Fig. 5.5. Calibration curve based on matrix-matched quantification approach for trimethoprim**

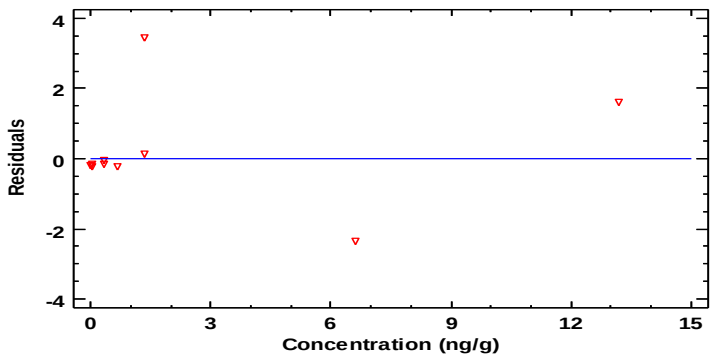


**Fig.5.6. Residuals for trimethoprim**

### 5.5. Coefficients of adjusted model

| | *Least-squares* | *Standard* | *Statistic* | |
|---|---|---|---|---|
| *Parameter* | *Estimated* | *Error* | *T* | *P-value* |
| Intercept | **928089,0** | 1,31899E6 | 0.703636 | 0.5016 |
| Slope | **20141,7** | 279.978 | 71.9403 | 0.0000 |

**Table 5.6. ANOVA**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *P-value* |
|---|---|---|---|---|---|
| Model | 6,70051E16 | 1 | 6,70051E16 | 5175,40 | **0.0000** |
| Residue | 1,03575E14 | 8 | 1,29468E13 | | |
| Total (Corr.) | 6,71087E16 | 9 | | | |

The **Lack-of-fit test** for trimethoprim (**fig. 5.7)** provided a P-value in the Analysis of Variance (ANOVA) (**Table 5.7**) higher than or equal to 0.05 and the model seemed to be adequate with a 95.0% confidence level.

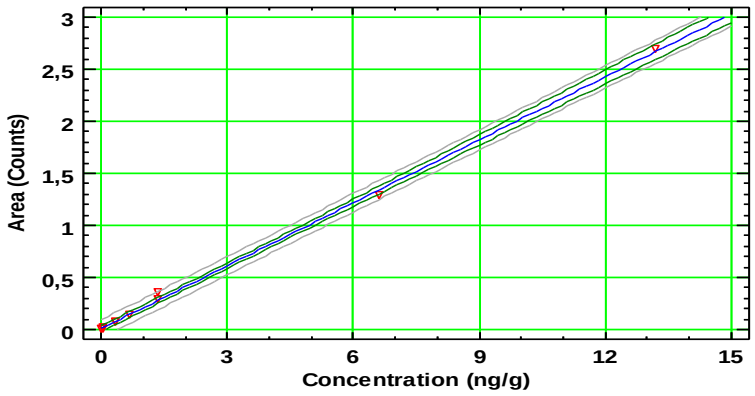


**Fig.5.7. Lack-of-fit test for trimethoprim**

**Table 5.7. ANOVA with lack of adjustment**

| *Source* | *Sum of squares* | *GF* | *Average square* | *F-reason* | *Value-P* |
|---|---|---|---|---|---|
| Model | 6,70051E16 | 1 | 6,70051E16 | 5175,40 | 0.0000 |
| Residue | 1,03575E14 | 8 | 1,29468E13 | | |
| Lack of adjustment | 7,77882E13 | 5 | 1,55576E13 | 1.81 | **0.3316** |
| Pure error | 2,57866E13 | 3 | 8,59554E12 | | |
| Total (Corr.) | 6,71087E16 | 9 | | | |

Finally, MLDs and MLQs were experimentally determined as the concentration providing a signal-to-noise ratio of 3 and 10, respectively, for each target analyte in each sample. MLDs were lower than 20 ng $g^{-1}$ and MLQs lower than 70 ng $g^{-1}$ for 23 of the target compounds in sludge samples **(Table 3.2)**. They were considered satisfactory for trace analysis of the target compounds in this type of matrix. The results also showed concentrations above 1,000 ng $g^{-1}$ corresponding to compounds of interest such as clofibrate (1,090 ng $g^{-1}$), sulfamethoxazole (1,267 ng $g^{-1}$), 4-hydroxybenzoic acid (4,027 ng $g^{-1}$), and erythromycin (12,875 n $g^{-1}$). However, other PPCPs reported concentrations below 10 ng $g^{-1}$ such as ethylparaben (2 ng $g^{-1}$) and naproxen (5 ng $g^{-1}$).

In summary, after the method development and optimization, 40 PPCPs from different physical-chemical properties, were validated for their analysis by online SPE-UHPLC-MS/MS in sewage sludge. Quantification method based on both matrix-matched and internal standard was successfully applyed. The main drawback of the internal standard quantification was its high cost and the lack of availability of isotopically-labelled standards for some cases. The results reported linear regression for 36 target compounds, including sulfadiazine and trimethoprim, as mentioned below. However, other target analites, such as carbamazepine, DEET, diclofenac, and 4-hydroxybenzoic acid, needed

a polynomial regression with degree 2. Finally, regarding the observed concentrations expressed in ng $g^{-1}$ level for dewatered digested sludge were found at concentrations between <MLQ-12,875 ng $g^{-1}$.

## 4. Conclusions

An analytical method for the determination of several groups of pharmaceuticals (analgesics/anti-inflammatories, antibiotics, lipid regulators, neuropharmaceuticals, anti-hypertensives, psychiatric and cardiovascular drugs, among others), preservatives, surfactants, plasticizers, stimulants, anti-parasatics, X-ray contrast agents and anti-itching drugs in dewatered digested sludge has been developed. The experimental conditions for sample pre-treatment consisted of 2-cycles UAE with a mixture of MilliQ® water/MeOH 95:5 (v/v) at pH 9 as extraction solvent, combined with an in-situ clean-up stage using 100.0 mg of activated $Al_2O_3$. Futhermore, a filtration step prior to the sample analysis was required using 0.45-µm. The instrumental part of the method consisted of an online SPE-UHPLC-MS/MS. The resulting environmentally friendly methodology decreased the use of expendable material (small amounts of reagents) and this fully automatized methodology was fast and analyst convenient to determinate PPCPs in sewage sludge samples with MLDs and MLQs below 30 ng $g^{-1}$ and 100 ng $g^{-1}$, respectively. The quantification method consisted of both internal standard and matrix-matched approaches. The calibration curves obtained for the compounds of interest reported linear equations for almost all of compounds, with linear ranges between MLQ and 2,491 ng $g^{-1}$ for all target analytes. Regarding occurrence, some PPCPs such as methylparaben, clofibric acid, ethylparaben, naproxen, crotamiton, sulfamethizole, tiamulin, and apramycin were found at concentrations below 30 ng $g^{-1}$ (d.w.) in dewatered digested sludge samples. In contrast, others like clofibrate, sulfamethoxazole, 4-hydroxybenzoic acid, and enrofloxacin were present at concentrations above 1,090 ng $g^{-1}$ (d.w.) for the same sample.

Currently, alternative sample pre-treatments (UAE using a MilliQ® water/methanol (MeOH) mixture, 95:5 (v:v) adjusted to pH 9 and extracts cleaned up by off-line SPE or not cleaned-up followed by a filtration step prior to analysis) are being examined to find out which one provides the best conditions for the determination of the variety of PPCP mentioned above.

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# Conclusions

The main conclusions obtained from the research carried out for the development of this Doctoral Thesis are shown as follows:

- A review of the different methodologies employed for the determination of PPCPs in sewage sludge samples has been carried out. Miniaturization and automation of analytical techniques are becoming a dominant trend as they eliminate the limitations of current analytical technologies.

- An analytical method for the determination of PPCPs in thickened mixed sewage sludge has been developed and validated. Ten PPCPs (methylparaben, clofibric acid, ethylparaben, ibuprofen, propylparaben, salicylic acid, naproxen, triclosan, diclofenac and bisphenol A) have been detected and quantified with MLDs and MLQs below 30 ng $g^{-1}$ and 100 ng $g^{-1}$, respectively.

- The optimal experimental conditions affecting sample pre-treatment have been carefully established. The optimal experimental conditions included a 1-cycle MAE combined with an in-situ clean-up stage using 100.0 mg of activated $Al_2O_3$ for the reduction or elimination of interferences associated with this type of environmental matrix. A MilliQ® water/MeOH 95:5 (v/v) mixture at pH 9 proved to be the best performing extraction solvent.

- A filtration step prior to the sample analysis was required. The instrumental part of the method consisted of an online DI-SPME-on derivation fiber derivatization-GC-MS. In addition, this fully automated methodology was fast and analyst convenient to determine the PPCPs in the sewage sludge.

- Real samples from both TH and AD pilot scale plants were analysed by the method developed. Some analytes such as methylparaben, clofibric acid, propylparaben and diclofenac were found at concentrations below 100 ng $g^{-1}$ (d.w.) in thermal hydrolysed samples. A different scenario was observed after AD treatment. Some analytes such as methylparaben, clofibric acid, ethylparaben, ibuprofen, salicylic acid and bisphenol A were found at concentrations below 50 ng $g^{-1}$ (d.w).

- The analytical method for the determination of PPCPs was successfully applied in dewatered digested sludge samples. Triclosan (702 ng $g^{-1}$) and bisphenol A (761 ng $g^{-1}$) were the compounds present with the highest values. Only propylparaben and ibuprofen presented a significant removal (around 60%).

- In the case of applying a thermal hydrolysis step prior to the digestion, the analytical method was also satisfactorily applied to determinate PPCPs. A significant degradation (from 31 to 76% removal) occurred of most of the compounds of interest during a TH pre-treatment followed by an AD process.

- The resulting environmentally friendly methodology for the determination of PPCPs in sewage sludge can be used in routine analysis laboratories around the world. In addition, it decreased the use of expendable material (small amounts of reagents, reusable SPME fiber and derivatizing agent, …), making it an environmentally friendly method.

- An alternative analytical method has been developed for the determination of sixty PPCPs (e.g., antibiotics, analgesics/anti-inflammatories, hormones, lipid regulators, psychiatric and cardiovascular drugs, preservatives, anti-parasitics, surfactants, plasticizers and antimicrobials.) in dewatered digested sludge. 40 PPCPs have been detected and quantified and 22 of them have been reported with MLDs and MLQs below 30 ng $g^{-1}$ and 70 ng $g^{-1}$, respectively.

- Sample pre-treatment consisted of 2-cyles UAE using a MilliQ® water/MeOH 95:5 (v/v) mixture at pH 9 and combined with an in-situ clean-up stage using 100.0 mg of activated $Al_2O_3$. A filtration step prior to the analysis was required using 0.45-µm. The instrumental part of the method was based on an online SPE-UHPLC-MS/MS. In addition, this fully automated methodology was fast and environmentally friendly for the determination the PPCPs in dewatered digested sludge.

# Appendix I

**Table A1: Compound, CAS number, molecular formula and other data of the target compounds**

| [1]IS | Analyte | Compound | [2]CAS | Molecular formula | [3]MW (g/mol) | $pK_a$ at 25°C | [4]$P_b$ (°C) at 101325 Pa | [5]Vp (Pa) at 25 °C | log [6]P at 25 °C |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | Methylparaben | 99-76-3 | C8H8O3 | 152.15 | Most acidic: 8.31 | 265.5 | 7.40E-01 | 1.882 |
| 1 | | Methylparaben-d4 | 362049-51-2 | C8H4D4O3 | 156.17 | N/A | N/A | N/A | N/A |
| | 2 | Clofibric acid | 882-09-7 | C10H11ClO3 | 214.65 | Most acidic: 3.18 | 324.1 | 1.37E-02 | 2.425 |
| 2 | | Clofibric acid-d4 | 1184991-14-7 | C10H7D4ClO3 | 218.67 | N/A | N/A | N/A | N/A |
| | 3 | Ethylparaben | 120-47-8 | C9H10O3 | 166.17 | Most acidic: 8.31 | 297.5 | 1.01E-01 | 2.391 |
| 3 | | Ethylparaben-d5 | 126070-21-1 | C9H5D5O3 | 171.20 | N/A | N/A | N/A | N/A |
| | 4 | Ibuprofen | 15687-27-1 | C13H18O2 | 206.28 | Most acidic: 4.41 | 319.6 | 1.85E-02 | 3.502 |
| 4 | | rac-Ibuprofen-d3 | 121662-14-4 | C13H15D3O2 | 209.30 | N/A | N/A | N/A | N/A |
| | 5 | 4-tert-octylphenol | 140-66-9 | C14H22O | 206.32 | Most acidic: 10.15 | 282.3 | 2.64E-01 | 5.18 |
| | 6 | Propylparaben | 94-13-3 | C10H12O3 | 180.20 | Most acidic: 8.23 | 294.3 | 1.24E-01 | 2.901 |
| 5 | | Propylparaben-d7 | 1246820-92-7 | C10H5D7O3 | 187.24 | N/A | N/A | N/A | N/A |
| | 7 | Salicylic acid | 69-72-7 | C7H6O3 | 138.12 | Most acidic: 3.01 | 336.3 | 5.93E-03 | 2.011 |
| 6 | | Salicylic acid-d4 | 78646-17-0 | C7H2D4O3 | 142.15 | N/A | N/A | N/A | N/A |
| | 8 | 4-nonylphenol | 104-40-5 | C15H24O | 220.35 | Most acidic: 10.15 | 330.6 | 1.14E-02 | 6.142 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 9 | Propranolol | 525-66-6 | C16-H21NO2 | 259.34 | Most acidic: 13.84; Most basic: 9.50 | 434.9 | 3.31E-06 | 2.9 |
| | 10 | Naproxen | 22204-53-1 | C14H14O3 | 230.26 | Most acidic: 4.84 | 403.9 | 4.01E-05 | 2.876 |
| 7 | | rac Naproxen-d3 | N/A | C14H11D3O3 | 233.28 | N/A | N/A | N/A | N/A |
| | 11 | Triclosan | 3380-34-5 | C12H7Cl3O2 | 289.54 | Most acidic: 7.80 | 344.6 | 4.35E-03 | 5.343 |
| 8 | | Triclosan-d3 | 1020719-98-5 | C12H4D3Cl3O2 | 292.56 | N/A | N/A | N/A | N/A |
| | 12 | Carbamazepine | 298-46-4 | C15H12N2O | 236.27 | Most acidic: 13.94; Most basic: -0.49 | 411 | 7.71E-05 | 1.895 |
| | 13 | Diclofenac | 15307-86-5 | C14H11Cl2NO2 | 296.15 | Most acidic: 4.18; Most basic: -2.26 | 412 | 2.12E-05 | 4.548 |
| 9 | | Diclofenac-d4 | 153466-65-0 | C14H7D4Cl2NO2 | 300.17 | N/A | N/A | N/A | N/A |
| | 14 | Bisphenol A | 80-05-7 | C15H16O2 | 228.29 | Most acidic: 10.29 | 400.8 | 7.12E-05 | 3.641 |
| 10 | | Bisphenol A-d8 | 92739-58-7 | C15H8D8O2 | 236.34 | N/A | N/A | N/A | N/A |

[1]IS: Internal Standard; [2]CAS: Compound Abstracts Service number; [3]MW: Molecular Weight; [4]$P_b$: Boiling point; [5]Vp: Vapor Pressure; [6]P: Partition coefficient

**Table A2: Parameters of Agilent 7890B GC System coupled to a 5977A MSD**

| | | |
|---|---|---|
| **Basic** | **Tool** | SPME 1 |
| | **Agitator** | Agitator 1 |
| | **Heat Agitator** | On |
| | **Incubation Time** | 10 min |
| | **Incubation Temperature** | 50ºC |
| | **GC Cycle Time** | 31.6 |
| | **Conditioning Port** | Front Inlet |
| | **Fiber Conditioning Station Temperature** | 270ºC |
| **Pre-Desorption** | **Conditioning Time** | 15 min |
| **Sample** | **Sample Vial Penetration Depth** | 60 mm |
| | **Sample Vial Penetration Speed** | 20 mm $s^{-1}$ |
| | **Sample Extraction Time** | 90 min |
| **Sample** | **Inlet Penetration Depth** | 50 mm |
| | **Sample Extraction Time** | 100 mm $s^{-1}$ |
| | **Inlet Penetration Depth** | 3 min |

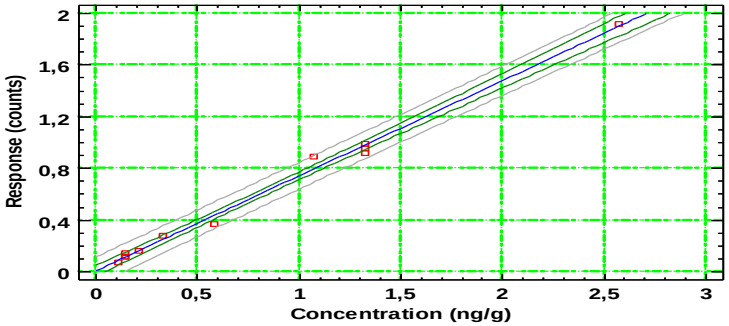
**Continued (Table A2)**

| | | |
|---|---|---|
| **Derivatizing** | **Derivatizing Agent Target** | Agitator 1 |
| | **Derivatizing Agent Adsorption Time** | 45 min |
| | **Derivatizing Agent Penetration Depth** | 45 mm |
| **Desorption** | **Conditioning Time** | 15 min |
| **Agitator** | **Agitator Speed** | 350 rpm |
| | **Agitator On Time** | 6 s |
| | **Agitator Off Time** | 20 s |
| **MMI-Front**<br>Agilent 5190-4048: 35 μL (Straight Ultra Inert Liner for SPME) | **Heater** | 270 ºC |
| | **Mode** | Split |
| **SSL-Back**<br>Agilent 5190-4048: 35 μL (Straight Ultra Inert Liner for SPME) | **Heater** | 250 ºC |
| | **Mode** | Pulsed Splitless |

**Continued (Table A2)**

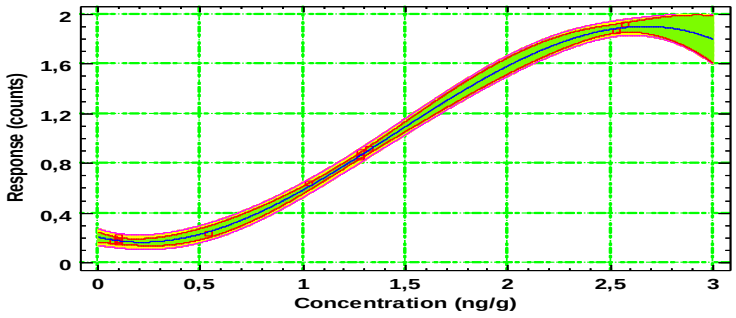
| | | |
|---|---|---|
| **Single Cuadrupole MS Method Editor** | **Tune Type** | Electron ionization |
| | **Solvent Delay** | 8 min |
| | **Adquisition Time** | SIM |
| | **Start Mass** | 50.00 |
| | **End Mass** | 850.00 |

Figures A3A to A3H show **lack-of-fit tests** based on **matrix-matched**, corresponding to eight PPCPs.



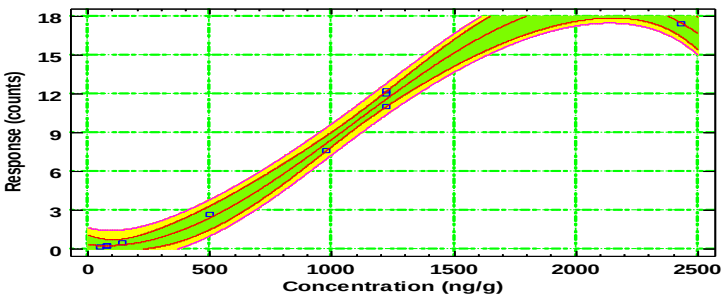

**Methylparaben**

**Fig. A3A. Lack-of-fit test for methylparaben**



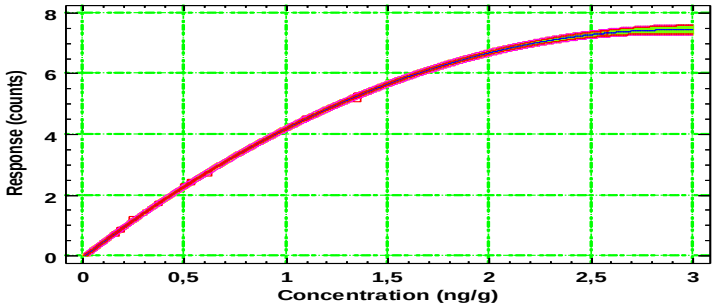

**Clofibric acid**

**Fig. A3B. Lack-of-fit test for clofibric acid**



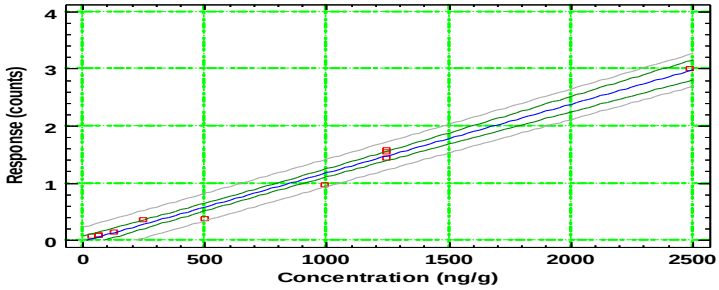

**Ethylparaben**

**Fig. A3C. Lack-of-fit test for ethylparaben**



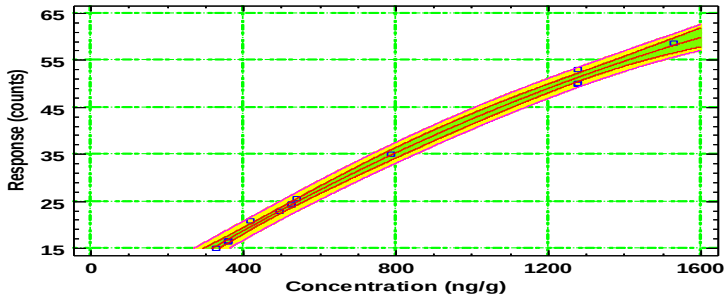

**Ibuprofen**

**Fig. A3D. Lack-of-fit test for ibuprofen**



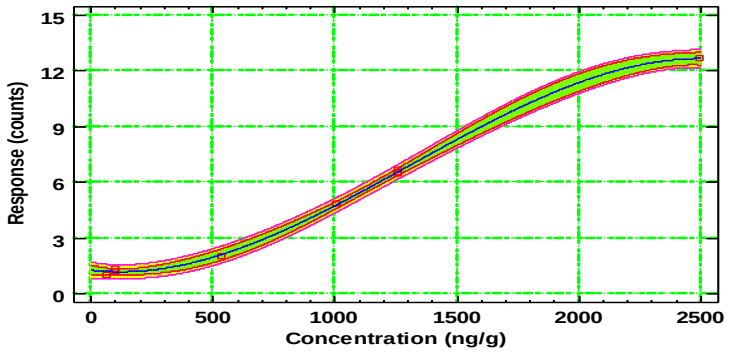

**Propylparaben**

**Fig. A3E. Lack-of-fit test test for propylparaben**



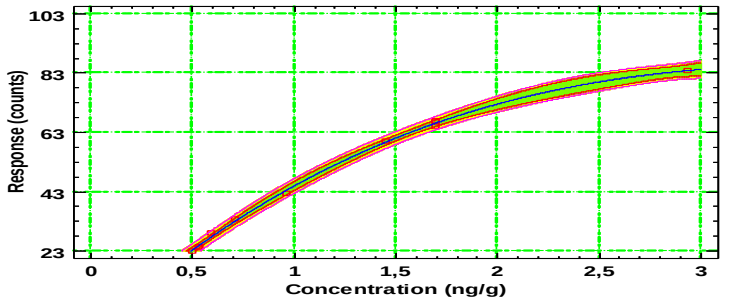

**Salicylic acid**

**Fig. A3F. Lack-of-fit test test for salicylic acid**




**Triclosan**

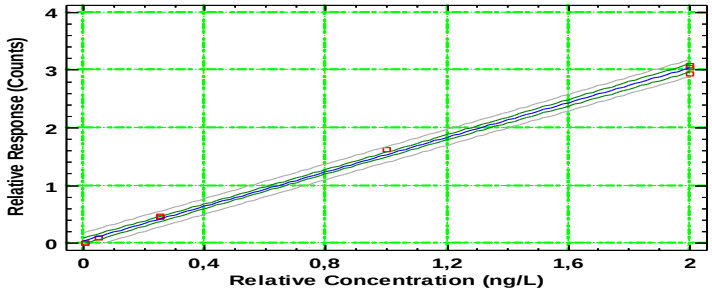
**Fig. A3G. Lack-of-fit test for triclosan**




**Bisphenol A**

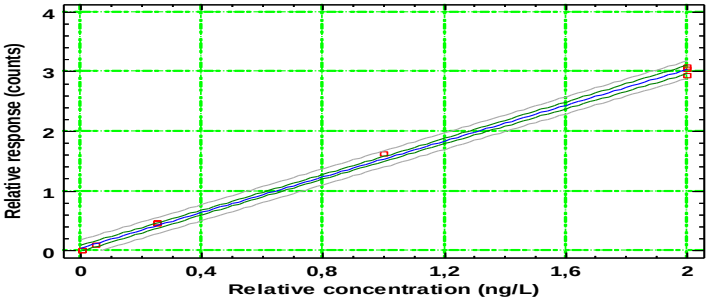
**Fig. A3H. Lack-of-fit test test for bisphenol A**

Figures A4A to A4H show **lack-of-fit tests** based on **internal standard**, corresponding to eight PPCPs.



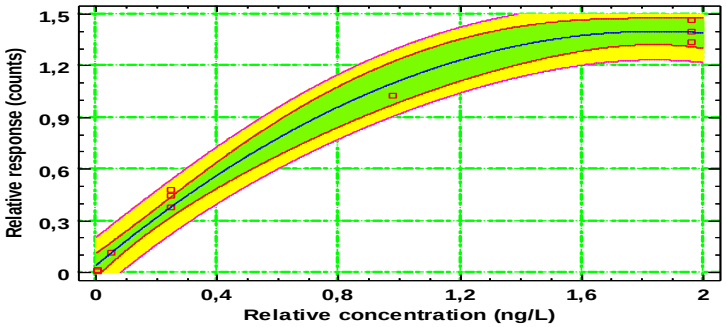

**Clofibric acid**

**Fig. A4A. Lack-of-fit test for clofibric acid**



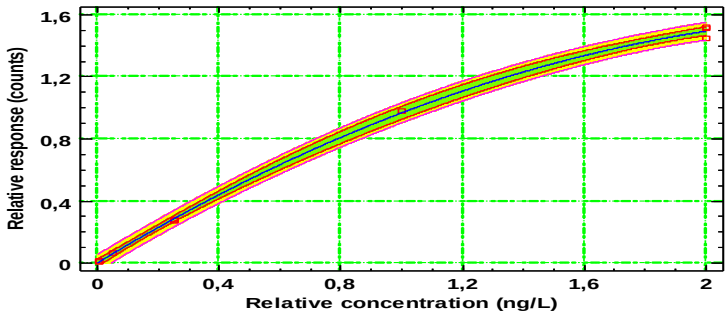

**Ethylparaben**

**Fig. A4B.Lack-of-fit test for ethylparaben**



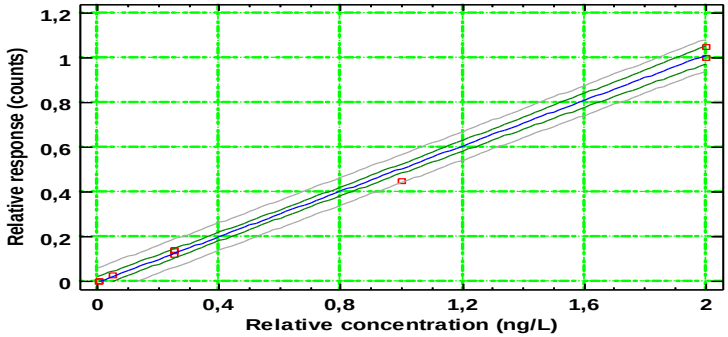

**Ibuprofen**

**Fig. A4C. Lack-of-fit test for ibuprofen**



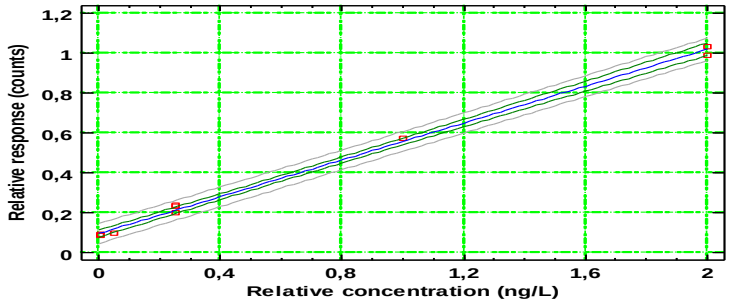

**Propylparaben**

**Fig. A4D. Lack-of-fit test for propylparaben**



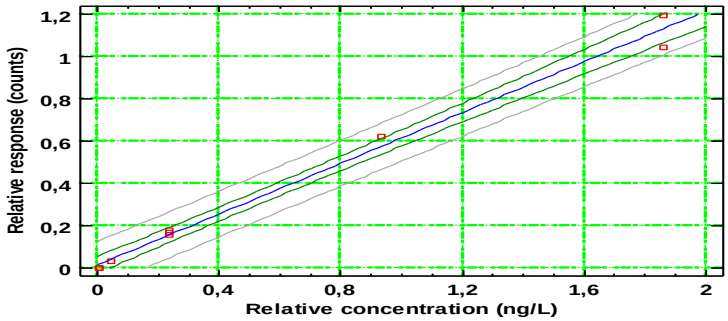

**Naproxen**

**Fig. A4E. Lack-of-fit test for naproxen**



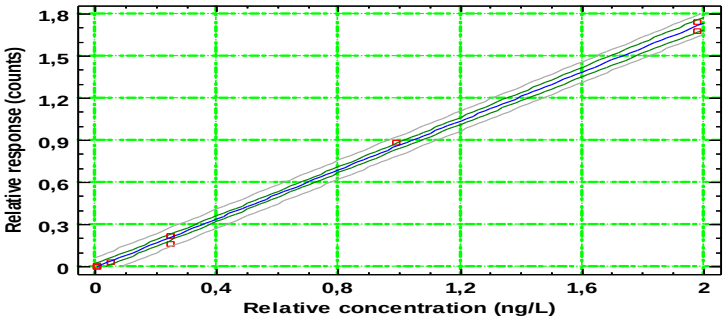

**Triclosan**

**Fig. A4F. Lack-of-fit test for triclosan**




**Diclofenac**

**Fig. A4G. Lack-of-fit test for diclofenac**




**Bisphenol A**

**Fig. A4H. Lack-of-fit test for bisphenol A**

**Table A3: Compound, CAS number, molecular formula and other data of the target compounds**

| [1]IS | Analyte | Compound | [2]CAS | Molecular formula | [3]MW (g/mol) | $pK_a$ at 25°C | [4]$P_b$ (°C) at 101325 Pa | [5]Vp (Pa) at 25 °C | log [6]P at 25 °C |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | Amoxicillin | 61336-70-7 | C16H19N3O5S | 365.4 | Most Acidic: 2.44<br>Most Basic: 7.14 | 743.2 | 3.39E-23 | 0.883 |
| | 2 | Penicillin G | 113-98-4 | C16H18N2O4S | 334.39 | Most Acidic: 2.45<br>Most Basic: -1.32 | 663.3 | 1.69E-18 | 1.918 |
| | 3 | Oxytetracycline | 2058-46-0 | C22H24N2O9 | 460.43 | Most Acidic: 4.50<br>Most Basic: 10.80 | 839.6 | 6.27E-30 | 0.479 |
| | 4 | Tetracycline | 60-54-8 | C22H24N2O8 | 444.43 | Most Acidic: 4.50<br>Most Basic: 11.02 | 790.6 | 2.40E-26 | 0.617 |
| | 5 | Doxycycline | 24390-14-5 | C22H24N2O8 | 444.43 | Most Acidic: 4.5<br>Most Basic: 10.84 | 762.6 | 1.90E-24 | 1.777 |
| | 6 | Marbofloxacin | 115550-35-1 | C17H19FN4O4 | 362.36 | Most Acidic: 6.02<br>Most Basic: 7.34 | 570.5 | 7.45E-14 | -0.641 |
| | 7 | Ciprofloxacin | 85721-33-1 | C17H18FN3O3 | 331.34 | Most Acidic: 6.43<br>Most Basic: 8.68 | 581.8 | 2.24E-14 | 1.625 |
| 1 | | Ciprofloxacin-d8 | 1130050-35-9 | C17H10D8FN3O3 | 339.13 | N/A | N/A | N/A | N/A |
| | 8 | Ofloxacin | 82419-36-1 | C18H20FN3O4 | 361.37 | Most Acidic: 5.19 | 571.5 | 6.70E-14 | 1.855 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 9 | Enrofloxacin | 93106-60-6 | C19H22FN3O3 | 359.39 | Most Basic: 7.37<br>Most Acidic: 6.43 | 560.5 | 2.13E-13 | 2.306 |
| 2 | | Enrofloxacin-d5 | 1173021-92-5 | C19H17D5FN3O3 | 364.43 | Most Basic: 7.76<br>N/A | N/A | N/A | N/A |
| | 10 | Danofloxacin | 112398-08-0 | C19H20FN3O3 | 357.38 | Most Acidic: 6.43 | 569.3 | 8.41E-14 | 1.811 |
| 3 | | Danofloxacin-d3 | 1217683-55-0 | C19H17D3FN3O3 | 360.15 | Most Basic: 9.00<br>N/A | N/A | N/A | N/A |
| | 11 | Sulfadiazine | 68-35-9 | C10H10N4O2S | 250.28 | Most Acidic: 6.81 | 512.6 | 1.28E-10 | -0.074 |
| 4 | | Sulfadiazine-d4 | 1020719-78-1 | C10H6D4N4O2S | 254.05 | Most Basic: 1<br>N/A | N/A | N/A | N/A |
| | 12 | Sulfathiazole | 72-14-0 | C9H9N3O2S2 | 255.32 | Most Acidic: 7.24 | 479.5 | 2.35E-09 | 0.05 |
| | 13 | Sulfamethizole | 144-82-1 | C9H10N4O2S2 | 270.33 | Most Basic: 2.19<br>Most Acidic: 5.51 | 504.9 | 2.56E-10 | 0.52 |
| | 14 | Sulfadimidine | 57-68-1 | C12H14N4O2S | 278.33 | Most Basic: 2.07<br>Most Acidic: 7.89 | 526.2 | 3.64E-11 | 0.296 |
| 5 | | Sulfadimidine-d4 | 1020719-82-7 | C12H10D4N4O2S | 282.08 | Most Basic: 1.69<br>N/A | N/A | N/A | N/A |
| | 15 | Sulfamethoxazole | 723-46-6 | C10H11N3O3S | 253.28 | Most Acidic: 5.81 | 482.1 | 1.87E-09 | 0.659 |
| 6 | | Sulfamethoxazole-d4 | 1020719-86-1 | C10H7D4N3O3S | 257.05 | Most Basic: 1.39<br>N/A | N/A | N/A | N/A |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 16 | Tylosin | 1401-69-0 | C46H77NO17 | 916.10 | Most Acidic: 13.06<br>Most Basic: 7.39 | 980.7 | 0 | 0.628 |
| 17 | Tiamulin | 55297-96-6 | C28H47NO4S | 493.74 | Most Acidic: 14.65<br>Most Basic: 9.74 | 563 | 5.06E-15 | 4.38E+00 |
| 18 | Apramycin | 65710-07-8 | C21H41N5O11 | 539.58 | Most Acidic: 12.91<br>Most Basic: 9.48 | 8.23E+02 | 2.11E-31 | -3.427 |
| 19 | Florfenicol | 73231-34-2 | C12H24Cl2FNO4S | 358.21 | Most Acidic: 10.73<br>Most Basic: -1.79 | 617.5 | 4.16E-16 | 1.175 |
| 20 | Trimethoprim | 738-70-5 | C14H18N4O3 | 290.32 | Most Basic: 7.04 | 526.0 | 3.74E-11 | 0.594 |
| 21 | Metronidazole | 443-48-1 | C6H9N3O3 | 171.15 | Most Acidic: 14.44<br>Most Basic: 2.58 | 405.4 | 2.64E-07 | -0.135 |
| 22 | Fenbendazole | 43210-67-9 | C15H13N3O2S | 299.35 | Most Acidic: 10.80<br>Most Basic: 5.25 | - | - | 2.364 |
| 23 | Dexamethasone | 50-02-2 | C22H29FO5 | 392.46 | Most Acidic: 12.13 | 568.2 | 2.81E-15 | 2.033 |
| 24 | Progesterone | 57-83-0 | C21H30O2 | 314.46 | - | 447.2 | 3.44E-08 | 3.827 |
| 25 | 1,4-Benzoquinone | 106-51-4 | C6H4O2 | 108.09 | - | 174.0 | 1.64E+02 | 0.394 |
| 26 | Acetaminophen | 103-90-2 | C8H9NO2 | 151.16 | Most Acidic: 9.86;<br>Most Basic: 1.72 | 387.8 | 1.91E-04 | 0.475 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 27 | Acetylsalicylic acid | 50-78-2 | C9H8O4 | 180.16 | Most Acidic: 3.48 | 321.4 | 1.65E-02 | 1.399 |
| 28 | Atorvastatin | 134523-00-5 | C33 H35FN2O5 | 558.64 | Most Acidic: 4.29; Most Basic: 0.38 | 722.2 | 9.12E-20 | 3.846 |
| 29 | Clarithromycin | 81103-11-9 | C38 H69NO13 | 747.95 | Most Acidic: 13.08 Most Basic: 8.16 | 805.5 | 5.06E-30 | 2.805 |
| 30 | Clofibrate | 637-07-0 | C12H15ClO3 | 242.7 | - | 274.8 | 7.04E-01 | 3.88 |
| 31 | Erythromycin | 114-07-8 | C37H67NO13 | 733.93 | Most Acidic: 13.09 Most Basic: 8.16 | 818.4 | 4.94E-31 | 1.909 |
| 32 | Levofloxacin | 100986-85-4 | C18H20FN3O4 | 361.37 | Most Acidic: 5.19 Most Basic: 7.37 | 571.5 | 6.70E-14 | 1.855 |
| 33 | Norfloxacin | 70458-96-7 | C16H18FN3O3 | 319.33 | Most Acidic: 0.16 Most Basic: 8.68 | 555.8 | 3.45E-13 | 1.744 |
| 34 | 4-hydroxybenzoic acid | 99-96-7 | C7H6O3 | 138.12 | Most acidic: 4.57 | 336.2 | 5.97E-03 | 1.401 |
| 35 | Nalidixic acid | 389-08-2 | C12H22N2O3 | 232.24 | Most Acidic: 3.45 Most Basic: 6.12 | 413.1 | 1.45E-07 | 0.025 |
| 36 | Sulfapyridine | 144-83-2 | C11H11N3O2S | 249.29 | Most Acidic: 8.54 | 473.5 | 3.90E-09 | 0.469 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Most Basic: 2.13 | | | |
| | 37 | Gemfibrozil | 25812-30-0 | C15H22O3 | 250.33 | Most Acidic: 4.75 | 158.5 | 6.13E-07 | 4.302 |
| | 38 | 17-α-Etinylestradiol (EE2) | 57-63-6 | C20H24O2 | 296.40 | Most Acidic: 10.24 | 457.2 | 3.74E-09 | 4.106 |
| | 39 | 17-β-estradiol (E2) | 50-28-2 | C18H24O2 | 272.38 | Most Acidic: 10.27 | 445.9 | 9.82E-09 | 4.146 |
| | 40 | Atenolol | 29122-68-7 | C14H22N2O3 | 266.34 | Most Acidic: 13.88<br>Most Basic: 9.43 | 508.0 | 3.82E-11 | 0.335 |
| | 41 | Atrazine | 1912-24-9 | C8H14ClN5 | 215.68 | Most Basic: 2.27 | 368.5 | 1.27E-05 | 2.636 |
| | 42 | Estrone (E1) | 53-16-7 | C18H22O2 | 270.37 | Most Acidic: 10.25 | 154 | 1.54E-08 | 3.624 |
| | 43 | Iohexol | 66108-95-0 | C19H26I3N3O9 | 821.14 | Most Acidic: 11.35<br>Most Basic: -2.72 | 891.5 | 3.95E-34 | -2.921 |
| | 44 | DEET | 134-62-3 | C12H17NO | 191.27 | Most Basic: -1.37 | 160 | 1.35E-03 | 2.419 |
| | 45 | Caffeine | 58-08-2 | C8H10N4O2 | 194.19 | Most Basic: 0.52 | 416.8 | 3.72E-07 | -0.628 |
| | 46 | Crotamiton | 483-63-6 | C13H17NO | 203.28 | Most Basic: 1.14 | 154 | 3.31E-03 | 2.464 |
| | 47 | Methylparaben | 99-76-3 | C8H8O3 | 152.15 | Most Acidic: 8.31 | 265.5 | 7.40E-01 | 1.882 |
| 7 | | Methylparaben-d4 | 362049-51-2 | C8H4D4O3 | 156.17 | N/A | N/A | N/A | N/A |
| | 48 | Clofibric acid | 882-09-7 | C10H11ClO3 | 214.65 | Most Acidic: 3.18 | 324.1<br>N/A | 1.37E-02<br>N/A | 2.425<br>N/A |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 8 | | Clofibric acid-d4 | 1184991-14-7 | C10H7D4ClO3 | 218.67 | N/A | | | |
| | 49 | Ethylparaben | 120-47-8 | C9H10O3 | 166.17 | Most Acidic: 8.31 | 297.5 | 1.01E-01 | 2.391 |
| 9 | | Ethylparaben-d5 | 126070-21-1 | C9H5D5O3 | 171.20 | N/A | N/A | N/A | N/A |
| | 50 | Ibuprofen | 15687-27-1 | C13H18O2 | 206.28 | Most Acidic: 4.41 | 319.6 | 1.85E-02 | 3.502 |
| 10 | | rac-Ibuprofen-d3 | 121662-14-4 | C13H15D3O2 | 209.30 | N/A | N/A | N/A | N/A |
| | 51 | 4-tert-octylphenol | 140-66-9 | C14H22O | 206.32 | Most Acidic: 10.15 | 282.3 | 2.64E-01 | 5.18 |
| | 52 | Propylparaben | 94-13-3 | C10H12O3 | 180.20 | Most Acidic: 8.23 | 294.3 | 1.24E-01 | 2.901 |
| 11 | | Propylparaben-d7 | 1246820-92-7 | C10H5D7O3 | 187.24 | N/A | N/A | N/A | N/A |
| | 53 | Salicylic acid | 69-72-7 | C7H6O3 | 138.12 | Most Acidic: 3.01 | 336.3 | 5.93E-03 | 2.011 |
| 12 | | Salicylic acid-d4 | 78646-17-0 | C7H2D4O3 | 142.15 | N/A | N/A | N/A | N/A |
| | 54 | 4-nonylphenol | 104-40-5 | C15H24O | 220.35 | Most Acidic: 10.15 | 330.6 | 1.14E-02 | 6.142 |
| | 55 | Propranolol | 525-66-6 | C16-H21NO2 | 259.34 | Most Acidic: 13.84 | 434.9 | 3.31E-06 | 2.9 |
| | 56 | Naproxen | 22204-53-1 | C14H14O3 | 230.26 | Most basic: 9.50 Most Acidic: 4.84 | 403.9 | 4.01E-05 | 2.876 |
| 13 | | rac Naproxen-d3 | N/A | C14H11D3O3 | 233.28 | N/A | N/A | N/A | N/A |
| | 57 | Triclosan | 3380-34-5 | C12H7Cl3O2 | 289.54 | Most Acidic: 7.80 | 344.6 | 4.35E-03 | 5.343 |
| | | | | | | N/A | N/A | N/A | N/A |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 14 | | Triclosan-d3 | 1020719-98-5 | C12H4D3Cl3O2 | 292.56 | | | | |
| | 58 | Carbamazepine | 298-46-4 | C15H12N2O | 236.27 | Most Acidic: 13.94 Most Basic: -0.49 | 411 | 7.71E-05 | 1.895 |
| | 59 | Diclofenac | 15307-86-5 | C14H11Cl2NO2 | 296.15 | Most Acidic: 4.18; Most Basic: -2.26 | 412 | 2.12E-05 | 4.548 |
| 15 | | Diclofenac-d4 | 153466-65-0 | C14H7D4Cl2NO2 | 300.17 | N/A | N/A | N/A | N/A |
| | 60 | Bisphenol A | 80-05-7 | C15H16O2 | 228.29 | Most Acidic: 10.29 | 400.8 | 7.12E-05 | 3.641 |
| 16 | | Bisphenol A-d8 | 92739-58-7 | C15H8D8O2 | 236.34 | N/A | N/A | N/A | N/A |

[1]IS: Internal Standard; [2]CAS: Compound Abstracts Service number; [3]MW: Molecular Weight; [4]$P_b$: Boiling point; [5]Vp: Vapor Pressure; [6]P: Partition coefficient

**Table A4: Conditions of Sciex Exion UHLC System connected to a Sciex 6500+ triple-quadrupole mass spectrometer from Sciex equipped with an ESI source.**

| Transitions | Analyte | [1]IS | Chemical name | [2]$R_t$ (min) | ESI mode |
|---|---|---|---|---|---|
| 1 | | | | | |
| 2 | 1 | | Amoxicillin | 0.41 | + |
| 3 | | | | | |
| 4 | 2 | | Atenolol | 0.8 | + |
| 5 | | | | | |
| 6 | 3 | | Metronidazole | 0.91 | + |
| 7 | | | | | |
| 8 | 4 | | Acetaminophen | 1.13 | + |
| 9 | | | | | |
| 10 | 5 | | Iohexol | 0.50 | + |
| 11 | | | | | |
| 12 | 6 | | Sulfadiazine | 0.49 | + |
| 13 | | 1 | Sulfadiazine-d4 | 0.58 | + |
| 14 | | | | | |
| 15 | 7 | | Sulfathiazole | 1.57 | + |
| 16 | | | | | |
| 17 | 8 | | Sulfapyridine | 1.66 | + |
| 18 | | | | | |
| 19 | 9 | | Trimethoprim | 1.94 | + |
| 20 | | | | | |
| 21 | 10 | | Marbofloxacin | 5.80 | + |
| 22 | | | | | |
| 23 | 11 | | Tetracycline | 3.06 | + |

Continued **(Table A4)**

| Transitions | Analyte | [1]IS | Chemical name | [2]$R_t$ (min) | ESI mode |
|---|---|---|---|---|---|
| 24 | | | | | |
| 25 | 12 | | Apramycin | 3.20 | + |
| 26 | | | | | |
| 27 | 13 | | Sulfamethizole | 3.19 | + |
| 28 | | | | | |
| 29 | 14 | | Oxytetracycline | 3.29 | + |
| 30 | | | | | |
| 31 | 15 | | Caffeine | 3.24 | + |
| 32 | | | | | |
| 33 | 16 | | Sulfadimidine | 3.33 | + |
| 34 | | 2 | Sulfadimidine-d4 | 3.26 | + |
| 35 | | | | | |
| 36 | 17 | | Ofloxacin | 3.37 | + |
| 37 | | | | | |
| 38 | 18 | | Levofloxacin | 3.37 | + |
| 39 | | | | | |
| 40 | 19 | | Norfloxacin | 3.40 | + |
| 41 | | | | | |
| 42 | 20 | | Ciprofloxacin | 3.57 | + |
| 43 | | 3 | Ciprofloxacin-d8 | 3.56 | + |

Continued **(Table A4)**

| Transitions | Analyte | [1]IS | Chemical name | [2]$R_t$ (min) | ESI mode |
|---|---|---|---|---|---|
| 44 | | | | | |
| 45 | 21 | | Sulfamethoxazole | 3.67 | + |
| 46 | | 4 | Sulfamethoxazole-d4 | 3.65 | + |
| 47 | | | | | |
| 48 | 22 | | Danofloxacin | 3.73 | + |
| 49 | | 5 | Danofloxacin-d3 | 3.72 | + |
| 50 | | | | | |
| 51 | 23 | | Enrofloxacin | 3.73 | + |
| 52 | | 6 | Enrofloxacin-d5 | 3.73 | + |
| 53 | | | | | |
| 54 | 24 | | Florfenicol | 3.70 | + |
| 55 | | | | | |
| 56 | 25 | | 4-hydroxybenzoic acid | 3.98 | - |
| 57 | | | | | |
| 58 | 26 | | Salicylic acid | 3.98 | - |
| 59 | | 7 | Salicylic acid-d4 | 3.94 | - |
| 60 | | | | | |
| 61 | 27 | | Bisphenol A | 4.73 | - |
| 62 | | 8 | Bisphenol A-d8 | 4.71 | - |
| 63 | | | | | |
| 64 | 28 | | Propranolol | 4.06 | + |

Continued **(Table A4)**

| Transitions | Analyte | [1]IS | Chemical name | [2]$R_t$ (min) | ESI mode |
|---|---|---|---|---|---|
| 65 | | | | | |
| 66 | 29 | | Methylparaben | 4.06 | + |
| 67 | | 9 | Methylparaben-d4 | 4.04 | + |
| 68 | | | | | |
| 69 | 30 | | Doxycycline | 5.36 | + |
| 70 | | | | | |
| 71 | 31 | | Tiamulin | 4.37 | + |
| 72 | | | | | |
| 73 | 32 | | Tylosin | 4.46 | + |
| 74 | | | | | |
| 75 | 33 | | Ethylparaben | 4.46 | - |
| 76 | | 10 | Ethylparaben-d5 | 4.44 | - |
| 77 | | | | | |
| 78 | 34 | | Nalidixic acid | 4.50 | + |
| 79 | | | | | |
| 80 | 35 | | Clarithromycin | 4.57 | + |
| 81 | | | | | |
| 82 | 36 | | Carbamazepine | 4.59 | + |

Continued **(Table A4)**

| Transitions | Analyte | [1]IS | Chemical name | [2]$R_t$ (min) | ESI mode |
|---|---|---|---|---|---|
| 83 | | | | | |
| 84 | 37 | | Penicillin G | 4.54 | + |
| 85 | | | | | |
| 86 | 38 | | Atrazine | 4.67 | + |
| 87 | | | | | |
| 88 | 39 | | DEET | 4.69 | + |
| 89 | | | | 4.69 | |
| 90 | 40 | | Propylparaben | | - |
| 91 | | 11 | Propylparaben-d7 | 4.68 | - |
| 92 | | | | | |
| 93 | 41 | | Erythromycin | 4.42 | + |
| 94 | | | | | |
| 95 | 42 | | Dexamethasone | 4.73 | + |
| 96 | | | | | |
| 97 | 43 | | 4-nonylphenol | 4.80 | - |
| 98 | | | Clofibric acid | | |
| 99 | 44 | | | 4.81 | - |
| 100 | 12 | | Clofibric acid-d4 | 4.80 | - |
| 101 | 45 | | Naproxen | 4.83 | - |
| 102 | | | | | |

Continued **(Table A4)**

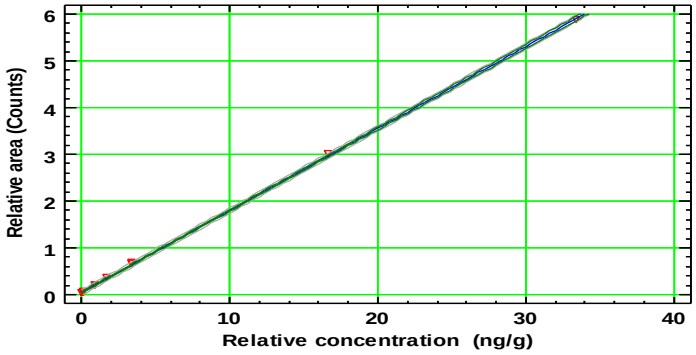
| Transitions | Analyte | [1]IS | Chemical name | [2]$R_t$ (min) | ESI mode |
|---|---|---|---|---|---|
| 103 | | 13 | Naproxen-d3 | 4.83 | + |
| 104 | | | | | |
| 105 | 46 | | Fenbendazole | 4.83 | + |
| 106 | | | | | |
| 107 | 47 | | 4-Octylphenol | 4.95 | - |
| 108 | | | | | |
| 109 | 48 | | Atorvastatin | 5.04 | + |
| 110 | | | | | |
| 111 | 49 | | Diclofenac | 5.08 | - |
| 112 | | 14 | Diclofenac-d4 | 5.07 | - |
| 113 | | | | | |
| 114 | 50 | | Ibuprofen | 5.10 | - |
| 115 | | 15 | Ibuprofen-d3 | 5.10 | - |
| 116 | | | | | |
| 117 | 51 | | Clofibrate | 5.10 | + |
| 118 | | | | | |
| 119 | 52 | | Progesterone | 5.13 | + |
| 120 | | | | | |
| 121 | 53 | | Triclosan | | - |
| 122 | | | | 5.25 | |
| 123 | | 16 | Triclosan-d3 | 5.24 | - |

Continued (Table A4)

| Transitions | Analyte | [1]IS | Chemical name | [2]$R_t$ (min) | [3]ESI mode |
|---|---|---|---|---|---|
| 124 | 54 | | Estrone (E1) | 4.89 | + |
| 125 | | | | | |
| 126 | 55 | | B-Estradiol (E2) | 4.90 | + |
| 127 | | | 17-α-Ethinylestradiol (EE2) | 4.91 | + |
| 128 | 56 | | | | |
| 129 | | | | | |
| 130 | 57 | | Crotamiton | 4.85 | + |
| 131 | | | | | |
| 132 | 58 | | Gemfibrozil | 5.22 | - |
| 133 | | | | | |
| 134 | 59 | | Acetylsalicylic acid | 5.67 | + |
| 135 | | | | | |
| 136 | 60 | | 1,4-Benzoquinone | 5.12 | + |

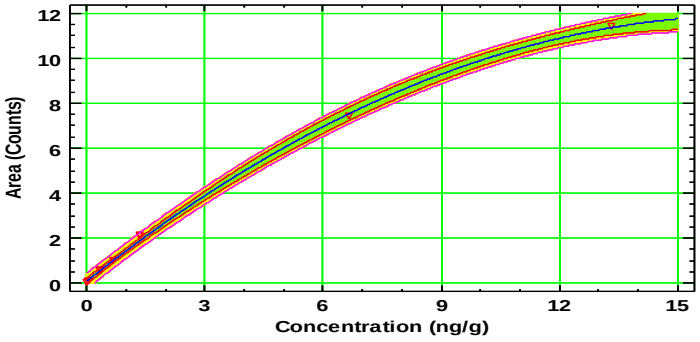
[1]IS: internal standard
[2]Rt: retention time
[3]ESI: electrospray ionization

Figures A5A and A5B show **lack-of-fit tests** based on both **internal standard** and **matrix-matched,** corresponding to naproxen and carbamazepine, respectively.




**Naproxen**

**Fig. A5A. Lack-of-fit test for naproxen**




**Carbamazepine**

**Fig. A5B. Lack-of-fit for carbamazepine**

# Appendix II. Publications

Analytica Chimica Acta 1083 (2019) 19–40

Contents lists available at ScienceDirect

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Review

# Analytical methodologies for the determination of pharmaceuticals and personal care products (PPCPs) in sewage sludge: A critical review

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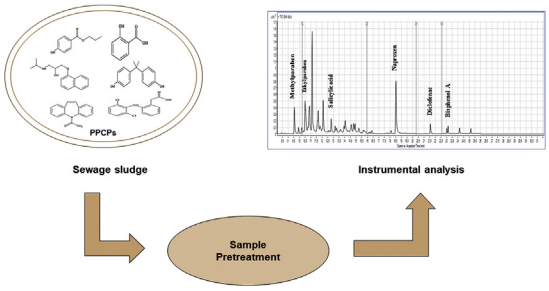
## HIGHLIGHTS

- A critical review on the determination of PPCPs in sewage sludge is presented.
- Analytical methodologies are discussed involving extraction, clean-up and instrumental analysis.
- UAE represents more than a half of the publications using extraction techniques.
- LC-MS/MS is the analysis technique more used to determinate PPCPs in sludge.
- Miniaturization and automation of analytical techniques is becoming a trend to analyze environmental.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Several analytical approaches have been developed for the determination of emerging pollutants (EPs), including pharmaceuticals and personal care products (PPCPs) in environmental matrices. This paper reviews the sample preparation and instrumental methods proposed in the last few years (2012–2018) to assess PPCPs in sewage sludge. Three main steps are examined: extraction, clean-up and analysis. Sample preparation is critical as target compounds are normally found at low concentrations in complex matrices. Most procedures include sewage sludge pretreatment mostly through ultrasound-assisted extraction (UAE) although other novel techniques such as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) or MSPD (matrix solid-phase dispersion) have been also employed. In one report, no differences in extraction efficiency were detected among the most commonly used extraction techniques such as ultrasound, microwave and pressurized liquid. Clean-up usually involves a conventional method such as solid phase extraction (SPE). This step is needed to appreciably reduce matrix suppression, and is followed by an instrumental analysis using techniques of preference such as gas chromatography (GC) or liquid chromatography (LC), mostly coupled to mass spectrometry (MS). A fully automated on-line system that includes extraction, chromatographic separation, and mass spectrometry in one-stage is here presented as a novel way of determining PPCPs in sewage sludge. This review also discusses the advantages and limitations of the different techniques used. Miniaturizing analytical techniques and the

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use of novel solid and liquid phase materials are emerging as efficient options that fulfill the principles of so-called "green chemistry".



**Contents**



## 1. Introduction

Emerging pollutants (EPs) are a great concern because of their detrimental effects on the health of human beings as well as aquatic and terrestrial life [1]. EPs include pharmaceuticals and personal care products (PPCPs) whose presence in the environment has not been yet regulated as stated in Directive 2013/39/EU on priority substances in the field of water policy [2].

PPCPs represent a large number of chemicals used in daily life including medicines, cosmetic and personal hygiene products. The active ingredients of PPCPs are products such as non-steroidal drugs like analgesics, antibiotics, antiepileptics, β-blockers, blood-lipid regulators, antiretroviral drugs and steroid drugs (hormones). As an example, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed pain medications. NSAIDs are used for the treatment of osteoarthritis, rheumatoid arthritis, inflammation, fever and sever and chronic pain and therefore improve quality of daily life [3]. Personal care products include cosmetic and personal hygiene products such as antimicrobials, fragrances, UV filters, and surfactants, among others. For instance, endocrine disrupting compounds such as parabens, are widely used as preservatives in PPCPs because their toxicity levels are theoretically low [4]. These drugs (active ingredients and preservatives), excreted in the environment via urine, feces, wastewater, sewage sludge and manure [5,6], are known to be persistent, bio-active and bio-accumulative as they are cleared at a faster rate than that of their natural degradation. These agents can pose a threat to drinking water supplies [7] and may be a health risk due to their estrogen activity and effects on the endocrine system [1,4,8,9]. PPCPs have been detected in water bodies throughout the world, even in Antarctic waters [10]. Moreover, in Europe, the rate of increase in the consumption and production of PPCPs has grown markedly in the last 20 years. Several studies examining the impacts of a wastewater treatment plant (WWTP) in Spain have shown that PPCPs contribute to water toxicity in a greater measure than traditional priority pollutants [11]. Conventional WWTPs are not designed to remove organic micropollutants. In fact, effluents from such plants are now considered to be a major point source of endocrine disrupting compounds and PPCPs in the receiving environment. For this same reason, PPCPs are commonly found in sewage sludge, as the residue left behind after the treatment of wastewater from various sources, including homes, industrial plants, and medical facilities [12]. The sewage sludge generated is often employed in agricultural and forestry activities, mainly due to its capacity to fertilize soils and the low economic impact of this practice [13], which leads to their spread in the environment.

In the past few years, numerous procedures for the determination of these emerging contaminants have been developed for use on the sewage sludge solid matrix. From an analytical perspective, sewage sludge (i.e., primary, secondary, digested sludge, compost) is challenging because of the complex nature of its matrix. In addition, its characteristics vary depending on the inputs to the WWTP.

In this study, the latest trends in methodology for the determination of PPCPs in sewage sludge are reviewed in detail. Focusing on the past six years after the last review published in 2012 (used as a reference for the present review) [14], 273 papers were identified, 67 of which deal explicitly with the determination of PPCPs in sewage sludge samples. A couple of recent general reviews have considered emerging contaminants in sludge samples [15] and aquatic ecosystems [16]. Martín-Pozo et al. recently provided a general overview of methodologies used to determine emerging contaminants in sewage sludge [15]. Here we present a holistic collection and critical review of all methodologies described to date that have been used for the determination of PPCPs throughout in sewage sludge. In effect, 85% of the literature gathered in this compilation has never been analyzed or discussed before.

The present article focuses on both current sample preparation procedures and instrumental analysis techniques including an assessment of the impact and efficiency of each stage and technique on several validation parameters. In addition, we discuss possible analytical perspectives for the future and provide novel information on the use of miniaturized and automated techniques as well as green chemistry approaches.

## 2. Analysis of sewage sludge samples

Studies worldwide have observed the presence of PPCPs in

several environmental matrices. Concentrations of some PPCPs such as diclofenac (NSAID), propranolol (antihypertension agent), triclosan (broad-spectrum antibacterial agent), triclocarban (antibacterial agent), and miconazole (azole antifungal agent) are commonly observed in the sewage sludge of most WWTPs. For instance, in Brazil, diclofenac has been found at concentrations of 25–60 ng/g, propranolol at 61.2–94.3 ng/g, triclosan at 2086–5466 ng/g and miconazole at 313–515 ng/g [93]. In India, propranolol has been detected in samples at concentrations of 46–54 ng/g, triclocarban at a mean concentration of 11.125 ng/g and miconazole averaged a concentration of 250 ng/g [59]. In France, diclofenac, triclosan and miconazole have been found at concentrations around 24 ng/g, 824 and 63 ng/g, respectively, and propranolol was observed at levels between 82 and 849 ng/g [100].

Sewage sludge is a complex matrix. It is not uniform in composition and concentrations of organic contaminants depend on the nature of inputs to the WWTP. Further, sludge contains substances that could interfere when trying to determine analytes of interest. Such interference may impact the whole analytical process, from sample preparation to instrumental detection. Thus, it is necessary to first remove these from samples using clean-up procedures.

Tables 1 and 2 present a summary of the references reviewed here. All types of sludge (i.e., primary, secondary, digested, and compost) were subjected to similar analytical approaches which roughly consisted of a sample pretreatment followed by an instrumental analysis. The different methods used are described in the following sections.

Despite similar analytical protocols (extraction, clean-up and analysis), differences did exist in terms of the quantity of sample treated or the amount of solvent in each matrix. Some of the studies reviewed used different amounts of sample and extraction solvent for different types of sludge with ultrasound as the extraction technique: Kopperi et al. [37] used 0.05 g of sample and 6 mL of solvent (acetonitrile) in composted sludge samples; Abril et al. [58] used 1 g of sample and 3 mL of solvent (methanol: acetic acid (1:1)) in digested sludge samples; Shafrir [49] used 2 g of sample and 10 mL of solvent (methanol: water (1:1) in secondary sludge samples; Lonappan et al. [31] used 0.5 g of sample and 20 mL of solvent (methanol) in primary sludge samples; and Yan et al. [40] used 2 g of sample and 10 mL of solvent (methanol/citric acid/$Na_2$EDTA (2:1:1)) in dewatered sludge samples.

Further, sample quantities and solvents also varied for different extraction techniques on the same type of sludge. Examples for digested sludge are 0.1 g [43], 1.5 g [76] or 3 g [64] and 6 mL of methanol:water (1:1) [43], 22 mL of hexane: dichloromethane (1:1) [76] or 20 mL of methanol:water (1:1)) [64] used in ultrasound [43], pressurized liquid [76], or microwave [64] extraction procedures, respectively.

The matrices associated with each type of sludge differ because their characteristics vary as the sludge goes through several treatment stages. For instance, major changes are produced by thickening, dewatering and digestion. In thickening and dewatering treatments, total solid (dry solids) concentrations increase and the volume of sludge is reduced. Following digestion treatment, the load of total solids is reduced (via the reduction of volatile suspended solids). Several sludge matrices should be, therefore, treated separately and their analysis should be viewed as a challenge to be addressed in future work.

### 2.1. Sample pretreatment

The sampling of different types of sludge is particularly important to assess the distribution of PPCPs along the sludge line. According to Tables 1 and 2, sampling sludge locations within WWTPs depends on the type of sewage sludge sample required for the subsequent analysis. In the literature reviewed, a large number of studies preferred sampling sewage sludge [92,100] (suspension with a dry solids content of 3–4% weight arising from the purification of wastewaters). Some authors sample the sludge after the final dewatering step to obtain a representative bulk product [22,78]. Other researchers carry out their sampling after the anaerobic digestion step in which some of the organic matter is removed [43,64]. However, few publications considered sampling in primary and secondary tanks [42,77].

Representative sludge samples can be collected from the WWTP sludge line. Sample volumes in the studies reviewed differed, e.g.: 1 L samples were collected weekly over a period of four weeks by Schoeman et al. [53]; random grab samples were pooled to provide a sample weighing about 500 g by Gago-Ferrero et al. [34]; and five grab samples collected daily were pooled to give a single sample (approximately 2 L) of sludge per day over three consecutive days by Jelic et at [74].

The materials used for sample collection also differed. Thus, one report describes the collection of solid pasty sludge using a metal bucket and the collection of liquid sludge using a sample probe. Thereafter, the samples were packed in glass bottles with a wide-mouth PTFE stopper [100]. Other materials such as 1 L clear Schott bottles [53] or antimicrobial plastic bags after sewage sludge dewatering [34] were also utilized for sample collection. These samples were then transported to the laboratory where they were frozen and lyophilized [53,59,88] or dried in air to room temperature [50], and passed through a 2 mm ∅ sieve and homogenized [50] or were macerated in a glass mortar for some minutes [93]. Finally, the lyophilized samples were stored at −20 °C [65] until their analysis.

Sample preparation takes up most of the analysis time. It usually includes a process of extraction followed by a clean-up step. A variety of techniques have been used to extract PPCPs from sewage sludge samples in the last 6 years. Besides traditional approaches such as Soxhlet [20,21] and ultrasound [28,34], other methods based on microwave [62,65] or pressurized liquid [72,74] are gaining popularity. Most extraction techniques are not sufficiently selective and clean-up procedures are also needed after extraction.

Figs. 1 and 2 show each of the extraction and clean-up techniques used, respectively, over the last 6 years (reviewed here) compared with the previous five-year period.

#### 2.1.1. Extraction

Solvent extraction of solid samples, commonly known as solid–liquid extraction, is one of the oldest techniques of solid sample preparation. This technique serves to remove and separate compounds of interest from insoluble high-molecular-weight fractions and other compounds that could interfere with subsequent steps of the analytical process [17]. Soxhlet is a reference extraction technique that belongs to that group. Some authors prefer this extraction procedure because of some advantages. For example, samples are repeatedly brought into contact with fresh portions of extractant, which facilitates displacement of the transfer equilibrium. In addition, filtration is not necessary after leaching, which increases sample yield. Further, several simultaneous extractions can be performed in parallel because of the low cost of basic equipment [18]. However, Soxhlet also has some shortcomings: it is time consuming, labor intensive and requires the use of large volumes of organic solvents (300–500 mL) and large samples (10–30 g). These features go against some of the main objectives of so-called “green chemistry” such as sustainable development and being environmentally friendly. Recent modifications have tried to bring the Soxhlet technique closer to these objectives. Hence, a technical version designated automated

**Table 1**
Determination of PPCPs in sewage sludge based on traditional extraction techniques (Soxhlet and UAE) [44].

| Analyte | Sample type | Extraction technique | Clean-up | Analysis | Quantification technique | LOQ (ng/g) | Recovery (%) | Precision (%) | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 3 NPE: NP, NP2EO, NP1EO | Primary and secundary sludge. (Freeze-dried 1g) | Soxhlet MeOH by DCM Overnight | The remaining extract was solvent-exchanged into cyclohexane for further cleanup prior to LC/MS analysis | LC-MS | Labeled internal standard (3) Non-labeled internal standard (2) | (0.4 (NP) 1.3 (NP1EO) 0.2 (NP2EO))[e] mg/kg | 94 (NP) 94 (NP1EO) 112 (NP2EO) | 1.3 (NP) 4.1 (NP1EO) 10.9 (NP2EO) | [20] |
| 2 PhACs (1-stearoyl-1h-1,2,4-triazole) | Sewage sludge (200 g d.w) | Soxhlet 300 mL DCM/acetone (1:1) 8 h | a | GC-MS | Qualification | d | d | d | [21] |
| 9 PBDE congeners: (BDE congeners 28, 47, 99, 100, 153, 154, 183, and 209) 2,2',4,4',5,5'Hexabromobiphenyl (BB-153) | Dewatered sludge (Oven dried 50ºC) (10g) | Soxhlet N-hexane and acetone (3:1) 16h | Draft Method 1614 for PBDE determination in wastewater and biosolid was employed with some modifications (USEPA 2007) | GC-ECD | d | d | >85% absolute recoveries | d | [22] |
| 6 Flame retardants (TBECH, BTBPE, DBDPE, EBTPI, TBBPA AE, TBBPA DBPE) | Sewage sludge (Freeze-dried) | Soxhlet MeOH 15 h | a | The extracts were divided in two parts, one for GC–MS analysis and one for HPLC–MS analysis. | Labeled ($^{13}$C) internal standard | 0.02 - 1.6 | d | d | [23] |
| 4 Benzotriazole UV stabilizers (UV 320, UV326, UV 327, UV 328) | Sewage sludge (Freeze-dried 2g) | Soxhlet (DCM:Hexane) (8:1) 6 h | SPE (Oasis HLB) | GC-MS | d | (0.0021-0.0087)[b] | (98-115)% | d | [24] |
| 12 PPCPs ( PhACs (IBP, NPX, DCF, SA, RAM) and PCPs (MP, EP, PP, CA, TCS, PHBA, BQ) | Digested sludge (0.8g) | UAE (2 cycles) 15 mL MilliQ water pH 9 30 min | a | Online DI-SPME – On-Fiber Derivatization – GC – MS | Matrix-matched Isotopically labelled internal standard (6) | <10[b] | (5.69-103.59)% absolute recoveries | < 10% | [28] |
| 10 EDCs and PPCPs (CBZ, SMX, TCS, 4-NP, BPA, OBZ), four estrogens (E1, E2, E3, EE2) | (Freeze-dried 0.5g) (Before and after anaerobic digestion) | UAE (2 cycles) 4 mL MeOH/Acetone (1:1)10 min | SPE (Oasis HLB) | LC-MS | Isotopically labelled internal standard (1) | 1.6-100 (pg absolute)) | (75-106)% | < 20% | [29] |
| 29 PPCPs (NSAIDs, antibiotics,stimulant, preservatives) | Return sludge (0.1g) | USEPA SPE method 1694[f] | SPE (Oasis HLB) | LC- MS/MS | Isotopic standards (5) | 0.1-5.0[c] | (31-93)% | <20% | [30] |
| 1 NSAID (DCF) | Wastewater sludge (Primary sludge, secondary sludge) (Lyophilized 0.5 g) | UAE MeOH 20 mL 15 min | SPE (Sep-Pak C18 plus Short Cartridges) | LDTD-APCI-MS/MS | Isotopically labelled internal standards (1) | 75[c] | (86 ± 4)% | 8.6% (repeatability) and 9.8% (reproducibility) | [31] |
| 18 Antibiotics (sulfonamides, tetracyclines, quinolones, macrolides, and β-lactams) | Dewatered sludge (Lyophilized and sieved sludge 0.5 g) | UAE 10 mL MeOH-EDTA-citrate buffer (3:1:2) | SPE (Oasis HLB) | UPLC-MS/MS | Standard addition Internal standard (1) | 0.3-3.2 | (60.1–92.7)% | (0.5-4.7)% | [32] |
| 8 PhACs (DCF, APh, NPX, GEM, CA, PH, CAF, Chol) | Sludge sample (dewatered) (25 g) | UAE MeOH 20 mL 30 min | SPE | Derivatization-GC-MS/MS | Internal standard (1) | (1.7-9.4) ng/L | (73-95)% | (8.9-20.4)% | [33] |
| 34 PhACs (antibiotics, analgesic and/or anti-inflammatory drugs, antiepileptics, benzodiazepines, antipsychotics, and antidepressants) | Freeze-dried sewage sludge (0.1g) | UAE 2 mL MeOH-MilliQ water 15 min 50ºC | a | LC-MS/MS | Standard additions Isotopically labeled internal standard (10) | < 55[c] | (16-119)% absolute recoveries | <20% | [34] |
| 153 compounds: pharmaceuticals, herbicides, antioxidants, intermediates, organic solvents and chemical raw materials | Sludge samples | UAE (3 cycles) DCM 20 min | Gel permeation chromatography (GPC) and a silica gel column | GC-MS | d | d | d | (5.8-14.9) % | [35] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 13 PhACs (4-AA, 4-AAA, 4-FAA, BZE, TBZ, VNF, CBZ, IRB, VAL, DCF, SA, ACE, FA) | Lyophilized sewage sludge (0.1g) | USE MeOH-MilliQ water 15 min 50ºC | a | (AMDIS) LC-MS/MS | Isotopically-Labeled Internal Standards (ILIS) (6) | 50-2000 | (70-120)% | < 20 % | [36] |
| 30 Steroidal compounds (androstanes, pregnanes, estrone, cholestanes) | Compost Dry sludge (0.05g) | USE (2 cycles) 6 mL AcCN 60 min | SPE (cartridges Strata-X and Florisil) | Derivatization-GCXGC-TOFMS | Internal standard (1) | d | >90% | d | [37] |
| 12 PhACs (ACE, FA, VAL, IRB, SA, DCF, CBZ, 4-AA, 4-AAA, 4-FA, VNF, BZE) | Lyophilized sludge (after anaerobic digestion) (0.1 g) | USE 2 mL MeOH-MilliQ water 15 min 50ºC | a | LC-MS/MS | Isotopically-Labeled Internal Standards (ILIS) (8) | <50 | (70-120)% | < 20 % | [38] |
| 1 NSAID (DCF) | Freeze dried sludge (0.1g) | UAE (2 cycles) 10 mL MeOH/Acetone (1:1) | SPE (Oasis HLB;MCX) | LC-MS/MS | Isotope labeled internal standard (ILIS) (1) | 5 | >80% | <20% | [39] |
| 21 compounds: PhACs (analgesics (IBP, DCF, ACM), sulfonamide antibiotics (SDZ, SM1, SM2, TMP), macrolide antibiotics (ERY, ROX, AZM), quinolone antibiotics (OFX, NOR, MOX), antiepileptics (MTP, ALP), cholesterol lowering statin drugs (ATT, SVT), lipid regulators (BZB, CA, GFB), and antihypersensitives (CBZ) | Dewatered sludge (Freeze-dried 2g) | UAE (3 cycles) 10 mL MeOH/Citric acid/ $Na_2EDTA$ (2:1:1) 15 min | SPE (Oasis HLB) | LC-MS/MS | Non-labeled internal standard (2) and labeled internal standard (2) | 0.17 - 5.83 | (46 –139)% | < 15 % | [40] |
| 62 PhACs (antibiotic, analgesic/anti-inflammatory, and antifungal compounds) | Dry biosolids (0.5 g) | UAE EPA method 1694 | SPE | LC-MS/MS | Isotopically labeled internal standard (16) | d | (20 – 150)% | < 22 % | [41] |
| 18 PhACs (antibiotics, analgesics, antiepileptics, antilipidemics and antihypersensitives) | Primary and secondary sludge (EPA Method (1694 USEPA, 2007 with some modifications) | UAE 10 mL MeOH/citric acid/$Na_2EDTA$ (2:1:1) 15 min <40ºC | SPE (Oasis HLB) | LC-MS/MS | Non-labeled internal standard (1) and labeled internal standard (2) | 0.17 - 5.83 | (54 – 139)% | < 13 % | [42] |
| 15 compounds: 5 artificial sweeterners and 10 PPCPs (analgesics, antibiotics and PCPs) | Digested sludge (Freeze dried 0.1g) | UAE 6 mL MeOH/water (5:3) 30 min | SPE ($C_{18}$ cartridges) | HPLC-MS/MS | Isotopically labeled internal standard (4) | 5-50 | (103 ± 24)% | < 14 % | [43] |
| 8 PhACs: NSAIDs (NPX, DCF, and IBP), lipid regulators (CA), and antibiotics (SFT, SP, SMT, and SMX) | Urban biosolids (Freeze-dried 0.2g) | UAE (3 cycles) 2 mL MeOH/water (1:1) 15 min | 0.2 µm nylon syringe filter | LC-MS/MS | Standard addition | 2 -12[b] ($ng.g^{-1}dw$) | (76-131)% absolute recoveries | 5-15% | [44] |
| 2 compounds: Lipid regulator (CA), and NSAID (DCF) | Raw mixed sludge (Oven dried 60-70º C; 0.02g) | UAE 8 mL MeOH/wáter (5:3) 5 mL MeOH (3 times) 30 min 50ºC | SPE | GC-MS | No internal standard or surrogate used | d | 101.8-105 % (CA) 98–104.3% (DCF) | d | [45] |
| 36 emerging contaminants (BTRs; BTHs; PFCs; NSAIDs and EDCs) | Dewatered sludge | UAE 5 mL MeOH/MilliQ water (1:1) 45 min | SPE | GC-MS (for EDCs and NSAIDs) UHPLC -MS/MS (for PFCs, BTRs and BTHs) | Non-labeled internal standard (3) Labeled internal standard (6) | From 0.14 (MTBTH) to 108 ng g−1 dw (BPA) | (64–115) % for most of the target compounds. Lower recoveries (26.4%–59.8%) were observed for longer PFCAs and PFASs | < 15 % | [46] |
| 15 compounds: 14 antidepressants along with their respective N-desmethyl metabolites and the anticonvulsive drug (CBZ) | Biosolids (Freeze dried 0.2g) | UAE 8 mL MeOH/acetic acid buffer solution (1:1) 15 min | SPE (cartridges Strata X-C) | LC-MS/MS | Standard addition Labeled internal standard (1) Non-labeled internal standard (1) | 0.2 ng g-[1](CBZ), 0.4 ng g -[1](FLX), 0.1ng g -[1](PAR) | 71 %, (CBZ), 97 % (FLX), 63% (PAR) | d | [47] |

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| 5 Chiral azole antifungals | Secundary sludge. Lyophilized and homogenized sludge | UAE<br>4 mL<br>MeOH (0.1% formic acid)<br>10 min | SPE<br>(Oasis HLB) | LC-MS/MS | Isotope-labeled internal standard (ILIS) (4) | (3–29) ng $g^{-1}$ d.w | (71-95)% | < 20% | [48] |
| 6 compounds: 4 antibacterial agents (SMX, SDM, TET, OXY) and 2 natural estrogens (E1, E2) | Secondary sludge and compost (Freeze-dried 2g) | UAE (2 cycles)<br>10 mL<br>MeOH/water<br>20 min | SPE<br>Antibiotics (Strata SAX; Oasis HLB)<br>Natural strogens (CarboPrep/NAX) | HPLC-MS/MS (ESI) source for antibiotics and (APCI) source for estrogens) | Standard addition | 1.1 - 17.1 | (17-59)% absolute recoveries for sludge<br>(11-50)% absolute recoveries for compost | d | [49] |
| 14 compounds: 4 EDCs (BPA, E1, NP and OP) and 10 PPCPs (ASA, CBZ, CA, DCF, GEM, IBP, KET, NPX, APAP, TCS) | Sewage sludge (1g) | UAE<br>5 mL<br>MeOH (1% formic acid)<br>20 min | SPE<br>(Oasis HLB) | Derivatization-GC-MS | Isotopically internal standard (2) | 4.7 -39 | (57.9-103.1)% absolute recoveries | (1.3–9.5)% | [50] |
| 27 BFRs | Sludge samples (primary treatment: primary sludge; secondary treatment or anaerobic digestion: biological sludge)<br>(Freeze dried 0.1g) | UAE<br>30 mL<br>EtAc/cyclehexane (5:2)<br>10 min | Florisil cartridges | GC-MS/MS | Non-labeled internal standard (2) | 28-575[c] | (79 -125)% | (3-26)% | [51] |
| 8 compounds: 4 BTRs and 4 BTHs | Dewatered sewage sludge. Additional sludge samples from the primary and secondary settlement tanks were also collected<br>(0.1g) | UAE<br>5 mL of acidified MeOH/Milli-Q water (1:1)<br>45 min | SPE<br>($C_{18}$ cartridges) | LC-MS/MS | Internal standard method (labeled internal standard (2))<br>and with a matrix-matched calibration standard prepared by spiking target analytes into a matrix prior to extraction | 0.04-13[b] ng/g d.w | Recoveries relative to BTR-d4 (64–116)% and to BTR-d5 (50–106)%.For 2-Me-S- BTH, the recovery values relative to BTR-d4 (>64%) both matrices | <15% | [52] |

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| 2 Pharmaceutical drugs (EFV and NVP) | Dried sludge (oven-dried for 48 h at 40 ºC ± 3ºC) (Dried sludge 1g) | UAE<br>15 ml<br>EtAc<br>45 min 50ºC | QuEChERS | GC-TOFMS | External standard<br>Internal standard (1) | 12900 (EFV)<br>11400 (NVP) | 104.6%(EFV)<br>80.9 %(NVP) | 3.3 % (EFV)<br>4.5 % (NVP) | [53] |
| 16 PPCPs: NSAIDs (DCF, FLU, NAP, KET), liquid regulators (BEZ, FEN, GEM), parabens (MP, EP, PP, BP), benzophenones (4-OH-BP, BP1, BP3, BP6, BP8) | Compost from sewage sludge (Freeze-dried 0.5g) | UAE (2 cycles)<br>5 mL<br>ACN:EtAc (1:1) containing 10% (v/v) of acetic acid<br>10 min | SALLE | UHPLC-MS/MS | Matrix–matched Surrogates (4) | 2 - 13 | (93-111)% | < 11% | [54] |
| 13 Quinolones (PIP , ENO, NOR, CIP, OFL, ENR, LOM, MOX, CIN, NAL, OXO, FLU, PIR) | Dried sewage sludge (Oven dried 60ºC; 0.5g) | UAE (2 cycles)<br>5 mL<br>MeOH/McIlvaine buffer,<br>(50:50)<br>15 min | a | LC-MS/MS | Matrix-matched Surrogates (2) | 4 - 18 | (97.9-104.8)% | The inter-and intra-day variability was >7% | [55] |
| 23 PhACs (sulfonamides, fluoroquinolones, tetracyclines, macrolides, trimethoprim, bet-blockers, anti-epileptics, lipid regulators, and stimulants) | Suspended solids (Freeze-dried sludge 0.5g) | UAE (3 cycles)<br>10 mL extraction solvent<br>MilliQ water<br>10 min | SPE<br>(Oasis HLB) | UHPLC-MS/MS | Isotopically labeled internal standard (3)<br>Non-labeled internal standard (1) | 0.02 - 1.00 | (54–130)% | Intraday <11%<br>Interday <13% | [56] |
| 10 PhACs and ECDs (triclosan, 2,4-dichlorophenol, 2,3,4-trichlorophenol, bisphenol A, estrone, 17-beta-estradiol, 17-alpha-ethinylestradiol, androsterone, 5α-androstan-17β-ol-3-one and 19-norethindrone) | Activated sludge (1g dry solids) | UAE<br>6 mL<br>MeOH<br>10 min | SPME | GC-MS | Matrix-matched | 4-50 | d | (2.19-12.10)% | [57] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 6 Perfluorinated compounds (5 perfluorocarboxylicacids and perfluorooctanesulfonic acid), the plasticizer BPA, four anionic surfactants (sodiumalkylsulfates), four preservatives (parabens), two antimicrobial agents (TCS and triclocarban TCC) and six UV-filters (benzophenones) | Digested sludge and compost (freeze-dried 1g) | UAE 3 mL MeOH: acetic acid (95:5) 7 min | d-SPE $C_{18}$ | LC-MS/MS | Isotopically-labelled internal standards (4) Matrix-matched | (0.01-6.2)[d] | (70-120)% | <21% | [58] |
| 29 PPCPs: 2 antischizophrenics 3 sedatives-hypnotics-anxiolytics, 3 antidepressants, 4antihypertensions, 1antimicrobial, 6 antibiotics, 4 analgesics,1 antihistamine, 1 antiplatelet, 1 UV-filter, 1 antihypercholesterolemic, and 1 stimulant | Activated sludge samples (0.1g freeze-dried) | UAE 6 mL MeOH:water (5:3) 30 min | SPE (Oasis HLB) | HPLC-MS/MS | Isotopically-labeled standards (7) | (0.5-50)ng/g | (77-122)% | d | [59] |

Abbreviations: Acesulfame (ACE), acetonitrile (ACN), 4-acetylaminoantipyrine (4-AAA), acetylphenylhydrazine (APh), acetylsalicylic acid (ASA), 4-aminoantipyrine (4-AA), amlodipine (ALP), atmospheric-pressure chemical ionization (APCI), automated mass spectral deconvolution -identification system (AMDIS), atorbastatin (ATT), azithromycin (AZM), bezafibrate (BZB), benzophenone 1 (BP1), benzophenone 3 (BP·), benzophenone 6 (BP6), benzophenone 8 (BP8), p-benzoquinone (BQ), benzothiazoles (BTHs), benzotriazoles (BTRs), benzotriazole UV stabilizers (BUVSs), benzoylecgonine (BZE), bezafibrate (BEZ), bisphenol A (BPA), 1,2-bis(2,4,6-tribromophenoxy) ethane (BTBPE), butylparaben (BP), brominated flame retardants (BFRS), caffeine (CAF), carbamazepine (CBZ), cholesterol (Chol), cinoxacin, (CIN), ciprofloxacin (CIP), clofibric acid (CA), decabromodiphenylethane (DBDPE), 1,2-dibromo-4-(1,2-dibromoethyl) cyclohexane (TBECH), diclofenac (DCF), dichloromethane (DCM), dispersive solid-phase extraction (d-SPE), efavirenz (EFV), electrospray ionization (ESI), endocrine disruptor compounds (EDCs), enoxacin (ENO), enrofloxacin (ENR), erythromycin-$H_2O$ (ERY), estrone (E1), 17β-estradiol (E2), estriol (E3), ethyl acetate (EtAc), ethylene bis(tetrabromophthalimide) (EBTPI), ethylparaben (EP), fenofibrate (FEN), fenofibric acid (FA), flumequine (FLU), 4-formyl aminoantipyrine (4-FAA), 4-formyl antipyrine (4-FA), gas chromatography -electron capture detector (GC-ECD), gas chromatography - mass spectrometry (GC-MS), gas chromatography -tandem mass spectrometry (GC-MS/MS), gas chromatography-time-of-flight mass spectrometry (GC-TOFMS), gel permeation chromatography (GPC), gemfibrozil (GEM), p-hydroxybenzoic acid (PHBA), 2,2′,4,4′,5,5′ Hexabromobiphenyl (BB-153), 4-hidroxy-benzophenone (4-OH-BP), high-performance liquid chromatography – tandem mass spectrometry (HPLC–MS/MS), ibuprofen (IBP), irbesartan (IRB), ketoprofen (KET)), liquid chromatography – tandem mass spectrometry (LC-MS/MS), laser diode thermal desorption—atmospheric pressure chemicalionization—tandem mass spectrometry (LDTD-APCI-MS/MS), limit of quantification (LOQ), liquid chromatography –triple quadrupole-tandem mass spectrometry (LC-QqQMS), lomefloxacin (LOM), methanol (MeOH), methylparaben (MP), moxifloxacin (MOX), nalidixicacid (NAL), naproxen (NPX), nevirapine (NVP), non-steroidal anti-inflammatory drugs (NSAIDs), nonylphenol (NP), nonylphenol ethoxylates (NPE), nonylphenol diethoxylate (NP2EO), nonylphenol monoethoxylate (NP1EO), norfloxacin (NOR), octylphenol (OP), ofloxacin (OFL), oxolinic acid (OXO), oxytetracycline (OXY), paracetamol (APAP), perfluorinated compounds (PFCs), pharmaceuticals (PhACs), pharmaceutical and personal-care products (PPCPs), phenacetin (PH), pipemidic acid (PIP), piromidicacid (PIR), polibrominated diphenyl ethers (PBDEs), propylparaben (PP), quick, easy, cheap, effective, rugged and safe extraction (QuEChERS), roxithromycin (ROS), salicylic acid (SA), salt-assisted liquid–liquid extraction (SALLE), solid-phase extraction (SPE), sulfadimethoxine (SDM), sulfamethazine (SMT), sulfamethoxazole (SMX), sulfapyridine (SP), sulfathiazole (SFT), symvastatin (SVT), tetrabromobisphenol A diallyl ether (TBBPA AE), tetrabromobisphenol A bis(2,3-dipropyl ether) (TBBPA DBPE), triclocarban (TCC), triclosan (TCS), ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), ultra-high-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS), ultrasound-assisted extraction (UAE), valsartan (VAL).

[a] Not clean-up.

[b] Limits of detection (LOD).

[c] Method detection limit (MDL).

[d] Not reported.

[e] Method quantification limit (MQL).

[f] Englert, B., 2007. Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS. US Environmental Protection Agency (EPA), EPA-821-R-08-002, pp. 1-72.

Soxhlet extraction was developed as a more competitive extraction technique. This was initially implemented with the commercial equipment Soxtec® System HT, which provided fundamental savings in time and extractant volume [19]. Automated Soxhlet extraction (Soxtec) uses a combination of reflux boiling and Soxhlet extraction in two extraction steps boiling and rinsing, followed by solvent recovery. Despite such developments, Soxtec does not improve on the scarce versatility of the conventional Soxhlet device. Only 7% of the reports reviewed here have employed the Soxhlet technique [20–24] (Table 1) as also observed in the previous review published in 2012 [14]. Despite the development of Soxtec, the publications mentioned above used Soxhlet as the extraction technique. Fig. 1 summarizes all the information analyzed.

Ultrasound-assisted extraction (UAE) is an alternative to Soxhlet extraction for solid matrices and has been widely used in PPCP procedures. Some of the latest examples are described in three of the reports reviewed here [28,53,54]. The cavitation of UAE reduces the extraction time in comparison with Soxhlet but, in contrast, it is less reproducible. This cavitation process consists of bubble formation, growth and implosion occurring during the propagation of an ultrasound wave in a liquid medium [25]. The principle of ultrasound cavitation is described in a diagram included in the publication [142].

The solvent is chosen based on physical criteria such as viscosity, surface tension and vapor pressure. All these parameters will affect the acoustic cavitation phenomenon [26]. Sonication extraction is faster than Soxhlet extraction (30–60 min per sample) but filtration is required after extraction. UAE is an environment-friendly technique in that it is energy- and time saving. Compared to Soxhlet, less solvent is required and the extraction time is shorter. Hence, using ultrasound, extractions can be completed in minutes, simplifying manipulation and work-up, and employing just a fraction of the energy usually required for a traditional extraction method such as Soxhlet [27]. As mentioned earlier, many studies in the last six years have examined this extraction technique (Fig. 1).

A more modern technique used to determine PPCPs in sewage sludge is microwave-assisted extraction (MAE). This approach uses microwave energy to directly heat the solvent to extract compounds of interest, thus accelerating the speed of extraction. The benefit of MAE is the use of small amounts of solvent compared to Soxhlet and sonication extraction (30 mL in MAE versus 300–500 mL for Soxhlet extraction) which enables the control of extraction parameters such as time, power or temperature [60]. In addition, this green technique offers protection for thermo-labile constituents. However, as UAE, MAE also has its shortcomings: a filtration step is required after extraction, and organic solvents and a subsequent extract cleaning-up step are needed. Further, the

**Table 2**
Determination of PPCPs in sewage sludge based on extraction techniques (MAE, PLE, MSPD and QuEChERS).

| Analyte | Sample type | Extraction technique | Clean-up | Analysis | Quantification technique | LOQ (ng/g) | Recovery (%) | Precision (%) | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 4 PhACs (ASA, NPX, IBP and GEM) | Sludge samples | MAE<br>5 mL MeOH<br>500 W 6 min | Oasis HLB | UHPLC-FLD | d | (1.16–86.4)[b] | 69% absolute recoveries | d | [61] |
| 52 PPCPs: 40 PhACs (steroid estrogens, antibacterials/ antibiotics, hypertension, NSAIDs, lipid regulators, B-blockers, anti-cancer, anti-depressans, anti-epileptics, analgesics), and 12 PCPs (UV-filters, parabens, plasticizers) | Digested sludge (Freeze-dried 0.5 g) | MAE<br>25 mL<br>water/MeOH (50:50)<br>110 °C 30 min | SPE<br>(Oasis MCX, MAX) | UPLC-MS/MS | Isotopically labeled internal standard (38) | <25 ng/g[e] | ˂45% absolute recoveries for majority of compounds | <10% | [62] |
| 17 Antimicrobials (quinolone antibiotics) | Sewage sludge<br>(freeze-dried 1 g) | MAE<br>15 mL<br>ACN:m-phosphoric acid (7:3)<br>5 min<br>120 °1000W | SALLE and d-SPE sorbent<br>(dispersive SPE sorbent.) | UHPLC-MS/MS | Matrix matched<br>Internal standard (1) | 0.5–1.5 | (95.3–106.2)% | <7% | [63] |
| 11 Chiral pharmaceuticals (AM, MA, MDMA, MDA, VNF, DVF, CTP, MTL, PPL, SOT, ALPR) | Digested sludge (1 g and 3 g) | MAE<br>20 mL<br>MeOH:water (1:1) 120 °C<br>30 min. | SPE<br>(Oasis HLB, MCX, MAX) | LC-MS/MS | Isotopically labeled internal standard (9) | 0.08–25.2 | (65–140)% | <30% | [64] |
| 22 compounds: 18 PhACs (analgesics, antibacterials, anti-epileptics, β-blockers, lipid regulators and non-steroidal anti-inflammatories), 1 personal care product and 3 hormones | Sewage sludge.<br>(Freeze-dried sludge 1 g) | MAE<br>10 mL<br>MeOH/water (3:2)<br>500W 6 min | Continuous SPE<br>(Oasis HLB) | Derivatization-GC-MS | Non-labeled internal standard (1) | (0.0008–0.0051)[b] | (91–101)% | ˂7% | [65] |
| 13 Quinolones (PIP, ENO, NOR, CIP, OFL, ENR, LOM, MOX, CIN, NAL, OXO, FLU, PIR) | Dried sewage sludge<br>(Oven dried 60 °C; 0.5 g) | MAE<br>10 mL MeOH/McIlvaine's Buffer (50:50)<br>1000 W<br>87 °C 17 min | a | LC-MS/MS | Matrix matched<br>Surrogates (2) | 4–18 | (97.9–104.8)% | The inter-and intra-day variability was >7% | [55] |
| 28 PhACs (analgesics and anti-inflammatory drugs, antihypertensive, anthelmintic, anti-$H_2$, calcium channel blockers, antibiotics, antiplatelet drug, contrast medium, diuretics, Psychiatric drugs) | Membrane biological reactor (MBR)<br>Sludge (Lyophilized 0.2 g) | ASE<br>MeOH/water (1:2)<br>3 cycles<br>15min<br>100 °C | SPE<br>(Oasis HLB) | UPLC-MS | Matrix matched<br>Isotopically labeled internal standards (1) | d | d | d | [13] |
| 1 NSAIDs (DCF) | Sewage sludge<br>(Lyophilized 1 g) | PLE<br>MeOH<br>15 min<br>100 °C 100 bar | SPE | LC-MS/MS | Standard addition | 1.2–68 | (81.0 ± 7.7- − 94.8 ± 9.6) | 5–17% | [71] |
| 9 PhACs (psychopharmaceuticals) | Raw influent<br>(Freeze dried sludge 2 g) | PLE<br>MeOH<br>5 min<br>3 cycles<br>80 °C 1500 psi | ENVI C18-DSK SPE disk | UHPLC-TOFMS | External matrix- matched | 2.0–25.0[e] | >80% absolute recoveries | <20% | [72] |
| 12 PhACs (2 analgesics (DCF, PNZ), 1antirheumatic agent (IBP), 1 antiepilepticdrug (CBZ), 4 antibiotics (SMX, CLR, RXM, ERY), 2 fibrates (BEZA, FA), 2 β-blockers (MTL, PPL) | Sewage sludge<br>(Lyophilized 1 g) | PLE<br>MeOH<br>15 min<br>100 °C 100 bar | d | LC-MS/MS | Standard addition | 1.2–68 | (22–106)% absolute recoveries | (5–17)% | [73] |
| 42 PhACs (analgesics and anti-inflammatory drugs, anti-ulcer agent, psychiatric drugs, antiepileptic drug, antibiotics, β-blockers, diuretics, lipid regulator and cholesterol lowering statin drugs and anti-histamines) | Thickened, digested and dewatered (treated) sludge<br>(Freeze dried) | PLE<br>MeOH/water (1:2)<br>3 cycles<br>15min<br>100 °C | SPE<br>(Oasis HLB) | LC-MS/MS | Isotopically labeled internal standard (28) | 0.2–16 (thickened) 0.2–14 (digested) 0.3–18 (treated) sludge | (31–136)% thickened; (35–126)% digested and (35–133)% treated sludge | 20% | [74] |
| 15 PhACs (TC, DMC, CTC, OTC, DOC, MCC, SDZ, SMR, SMZ, | | PLE<br>ACN/water (70:30) | SPE<br>(Oasis HLB) | LC-MS/MS | External standard | 2–487 | (49–95)% absolute recoveries | ˂10% | [75] |

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|---|---|---|---|---|---|---|---|---|
| TYL, AMP, ERY, LCM, CBZ, CAF) | Sludge samples (primary sludge, waste sludge) (Freeze-dried sludge 0.5 g)) | 15 min<br>100 °C 1500 psi | | | | | | |
| 19 Brominated compounds: 8 PBDEs, 8 MeO-PBDEs, BFRs (HBB, PBEB, DBDPE) | Digested sludge (Freeze-dried 1.5 g d.w) | PLE<br>22 mL<br>Hexane: DCM (1:1)<br>2 cycles<br>10 min<br>100 °C 1500 psi | SPE<br>(Silica cartridges and alumina cartridges) | GC-MS/MS | d | 0.17 and 9.26 ng/g dw | PBDEs (52–67) %<br>MeO-PBDEs (53–68) %<br>Finally, HBB, PBEB, and DBDPE<br>(52–66)% absolute recoveries | <20% | [76] |
| 7 Antibiotics (4 tetracyclines, 3 sulfonamides) | Primary sludge (after primary clarifier), waste sludge (after secondary clarifier) and dewatered sludge (after dewatering system) (Freeze-dried 0.5 g) | PLE<br>ACN/water (7:3)<br>3 cycles<br>15 min<br>100 °C 100 bar | SPE<br>(Oasis HLB) | LC-MS/MS | External standard | 0.6 μg/kg d.w (sulfonamide)[b] and 146 μg/kg dw (tetracycline)[b] | (49–95)% absolute recoveries | (1.1–5.4)% | [77] |
| 14 PhACs (antibiotic, anti-inflammatory, antilipidemic, anti-hypertensive, anticonvulsant) | Dewatered sludge | PLE<br>MeOH/McIlvaine buffer (1:1)<br>2 cycles<br>15 min<br>100 °C 100 bar | SPE | HPLC-MS/MS | Isotopically labeled internal standard (6) | 0.6–19.4 | (70–120)% | <19% | [78] |
| 59 Emerging nonpolar halogenated micropollutants | Primary sludge, and secondary sludge matrices (freeze-dried 1 g) | PFE<br>U.S. EPA Method 3545A. | SPE | GC-TOFMS | Non-labeled internal standard (2) | <10 | (70–130)% | <30% | [79] |
| 30 PhACs (anti-infective, antiperitic, analgesics) | Dewatered sewage sludge | PLE<br>Methanol/EDTA-McIlvaine buffer (50/50)<br>2 cycles<br>15 min<br>100 °C 100 bar | SPE<br>(Oasis HLB) | HPLC-MS/MS | Isotopically labeled internal standard (22) | 1–30 | (64.0 ± 6.1<br>−324.5 ± 44.1)% | d | [80] |
| ECDs: Natural and synthetic estrogens and their conjugates, antimicrobials, parabens, bisphenol A, alkylphenolic compounds, benzotriazoles, organophosphorus flameretardants | Sewage sludge (Lyophilized samples 1 g d.w) | PLE<br>water:methanol:acetone (1:2:1) 25 min<br>50 °C 1500 psi | SPE<br>(Oasis HLB) | TFC–LC–MS/MS | Isotopically labeled<br>Internal standard (7) | 0.10–125) | (64–115)% | <10% | [81] |
| 13 Quinolones (PIP, ENO, NOR, CIP, OFL, ENR, LOM, MOX, CIN, NAL, OXO, FLU, PIR) | Dried sewage sludge (Oven dried 60 °C; 0.5 g) | PLE MeOH/McIlvaine buffer (50:50, pH = 3) 5 cycles 5 min 86 °C 1000 psi | a | LC-MS/MS | Matrix matched<br>Surrogates (2) | 4–18 | (97.9–104.8)% | The inter-and intra-day variability was >7% | [55] |
| 4 NSAIDs (NPX, KET, DCF, IBP) | Digested sludge (0.5 g) | PHWE<br>NaOH in water<br>5 cycles<br>5 min<br>120 °C 100 bar | HF-LPME | LC-MS | Standard addition | 1.5–12.2 | (101–109)% spiked; (38.9–90.3)% native; absolute recooveries | <13.1% | [87] |
| 23 Pharmaceuticals, antibiotics and hormones | Sewage sludge (Lyophilized sludge 0.2 g) | The PHWE system consisted of a Waters Alliance 2690 HPLC system (Waters, Milford, MA, USA). | SPE<br>(HLB cartridges) | UPLC-MS/MS | Isotopic Labeled internal standard<br>(8) | d | (17–45)% | <25% | [144] |
| 5 NSAIDs (Valdecoxib, Etoricoxib, Parecoxib, Celecoxib and 2,5-Dimethylcelecoxib) | Sewage sludge (Freeze dried 0.2 g) | MSPD<br>Florisil (1 g)<br>Silica (3 g)<br>Hexane (acetone (1:2)<br>15 mL | d | LC-QTOF-MS | Standard additions | 0,005–0,05 | (86–105)% absolute recoveries | <4% | [91] |
| 45 PPCPs: 34 PhACs (antibiotics, nonsteroidal anti-inflammatory drugs, β-blockers, antidepressants), 11 PCPs (antimicrobial agents, preservatives, UV filters) | Sewage sludge (Dewatered sludge)<br>(Freeze-dried 0.1 g) | MSPD<br>$C_{18}$-bonded silica (0.4 g)<br>6 mL MeOH and 10 mL ACN/5% oxalic acid (8/2) | d | LC-MS/MS | Matrix-matched | 0,117–5,55 | (50.3–107)% absolute recoveries | <15% | [92] |
| 23 PPCPs: 19 PhACs and 4 PCPs | Sewage sludge (Freezed-dried 2 g) | MSPD<br>Maceration of the sample for 5 min.<br>Addition 5 mL MeOH and vortexing<br>for 1 min. Centrifugation for 5min. | d | HILIC-MS/MS | Matrix-matched | 1,25-1250 | (50–120)% absolute recoveries | <20% | [93] |
| 4 Antimycotic drugs (tioconazole, sertaconazole, fenticonazole, and | (Freeze-dried sludge 0.5 g) | MSPD<br>$C_{18}$ (2 g)<br>20 mL<br>MeOH: formic acid, (99:1). | SPE<br>(SCX sorbent) | LC-QTOF/MS | Isotope-labeled internal standard (1) | 2 ng/g | (75–124)% absolute recoveries | 13% | [94] |

(continued on next page)

**Table 2** (*continued*)

| Analyte | Sample type | Extraction technique | Clean-up | Analysis | Quantification technique | LOQ (ng/g) | Recovery (%) | Precision (%) | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| itraconazole), the fungicide imazalil | | | | | | | | | |
| 9 compounds: PFAESs (PFSAs, Cl-PFAESs, FTSAs) | Freshly digested sludge (lyophilized 0.5 g) | DSPE with slight modifications<br>3 mL of ACN and<br>160 μL NaOH 50 °C 120 min | d | UPLC-MS/MS | External standard with correction of 2 isotope-labeled internal standards | 0.043 | (84–137)% | <20% | [95] |
| 9 Parabens (MP, EP, PP, BP, PhP, IsBP, IsPP, BzP, PeP) | Drinking water sludge samples<br>(10 g) | QuEChERS<br>10 mL ACN 1% formic acid.<br>4 g $MgSO_4$ and 1 g NaCl | $MgSO_4$ | LC-MS/MS | Matrix-matched | 5–500 | (62–119)% absolute recoveries | <20% | [98] |
| 5 Acid pharmaceutical drugs (CA, IBP, ASA, NPX, FLB) | Sewage sludge<br>(2 g) | QuEChERS/automated online<br>2.0 mL deionized water and 10 mL polypropilene<br>1.2 g NaCl | a | IC-FLD | Matrix-matched | 0.082–29 | (81.1–112.7)% absolute recoveries | <17,8% | [99] |
| 136 compounds: 119 PhACs, 17 hormonal steroids. | Sewage sludge (Freeze-dried 2 g) | QuEChERS<br>10 mL EDTA and 10 mL ACN + acetic acid 1%<br>1 mL heptane and 10 metal balls<br>Acetate buffer (1.5 g NaOAc and<br>6 g $MgSO_4$, whereas the citrate buffer contained 1 g sodium citrate,<br>4 g $MgSO_4$, 1 g NaCl and 0.5 g disodium citrate sesquihydrate) | a | LC-TOF/MS | Standard addition | 1–2500 | (15–131)% absolute recoveries | Intra-day (<20%)<br>Inter-day (<28%) | [100] |
| 27 PPCPs (21 PhACs, 6 PCPs) | Drinking-water sludge samples<br>(10 g) | QuEChERS<br>10 mL ACN acidified with 100 μL acetic acid.<br>4 g $MgSO_4$ and 1 g NaCl | SPE (PSA) | UPLC-MS/MS | Standard addition | (0.5–10)[e] | (50–93)% absolute recoveries | <10% | [101] |
| 13 SMCs (6 polycyclic, 2 macrocyclic and 5 nitromusks) and 6 ultraviolet-filters (UVFs) | Sewage sludge<br>(Freeze dried 0.5 g) | QuEChERS<br>10 mL ACN<br>15 min in a 420W ultrasonic bath<br>500 mg $MgSO_4$,<br>315 mg PSA and 410 mg $C_{18}$ | d-SPE | GC-MS/MS | Isotopically labeled internal standard (3) | (0.003–25) pg | (75–122)% | <10% | [102] |
| 8 PCPs (macrocyclic musk fragrances) | Mixture of primary and secondary sewage sludge<br>(freeze-drying 0.25 g d.w) | HS-SPME<br>0.5 mL ultrapure water<br>45 min<br>750 rpm 80 °C | d | GC-MS | Matrix-matched | 0.89 pg/g[c] | d | 1–15% | [105] |

Abbreviations: accelerated solvent extraction (ASE), acetaminophen (AMP), acetonitrile (ACN), acetylsalicylic acid (ASA), alprenolol (ALPR), amphetamine (AM), bezafibrate (BEZA), brominated flame retardants (BFRS), butylparaben (BP), caffeine (CAF), carbamazepine (CBZ), chlortetracycline (CTC), cinoxacin, (CIN), ciprofloxacin (CIP), citalopram (CTP), clarithromycin (CLR), chlorinated Polyfluoroalkyl Ether Sulfonates (Cl-PFAESs) clofibric acid (CA), cyclooxygenase-2 (COX-2) cyclooxygenase inhibitors (COXIBs), decabromodiphenylethane (DBDPE), demeclocycline (DMC) o-desmethylvenlafaxine (DVF), diclofenac (DCF), dispersive solid-phase extraction (d-SPE), doxycycline (DOC), emerging contaminants (ECs), endocrine disruptors (EDCs) enoxacin (ENO), enrofloxacin (ENR), erythromycin (ERY), ethylparaben (EP), fenofibric acid (FA), flumequine (FLU), flurobrofen (FLB), luotelomer Sulfonates (FTSAs), gas chromatography-mass spectrometry (GC-MS), gas chromatography-tandem mass spectrometry (GC-MS/MS), gas chromatography-time-of-flight mass spectrometry (GC-TOFMS), gemfibrozil (GEM), hexabromobenzene (HBB), high performance liquid chromatography −tandem mass spectrometry (HPLC-MS/MS), hollow fibre liquid-phase microextraction (HF-LPME), ibuprofen (IBP), ionic-chromatography-fluorescence detector (IC-FLD), ketoprofen (KET), liquid chromatography−hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), liquid chromatography − mass spectrometry (LC-MS), liquid chromatography -tandem mass spectrometry (LC−MS/MS), liquid chromatography-time-of-flight (LC-TOF), liquid chromatography −triple quadrupole (LC-QQQ), lomefloxacin (LOM), matrix solid-phase dispersion (MSPD), meclocycline (MCC), methamphetamine (MA), metanol (MeOH), methoxylated-polybrominated diphenyl ethers (MeO-PBDEs), 3,4-methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylparaben (MP), 2-methylpropyl paraben (IsBPB) metoprolol (MTL), microwave-assisted extraction (MAE), moxifloxacin (MOX), nalidixicacid (NAL), naproxen (NPX), non-steroidal anti-inflammatory drugs (NSAIDs), norfloxacin (NOR), octadecyl-silica (C18), ofloxacin (OFL), oxolinic acid (OXO), oxytetracycline (OTC), pentabromoethyl benzene (PBEB), perfluoroalkyl Sulfonates (PFSAs) personal care products (PCPs), pentyl paraben (PePB), pharmaceuticals (PhACs), pharmaceutical and personal-care products (PPCPs), phenazone (PNZ), phenylparaben (PhP), pipemidic acid (PIP), piromidicacid (PIR), polybrominated diphenyl ethers (PBDEs), polyfluorinated ether sulfonates (PFAESs)), pressurized fluid extraction (PFE), pressurized hot water extraction (PHWE), pressurized liquid extraction (PLE), primary and secondary amine exchange bonded silica sorbent (PSA) propan-2-yl paraben (IsPPB), propranolol (PPL), propylparaben (PP), quick, easy, cheap, effective, rugged and safe extraction (QuEChERS), roxithromycin (RXM), solid-phase extraction (SPE), sotalol (SOT), sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethoxazole (SMX), syntheticmusk compounds (SMCs), flow chromatographyfollowed by liquid chromatography coupled to tandem mass spectrometry (TFC-LC-MS/MS), tylosin (TYL), ultra-high-performance liquid chromatography-fluorescence detector (UHPLC-FLD), ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), SALLE (salt-assisted liquid−liquid extraction), ultra-high performance liquid chromatography −mass spectrometry (UPLC-MS), ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-TOF-MS), ultraviolet-filters (UVFs), venlafaxine (VNF).

[a] Not clean-up.

[d] Not reported.

[f] Englert, B., 2007. Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by HPLC/MS/MS. US Environmental Protection Agency (EPA), EPA-821-R-08-002, pp. 1-72.

[b] Limits of detection (LOD).

[c] Method detection limit (MDL).

[e] Method quantification limit (MQL).

equipment for MAE is relatively expensive. Thus, probably because of all these downfalls, only a small number of studies addressing MAE have been reported in the literature reviewed [55,61–65] over the last 6 years (Table 2). However, the number of studies reviewed is still higher compared to the previous review [14], which only mentioned four references [66–69].

Another extraction method is pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE). This is a fully automatic technology which uses low volumes of liquid extractants such as hexane, ethanol and acetone at high pressure (usually up to 200 bar) and temperature (usually up to 200 °C) without reaching the critical point to recover those target analytes with short extraction times [70]. PLE has proven very effective for extracting target analytes. However, extracts usually contain a complex matrix as well. Thus, a clean-up procedure is often needed after extraction to remove interferences. Solid phase extraction (SPE) with a great variety of sorbents has been the most common clean-up technique when PPCPs are the target analytes [13,71–81]. However, gel permeation chromatography (GPC) has also been used to purify organic pollutants [35]. PLE has many advantages over traditional extraction techniques as efficient ways of increasing automation, shortening the extraction time and reducing the amount of organic solvents. PLE usually entails extraction times of around 15 min per sample and uses between 15 and 40 mL of solvent. In addition, the instrumentation allows for extraction in an unattended operation. It is regarded as reasonably easy and exhaustive, offering quantitative recoveries with little spare time spent on method development [70]. All these attractive features have meant that many of the works reviewed used PLE to extract PPCPs from sewage sludge. Some of the most relevant examples are [13,55,71,80,81]. The number of recent publications is comparable to those reported [82–84] (Table 2) in the previous review published in 2012 [14] (Fig. 1).

An even more environmentally-friendly technique is pressurized hot water extraction (PHWE). This technique uses pressurized water as an extraction fluid at elevated temperature. Water has several positive features such as easy access, safety and can be recovery or disposed of with minimal environmental concerns [85]. Temperature is the most important parameter to optimize in this technique as it affects extraction efficiency and selectivity. Elevated temperatures provide certain advantages such as high diffusion, low viscosity and surface tension [85]. The best features of PHWE are the use of small amounts of organic solvents [86] and its low cost. In the future, this green extraction technique is expected to help manipulate large sample sizes for industrial applications. Despite these commented advantages, only two references of the use of PHWE as the extraction technique was found in the last 6-years reviewed [87,144] along with one more [88] in the previous five-year period [14].

Recently some authors have replaced the more traditional extraction techniques such as UAE or Soxhlet and also MAE or PLE with novel methodologies including MSPD (matrix solid-phase dispersion) or QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). These approaches have as their main goal to improve the method's sensitivity and selectivity as isolation and purification are combined in a single step. The main sources of error of most analytical methodologies are avoided. Main benefits are the short time required for sample preparation and their efficiency in cleaning-up the extract [93,101].

MSPD for the extraction of PPCPs in sewage sludge was introduced in 1989 and applied to the extraction of solid, semisolid or viscous samples. It consists of homogenizing the sample with a dispersing agent (abrasive solid) onto a solid support, allowing the disruption of the sample and the extraction of target analytes by means of a suitable elution solvent [89]. The great interest in MSPD may be attributed to the advantages it offers and its simplicity and flexibility which have contributed to its choice over more classical sample preparation methods [90]. MSPDE is rapid, scarcely manual-intensive and eco-compatible. After extraction, depending on the nature of target analytes and the instrumentation used for their detection, a clean-up procedure may or not be needed. This technique and PLE have sometimes been employed together as the solvents used at high pressures and temperatures increase analyte recoveries when interactions of the analytes with the solid matrix are really strong. The method's selectivity is related to the elution solvent utilized and the nature of the sorbent materials. Lipophilic sorbent materials such as $C_{18}$-bonded silica or $C_8$-bonded silica are employed in numerous applications, although the latter is used less frequently [90]. The solvents chosen for elution depend on the nature of the solid material. Organic solvent mixtures are mainly used, however, hot water offers excellent results in certain applications (mostly in PLE procedures). MSPD extraction has several benefits such as reduced amounts of solvents and sample, short extraction times, low cost and good performance at room temperature and atmospheric pressure with acceptable yields and selectivity. The technique is suitable for a great variety of analytes and environmental matrices due to its flexibility and versatility. Some reports exist in the literature [91–95] (Table 2) for the last 6 years. In contrast, only one study was found in the previous period from 2008 to 2012 [96]. This indicates a large increase in the use of this technique.

Finally, one of the most novel techniques employed to determine PPCPs in environmental matrices is QuEChERS. This procedure offers benefits such as the use of a small content of organic solvents, scarcely time consuming, good recoveries and high selectivity. It mainly consists of two steps, salting-out liquid-liquid extraction (SALLE) and dispersive solid-phase extraction (DSPE) for extract clean-up [97].

QuEChERS encompasses both extraction and clean-up steps for complex environmental matrices. This reduces sample preparation to approximately 20 min. The technique uses less solvent than ASE (usually up to 10 mL), and entails minimal times and costs. Some reports of QuEChERS applications exist in the literature reviewed here [98–102] (Table 2) but no studies addressed this issue in the five-year period before 2012 [14].

Overall, as depicted in Fig. 1, UAE emerges as the most popular extraction technique (49%), followed by PLE (19%) and MAE (9%). Thus, the trend observed until 2012 reviewed by Ref. [14] has been maintained in the last six years. Nonetheless, UAE seems to have lately experienced a boost, most probably because of its simplicity and high performance as well as affordability and availability at most of laboratories around the world.

### 2.1.2. Clean-up

Most extraction techniques for PPCPs in sewage sludge are not sufficiently selective and a clean-up step is usually subsequently necessary. Some of the most common interfering constituents of sludge are compounds such as lipids and substances added to sewage sludge during processing such as surfactants and polymer colloids, among others. Although interference can occur at any stage of the analytical process, instrumental analysis based on liquid chromatography interphase to mass spectrometry by electrospray ionization is especially sensitive to matrix effects [55].

$C_{18}$ is a clean-up agent commonly used to remove interfering lipids and lipophilic compounds in extracts contained in organic solvents. PSA (primary and secondary amine) has also proved effective for the removal of acidic interferences such as humic and fulvic acids (main components of compost) among others [55]. $C_{18}$ and PSA (primary and secondary amine) are examples of some clean-up agents commonly used in dispersive solid-phase

extraction (d-SPE) [102]. Thus, the choice of sorbent must be adequate to retain interferences present in each particulate sludge matrix. Deficiencies in the extraction process have been also attributed to the presence of co-extracted matrix components [34].

Solid-phase extraction (SPE) is the most popular technique for the clean-up of PPCPs after extraction from sewage sludge, and from environmental samples in general [28,30,54,78]. This procedure is quick and simple to operate and can be easily automated and coupled to instrumental techniques such as liquid chromatography (LC) [103].

There are three general extraction mechanisms used in SPE: polar, non-polar and ion exchange. More than half of the works found in the literature during the last 6 years have employed reverse-phase SPE (63%). The retention mechanism is the interaction of non-polar groups of the analytes of interest and the non-polar functional groups on the sorbent (Van der Waals forces) [104]. In many cases, extraction was performed in a polar solvent [13,24,39–43,45–48,50,52,56,59,62,64,74,75,77,80,81]. Mixed-mode SPE is an extraction approach involving sorbents which are designed to exhibit two or more primary interactions for analyte retention. Most mixed-mode sorbents include hydrophobic functional groups in combination with ion-exchange functional groups. In some cases, Oasis MCX (Mixed-Mode Cation-eXchange) has been used for the clean-up of extracts containing acidic pharmaceuticals [39,62,64]. Oasis MAX (Mixed-Mode Anion-eXchange) has been also used in other cases [62,64]. However, the reverse phase sorbent patented in Oasis HLB (Hydrophilic-Lipophilic Balanced) has been the preferred option over the last six years [13,24,29,30,32,39,40,42,48–50,56,59,64,74,75,77,80,81]. It is a universal polymer reversed-phase sorbent that was developed for the extraction of a wide range of acidic, basic and neutral compounds from various matrices. Another type of adsorbent is based on $C_{18}$-silica and used to adsorb analytes of even weak hydrophobicity from aqueous solutions [43,52]. In the 1990s, a miniaturized variation of SPE emerged as a solid-phase microextraction technique (SPME). This method involves an alternative preconcentration technique to LLE or SPE. It consists of a silica fiber coated with a thin layer of an extractant polymer, which can be placed in the head space (HS-SPME) or subjected to direct immersion (DI-SPME) in solid, liquid or gaseous samples. As the fiber is desorbed in the injection port of a gas chromatography system, the use of solvents is eliminated and possible losses of analytes and contamination of the samples are reduced [28,57]. are examples found in the literature reviewed here.

Gel permeation chromatography (GPC), also known as size-exclusion chromatography (SEC), is a method in which component separation is based on differences in molecular weight or size. It requires short analysis times and small volumes of mobile phases. It has been widely employed to isolate and analyze biomacromolecular substances such as sugar, peptides, proteins, rubbers, and others, on the basis of their size. GPS has been also applied to PPCPs, usually in combination with other clean-up techniques. In particular [35], made use of GPC along with a silica gel column to clean up 153 pharmaceuticals, herbicides, antioxidants, intermediates, organic solvents and chemical raw materials. Three studies reviewed by Ref. [14] for the period 2008 to 2012 included GPC and normal-phase SPE used together as the clean-up procedure [106–108].

Liquid–liquid extraction (LLE) is an effective separation method for compounds having different solubility in two immiscible liquids. These two liquids are generally water, with or without additives, and a nonpolar organic solvent. Polar compounds prefer the aqueous layer while nonpolar compounds are extracted into the organic layer. In salting-out systems, water-miscible solvents have been investigated for the extraction or concentration of analytes that cannot be extracted by conventional LLE methods. This salting-out often occurs at high salt concentrations [109]. However, LLE extracts are not particularly clean in comparison with other more intensive sample preparation procedures. The first applications of this technique to PPCPs in sludge were reported by Refs. [54,63].

Overall, the vast majority of publications, 60% of the reports reviewed here, chose SPE as the clean-up approach, as shown in Fig. 2. Only isolated examples of other techniques have been found such as florisil [51], silica [90] or $MgSO_4$ [98].

### 2.2. Instrumental analysis

Instrumental analysis for PPCPs in sewage sludge is basically based on chromatographic separation coupled to mass spectrometry. PPCPs are mostly polar compounds with limitations of volatility and/or thermal stability for their analysis by gas chromatography (GC) [28]. Nonetheless, these limitations have been overcome by derivatization processes such as acylation (acetylation), alkylation [33] and silylation [28,37,50,65]. GC is a relatively inexpensive instrumental technology which enables this kind of analysis to be carried out by a wide range of laboratories around the world, including those in developing countries [20,53]. Overall, 25% of the reports reviewed chose GC-based on instrumental techniques. In comparison to the period reviewed by Ref. [14], there seems to have been a decline in the popularity of GC (Fig. 3). Most GC approaches are coupled to mass spectrometry (MS) detection in both a single and tandem (MS/MS) modality. Other detection approaches were found coupled to GC such as electron capture detector (ECD) [22]. Triple quadrupole (QqQ) is the most common analyzer mainly used in selected reaction monitoring (SRM) mode for quantitative analysis [51,76]. However, some examples of target analysis in high resolution by quadrupole to time-of flight (Q-TOF) couplings have been also found in the literature [37,53,79]. As pointed out in the previous section, SPME is a pretreatment technique which allows automation when coupled to GC and was employed by Refs. [28,129] for the analysis of 12 PPCPs and 8 macrocyclic musk fragrances in sewage sludge respectively. This constitutes the only examples of pretreatment coupling to instrumental analysis in our realm.

However, despite the above, LC-based on instrumental analysis has become the most popular technique (Fig. 3) in the determination of PPCPs in environmental matrices including sewage sludge. This is probably because of its higher versatility as a larger spectrum of compounds can be readily analyzed with no prior derivatization or alike. Again, mass spectrometry is the preferred detection option, but some examples (2) of coupling to fluorescence detection have been also found [61,99]. This repeats the scenario as in the period reviewed by Ref. [14] where a single example of this coupling was cited [110]. In contrast, ultraviolet (UV) detection cited years ago [111] is no longer an interesting option. Within MS modalities, MS/MS was found to have the greatest applicability, in particular using QqQ in SRM mode for target analysis. Hence, 63% of the LC works reviewed fit this classification. Nevertheless, interest in the use of other tandem combinations such as Q-TOF has been recently sparked due to improvements in the dynamic range and sensitivity of TOF. In addition, TOF analyzers offer a high resolution capacity. This ensures high selectivity and reduces the probability of false positive results. In addition, they open the possibility of qualitative analysis of un-known compounds, which is not readily available in QqQ. Electrospray ionization (ESI) is the most commonly used ionization approach as it allows mild ionization of the target analyte and molecular ions usually remain un-fragmented [47,75,100]. Nonetheless, apolar compounds might undergo poor ionization by ESI, and atmospheric pressure chemical ionization (APCI) is then

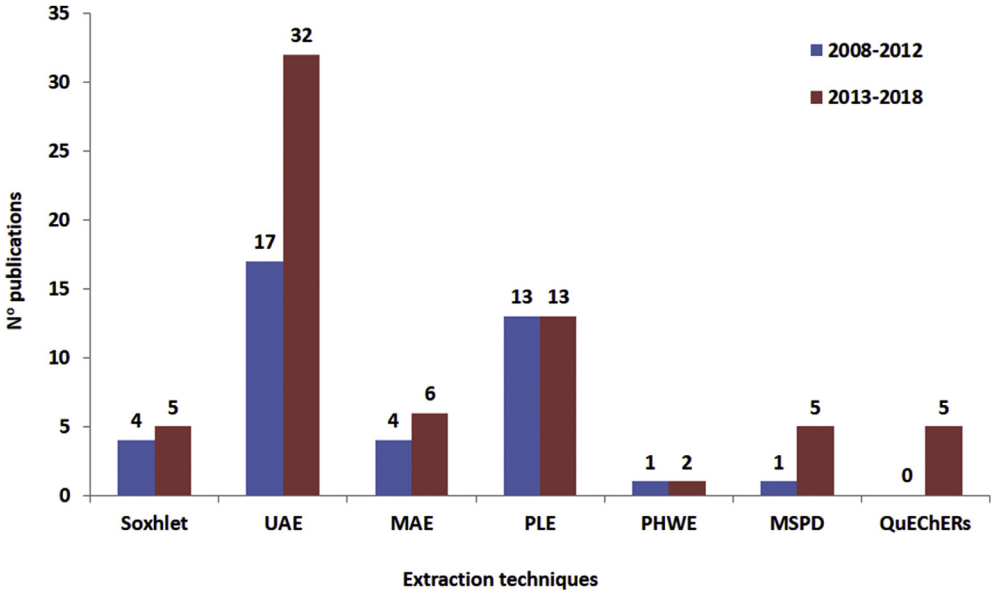


**Fig. 1.** Extraction techniques for PPCPs in sewage sludge.

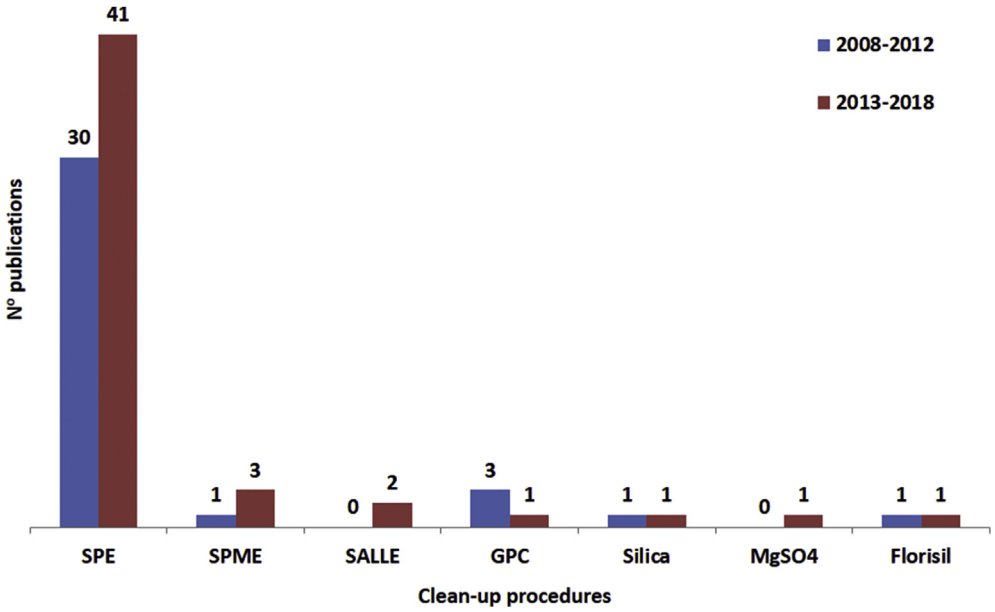


**Fig. 2.** Clean-up techniques for PPCPs in sewage sludge.

recommended as in Refs. [31,49]. Weak acids and bases such as formic acid and ammonium acetate are usually used as mobile phase modifiers when working at + ESI and −ESI respectively. Moderate acidic (~3) and basic pHs (~8) are provided by formic acid and ammonium acetate respectively. In this regard, a larger number of PPCPs contain basic functional groups (such as amines) with pKa values above pH 3 rather than acidic functional groups (such as alcohols) with pKa values below pH 8. Therefore, PPCPs are more prone to be positively ionized and are more efficiently analyzed by + ESI rather than -ESI.

Within LC, fast chromatography has emerged as an improved modality over high performance liquid chromatography (HPLC). The ultra-high version (UHPLC) was introduced under the trade mark UPLC™ in 2004 and triggered many advances in instrumentation and column technology, which have led to a significant increase in resolution, speed and sensitivity. Column efficiency increases with reduction of stationary phase particle size (usually <1.7 μm) and mobile phase delivery is done at ˂15,000 psi (about 1000 bar) [112]. Separations are mostly completed in less than 10 min and some even in under 2 min [32,62,72]. UHPLC often provides narrow peaks (in few seconds or even less) offering a high-speed detection response (>100Hz) [112].

Over these past 6 years, out of 47 of the applications using LC, 14 were fast chromatography. This in comparison to the previous 5-year period reviewed by Ref. [14], in which only 8% of studies examined this kind of liquid chromatography, reveals a clear upward trend in the use of UHPLC likely attributable to its many benefits mentioned. Overall, as depicted in Fig. 3, LC has been the most popular instrumental technique (73%) for the determination of PPCPs in environmental matrices including sewage sludge.

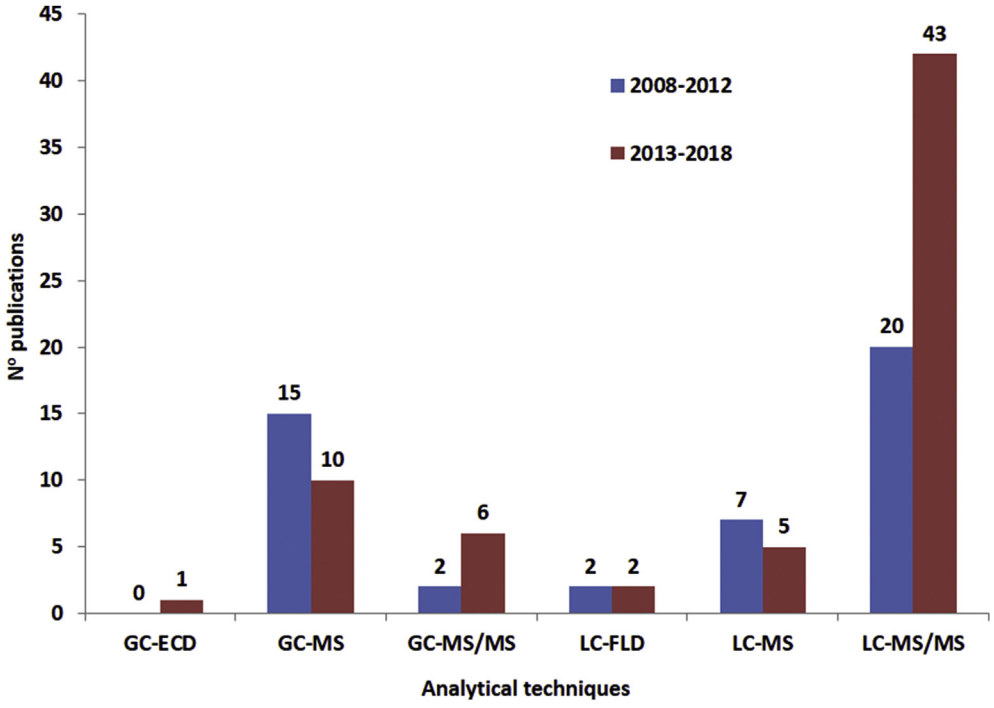


**Fig. 3.** Instrumental analysis techniques for PPCPs in sewage sludge.

Hence, the trend observed until 2012 and reviewed by Ref. [14] has been maintained over the last six years.

### *2.3. Current trends and future perspectives in the determination of PPCPs in sewage sludge*

The concept of "green chemistry", otherwise known as sustainable chemistry, was introduced 20 years ago and refers to the design of chemicals and processes that reduce and eliminate the use or generation of hazardous substances. When applying and proposing new methods and processes of analysis, sustainability should be considered a necessary characteristic. By automatizing a technique, the use of resources, including time, usually becomes more efficient. In addition, human error and analyst exposure to hazards are minimized [113]. Besides automation, miniaturization in analytical chemistry has also become a dominant trend recently replacing traditional sample preparation. The goal is to provide high extraction efficiencies in short times and minimize the amount of sample and so reduce the consumption of reagents and solvents. In addition, after automation and miniaturization, many sample preparation methodologies are susceptible to being incorporated into instrumental analysis systems such as GC or LC [113]. Hence, in the early 2000s, a research group developed simple procedures based on SPME or USAEME (ultrasound-assisted emulsification-microextraction) for the analysis of allergenic fragrances, synthetic musks, phthalates and preservatives in water samples [114–116]. While the use of miniaturized and automatized methodologies for the determination of PPCPs in water matrices is a reality [117,118], the reports reviewed here barely show the use of miniaturization techniques for the determination of the contaminants of interest in sewage sludge. Only two studies found in the literature offer an analytical method for the determination PPCPs and PCPs in sewage sludge by DI-SPME-On-fiber derivatization-GC-MS [28] and HS-SPME-GC-MS [129] respectively. Interest in microextraction processes has been renewed due to the incorporation of new materials, either as suitable substitutes for conventional halogenated organic solvents or other types of toxic reagents [113]. At present sufficient technology already exists for research groups to develop miniaturized and automatized analytical methods for the determination of PPCPs in sewage sludge.

Additionally, there are concerns in the scientific community over the presence of transformation products (TPs). Many of these TPs have shown to be as pernicious as the parent PPCPs they come from. Clear efforts are currently focusing on the identification in environmental water samples of metabolites and other TPs generated over the PPCP life cycle, such as during treatment processes in WWTPs [28,36]. However, there is no evidence in the literature yet of this trend in relation to sewage sludge.

Many PPCPs consist of chiral molecules and each enantiomer usually exerts different toxicity according to its biological properties [119]. Hence, reports exist of the determination of chiral pharmaceuticals by chiral LC-MS/MS [64,120] in sewage sludge samples. Nonetheless, much more work is still needed in this area.

Future perspectives related to the development of new sample preparation methods differ depending on the type of the pollutant. There is increasing interest in nanotechnology in important sectors of science and technology such as engineering, medicine or agriculture, among others. Nanotechnology is making progress in technologies for protecting the environment too. However, nanotechnology's unique characteristics can lead to unforeseen environmental problems [121]. In parallel, the use of novel solid and liquid phase materials has increased in the last years including nanomaterials (NMs), ionic liquids (ILs) or supramolecular solvents (SUPRAS) used in the analysis of environmental samples. Engineered nanomaterials (ENMs) are materials or chemical substances with particle sizes between 1 and 100 nm in at least one dimension [122]. There is great interest in innovations produced in the industrial, commercial and medical sectors due to the physical and chemical properties of these materials. However, some of their properties (chemical reactivity, surface area and particle size) pose a risk to health and the environment [123]. Some works have described applications of nanoadsorbents in environmental water

samples [124,125]. In the near future, NMs could be applied to sewage sludge samples. SUPRAS are nanostructured liquids generated from compounds with both hydrophilic and lipophilic properties (amphiphiles) [126]. SUPRAS have been employed for the extraction and preconcentration of emerging pollutants in environmental water samples [127]. However, there are still no reports of applications of SUPRAS to sewage sludge. ILs are salts whose ions are poorly coordinated, which makes these solvents liquid at temperatures below 100 °C, or even at room temperature (room temperature ionic liquids) [128]. One publication reports on the determination of musk fragrances in sewage sludge based on IL–HS–SPME followed by GC-MS/MS [129].

## 3. Data processing

Environmental sample matrices are complex and their analysis and subsequent data processing are extremely difficult. For many years, a traditional approach offering reliable rapid identification and quantification of target compounds has been used [130]. In total, 98% of the reports reviewed employed target analysis to determine PPCPs in sewage sludge samples. However, target analysis has the drawback that only a limited number of compounds can be determined and many pollutants present are ignored [131].

A comprehensive picture can be obtained by non-target analysis which does not require "a priori" selection of contaminants. This approach is able to detect any analyte present above the MDL. In addition, retrospective analysis is possible [131]. Anthropogenic compounds such as pharmaceuticals and personal care products, flame retardants, plasticizers, polymer additives and other well-known persistent organic pollutants can be identified using this approach. Suspect screening is a non-target analysis. Both suspect and non-target analysis are based on the power and development of high-resolution mass spectrometric instruments. These techniques serve to acquire full scan spectra and allow a retrospective analysis of emerging contaminants after the data has been acquired, while providing two essential factors for non-target analysis: accurate-mass and high-resolution [131]. The most common MS analyzers used for this purpose, such as Orbitrap or the Fourier transform ion cyclotron resonance (FT-ICR) device, can be linked to different ionization sources (ESI, APPI and APCI) and different separation techniques (GC, LC and GCxGC) depending on the class of compounds to be examined [128]. However, in the past 6 years, only one study has used this method to determine emerging contaminants in sewage sludge. This study [37] described a non-targeted approach based on GCxGC-TOFMS. In contrast, numerous reports exist of a non-target approach for the determination of these contaminants in wastewater; some examples being [132,133].

Target methods are usually quantitatively more powerful as they show a greater sensitivity and dynamic range than untargeted methods. Regardless, analyte quantification is usually performed through the use of authentic chemical standards and the construction of calibration curves. Calibration curves are used to understand the instrumental response to an analyte and to predict its concentration in a sample. Over the past six years, the calibration methods reported in the literature to determine PPCPs have been based on approaches including an internal standard, standard additions, matrix matched or external standard. The choice of a specific calibration method depends on a number of factors such as affordability, matrix complexity, and number of samples, among others. External standard calibration has been one of the most commonly used calibration approaches among the reports reviewed here. This approach is inexpensive as well as quick and easy to set up. On the downside, it is greatly affected by the stability of the chromatographic detector system and the presence of chromatographic interferences in the sample. Some of the publications reviewed make use of this quantification approach [53,75,77,95] (Tables 1 and 2). When matrix problems are suspected, a more reliable calibration may be obtained via matrix-matched calibration. This may make up for matrix effects although it does not eliminate the underlying cause because the effect intensity may differ from one matrix or sample to another, and can be also affected by the matrix concentration. In fact, matrix-matched calibration is a particular type of external calibration in which the calibration standards are prepared using a simulated sample that initially does not contain the analyte. Of the reports reviewed, 22% chose matrix-matched as calibration method (Tables 1 and 2), which represents an increase in comparison with the period reviewed by Ref. [14], in which only 6% of the publications selected the matrix-matched method [134–136]. Another calibration alternative is based on standard addition. This method is more accurate and precise and overcomes more matrix effects than external and matrix-matched calibration approaches, as it uses the sample itself to build the calibration curve. However, it entails the preparation of a different calibration curve per sample. It is therefore labor intensive, time-consuming, and requires large sample amounts, which is usually in disagreement with green chemistry principles. Overall, 14% of the publications reviewed here used this calibration method (Tables 1 and 2). In contrast, previous publications reviewed by Ref. [14] reported this calibration approach less frequently (9%). Finally, an internal standard (IS) is a reference species with similar physicochemical properties and similar analytical behavior to the compounds of interest not expected to be found in the samples. This calibration method is not as useful for GC and HPLC methods involving non-MS detectors unless the internal standards can be separated from target compounds chromatographically. The advantage of this calibration method is that fluctuations are monitored in every sample. It assumes that the behavior of the IS is identical to that of the analyte. Thus, the selection of a suitable IS is mandatory. The use of internal standard calibration approaches has experienced a boom in the last few years. In effect, 47% of the reports reviewed selected this procedure (Tables 1 and 2) versus 4% reported in the prior review [14]. In particular, the use of stable-isotope-labeled analogues of the analytes has become popular because of its efficiency and reliability to compensate for any alteration in the signal due to casualties across the whole analytical process. However, for highly multi-component applications, it requires a significant economic investment, unaffordable for many laboratories.

Fig. 4 depicts the frequency of each calibration method used in the reports reviewed. The use of isotopically labeled analogues in internal standard calibrations has been the most popular choice.

## 4. Validation

The purpose of validation of an analytical procedure is to confirm that the analytical method used for experimental tests is suitable for that purpose. Method validation was established in analytical laboratories in the late 1970s, recognizing its importance in obtaining standard methods. The United States Food and Drug Administration (FDA) [137] and Eurachem [138] have published guidelines for methods validation.

To a large extent, the reliability and capacity of analytical methods have improved to a large extent as a result of recent technical advances [139]. The main validation parameters provided in the publications are (Tables 1 and 2):

a. Accuracy is the closeness of agreement between test results across the specified range and an accepted reference value. In our particular case, it is expressed as the percentage recovery of

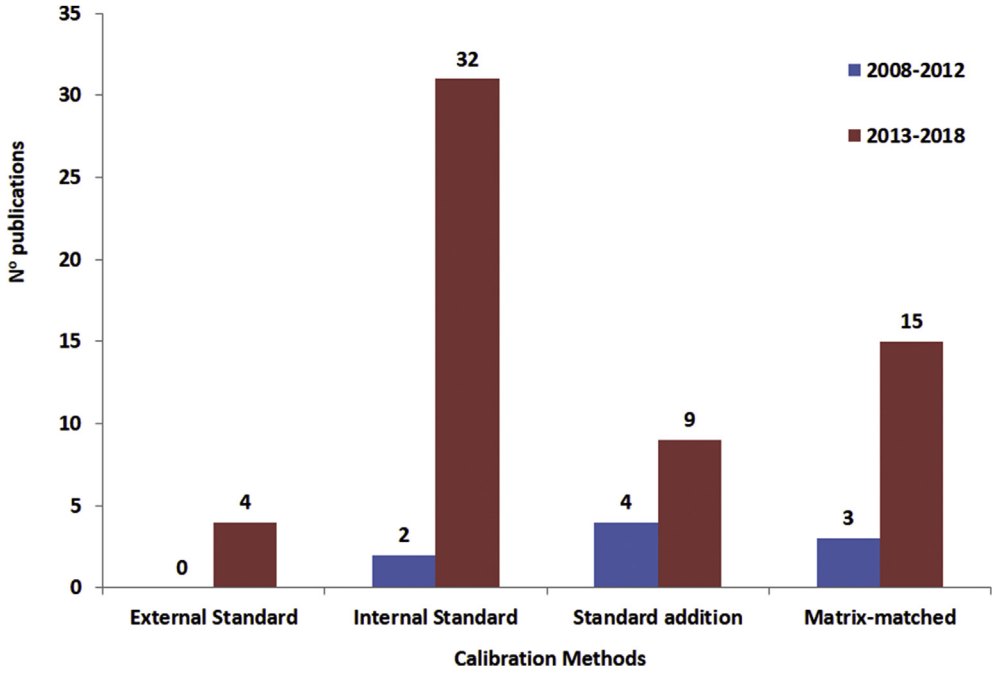


**Fig. 4.** Calibration methods used in the quantification of PPCPs in sewage sludge.

each analyte after the whole analytical protocol (absolute recovery). Some authors also provide improved recovery values after adjusting for method deficiencies when applying an internal standard calibration approach (relative recovery). The reports reviewed showed considerably high analyte relative recoveries. Thus, 35 out of 67 publications showed percentages higher than 70% and 22 out of 67 publications obtained values below 70%. In contrast, 17 out of 47 publications were found for the five years before 2012 with percentages higher than 70% and 13 studies with values below 70%.

b. Precision is the closeness in agreement between individual results obtained for a repeatedly applied procedure on a homogeneous sample, comprising repeatability and intermediate precision. In our particular case, method repeatability is usually expressed as the standard deviation, relative standard deviation or coefficient of variation. Overall, 72% of the reports reviewed cited values below 20%. In comparison, for the period reviewed by Ref. [14], 23 out of 47 publications reported values below 20%.

c. Limits of detection (LOD) and limits of quantification (LOQ) can be directly obtained from the linearity test in the validation protocol. Hence, the lowest amount of analyte that can be detected under the stated experimental conditions is the LOD, while LOQ is the lowest amount of analyte that can be quantitatively determined with precision and accuracy under the stated experimental conditions. Among the publications included in the present review, 35% obtained LOQs below 100 ng/g. Additionally, 16 and 22 out of 67 publications obtained LOQs below 50 and 10 ng/g, respectively. These figures reflect the improvement in signal to noise ratios of current analytical methodologies produced over the last few years. Effectively, LOQs levels were commonly reported as LODs in studies conducted before 2012.

d. The matrix effect is attributable to components of the sample matrix that co-elute with the compound(s) of interest and interfere with the ionization process in the mass spectrometer. This may cause ionization suppression or enhancement and negatively affect method accuracy. It is usually expressed as the percentage of signal suppression, and consequently negative values are interpreted as signal enhancement. In most cases, signal suppressions were measured. In contrast to that observed in the review of 2012 [14], strong effects of signal suppression were described including values from 14 to 100% [140] or higher than 30% [141].

In one study [34], 148 pharmaceuticals and illicit drugs were analyzed in sewage sludge and the matrix effect assessed. For 12 out of the 148 target compounds, a signal enhancement in the range -11 to -90% was reported, and for 136 target compounds, signal suppression was reported in the range 3-92%.

e. The dynamic range is closely related to the response of the instrumental detector, and describes the concentration span, in orders of magnitude, over which the method provides a response proportional to the concentration of a given compound. Accordingly, linearity ranges of 3 orders of magnitude are usually reported for single quadrupole [28] and TOF [100] MS detectors, and of 5 orders of magnitude for triple quadrupole [102] MS detectors.

Tables 1 and 2 summarize the validation values cited in the 67 reports reviewed for the determination of PPCPs in sewage sludge samples from 2012 to the present.

## 5. Impacts of sewage sludge analytical procedures on validation parameters

Each stage in the analytical procedure (extraction, clean-up, instrumental analysis, etc.) may to some extent have an effect on the validation parameters examined.

Extraction and clean-up steps are thought to be the main contributors to absolute recovery [55]. In the literature reviewed, various studies have addressed the determination of PPCPs both in sewage sludge and sewage. In many of those cases, methodology was common for both matrices but an extraction step was added at the beginning of the protocol for the sludge samples. For instance, Křesinová et al. [72] used PLE followed by SPE with ENVI C18-DSK SPE disk and LC-ToFMS for the determination of PPCPs in sludge.

The same methodology was employed when these PPCPs were determined in water samples, but a PLE extraction step did not precede the protocol. This extra step for the solid samples led to lower absolute recoveries for most of the compounds, indicating how extraction influences method accuracy. Accordingly, amitriptyline, 2-chloroprothioxanthen-9-one and melitracene carbinol rendered recoveries of 97.6%, 96.7%, 88.1%, respectively. These percentages decreased to 92.8%, 89.5% and 86.8%, for the same compounds in solid samples [72]. Additionally, López-Serna et al. [28] showed how dramatic the impact of the extraction step can be on the accuracy. These authors employed a fully automated method based on online extraction by DI-SPME followed by on-fiber derivatization coupled to GC-MS for sewage samples. In sewage sludge samples, UAE preceded the sewage methodology. The absolute recoveries reported in this paper for compounds such as ibuprofen, salicylic acid and diclofenac were 77.77%, 21.43%, and 83.07%, respectively, in sewage samples. However, in sludge, these recoveries dropped to 18.18% for ibuprofen, 17.92% for salicylic acid, and 65.89% for diclofenac. Among the different extraction techniques discussed in the present paper, UAE, MAE and PLE seem the most popular. PLE is considered to be much more effective at extracting analytes from solid samples than UAE or MAE, leading to higher real recoveries. However, PLE is also described to extract more components of the matrix along with the analytes of interest. This means the associated matrix effect diminishes the given absolute recovery [70]. Nonetheless, PLE and MAE are usually shown to be slightly more efficient than UAE for extracting PPCPs from sludge as observed by Dorival-García et al. [55]. For instance, Gao et al. [77] tested a method based on PLE-SPE-LC-MS/MS and the absolute recoveries reported for compounds such as sulfamethoxazole, tetracycline and oxytetracycline in sludge samples were 78%, 54%, and 52%, respectively. Similarly, Shafrir et al. [49] used a method based on UAE-SPE-LC-MS/MS and reported absolute recoveries such as 17%, 22%, and 17% for sulfamethoxazole, tetracycline and oxytetracycline, respectively. Gago-Ferrero et al. [34] developed a method that combined UAE and LC-MS/MS, and absolute recoveries reported in this paper for compounds such as propranolol, diclofenac and sulfamethoxazole in sewage sludge samples were 53%, 27%, and 63%, respectively. In contrast, Eyser et al. [73] made use of a method based on PLE followed by LC-MS/MS and reported recoveries of up to 96% for propranolol, 85% for diclofenac, and 33% for sulfamethoxazole in sewage sludge samples.

The presence of the analytes of interest along with matrix components in the sample influences every step of the analysis. GC combined with EI ionization MS operating in SIM mode did not cause any apparent matrix effect during the determination of PPCPs in sewage sludge [50]. In LC, the matrix effect differs when it is interphased with MS by ESI or APCI. Lonappan et al. [31] compared the use of LC-ESI-MS/MS and LDTD-APCI-MS/MS to quantify diclofenac in wastewater sludge samples. These authors reported that matrix effects due to interactions between diclofenac and co-extracted compounds could cause signal suppression in the ESI source. In fact, competition for ionization could exert signal enhancement or suppression phenomena [50,73]. However, they reported that matrix interferences in LDTD-APCI-MS/MS did not significantly affect the signal [31]. Additionally, Luque-Muñoz et al. [54] used UHPLC-MS/MS in their instrumental analysis and reported matrix effect values such as −25% for propylparaben or −37% for benzophenone-6. However, Abril et al. [58] reported matrix effects of −79% for propylparaben and −81% for benzophenone-6 for HPLC-MS/MS. This lesser matrix effect might be attributed to the better resolution capacity of UHPLC. While in conventional HPLC, analytes could co-elute with the matrix compounds, in UHPLC they may reach the detector at different retention times. Sample preparation usually includes a clean-up step that partially removes interferences from the matrix [73]. SPE has been the preferred method among those examined here due to its simplicity and the use of small volume of organic solvents. However, these clean-up procedures might have marked performance deficiencies in multi-residue-methods [73]. Oasis HLB SPE cartridges are based on a co-polymer which is very efficient at recovering a wide range of compounds in environmental matrices. Nonetheless, it is not highly selective and matrix interferences may not be successfully reduced [62]. Petrie et al. [62] observed that Oasis MCX and MAX reduced matrix suppression more satisfactorily. These authors reported matrix suppression values of 59.2% for diclofenac, 88.6% for naproxen and 80.0% for ibuprofen using MCX SPE [62]. Other authors such as Gago- Ferrero et al. [33] reported matrix enhancement values for the same pollutants: −18% for diclofenac, −36% for naproxen and −43% for ibuprofen without the use of any clean-up step. After comparing examples from the literature for sewage samples, we found that Klančar et al. [143] employed Strata X cartridges for SPE combined with LC-MS/MS and reported matrix effect values of 83% for naproxen, 79% for propranolol and 96% for tramadol. These matrix effect values are substantially higher than those observed by Petrie at al [62]. who used Oasis HLB-based SPE followed by LC-MS/MS and reported percentages of around 30%, 57% and 62% respectively for the same compounds.

Precision (expressed as repeatability) is usually affected by the number of stages included in the analytical procedure. A strategy to achieve good precision has been to automatize some of the method stages (e.g., PLE, SMPE) to minimize the human error impact. In the literature, two fully automated methods DI-SPME − on fiber derivatization-GC-MS [28] and HS-SPME-GC-MS [129] have been used to determine PPCPs and PCPs in sewage sludge, respectively. López-Serna et al. [28] reported satisfactory intra-day repeatability (expressed as %RSD) values such as 0.87% for propylparaben, 1.59% for naproxen and 2.99% for triclosan, among others. Vallecillos et al. [129] also reported good intra-day repeatability results such as 1% for exaltone, 8% for muscone, and 9% for exaltolide, among others. However, SPME fibers used for a large number of samples might lead to significant carry over effects. López-Serna et al. [28] reported carry over values of up to 10% and 13% for diclofenac and triclosan, respectively.

Sensitivity and signal to noise ratios are mainly affected by the instrumental analysis technique employed [28]. In the revised literature, different groups have examined the use of similar methods with different detectors such as FL [61], Q-MS [13], QqQ-MS [62], or QToF-MS [72] for the determination of PPCPs in sludge samples. For instance, Morales-Toledo et al. [61] developed a method based on MAE and SPE combined with UHPLC-FLD for the determination of pharmaceuticals in sludge samples, and reported method LODs for naproxen and ibuprofen below 86.5 ng/g. Much lower LODs were observed by Petrie et al. [62] for a similar method based on MAE and SPE followed by UHPLC-MS/MS. In particular, they reported method LODs of 0.07 ng/g for ibuprofen and 0.60 ng/g for naproxen. Among the analyzers used in mass spectrometry, QqQ has usually provided lower LODs than QToF. Hence, Peysson et al. [100] made use of a method based on QuEChERs followed by UPLC-QToF and reported LODs as low as 17 ng/g for sulfamethoxazole and 3 ng/g for propranolol, among others. Even lower limits of 0.6 ng/g and 0.3 ng/g respectively for the same compounds were reported by Cerqueira et al. [101] for a similar pretreatment method followed by UHPLC-QqQ-MS. The use of GC usually leads to higher LODs in comparison to LC, even when the detection method is MS. This is usually attributed to incomplete derivatization of the non-volatile PPCPs and/or a poorer ionization rate of the resulting substance. UHPLC provides narrower chromatographic peaks than

conventional HPLC and improve signal to noise ratios. Accordingly, the same area will offer a greater height, which entails an increase in signal intensity, and so sensitivity. For instance, Gago-Ferrero et al. [34] achieved LOQs of 4.1 ng/g for diclofenac and 9.8 ng/g for salicylic acid by applying a method based on UAE and UHPLC-MS/MS. In contrast, Boix et al. [38] reported poorer limits (eg., 63 ng/g for diclofenac and 35 ng/g for salicylic acid) using a similar method but with HPLC as the chromatographic stage.

Selectivity and throughput (multiresiduality) are usually improved following the same pattern as sensitivity. Thus, the probability of providing false negatives or positives is decreased when a MS detector is used, especially if in a tandem configuration (QqQ or QToF). Gago-Ferrero et al. [34] used LC-MS/MS as the instrumental analysis technique for the simultaneous determination of 148 pharmaceuticals and illicit drugs in sewage sludge. Similarly, Peysson et al. [100] used LC-ToF/MS to determine 136 pharmaceuticals and hormones in sewage sludge. In contrast, Morales-Toledo et al. [61] only determined four substances (acetylsalicylic acid, ibuprofen, naproxen and gemfibrozil) in sludge samples by LC-FLD.

Differences in linearity range have been reported depending on the instrumental detector. Hence, for instance methods including QqQ usually attain 5 orders of magnitude [102]. However, up to 3 orders are reported for QToF-based methods [100].

Regardless of these factors, through the use of quantification approaches such as internal standard with isotope dilution, standard addition or matrix-matched techniques most technical deficiencies during extraction, clean-up, instrumental analysis, etc. may be circumvented, compensated and corrected. This means that a partial, non-optimal method developed for the pretreatment and instrumental stages might still be sufficient to achieve a methodology capable of fulfilling analytical requirements, provided sensitivity is appropriate and the quantification approach is powerful.

## 6. Conclusions

The studies reviewed here examining the determination of PPCPs in sewage sludge consider a wide variety of emerging pollutants in environmental matrices. The most frequently investigated PPCPs belong to the class of pharmaceutical products. In effect, 49 out of the 67 reports reviewed focused on the detection and quantification of pharmaceuticals in sewage sludge.

In some studies, traditional sample pretreatment techniques such as Soxhlet were replaced with more modern techniques such as MAE or PLE, or alternative techniques like QuEChERS or MSPD. However, UAE emerged as the most popular extraction technique for determining PPCPs in sewage sludge reported in almost half of the publications. This method provides safe, fast and easy sample preparation. It also makes use of small sample sizes and amounts of solvents. Usually after the extraction step, a clean-up protocol is needed as extraction is never completely selective. For this purpose, SPE was the technique most frequently used on pollutants after their extraction from environmental samples. For the determination of PPCPs in sewage sludge, LC and GC coupled to MS were the techniques of choice. Among the LC procedures, several studies chose UHPLC over HPLC because of its better resolution and shorter run times as well as its lesser demands in terms of solvent and sample quantities.

In recent years, novel solid and liquid phase materials and miniaturization and automation of the analytical techniques are becoming a dominant trend as they eliminate the limitations of current analysis technologies. Minimizing sample size decreases the consumption of expensive and toxic reagents and solvents, thus fulfilling the principles of green chemistry.

Most reported studies employed a target analysis to determine PPCPs in sewage sludge samples. Only one of the studies reviewed applied a non-target quantification method. Thus, a challenge to be addressed in the near future might be the individual treatment of each sludge-associated matrix. A boost in non-targeted approaches is expected for the determination of PPCPs in sewage sludge, as occurred for their analysis in aqueous matrices.

Finally, this review reports improved validation parameters in comparison with previously reviewed periods, especially regarding precision and LODs. This is mostly attributed to developments in analytical instrumentation.

## Conflict of interest

The authors declare no conflicts of interest.

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# Sample pre-treatment and analytical methodology for the simultaneous determination of pharmaceuticals and personal care products in sewage sludge

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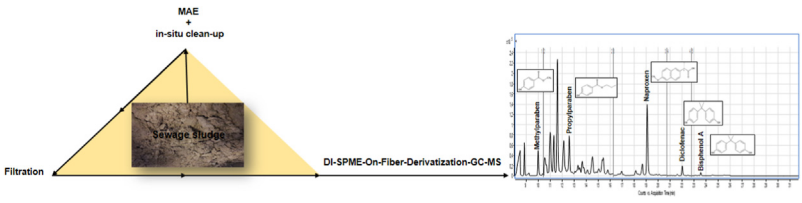
## HIGHLIGHTS

- A method for the determination of PPCPs in sewage sludge was optimized and validated.
- Microwave-assisted extraction combined with in-situ clean-up and filtration were presented.
- Online-DI-SPME-On-Fiber-Derivatization-GC-MS was used for analysis of PPCPs in sludge samples.
- The analytical method was successfully applied to different real samples.
- PPCPs were detected in concentration between 48 and 9355 ng $g^{-1}$.

## GRAPHICAL ABSTRACT

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## ABSTRACT

This work describes the design, optimization and validation of an analytical method for the simultaneous determination of 14 pharmaceuticals and personal care products (PPCPs) in sewage sludge. A thorough optimization of the sample pre-treatment was carried out. As a result, microwave-assisted extraction (MAE) was combined with an in-situ clean-up stage and a filtration step. A combination of MilliQ® water/MeOH 95:5 (v/v) adjusted to pH 9 turned out to be the optimal solvent mixture for extraction. The instrumental part of the method presents a significant novelty based on a fully automated sample preparation for the analysis of PPCPs. It consisted of a direct immersion solid phase microextraction followed by on-fiber derivatization, online coupled to gas chromatography - mass spectrometry (DI-SPME-On-fiber derivatization - GC-MS). An isotope dilution approach was used for quantifying, which conferred high reliability to the method. This methodology was validated for 10 compounds with good analytical performance, limit of detection below 20 ng $g^{-1}$ and absolute recovery in the range of 30–70% for most of the compounds. It supposes an ecological analytical alternative for many routine analysis laboratories around the world. The developed method was applied to different real samples generated in

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both a pilot-scale thermal hydrolysis treatment plant and an anaerobic digester operated in mesophilic conditions. Salicylic acid and naproxen were found at concentrations above 1000 ng $g^{-1}$.


## 1. Introduction

Chemical pollution is one of the most important problems that impact on our planet. It is a cyclical process that affects all types of environment (air, water and soil) as well as living beings, both emitters and receivers of pollutants (Bolong et al., 2009).

The World Health Organization, the Environmental Protection Agency or the European Commission are the main bodies dedicated to the protection of public and environmental health. Within their priority lines of research, the study of the so-called "emerging pollutants" (EPs), compounds of various origin and chemical nature, can be outstanding. Their presence and consequences on the environment have gone unnoticed until recently (Kallenborn et al., 2018). In accordance with the Directive 2013/39/EU, EPs are those that are not included in the systematic monitoring programmes of the European Union at present. However, they present a significant risk because of a continuous exposure can cause potentially adverse effects because of the bioaccumulation such as endocrine disruption or chronic toxicity even though their concentrations in the environment are relatively low, range from ng $L^{-1}$ to μg $L^{-1}$ (European Commission, 2013).

A wide variety medicines, cosmetics, fragrances, clean-up products and synthetic or natural hormones are considered as EPs. These pharmaceutical and personal care products constitute a heterogeneous group with large differences in structure, function and properties (Daughton and Ternes, 1999). Pharmaceuticals have attracted the most interest and have been the subject of the most in-depth studies, particularly in the 1990s.

One of the essential problems is that Wastewater Treatment Plants (WWTPs) are not capable of removing many EPs since they were designed to eliminate organic matter and nutrients in concentrations higher than mg $L^{-1}$ (Joss et al., 2008). Therefore, these contaminants are present in surface water and groundwater as well as in drinking water. In addition, the primary degradation of some of them in WWTPs or environment can produce even more persistent and dangerous products (Giger et al., 1984). Thus, soils that are fertilised with sewage sludge might end up accumulating PPCPs and the underlying aquifers become contaminated.

In recent years, a large number of methodologies have been developed for the determination of EPs in solid matrices as sewage sludge. Traditional sample preparation is being replaced by miniaturized and automated techniques. In addition, some sample preparation methodologies can be directly incorporated into liquid chromatography (LC) or gas chromatography (GC) (Pérez-Lemus et al., 2019). In the 1990s, Pawliszyn and colleagues developed a miniaturized solid phase extraction technique known as Solid-Phase Microextraction (SPME) (Arthur and Pawliszyn, 1990). This sample preparation technique is fast, simple, effective and can be coupled to GC or LC. The static procedure "fiber SPME" is the most common format and presents great popularity due to advantages such as simplicity of operation, solvent-free nature, moderately short extraction time, complete automation and simple coupling with chromatography (Li et al., 2015). However, the analysis of polar compounds in environmental samples has not been so much explored with it, particularly when the pre-treatment of the sample is followed by GC. This is probably due to the fact that a derivatization step is necessary for the analysis of non-volatile and/or thermolabile compounds.

This study aimed to contribute to the detection and quantification of 10 PPCPs in sewage sludge samples thanks to development and optimization of an analytical methodology with a fully automated analysis method based on online DI-SPME-On-fiber derivatization-GC-MS. To the best of the authors' knowledge, only another scientific paper has been found suggesting the use of this technique for the analysis of PPCPs in sludge samples (López-Serna et al., 2018). Thanks to automatized sludge extraction, matrix in-situ clean-up and isotope dilution quantification approach, the resulting methodology here presented stands out for its robustness, short time consumption and environmental and analyst safety.

## 2. Experimental procedures

### 2.1. Standards and reagents

The standards for all PPCPs (Table S1 as Supplementary material data) were of high purity grade (>95%, Sigma-Aldrich, Madrid, Spain) as neutral non-solvated molecules, except for diclofenac (sodium salt).

Ten internal standards, such as the isotopically labelled rac-ibuprofen-d3, rac-naproxen-d3, propyl-d7-paraben, salicylic acid-d4, triclosan-d3, diclofenac-d4, methylparaben-d4, ethylparaben-d5, clofibric acid-d4 and bisphenol A-d8 (LGC Standards, Barcelona, Spain) (Table S1), were used.

Individual stock solutions at 1000 mg $L^{-1}$ for both PPCPs standards and isotopically labelled internal standards were prepared in methanol (MeOH). From them, a stock solution with all the analytes was prepared in MeOH at 20 mg $L^{-1}$. Fresh serial dilutions (2, 0.5, 0.05, 0.005) mg $L^{-1}$ in acetone were subsequently prepared from it when need them. A mixture of isotopically labelled internal standards in MeOH and their corresponding serial dilutions in acetone (2, 0.5, 0.05, 0.005) mg $L^{-1}$ were also prepared. All solutions were stored at −20 °C in darkness.

High purity solvents, i.e., LC-MS Chromasolv® Ethyl Acetate (EA) grade from Fluka (Madrid, Spain), SupraSolv® GC-MS MeOH grade by Merck Millipore (Madrid, Spain), Sodium Chloride (NaCl) and Hidrochloric acid (HCl) with 37% purity were supplied by Panreac (Barcelona, Spain). Aluminium oxide by Sigma-Aldrich (Tres Cantos, Madrid, Spain). Acetone ($C_3H_6O$), with 99% purity, was supplied by Cofarcas (Burgos, Spain). N-terc-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with a purity greater than 99% was obtained from Sigma-Aldrich (Tres Cantos, Madrid, Spain). The Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fibres were acquired from Supelco (Tres Cantos, Madrid, Spain). All aqueous solutions were prepared in deionized water with a resistivity not less than 18 MΩ cm. Helium (He) with 99.999% purity was acquired from Abelló Linde S.A. (Alcalá de Henares, Madrid, Spain).

### 2.2. Sewage sludge analytical methodology

Sewage sludge samples were collected from a WWTP in Valladolid (Spain). This WWTP serves a population of 344,600 inhabitants. The wastewater treatment consists of a primary purification step (primary sludge) followed by a biological

treatment consisting of a conventional active sludge process (secondary sludge). The mixture of the generated sludge is treated in a thickening step that reduces the volume of the sludge by concentrating or partially eliminated water. The WWTP of Valladolid treats approximately 101,000 $m^3$ $d^{-1}$ of wastewater. It generates around 9600 $m^3$ $d^{-1}$ of biogas by digesting 2500 $m^3$ $d^{-1}$ of sludge. The resulting thickened mixed sewage sludge is used as fertilizer in substitution of chemical alternatives, as recommended the European Commission (European Commission, 2001).

The proposed method for sludge analysis consisted of the following stages:

1. Sampling. Grab samples of thickened mixed sludge were randomly collected and combined to provide a final sample of approximately 25 kg. The samples were collected in high density polyethylene (HDPE) drums with polypropylene screw caps. Then, they were properly sealed and taken to the laboratory under conditions of refrigeration and darkness.
2. Centrifugation. Immediately after arrival to the lab, 200 mL of the homogenized sewage sludge were centrifuged at 10,000 rpm for 10 min in a Thermo Sorvall Legend RT + Refrigerated Benchtop Centrifuge (Madrid, Spain). The solid phase was then collected and stored in the dark at −20 °C.
3. Freeze-Drying. After two days of congelation, an amount around ~25–30 g of solid phase was freeze-dried and stored at −20 °C in darkness until analysis.
4. Spiking. An exact amount of freeze-dried sewage sludge (~0.8 g) was placed in a vessel and spiked with 200 μL of a solution at 2 mg $L^{-1}$ in acetone containing a mixture of all isotopically labelled internal standards and homogenized. Then, it was kept in contact overnight in the extraction hood to allow solvent evaporation and internal standard fixation. Sample size was chosen by recommendations found in the literature for similar matrixes (Pérez-Lemus et al., 2019).
5. Pre-treatment for desorption of the analytes to aqueous phase. The sample underwent, then, MAE in a Milestone START-D Microwave Digestion System (Madrid, Spain) at 110 °C during 30 min to facilitate the desorption of the analytes. Twenty-four millilitres of a MilliQ® water/MeOH water mixture, 95:5 (v/v) at pH 9 were used as extracting solvent. At this pH, all the target compounds were supposed to be as negative ions (Table S1), increasing their affinity for the liquid phase. Subsequently, 100.0 mg of activated alumina ($Al_2O_3$ at 100 °C for 48 h) were added for matrix in-situ clean-up.
6. After MAE centrifugation. The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was collected (20–22 mL) with a glass pipette and transferred to a 25-mL glass beaker. The total of supernatant was saturated with ~7.5 g NaCl (solubility in water at 25 °C is 359 g $L^{-1}$) at 36% (weight/volume) to increase the ionic strength. The resulting sample was also pH-adjusted to 3 with HCl, in order to increase the analyte lipophilia by shifting their acid-base equilibrium into neutral molecules. Finally, the extract was filtered through a 0.7-μm glass fiber (GF) syringe filter and 17.0 mL of the filtrate was collected in a 20.0 mL SPME glass vial.
7. Instrumental analysis. It consisted of automatized DI-SPME, followed by on-fiber derivatization, coupled to GC (Agilent 7890B) and detected by MS (Agilent 5977A). This method was based on another one published elsewhere (López-Serna et al., 2018). However, important upgrades were implemented. Hence, 90 min sample extraction at a penetration depth of 60 mm, 45 min derivatization step at a penetration depth of 45 mm, orbital agitation at 350 rpm with a stirring regime of 6 s on/20 s off were implemented to increase SPME fiber life time (Table S2). In fact, these adjustments extended average fiber lifespan beyond 80 and up to 130 injections with no signs of performance deterioration, which entails a 62% lifespan increase. A DVB/CAR/PDMS SPME fiber was utilized for the analysis. Chromatographic separation was achieved on a capillary HP-5MS GC column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) with He as carrier gas at a constant flow rate of 1.2 mL $min^{-1}$. Injector temperature was set at 250 °C, while the GC oven temperature increased from 70 °C (held for 3 min during fiber desorption) to 150 °C at 50 °C $min^{-1}$, to 220 °C at 5 °C $min^{-1}$ and finally to 300 °C (held for 5 min) at 10 °C $min^{-1}$. The total analysis time for each injection was 31.6 min. Mass detection was obtained in electron impact ionization mode (70 eV) with selected ion monitoring (SIM) and a filament delay of 8 min. The GC–MS interface, ion source and quadrupole temperatures were set at 280, 230 and 150 °C, respectively. Target compounds were recorded in six acquisition windows along the run time. Acquisition stopped at 26 min. Data acquisition and evaluation were performed by Agilent Technology Mass Hunter B.07.03.2129 software. Table 1 shows the primary ions (in black) and two secondary ions monitored for each compound.

### 2.3. Optimization of analytical method parameters for sewage sludge

Fourteen PPCPs belonging to diverse categories (i.e., pharmaceuticals, endocrine disruptors, preservatives and fungicides) were initially selected as target analytes. The selection criteria were based on their high use in daily life, ubiquity in aquatic environments and/or recognized toxicity. The most significant physical-chemical properties are reported in Table S1.

Thickened mixed sludge aliquots, spiked at 1500 ng $g^{-1}$ in triplicate, were used in a one-factor-at-a-time approach method optimization.

#### 2.3.1. Ultrasonic extraction method

An exactly known amount (~0.8 g) of freeze-dried sludge was weighed into a polypropylene centrifuge tube (50 mL). Then, considering the final volume needed for the instrumental analysis, 12.0 mL of an extraction solution was added to the tube. After reviewing related literature (Pérez-Lemus et al., 2019), MilliQ® water at pH 9 and MilliQ® water/MeOH, 95:5 (v/v) at pH 9 were considered as tentative extraction solvents. Subsequently, an in-situ clean-up stage was performed by adding 100.0 mg of activated $Al_2O_3$ at 100 °C during 48 h. The centrifuge tube was then vortex-stirred for 1 min and the extraction was carried out for 30 min at room temperature in a JP Selecta Univeba ultrasound bath of 50 W and 60 Hz (Barcelona, Spain). The extract was centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a 25-mL glass beaker. Subsequently, 12.0 mL of the extraction solvent was added again and a new extraction cycle was carried out. The total volume of supernatant collected was measured (20–22 mL) and a saturation with 36% NaCl (weight/volume) was performed. Variations to the described 12 + 12 mL volume combinations for the extraction solvent were not tested as they were not expected to significantly influence the extraction performance. Then, the pH was measured by a Crison pH-Meter Basic 20 and adjusted to 3 by adding a few drops of diluted solutions of HCl (10%, 1% and/or 0.1%) as needed. The total supernatant volume was filtered through a 0.7-μm GF syringe filter and 17.0 mL was collected in a 20.0 mL SPME glass vial. The resulting solution was analysed by online DI-SPME - on-fiber derivatization – GC-MS.

**Table 1**
MS parameters for the final target compounds and internal standards.

| Analyte | [a]IS | Chemical name | Adquisition window | [b]$t_R$ (min) | [c]SIM ions, *m*/*z* | | |
|---|---|---|---|---|---|---|---|
| 1 | | Methylparaben | 1 | 9.531 | **209.1** | 210.1 | 135.1 |
| | 1 | Methylparaben-d4 | | 9.524 | **213.2** | 214.2 | 139.1 |
| 2 | | Clofibric acid | 2 | 10.449 | **143.1** | 271.1 | 185.1 |
| | 2 | Clofibric acid-d4 | | 10.427 | **143.1** | 275.1 | 75.1 |
| 3 | | Ethylparaben | | 10.536 | **223.1** | 224.1 | 151.1 |
| | 3 | Ethylparaben-d5 | | 10.463 | **228.2** | 229.2 | 230.2 |
| 4 | | Ibuprofen | | 11.059 | **263.2** | 264.2 | 117.1 |
| | 4 | rac Ibuprofen-d3 | | 11.074 | **266.2** | 267.2 | 164.2 |
| 5 | | Propylparaben | 3 | 12.062 | **237.2** | 238.2 | 151.1 |
| | 5 | Propylparaben-d7 | | 11.989 | **244.2** | 245.2 | 152.1 |
| 6 | | Salicylic acid | | 12.760 | **309.2** | 310.2 | 195.1 |
| | 6 | Salicylic acid-d4 | | 12.751 | **313.2** | 314.2 | 312.2 |
| 7 | | Naproxen | 4 | 18.508 | **287.2** | 305.1 | 288.2 |
| | 7 | rac Naproxen-d3 | | 18.459 | **290.2** | 188.1 | 207.1 |
| 8 | | Triclosan | | 19.311 | **347.0** | 345.0 | 200.0 |
| | 8 | Triclosan-d3 | | 19.309 | **350.0** | 348.0 | 200.0 |
| 9 | | Diclofenac | 5 | 21.571 | **352.1** | 214.1 | 354.1 |
| | 9 | Diclofenac-d4 | | 21.528 | **356.1** | 218.1 | 158.1 |
| 10 | | Bisphenol A | 6 | 23.096 | **441.0** | 207.0 | 442.0 |
| | 10 | Bisphenol A-d8 | | 23.036 | **449.4** | 211.2 | 450.4 |

[a] IS: internal standard.
[b] $t_R$: retention time.
[c] SIM: selected ion monitoring.

*2.3.2. Microwave extraction method*

An exactly known amount (~0.8 g) of freeze-dried sludge was weighed into a microwave equipment vessel. Then, considering the final volume needed for the instrumental analysis, 24.0 mL of the extraction solution (MilliQ® water at pH 9 or MilliQ® water/MeOH, 95:5 (v/v) at pH 9) were added. Consequently, an in-situ clean-up stage was performed by adding 100.0 mg of activated $Al_2O_3$ at 100 °C during 48 h. Up to 12 samples were able to be prepared simultaneously. The vessel was then vortex-stirred for 1 min and the extraction was carried out in a computer-controlled microwave heater with fibre optic temperature registration (Milestone START-D Microwave Digestion System). The extraction process, which was carried out at 110 °C and 500 W, lasted 60 min in total (10 min until reaching a temperature of 110 °C, 30 min of extraction and 20 min of cooling). After microwave irradiation, the vessels were cooled off by an air current (<45 °C). The resulting extract was, then, centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a 25-mL glass beaker. The total volume of supernatant collected was measured (20–22 mL). Then, a saturation with 36% NaCl (weight/volume) was performed and the pH was adjusted to 3 by adding a few drops of diluted solutions of HCl (10%, 1% and/or 0.1%) as needed. The total supernatant volume was filtered through a 0.7-μm GF syringe filter and 17.0 mL was collected in a 20.0 mL SPME glass vial. The resulting solution was analysed by online DI-SPME - on-fiber derivatization − GC-MS.

## 3. Results and discussion

Several operational parameters were evaluated and optimized to achieve the best analytical conditions for all PPCPs. For this purpose, 0.8-g freeze-dried samples were spiked with 600 μL of a freshly made solution containing all the analytes at 2 mg $L^{-1}$ in acetone, i.e., at a spiking concentration of 1500 ng $g^{-1}$.

Some of the target parameters, i.e., extraction solvent, extraction technique, type and amount of adsorbent and filtration method, among others were discontinuous. Therefore, some precautions were taken into consideration during the experimental design.

Optimized sensitivity was the proposed goal for the method development. Thus, total signal-to-noise (TS/N), i.e., the sum of the individual S/N ratio for each target compound, was selected as the response variable during the statistical study in order to get a compromise among the performance of all the compounds.

The distribution of the optimizing parameters along the method phases is shown in Fig. 1., and were as follows (1) solvent extraction, (2) extraction technique and number of cycles of the extraction technique, (3) type of adsorbent and amount used in the clean-up stage, (4) most suitable filtration method. The influence of each parameter was evaluated in triplicate. Total method sensitivity, based on TS/N for all target analytes, was the criterion selected as mentioned to achieve an optimum multicomponent method.

### 3.1. Extraction solvent

Two solvents such as MilliQ® water at pH 9 and MilliQ® water/MeOH, 95:5 (v/v) at pH 9 were tested. These solvents were examined for their ability for the extraction of PPCPs in sewage sludge samples. The TS/N results showed that MilliQ® water/MeOH, 95:5 (v/v) at pH 9 reported a 4% improvement in TS/N compared to MilliQ® water at pH 9. Therefore, MilliQ® water/MeOH, 95:5 (v/v) at pH 9 was selected as the extraction solvent.

### 3.2. Extraction technique

The performance of ultrasound-assisted extraction (UAE) and MAE were compared. MAE offers benefits such as automation and shorter extraction in comparison with UAE (Pérez-Lemus et al., 2019).

An 18% improvement in TS/N was achieved by applying the MAE technique over UAE. In addition, effectiveness of a solid-liquid extraction for a given extraction volume, usually improves by dividing it into several extraction cycles. On the other hand, the experimental error may increase. Hence, the TS/N was evaluated after 1 vs 2 MAE cycles were carried out. Extraction solvent volume varied from 12.0 mL per cycle to 24.0 mL in 2 and 1 MAE cycle performances, respectively. The results reported that a 1 MAE cycle rendered a 31% improvement in TS/N compared to 2 MAE cycles. Therefore, a decrease in the analysis time and number of cycles was justified.

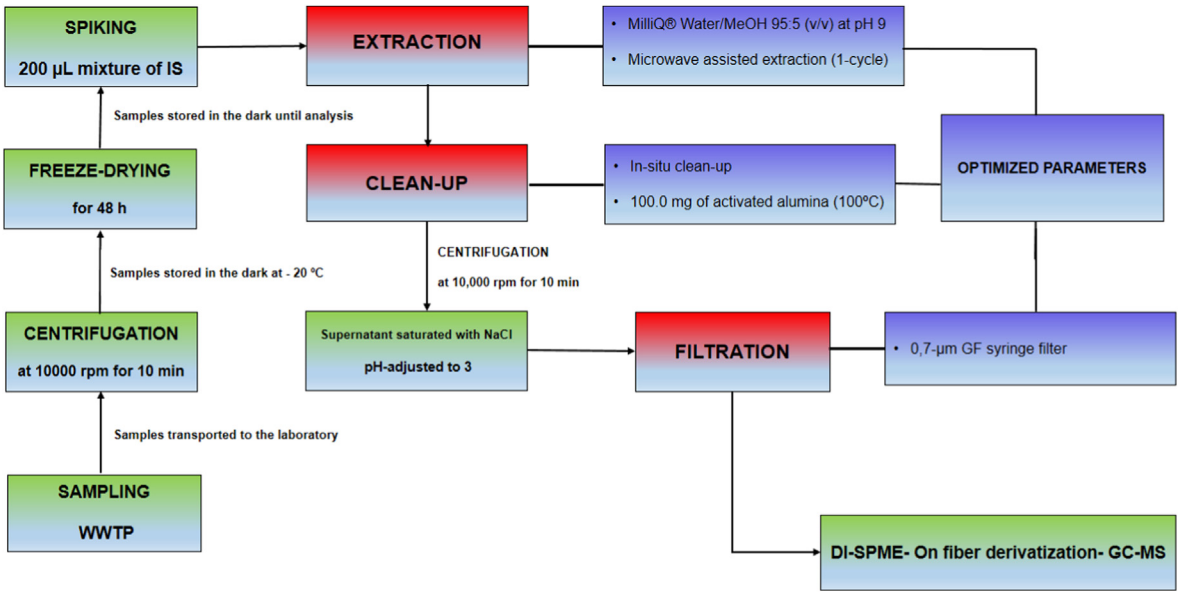


**Fig. 1.** Distribution of the optimizing factors within the pre-treatment stages during the PPCPs analysis.

### 3.3. Clean-up stage

The extraction stage included an extract clean-up to increase extraction efficiency, sensitivity and minimization or elimination of interferences that may affect the determination of compounds of interest (Pérez-Lemus et al., 2019).

Some preliminary tests focused on the assessment of the clean-up type. More specifically, in-situ and non in-situ clean-up were compared. The results showed that in-situ clean-up provided with a 21% improvement in TS/N over non in-situ clean-up. Therefore, the choice of an in-situ clean-up was justified.

In order to select the clean-up agent, 100.0 mg of different adsorbents such as activated $Al_2O_3$ (at 100 °C for 48 h), activated silica gel ($SiO_2$ at 100 °C for 48 h) and 5.0 mL of hexane were individually tested, by adding them along the sample and the extraction solvent. Afterwards, the extraction was carried out as discussed in the previous section 2.3. Activated $Al_2O_3$ obtained a 32% and 46% improvement in TS/N compared to activated $SiO_2$ and hexane, respectively. Therefore, activated $Al_2O_3$ was chosen as the best clean-up adsorbent.

Once selected the clean-up agent, three different amounts (100.0, 500.0 and 1000.0) mg of activated $Al_2O_3$ were tested for the in-situ clean-up task. The best results were observed for 100.0 mg activated $Al_2O_3$. In fact, a 24% and 22% improvement were reported in TS/N compared to 500.0 mg and 1000.0 mg, respectively.

### 3.4. Filtration

Two extract filtration modes were assessed. When possible along the whole analytical method, glass material was selected over any kind of plastic in containers and utensils. This preference was extended to the filtration steps too. Hence, 0.7-µm GF syringe filtration (2.5 cm diameter) was compared to 0.7-µm GF membrane vacuum filtration. The TS/N results showed that syringe filtration obtained a 14% improvement in TS/N compared to the vacuum filtration, most probably due to the elimination of sample transferences. Syringe filtration is a faster and more suitable approach for small volumes.

The results obtained for the TS/N during the optimization are collected and depicted in Fig. 2.

After the optimization, four of the initial PPCPs of interest (propranolol, 4-tert-octhylphenol, 4-nonylphenol and carbamazepine) proved to be inadequate for their analysis by online DI-SPME – on-Fiber Derivatization – GC-MS as they presented a very low TS/N ratio (<5.00) even at the optimized method conditions. Therefore, they were ruled out and the final method included 10 PPCPs and is described in section 2.2.

Fig. 3 and Fig. 4 show representative SIM chromatograms obtained from hydrolysed and anaerobically digested thickened mixed sludge, respectively.

## 4. Validation of the developed method and applications

### 4.1. Method validation

Several regulatory bodies have published guidelines for method validation. Methodologies for the analysis of PPCPs in sewage sludge have not followed a homogenous criterion. Hence, Dorival-García et al. (2015) and Luque-Muñoz et al. (2017) followed the American Food and Drug Administration (U.S. Department of Health and Human Services, 2001) and Azzouz and Ballesteros (2012) and Peysson and Vulliet (2013) selected the International Association of Official Analytical Chemists (AOAC, 2002).

Authors such as Cristale and Lacorte (2013) and Evans et al. (2015) as well as this present study used as a reference for the method development and validation a directive executed by the European Union (Commission Decision, 2002), concerning products of animal origin due to the absence of specific guidelines.

The following validation parameters were determined for the 10 PPCPs that showed sufficient sensitivity as explained in the previous section (methylparaben, ethylparaben, clofibric acid, ibuprofen, propylparaben, salicylic acid, naproxen, triclosan, diclofenac and bisphenol A) in thickened mixed sludge. Each test was performed in triplicate ($n = 3$) and spiked at two significant concentration levels of 1000 ng $g^{-1}$ and 1500 ng $g^{-1}$ with the optimized method and average results are shown in Tables S3A and S3B.

1. Accuracy: In our specific case, it was expressed as absolute recoveries (%). They were calculated by comparing the peak areas obtained from spiked samples employing the optimized method

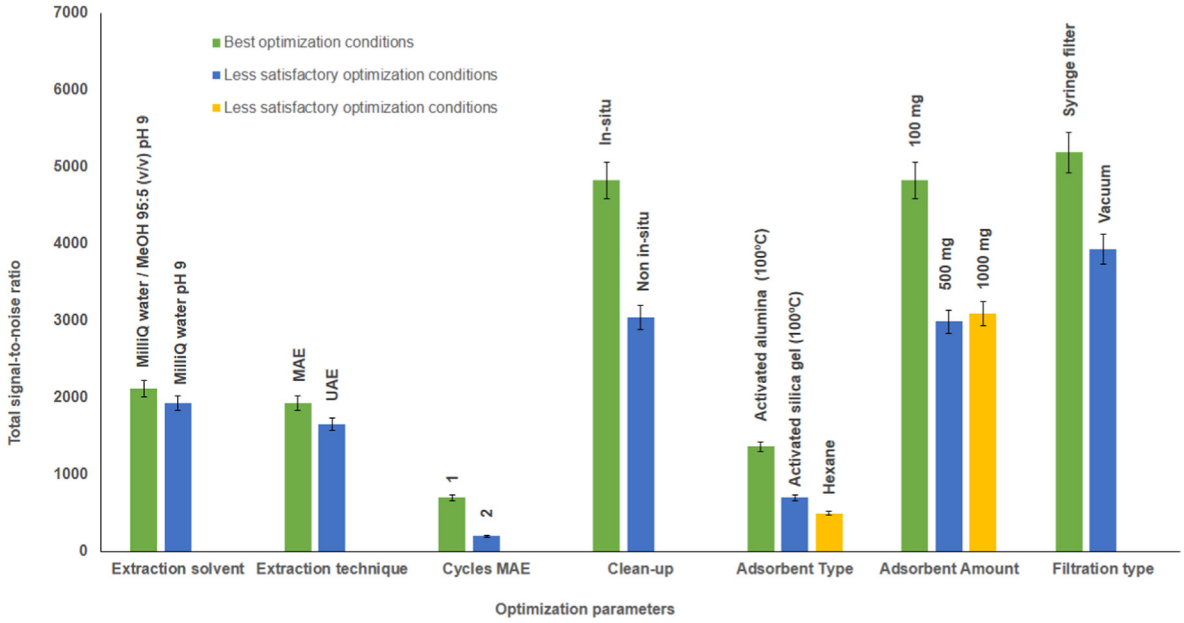


**Fig. 2.** Optimization of the sample pre-treatment parameters.

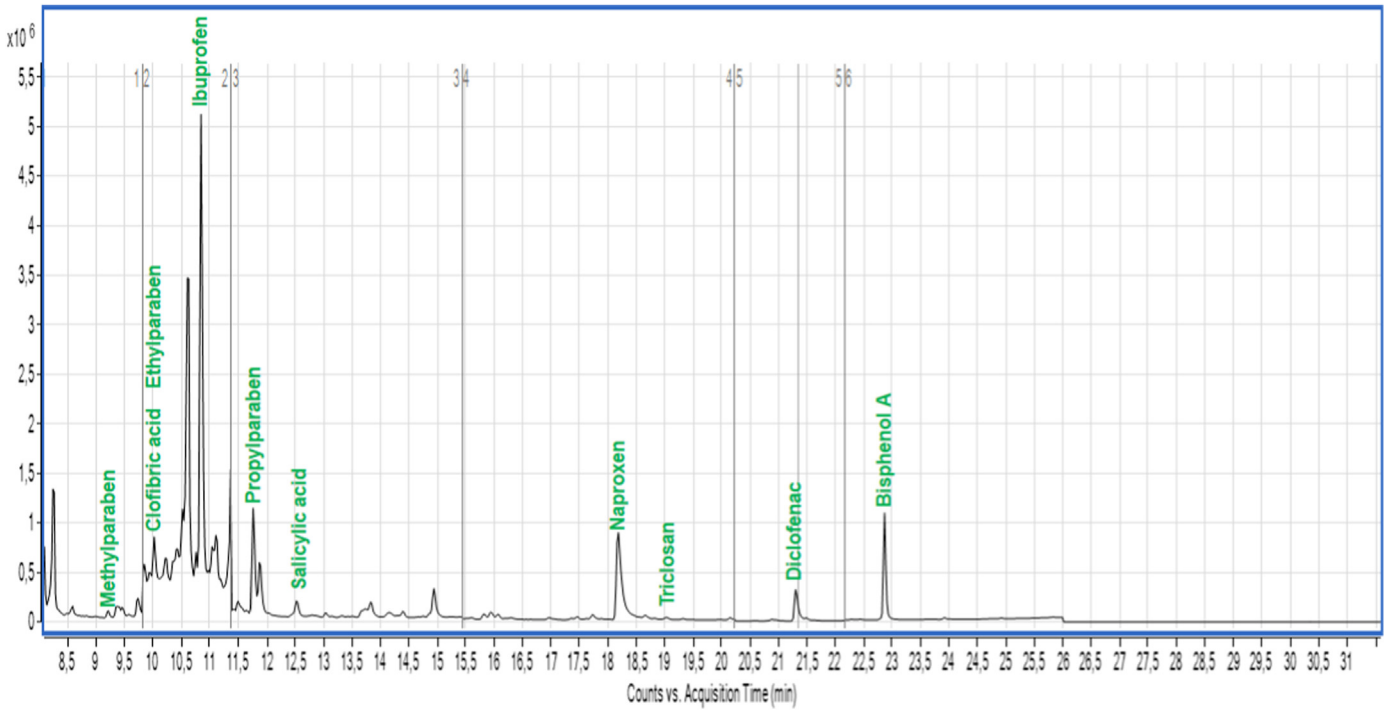


**Fig. 3.** Chromatogram from a hydrolysed thickened-mixed sludge sample after applying the optimized method.

with the peak areas of direct injections (2 μL) of equivalent amounts of the standards in EA solutions. Quantification method was based on an isotope dilution (10 isotopically labelled analogues) calibration curve. It was prepared with MilliQ® water samples saturated in NaCl at pH 3 adjusted and filtered through 0.7-μm. These samples were spiked at different levels of concentration and 10 internal standards (isotopic analogues to 10 of the target analytes) were also added. Observed absolute recoveries were below 70% for all target compounds (Table S3A) in sewage sludge. These absolute recoveries were very similar to those reported in other studies for the analysis of sewage sludge samples (Yu and Wu, 2012; Petrie et al., 2016). Nonetheless, these deficiencies were properly corrected by the isotopic dilution quantification approach. In fact, relative recoveries for all target compounds, which were calculated as the ratio between the absolute recoveries for each compound and the recoveries of their corresponding internal standard, were obtained in the range 86–108% (Table S3A).

2. Matrix effect: It refers to the impact the components of the sample matrix may exert on the analysis of the analytes of

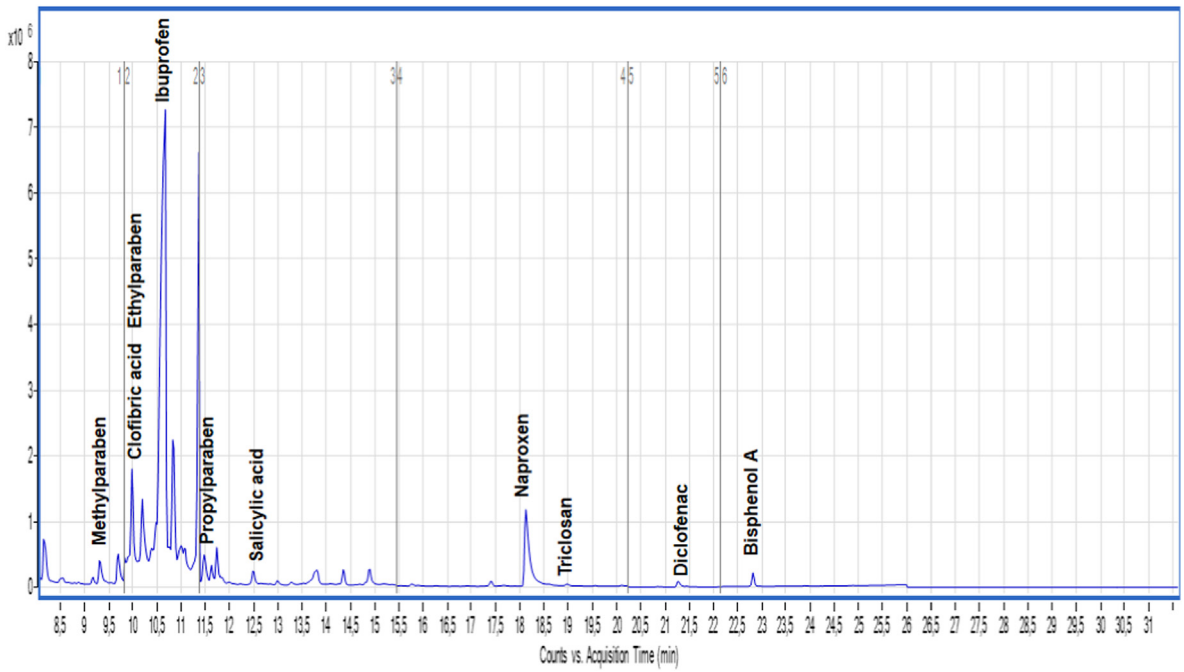


**Fig. 4.** Chromatogram from an anaerobically-digested thickened-mixed sludge sample after applying the optimized method.

interest. More specifically, it is mainly due to the fact that co-eluting matrix elements may hamper the ionization process of the analytes in the mass spectrometer (Pérez-Lemus et al., 2019). Matrix effect is typically expressed as the percentage of signal suppression. In our particular case, to determine the matrix effect associated to sewage sludge samples, empty glass vials were similarly spiked as the validation samples and underwent the same optimized methodology. The matrix effect corresponds to the differences between the areas obtained in the samples with and without matrix. The results reported in the sewage sludge (Table S3A) were close to 100% in signal suppression for many of the compounds like in other reported studies (Jelić et al., 2009). However, these deficiencies were included within the accuracy of the method discussed above and corrected by the use of isotope dilution quantification. That showed that the clean-up and automation here proposed not only reduced drastically the analysis time, analyst exposure and disposable material consumption, but also maintained the efficiency of the conventional methods in terms of matrix effect.

3. Precision: It refers to method repeatability and was expressed as the relative standard deviation (%RSD) of the area observed for analogous samples prepared in triplicate with the optimized method. The analyses were performed in the same day (intra-day) as well as in different days (inter-day). The overall method repeatability was acceptable for the sludge samples. The %RSD values were lower than 10% for most of the compounds when the analyses were performed in the same day (intra-day precision). In addition, the %RSD values in different days (inter-day precision) were lower than 21% for most the compounds (Table S3A). These results reported a precision similar to previous methodologies for sludge samples (Yu and Wu, 2012; Gago-Ferrero et al., 2015; Petrie et al., 2016).
4. Method limits of detection (MLDs) and quantification (MLQs) were experimentally calculated as the concentration providing a total-signal-to-noise ratio of 3 and 10, respectively, for each target analyte in each matrix. MLDs were lower than 20 ng $g^{-1}$ and MLQs lower than 65 ng $g^{-1}$ for most of the target compounds in sludge samples (Table S3A). They were considered acceptable for trace analysis of target compounds in this type of matrix. In addition, these values were similar to, or even lower than, values reported in analogous multicomponent methods based on GC-MS (Petrie et al., 2016; López-Serna et al., 2018) and even LC-MS/MS (Boix et al., 2016a). On the other hand, an alternative method for calculating MLDs and MQLs based on USEPA guidelines, described in Glaser et al. (1981) for wastewaters, was also used, and the results are shown in Table S3A. MDLs and MQLs from both methods tuned out being very similar or slightly higher for the latter.
5. Instrumental carry over: An irrelevant carryover effect was observed during the instrumental analysis despite the reuse of the derivatizing agent MTBSTFA and SPME fiber for a considerable number the samples (~100). MilliQ® water samples saturated in NaCl and pH 3 adjusted and filtered through 0.7-μm (blanks) were run under the optimized instrumental method right after spiked sludge samples at different levels of concentration. The peak areas from both the blanks and the spiked samples were then compared. Most of the blanks contained less than 4% of the previous signal from the sludge samples (Table S3B). Therefore, the carryover effect was considered insignificant and desorption and fiber conditioning were adequately validated. This constituted an important achievement over related methodologies such us López-Serna et al. (2018).

6. Dynamic range: The quantification method was based on an internal standard approach. Eight-point calibration curves were built by spiking equal sludge samples covering the range from 30 to 2500 ng $g^{-1}$, for all target compounds. The reported calibration curves (Table S3B) corresponded to linear equations with correlation coefficients ($R^2$) above 0.99 within the indicated concentration range. Up to 3 orders of magnitude were observed. Linearity ranges up to 3 (López-Serna et al., 2018) and 2 (Yu and Wu, 2012) orders of magnitude have been reported elsewhere.

In summary, the method has been successfully validated for 10 PPCPs with different physical-chemical properties.

### 4.2. Application of the method to the analysis of sludge samples

The proposed method was successfully applied to samples of different types of sludge from two indoor pilot scale reactors run at the Department of Chemical Engineering and Environmental Technology of the University of Valladolid (Spain): thermally pre-treated mixed sludge and digested sludge. The experimental devices to treat the sludge were: a 2-L thermal hydrolysis (TH) treatment plant treating thickened mixed sludge at 180 °C during 30 min, and a 5-L continuous anaerobic digester operating in mesophilic conditions. Both reactors were daily supplied with thickened mixed sludge from the WWTP in Valladolid, whose details were described in section 2.2. TH is a pre-treatment that reduces the viscosity of the sludge, increases its organic load and improves both the dehydratability and degradability of the treated sludge (Barber, 2016). Anaerobic digestion (AD) decomposes organic matter with the aid of different microorganisms and the final product includes added-value products such as biogas (60–70%) and biomass that could be used as fertilizer (Jain et al., 2015).

One litre samples were grabbed in HDPE drums for the inlets of both reactors at the beginning of the experiments. The same amount of sample was grabbed for the outlets after 2 and 24 h of treatment of TH and AD, respectively. All samples were promptly centrifuged and the solid phase was stored at −20 °C and darkness until analysis.

The results, which are displayed in Table S4, showed a significant degradation (range in 33–90%) of most of the compounds of interest during the TH process. However, the concentration of some PPCPs (propylparaben and bisphenol A) increased slightly after this treatment. Similarly, some concentrations remarkably decreased after the AD (Table S5). This was the case of salicylic acid (99.9%), triclosan (48%), diclofenac (22%) and bisphenol A (32%). In contrast, clofibric acid, propylparaben and naproxen increased their concentrations after AD. An explanation for these augmentation events could be related to compound adsorption phenomena onto the solid residue during the sludge treatment. In addition, non-monitored pro-drugs and metabolites such as glucuronides could easily turn into the target analytes after the tested processes (López-Serna et al., 2013; Pedrouzo et al., 2011). Authors such as Boix et al. (2016b) also reported similar increases in the studied contaminants after urban sewage sludge AD.

A significant correlation between lipophilicity and the persistence of pharmaceutical residues was observed by Malmborg and Magnér (2015) during AD. This mentioned correlation was also observed for naproxen in the present study.

Regardless, observed concentrations for the inlet and outlet sewages sludge samples were in the ng $g^{-1}$ level, in all cases. In particular, the compounds of interest were found at concentrations between < MLQ-8332 ng $g^{-1}$ in the inlets. Ranges of 15-1675 ng $g^{-1}$ and <MLQ-9355 ng $g^{-1}$ were determined for TH and AD outlets, respectively.

## 5. Conclusions

An improved analytical method for the determination of PPCPs in urban sewage sludge has been designed, optimized and validated, which can be used in routine analysis laboratories around the world. The optimum sample pre-treatment included a 1-cycle MAE combined with an in-situ clean-up stage using 100.0 mg of activated $Al_2O_3$ for the reduction or elimination of interferences associated with this type of environmental matrices. A mixture of MilliQ® water/MeOH 95:5 (v/v) at pH 9 turned out being the best performing extraction solvent. In addition, a filtration step prior to the sample analysis was required. The instrumental part of the method consisted of an online DI-SPME-on fiber derivatization-GC-MS. The resulting environmentally friendly methodology decreased the use of expendable material (small amounts of reagents, reusable SPME fiber and derivatizing agent, …) and was successfully validated for 10 PPCPs (methylparaben, clofibric acid, ethylparaben, ibuprofen, propylparaben, salicylic acid, naproxen, triclosan, diclofenac and bisphenol A), with MLDs below 30 ng $g^{-1}$. The isotope dilution (10 isotopically labelled analogues) quantifying approach provided with high reliability to the method. In addition, this fully automatized methodology was fast and analyst convenient to determinate PPCPs in sewage sludge.

Real samples from both TH and AD pilot scale plants were analysed. Some PPCPs such as methylparaben, clofibric acid, propylparaben and diclofenac were found at concentrations below 100 ng $g^{-1}$ (d.w.) in thermal hydrolysed samples. In contrast, another as salicylic acid presented a concentration above 1000 ng $g^{-1}$ (d.w.) for the same matrix. A different scenario was observed after AD treatment. Some PPCPs such as methylparaben, clofibric acid, ethylparaben, ibuprofen, salicylic acid and bisphenol A were found at concentrations below 50 ng $g^{-1}$ (d.w). However, naproxen presented a concentration above 9355 ng $g^{-1}$ for the same matrix.

## Author contributions

The corresponding author is responsible for ensuring that the descriptions are accurate and agreed by all authors. Authors have been contributed in multiple roles: Conceptualization, Methodology, Formal Analysis, Investigation, etc.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127273.

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# ANÁLISIS DE COMPUESTOS FARMACÉUTICOS Y PRODUCTOS DE CUIDADO PERSONAL (PPCPs) EN LODOS DE DEPURADORA

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## 1. Introducción

El desarrollo de métodos de análisis nuevos y más sensibles que se han producido en los últimos años, han permitido alertar de la presencia de compuestos, potencialmente peligrosos, de origen antropogénico a niveles de trazas en aguas residuales, denominados "contaminantes emergentes" (CEs). Estos contaminantes son compuestos de diverso origen y naturaleza química; retardantes de llama, parafinas cloradas, pesticidas, compuestos perfluorados, compuestos farmacéuticos (PhACs), productos de cuidado personal (PCPs) y drogas de abuso, entre otros. La gran mayoría de estos contaminantes no se encuentran regulados por ninguna legislación, tanto europea como española, pero se consideran perjudiciales para el medio ambiente y la salud humana ya que pueden causar diversos efectos nocivos en los organismos, como toxicidad crónica, disrupción endocrina y bioacumulación [1].

Uno de los principales problemas de este tipo de contaminantes se encuentra en que la mayoría de ellos no se eliminan de forma adecuada y eficiente con los tratamientos actuales en las Estaciones de Depuración de Aguas Residuales (EDARs) ya que no están diseñadas para eliminar dichos contaminantes presentes a bajas concentraciones (ng $L^{-1}$ a µg $L^{-1}$), siendo una fuente de contaminación. Por ejemplo, los lodos de depuración generados son, a menudo, empleados en actividades agrícolas y forestales, principalmente debido a su capacidad para fertilizar los suelos y el bajo impacto económico de esta práctica [2], lo que lleva a su propagación en el medio ambiente.

La lista de CEs incluye una gran variedad de productos de uso diario con aplicaciones tanto industriales como domésticas. Dentro de esa gran variedad, encontramos los compuestos farmacéuticos y productos de cuidado personal, conocidos como PPCPs ("pharmaceuticals and personal care products"), un amplio grupo empleado en el cuidado personal de la salud humana y animal. Los PPCPs engloban una amplia variedad de sustancias químicas como son los medicamentos terapéuticos o veterinarios, las fragancias y los cosméticos, empleados en el cuidado estético, en el bienestar y salud personal, así como en la industria agroalimentaria para mejorar la salud y crecimiento de los animales. Los compuestos farmacéuticos (PhACs) son los que más interés han generado, cogiendo fuerza en la década de los 90. Su uso generalizado da lugar a una descarga continua al medio ambiente, pudiendo llegar a quedarse retenidos en el entorno o incluso acumularse, afectando al ecosistema y a los seres humanos a través de la cadena trófica [3].

Se ha observado la presencia de PPCPs en lodos de depuradora de la mayoría de las EDARs en diferentes partes del mundo. Entre los PhACs se pueden encontrar antiinflamatorios no estereoideos (AINE) como ibuprofeno [4,5], naproxeno [4,5] o diclofenaco [4,5], antibióticos como enrofloxacina [6] y doxiciclina [6], anticonvulsionantes o reguladores de lípidos como carbamazepina [5] y ácido clofíbrico [4], respectivamente. Un caso muy particular es el de los compuestos citostáticos como vinblastina [7] o vincristina [7], diseñados y utilizados para causar la disfunción celular porque son capaces de inhibir el crecimiento desordenado de células, alterar la división celular y destruir las células que se multiplican de forma rápidamente. Por otro lado, entre los productos de cuidado personal (PCPs) se pueden encontrar conservantes como metilparabeno [4], etilparabeno [4] o propilparabeno [4], agentes antibacterianos como triclosán [4,8] o triclocarbán [8] y filtros UV como benzofenona-1 [8,9] y benzofenona-2 [8,9], considerados disruptores endocrinos ya que alteran el sistema endocrino del organismo [1].

## 2. El análisis de las muestras de lodo de depuradora

Diferentes estudios realizados en distintas partes del mundo han observado la presencia de PPCPs en diferentes matrices ambientales. Algunos PPCPs como diclofenaco (AINE), triclosan (agente antibacteriano de amplio espectro), triclocarbán (agente antibacteriano), propranolol (agente antihipertensivo) o el miconazol (agente antifúngico) son frecuentemente observados en lodos de depuradora de la mayoría de las EDARs [10].

Los lodos de depuradora se caracterizan por ser un residuo prácticamente líquido (más de un 95% de agua) y su composición va a depender de la carga de contaminación del agua residual inicial y de las características de los diferentes tratamientos aplicados en las aguas residuales. La matriz asociada es muy compleja, no es uniforme en su composición y, además, el lodo contiene ciertas sustancias que podrían interferir en la determinación de los compuestos de interés. Esas interferencias pueden afectar a todo el proceso analítico desde la preparación de la muestra hasta la detección instrumental. Por lo tanto, es necesario eliminarlos de las muestras mediante procedimientos de limpieza [10].

### 2.1. Pre-tratamiento de muestra

Las muestras de lodo recogidas de las EDARs son congeladas y liofilizadas para poder eliminar el contenido de agua que contienen las mismas y, posteriormente, almacenadas a una temperatura de -20ºC hasta su análisis. La preparación de las muestras conlleva la mayor parte del tiempo de análisis y, por lo general, incluye un proceso de extracción seguido de un paso de limpieza [10].

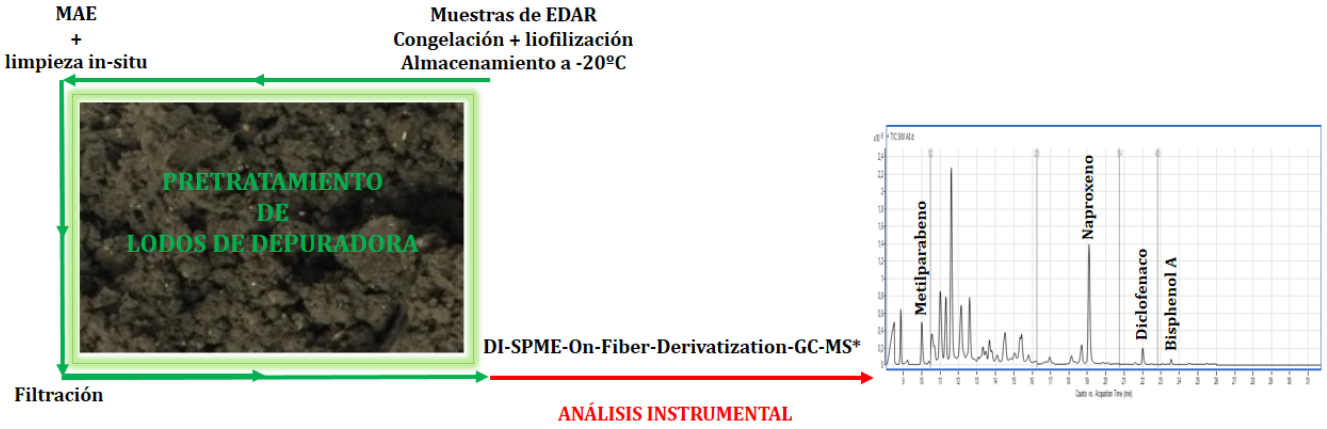


**Fig.1. Metodología analítica desarrollada por Pérez-Lemus et al. (2020) [4] para la determinación de PPCPs en lodos de depuradora**

#### 2.1.1. Etapa de extracción

La extracción permite separar los analitos de interés de la muestra para un análisis posterior más sencillo ya que los lodos son muestras sólidas complejas con gran cantidad de especies capaces de interferir con los propios analitos a la hora de ejecutar su análisis. Se pueden encontrar diferentes técnicas empleadas para conseguir una extracción satisfactoria. Las técnicas utilizadas presentan las ventajas de tiempos de extracción cortos y el uso de pequeñas cantidades de disolvente, por lo que se consideran técnicas amigas del medio ambiente. La más empleada es la extracción asistida por ultrasonidos (UAE) [8,9,11]. Es un método relativamente económico en comparación con otros y de una gran simplicidad. Otras técnicas más modernas son la extracción asistida por microondas (MAE) [4,7] y la extracción de líquido presurizado (PLE) [5], siendo ambas técnicas automatizables. En el caso de MAE, permite la reducción de muestras y energía, consiguiendo reducir la generación de residuos [12]. En el caso de PLE, se considera una técnica de alto rendimiento para la determinación de una gran cantidad de analitos en muestras ambientales. Se trata de una técnica muy eficaz a la hora de extraer los analitos de interés, aunque también extrae otros compuestos presentes en la muestra, lo que implica la necesidad de una etapa de limpieza posterior a la extracción. PLE es una técnica más rápida y se obtienen mayores rendimientos en comparación con otros procedimientos de extracción convencionales. El inconveniente es el empleo de temperaturas elevadas y las extracciones poco selectivas [13]. Y sobre todo, tanto MAE como PLE presentan el elevado precio del equipo. Una técnicas alternativa y mucho más novedosa es la extracción de la matriz en fase sólida (MSPD) [14]. Esta técnica implica un proceso permitiendo la extracción y limpieza simultánea de muestras sólidas o semisólidas con una reducción significativa del consumo de disolventes y sin requerir instrumentación particularmente costosa [15,16].

#### 2.1.2. Limpieza

En la mayoría de los casos, se necesita una etapa de limpieza posterior a la extracción, ya que algunas técnicas de extracción no son lo suficientemente selectivas como para extraer únicamente los compuestos de interés, sino que también extraen otros compuestos presentes en la muestra conocidos como interferentes, ya que interfieren y complican el análisis de los compuestos de interés de una muestra [10]. La extracción en fase sólida (SPE) es la técnica de limpieza más empleada previa al análisis debido a la poca selectividad de las técnicas de extracción empleadas o para una mejora de los propios resultados [9,11]. Esta técnica permite concentrar y separar analitos de una matriz compleja mediante una fase sólida estacionaria. Se consigue eliminar los interferentes que no han quedado retenidos y, posteriormente, los analitos de interés se analizan con la técnica analítica adecuada [10]. Algunas ventajas son el tamaño de muestra, la pequeña cantidad de volumen de elución, las reducidas limitaciones en la utilización de disolventes, el poco consumo de disolventes y, por último, las pocas posibilidades de contaminación. Una alternativa a la SPE, es la extracción dispersiva en fase sólida (d-SPE) [8], una técnica simple, de fácil manejo y adaptable. Además, es

una técnica selectiva, robusta, versátil y de bajo coste en comparación con técnicas clásicas. Entre sus ventajas, destacan la reducción del tiempo en el tratamiento de la muestra, permitiendo así analizar más cantidad de muestras en menos tiempo y la poca cantidad de disolvente requerida.

### 2.2. Análisis instrumental

Para valorar el comportamiento de los PPCPs en los diferentes tratamientos de muestra empleados es necesario el desarrollo de metodologías que permitan su identificación y cuantificación en distintas matrices ambientales. Las técnicas de análisis de estos contaminantes más empleadas corresponden a la cromatografía de líquidos con detector de masas en tándem (LC-MS/MS) [8,12] o cromatografía de alta presión (UHPLC) [6,7]. El sistema UHPLC ha permitido un aumento de la resolución, la velocidad y la sensibilidad [17]. El inconveniente es que estas técnicas requieren una instrumentación compleja que no está al alcance de todos los laboratorios y, a pesar de que una gran cantidad de estos compuestos son polares y no pueden analizarse de manera sencilla por cromatografía de gases, la técnica de cromatografía de gases con detector de masas (GC-MS) puede ser efectiva para su determinación en matrices ambientales tras una derivatización de las muestras [4]. Además, la microextracción en fase sólida mediante inmersión directa es la técnica cada vez más utilizada en la actualidad para la extracción de estos contaminantes en muestras ambientales como el caso de lodos de depuradora y los últimos avances van encaminados hacia su empleo de forma automática [4].

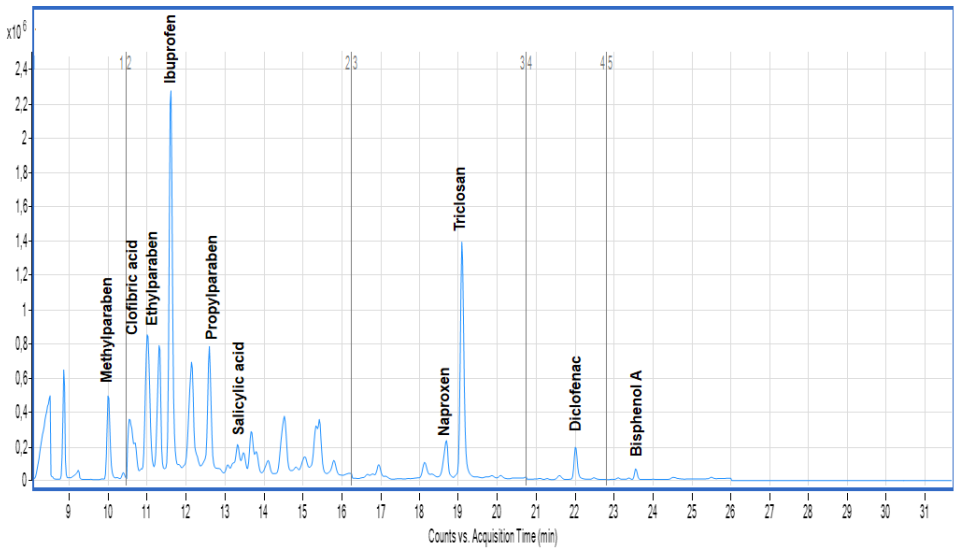


**Fig. 2. Cromatograma de una muestra de lodo de depuradora después de su análisis por DI-SPME-on-fiber-derivatization-GC-MS [4]**

## 3. Conclusiones

La mayoría de estudios realizados en los últimos años se centraron en la determinación de PPCPs en muestras de lodo de depuradora, concretamente en PhACs, debido a que gran parte de ellos son continuamente liberados al medio ambiente, convirtiéndolos en agentes contaminantes. En ciertos casos, técnicas de pre-tratamiento de muestra como MAE o PLE, o incluso más novedosas como MSPD se emplearon para la extracción de los analitos de interés, sin embargo, UAE sigue siendo la más popular ya que proporciona una muestra segura, rápida y fácil de preparar. Habitualmente, después del paso de extracción, una etapa de limpieza es necesaria ya que la extracción no es completamente selectiva, siendo SPE la técnica más empleada después de la extracción de los PPCPs en este tipo de matrices. Para el análisis de los contaminantes en muestras de lodo de depuradora, LC acoplada a MS/MS fue la técnica principalmente seleccionada. Sin embargo, GC acoplada a MS es otra técnica empleada para el análisis de PPCPs tras una etapa de derivatización de las muestras. En la actualidad, se han desarrollado métodos analíticos cada vez más sensibles que permiten identificar y cuantificar CEs, especialmente, en muestras ambientales, donde su presencia es considerablemente baja. Además, los nuevos métodos y procesos que se están desarrollando permiten reducir o incluso eliminar el uso de sustancias extremadamente nocivas, permitiendo seguir haciendo química, pero de forma sostenible y cuidando nuestra salud y la de nuestro planeta.

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